MOLECULAR REGULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 AND ITS ROLE IN RIBOTOXIC STRESS-INDUCED APOPTOSIS

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ABSTRACT OF THE DISSERTATION

Molecular Regulation of Insulin-like Growth Factor Binding Protein-3 and its Role in Ribotoxic Stress-induced Apoptosis

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Insulin-like growth factor binding protein-3 (IGFBP-3) is one of six high affinity binding proteins for IGF-I. However, new and exciting IGF-independent functions have begun to emerge for IGFBP-3 in the regulation of cell growth. Specifically, the biological role of IGFBP-3 in apoptosis has been found to be extremely complex, with a number of possible functions suggested, but few well-characterized mechanisms determined. The overall goal of this work was to elucidate the role of IGFBP-3 within the context of ribotoxic stress-induced apoptosis, with specific aims to determine how ribotoxic stressors regulate IGFBP-3 expression and how IGFBP-3 influences the apoptotic pathway. Studies involving both chemical inhibitors and siRNA determined that the ribotoxic stressors anisomycin (ANS) and ricin A chain (RTA) both induced IGFBP-3 expression through the p38 signaling pathway, and that both stressors enhanced IGFBP-3 mRNA stability through a 3'untranslated region-mediated, p38 dependent mechanism. Additional studies demonstrated that RTA also activated the IGFBP-3 promoter in a p38-dependent manner. Subsequent work allowed for the conclusion that IGFBP-3 is specifically required for ANS and RTA-induced apoptosis. Knockdown of

IGFBP-3 with siRNA prevented ANS-induced cytochrome c release, caspase activation and PARP cleavage. Knockdown of JNK-2 also inhibited caspase activation and PARP cleavage, suggesting that it and IGFBP-3 were involved in the same apoptotic mechanism. These findings led to the discovery that the orphan nuclear receptor Nur77 translocated to the cytosol following ANS treatment, where it is known to induce apoptosis. Additionally, knockdown of either IGFBP-3 or JNK-2, pre-treatment with the nuclear export inhibitor leptomycin B (LMB) or treatment with IGF-I could both prevent this translocation and prevent ANS-induced apoptosis. In conclusion, this work describes the novel regulation of IGFBP-3 by the p38 pathway, and helps to shed light on the complex role that IGFBP-3 plays in apoptosis. These findings suggest that IGFBP-3 may be an integral part of the inherent apoptotic machinery in normal cells and may play a crucial role in the response of normal cells to toxic stressors.

DEDICATION

To My Parents

For unwavering support

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CHAPTER I

REVIEW OF LITERATURE

I. THE INSULIN-LIKE GROWTH FACTOR SYSTEM

The insulin-like growth factor (IGF) axis influences a wide range of cellular activities, including proliferative, migratory, metabolic and survival functions. As a result of these diverse actions, IGF plays important roles throughout growth and development in most tissues of the body. In addition to normal regulatory functions, disregulation of the IGF system is proposed to be involved in the development and progression of a variety of cancers, including, but not limited to thyroid, prostate and breast, due in large part to its strong anti-apoptotic and mitogenic functions [1-4]. The IGF axis consists of three ligands, IGF-I, IGF-II and insulin, which are all single chain polypeptide hormones. In addition, the IGF system contains a specific, high affinity, cell surface receptor for each ligand, as well as six IGF binding proteins (IGFBP) [5]. IGF-I and –II contain A and B domains that are homologous to the A and B chains of insulin, but also contain additional C and D domains that are absent from mature insulin. The mature IGF peptides share approximately 50% sequence homology to mature insulin [6, 7].

The type I and II IGF receptors (IGF-IR and IGF-IIR) and insulin receptor (IR) bind to IGF-I, IGF-II and insulin, respectively, with high affinity, though IGF-II and insulin can also bind to IGF-IR with approximately 3-fold and 100-fold lower affinity, respectively, than IGF-I [8]. Studies have found that the IGF-IR is responsible for most of the biological actions of IGF, with respect to growth and development [9]. The IGF-IIR, which cloning studies found to be identical to the mannose-6-phosphate receptor

(M6P), is involved in trafficking lysosomal enzymes and endocytosis [10]. It also can serve as a sink for IGF-II, helping to control local levels [11]. However, its role in signal transduction is thought to be limited, though some data suggest that it can associate with G-proteins [12, 13].

As stated above, the IGF-IR is believed to be most responsible for conveying mitogenic signals from IGF ligands into the cell. The IGF-IR is a heterotetramer composed of two extra-cellular, ligand binding alpha subunits, and two transmembrane beta subunits that contain cytosolic tyrosine kinase domains. Ligand binding causes a conformational change that results in autophosphorylation within the tyrosine kinase domains, recruitment of intracellular adapter and docking molecules, and subsequent stimulation of downstream signaling [14-16].

Prior to any initiation of signaling, IGF ligands have to be available to the receptor, and this process is regulated by the six IGFBP [17]. The IGFBP possess high affinities for IGF, generally exceeding that of IGF-I for its receptor [18]. The IGFBP have conserved amino and carboxy terminal regions, with the amino terminus being most responsible for IGF binding, and the carboxy terminus being necessary for both IGF binding as well as interactions with other proteins and, in some cases, the cell surface. The central domains provide the greatest variation across IGFBP and are important for providing diversity of both structure and function. Central domain residues are sites for post-translational modifications that can affect IGFBP function to varying degrees [19]. Specific post-translational modifications including phosphorylation (IGFBP-1, -3, and –5), glycosylation (IGFBP-3, -4, -5, and –6) and proteolysis (all six IGFBP) have all been described [20].

In addition to regulating IGF bioavailability in local tissues, the IGFBP serve to stabilize and transport IGF in the circulation [17]. The vast majority of circulating IGF-I is found in a 150 kDa ternary complex composed of one molecule each of IGF-I, IGFBP, and acid labile subunit (ALS). Generally, IGFBP-3 is found in this complex, but IGFBP-5 has also been identified as the IGFBP unit [21]. When complexed with an IGFBP and ALS, the half-life of IGF in the circulation increases from approximately 30 min to over 15 h [22]. Locally, the IGFBP/IGF complexes continue to stabilize the levels of IGF, as well as potentially regulating access to receptors [23].

Within specific tissues, the roles of individual IGFBP have been difficult to determine. All six IGFBP have been found to inhibit IGF actions, but IGFBP-1, -3, and –5 have also been found to enhance IGF action. Additionally, several IGFBP have been found to exert effects independent of IGF binding [20]. Local protease production, extra-cellular matrix (ECM) composition and integrin expression can all potentially influence IGFBP activity as well [24-27]. Combined with the fact that many cell types make multiple forms of IGFBP, the study of individual functions of these pluripotent proteins has been difficult.

ROLE OF IGF AND IGFBP IN GROWTH AND DEVELOPMENT

Mice possessing *igf1*-/-, *igf2*-/-, *igfr1*-/- and *igfr2*-/- genotypes have all been produced, and the resultant phenotypes provided clear evidence for both *in utero* and post-natal dependence on IGFs for normal growth [9, 28]. *Igf1* and *igf2* null mutations caused approximately 40% reductions in prenatal growth weights, while *igfr1* knockouts had an even more dramatic phenotype, with 55% growth reduction and respiratory

failure-induced death shortly after birth. Mice lacking the *igf2r* gene exhibited fetal over-growth and *in utero* lethality. While *igf1*-/- mice had continued post-natal growth retardation, *igf2*-/- mice had essentially normal post-natal growth rates, suggesting that IGF-II is required primarily for early development [29].

The pre-natal and post-natal roles for IGF-I in growth have been studied for more that 50 years, with the first somatomedin hypothesis, formulated in 1957, stating that growth hormone stimulates liver production of IGF-I, which is then distributed throughout the body via the circulation, where it acts on local tissues to mediate the effects of growth hormone [30]. Liver-specific deletion of *igf1* in mice (LID) led to a 75% reduction in circulating IGF-I, which supported the somatomedin hypothesis in terms of the liver being a primary site of IGF-I production. However, these mice did not have post-natal growth deficiency, which suggested that local IGF-I production was also important, and that the regulation of IGF-I was more complex than the somatomedin hypothesis originally proposed [31, 32]. To further explore this issue, a means of reducing circulating IGF even more dramatically was sought. Since circulating IGF-I exists primarily in a ternary complex with IGFBP-3 and ALS, a genetic model targeting the ALS gene was used to produce ALS knockout (ALSKO) mice. Similar to the LID mice, these mice had a 65% reduction in circulating IGF-I, with minimal post-natal growth retardation [33]. However, when crossed with the LID mice, the new ALSKO+LID double knockout mice showed greater than 85% reduction in circulating IGF-I and significantly reduced linear growth, as well as decreased bone density [34]. These data forced the somatomedin hypothesis to be revised to include a role for

circulating IGF-I in postnatal growth, but still allow for potential roles of locally produced IGF-I.

In contrast to genetic models of IGF or IGF-IR gene disruption, where overt growth phenotypes were observed, systemic knockouts of IGFBP have provided less clear information. It's believed that this is due to compensatory behavior of other IGFBP in the absence of one specific form [35]. For example, in IGFBP-3 knockout mice, post-natal growth was not affected, possibly due to the ability of IGFBP-5 to substitute in the circulating IGF/IGFBP/ALS ternary complex [35]. However, some phenotypic information has been gleaned from IGFBP single gene knockout models. In IGFBP-2 knockout mice, levels of IGFBP-1, -3, and –4 were all found to be increased, and spleen weight was reduced in comparison to wild-type littermates [36]. Additionally, IGFBP-1 deficient mice showed impaired liver regenerative activity, in association with altered IGF signaling [37]. As for direct growth alterations, only the IGFBP-4 knockout mice displayed a growth phenotype, with approximately 15% reductions in birth weight. Interestingly, this suggests a role for IGFBP-4 during prenatal growth that cannot be easily compensated for by other IGFBP [35]. It was not until combinatorial IGFBP knockouts were produced that more substantial phenotypes were observed. Mice with disruptions in IGFBP-3, -4, and –5 showed the same phenotype at birth as IGFBP-4 knockout mice, but then demonstrated a variety of additional phenotypes later in life. They were significantly smaller in total body growth, had smaller quadriceps muscles, and had substantially reduced fat pad accumulation. Additionally, they had an increased number of pancreatic beta cells, leading to greater insulin secretion in response to a glucose challenge, which in turn led to more rapid glucose clearance. Circulating IGF-I

levels decreased 55%, but, in contrast to the LID mice, free IGF levels also decreased, leading to significant reductions in IGF bioactivity [35].

Where knockout studies have, for the most part, underachieved, transgenic studies that over-express IGFBP have provided a wealth of functional information. Transgenic mice expressing IGFBP-1, -2, -3, or –5 all displayed impaired growth [37, 38]. Systemic over-expression of IGFBP-1 led to brain abnormalities, as well as impaired glucose homeostasis and reproductive deficiencies [39-42]. In addition to diminished overall growth, IGFBP-3 over-expression led to selective organomegaly, with spleen, liver and heart weight all increased, as well as some reproductive deficiencies characterized by decreased litter sizes [43, 44]. Local over-expression of IGFBP-4 led to smooth muscle hypoplasia, and correspondingly lower weights of organs containing high percentages of smooth muscle, including bladder, intestine, stomach and aorta [45]. IGFBP-5 transgenic mice displayed a myriad of compromised phenotypes, including increased neonatal mortality and decreased muscle development, as well as some gender specific phenotypes including reduced female fertility and reduced bone mineral density in males [38, 46].

IGF AXIS IN MAMMARY GLAND DEVELOPMENT

The IGF axis is thought to be involved in each major stage of postnatal mammary gland biology, from early pubertal development to pregnancy, lactation and involution [47]. At birth, the mammary gland consists of a fat pad containing deposits of secretory epithelial cells that are surrounded by stromal tissue, though the two are separated by a basement membrane of extracellular matrix (ECM) proteins derived primarily from the stroma [48]. A rudimentary system of ducts is present and develops slowly until puberty.

With the onset of puberty, epithelial cell proliferation accelerates, leading to greater expansion of the ductal tree into the mammary fat pad, and terminal end-bud (TEB) formation. During pregnancy, ductal branching continues and alveolar formation progresses, leading to terminal differentiation of these milk-secreting cells by the end of gestation. Following lactation, the mammary gland undergoes involution, during which time much of the epithelial structure undergoes apoptosis and the gland regresses to a stage similar to that of the mature virgin animal [49].

Ruan and Kleinberg, using igf1-/- mice, elegantly demonstrated a requirement for IGF-I in early mammary development. These mice exhibited dramatically reduced mammary growth compared to wild-type litter mates, in terms of both gland size and TEB formation [50]. Additionally, treatment of these mice with either growth hormone (GH) or IGF-I, in combination with estrogen, showed that it was IGF-I, and not GH, that was responsible for TEB development. Daily subcutaneous administration of IGF-I plus estrogen led to restored TEB formation, while GH plus estrogen treatment failed to do so. Taken together with data showing that GH induces IGF-I expression in mammary stromal tissues, these studies suggest that TEB formation results from GH stimulation of local IGF-I production in the stroma, followed by IGF-I acting on the epithelium in a paracrine fashion [51-53]. Richards and colleagues gave further credence to the role of local IGF in TEB development and ductal branching with the comparison of LID mice to those with a whole body *igf1* mutation (*igf1*$^{m/m}$). *igf1*$^{m/m}$ mice do not have an *igf1* gene deletion, but instead have an intronic disruption of exon 3 that causes a 70% reduction in circulating IGF, as well as a corresponding decrease in local mammary derived IGF-I. LID mice had normal ductal development, while the *igf1*$^{m/m}$ mice were severely impaired. Since

circulating levels of IGF-I were comparable in the LID mice and the *igf*$^{m/m}$ mice, this indicated that reduced circulating IGF-I was not responsible for the phenotype observed by Ruan and Kleinberg, but it was in fact the loss of local IGF-I that was the culprit [54].

The role of IGF-I during lactation is less well understood. In order for lactation to occur following pregnancy, the epithelial cells of the mammary gland must differentiate into polarized cells, connected by tight junctions, with distinct apical and basolateral surfaces. Additionally, the organelle composition of these cells changes to accommodate the new output requirements. This leads to an increase in Golgi and rough endoplasmic reticulum, which are both involved in the protein secretion pathway [55]. However, it is unclear whether IGF directly influences the differentiation process. There is some evidence that IGF expression is associated with more differentiated human mammary tumors, while low or absent IGF expression is associated with more malignant tumors [56]. In terms of milk production, IGF has been suggested to possibly play two roles. First, its anti-apoptotic functions lead to ductal hypertrophy and inhibition of the cell death associated with involution [57]. Second, there is some evidence that IGF can stimulate the expression of some genes coding for milk proteins, although there are some conflicting data on this front [58-60].

Following cessation of lactation, involution occurs. During this time the mammary gland undergoes a dramatic regression to once again resemble the virgin gland. Not surprisingly, IGF expression decreases substantially during early involution, as the milk secreting cells undergo significant apoptosis, but returns later as extensive ECM remodeling takes place during the reorganization of the gland [61]. In contrast to IGF-I, IGF-II levels increase during involution, but do so correspondingly with IGFBP-6, which

is proposed to sequester the IGF-II away from the IGF-IR [61]. This possibility is supported by evidence that IGFBP-6 can be pro-apoptotic in some systems, along with IGFBP-6 having far great affinity for IGF-II than IGF-I [62].

II. APOPTOSIS

Apoptosis is the natural process of programmed cell death, whereby a cell activates enzymes that degrade proteins and cleave DNA, causing the cell to die without an associated inflammatory response [63]. Apoptosis functions as both a homeostatic mechanism to control cell numbers within specific tissues, and as a defense mechanism to destroy cells damaged by either physiological or pathological stimuli [64]. There are several characteristic morphological changes that occur during apoptosis that can be observed via microscopy. Upon apoptotic initiation, cells round up and shrink, leading to denser cytoplasm and more closely packed organelles, as well as chromatin condensation [65]. As apoptosis progresses, the plasma membrane undergoes a process called blebbing where the cells become abnormally shaped and fragment into smaller "buds" containing densely packed organelles and nuclear fragments [65, 66]. These buds, called apoptotic bodies, are then quickly phagocytosed, mostly by macrophages, thus preventing their cytosolic components from being released into the surrounding interstitial fluid and causing inflammation [67].

Apoptosis is generally initiated via one of two main pathways, referred to as the extrinsic, death receptor mediated pathway (summarized in Figure 1), and the intrinsic, mitochondrial mediated pathway (summarized in Figure 2) [63]. In either case, a family of proteases called caspases is activated early and ultimately carries out the execution of apoptosis through cleavage of cytoskeletal proteins, nuclear proteins and nucleic acids. The extrinsic pathway begins with transmembrane receptors from the TNF-α receptor family that contain both extra-cellular ligand binding domains and intra-cellular death

domains [68, 69]. There are several members of the death receptor signaling family, including Fas (CD95, Apo1), TNFR-1 (p55, CD120a), DR3 (Apo3, TRAMP, LARD), DR4 and DR5 [70]. These receptors all contain cytosolic death domains, where downstream adapter proteins are recruited. The best characterized of these death receptor pathways are those that stem from TNFR-1 and Fas.

When TNF-α binds TNFR-1, trimerization of the intracellular death domains occurs, allowing an adaptor protein called TRADD to bind to this region. TRADD associates with two other proteins, FADD and RIP [71]. This complex then recruits procaspase-8 via interaction between the death effector domains (DEDs) of FADD and the amino terminal of procaspase-8 [72, 73]. This complex is referred to as the death-induced signaling complex (DISC), and results in autolytic cleavage and activation of caspase-8 [74]. Upon activation, caspase-8 is rapidly released from the DISC complex and proceeds to activate downstream caspases or other cellular targets. An alternate pathway can be activated when TNFR-1 recruits the adapter molecule RAIDD, which leads to activation of caspase-2 [75]. When Fas ligand (FasL) binds to its receptor, the same characteristic trimerization of the cytosolic death domains occurs. However, unlike TNFR-1, Fas only recruits FADD, not TRADD, via interaction of their DEDs [76]. This complex then recruits either procaspase-8 or procaspase-10 to form DISC. As with TNFR-1, DISC formation leads to autolytic cleavage of the procaspase, its rapid release, and progression of the apoptotic pathway [73, 77, 78]. In either TNFR-1 or Fas death receptor signaling, the Flice-inhibitory protein (FLIP) can act as a negative regulator of apoptotic progression. FLIP can suppress apoptosis by direct interaction with the DED of FADD, thus preventing recruitment of procaspase-8, or via stimulation of the NF-κB

survival pathway [70]. As evidence of this process, Aggarwal et al. showed that stable over-expression of FLIP led to resistance to death receptor mediated apoptosis [79].

The intrinsic pathway can be initiated by a variety of non-receptor mediated stimuli. Cellular homeostasis involves a delicate balance of positive and negative growth signals, and the intrinsic apoptotic pathway can be initiated by changes in both types of stimuli. Exposure to negative factors such as toxins, viruses, radiation, free radicals or a number of other inducers can trigger this pathway. Additionally, this pathway can be induced by the withdrawal of cell survival signals, like certain growth factors and hormones, which may suppress components of the apoptotic machinery [66]. In either case, apoptosis begins with the loss of mitochondiral membrane integrity, and the subsequent release of two groups of pro-apoptotic proteins that are normally sequestered safely within the mitochondria [80]. The first proteins released include cytochrome c, second mitochondrial activator of caspases/direct IAP binding protein with low PI (Smac/DIABLO) and the protease HtrA2/Omi, which aid in the activation of caspase-9 [81-84]. Two cytochrome c molecules bind to two apoptotic protease activating factor-1 (Apaf-1) and two procaspase-9 molecules to form the apoptosome, which leads to cleavage and activation of the proteases [85]. Smac/Diablo and HtrA2/Omi support this process by inhibiting an anti-apoptotic group of proteins called IAP (inhibitors of apoptosis proteins) [83, 86]. This caspase-9 initiation pathway is sufficient to elicit cell death, but to further insure efficient apoptosis a second group of proteins is released later. This second group contains three proteins, AIF, endonuclease G and CAD that translocate to the nucleus and participate in chromatin condensation, oligonucleosome formation and ultimately DNA fragmentation [87, 88]. AIF and endonuclease G are

functional upon release, while CAD activation requires cleavage of its inhibitor, ICAD, by caspase-3 [89].

The whole process of mitochondrial protein release can be turned on or off at the initial stages by one or more of the 25 known members of the B-cell lymphoma protein-2 (Bcl-2) family [90, 91]. These proteins fall into two distinct groups, with members such as Bcl-2, Bcl-XL and Bcl-XS working to prevent cytochrome c release, while members such as Bax, Bak, Bad, Bid, Bim and Bik promote mitochondrial membrane permeability and apoptosis [66]. A great deal of study has gone into understanding how this family of proteins governs the intrinsic pathway. Bad has been extensively studied and some of its properties have been determined. Serine phosphorylation of Bad allows it to bind to 14-3-3 and be inactivated via sequestration in the cytosol. Dephosphorylation frees Bad from 14-3-3, allowing it to translocate to the mitochondria and facilitate cytochrome c release [92]. Bcl-2 and Bcl-XL normally work to prevent cytochrome c release, but this action can be inhibited by heterodimerization with Bad, which suppresses their protective effects [93]. Bcl-2 can also have its pro-survival functions reversed when it binds with the orphan nuclear receptor, Nur77. After nuclear export and mitochondrial translocation, Nur77 can bind to Bcl-2 and cause a conformational change, thus turning Bcl-2 into a pro-apoptotic molecule and facilitating cytochrome c release [94].

Bax has also been extensively studied. With the induction of apoptosis, cytosolic Bax oligomerizes into large complexes that rapidly translocate to the mitochondria and insert directly into the outer membrane [95-99]. This mechanism relies in part on Bid, thus providing an excellent example of cross-talk between the extrinsic and intrinsic pathways. Upon Fas or TNFR-1 activation, caspase-8 is activated and one of its cleavage

targets is Bid, producing an activated form called t-Bid [100, 101]. Bax and t-Bid can each induce cytochrome c leakage from mitochondria, but following a proposed direct interaction with each other, they produce a synergistic effect [102-105]. However, while their combined effect on mitochondrial membrane permeability is not in question, their physical interaction remains controversial. This has led to what is known as the kiss and run hypothesis, which suggests that they physically interact very briefly, facilitating a conformational change in Bax and enhancing its activity [106]. However, there is also emerging evidence that Bax and Bak can induce the mitochondria to fracture into smaller units, thus facilitating cytochrome c release that is not dependent on oligomerized Bax-induced pore formation [107, 108].

Both the extrinsic and intrinsic pathways converge on what is known as the execution pathway. It is at this point that the downstream effector, or executioner, caspases (caspase-3, -6, -7) become active and begin to carry out the final phases of cell death. They cleave a variety of cellular targets including cytokeratins, PARP, and cytoskeletal proteins. Slegh and colleagues described this process quite accurately as the "demolition phase" of apoptosis [109]. Of these executioner caspases, caspase-3 is generally thought to be most important, and can be activated by any of the upstream initiator caspases. In addition to activating CAD as described above, caspase-3 also targets PARP, to prevent DNA repair, and the actin binding protein gelsolin, to facilitate cytoskeletal breakdown [110, 111]. While it is still generally accepted in the field that caspase-3 is a downstream harbinger of cell death, a 2006 study by Lakhani et al. found that a double knockout of caspase-3/7 in MEFs led to inhibition of initiation of the mitochondrial pathway, suggesting an upstream role for these proteases [112]. However,

there may be some questions as to the tissue specificity of this effect, since the major findings of the study were not all observed in the same cell type [113].

As mentioned above, the caspases play a key role in the execution of apoptotic cell death. This family of cysteine proteases has 14 known members, though their functions are divided into two major categories [114]. Caspase-2 [115], -3, -6, -7, -8 [116], -9 [117] and –10 [70] have been demonstrated to be apoptosis related, while caspase-1 [118], -4 [119], -5 [120], -11, -12, -13, and –14 [121] are classified as cytokine processing enzymes that function mostly in the immune system. Caspases are nearly ubiquitously expressed throughout the tissues of the body, and exist almost exclusively as zymogens [122]. The caspases contain a highly homologous protease domain, as well as a more variable amino terminal peptide that tends to be substantially longer in upstream, apoptotic initiator caspases (caspse-2, -8, -9, -10) and the cytokine processing caspases, than in the downstream, apoptotic effector group [121, 123]. These long amino terminal domains, or prodomains, can contain important sequence motifs for DEDs and caspase recruitment domains (CARD), which play significant roles in caspase activation via association with adapter proteins. Procaspase-8 and procaspase-10 contain DEDs in their amino terminal prodomains, while CARDS are present in this domain of caspase-1, -2, -3, -4, -5, and –9 [124-126]. Activation of the caspases requires proteolysis of the prodomain and its inter-domain linker [127, 128], which may occur via oligomerization [129, 130], transactivation by the death receptor or mitochondrial apoptotic pathways [131, 132] or by direct proteolysis by granzyme B, cathepsin G, calpains or p24 [117, 133].

Use of genetic models, specifically gene knockout models, has helped elucidate the roles of the caspases in whole organism development. Gene disruption studies have shown that caspase-2 knockout mice develop essentially normally, while caspase-8 and caspase-9 deficient mice have problems ranging from abnormal brain development (caspase-9) to embryonic lethality (caspase-8) [115, 134-136]. Caspase-3 knockout mice survived to birth, but began dying shortly thereafter due to incomplete brain development [137, 138]. Caspase-6 and caspase-7 are both required for normal apoptotic cell death, but as with the other knockouts, cell death can proceed without them, albeit in somewhat different forms [139, 140].

As mentioned above, apoptosis is a natural response to both physiological and pathological stimuli. In normal physiology, apoptosis is the reaction to mitosis' action, or vice versa, providing an elegant biological representation of Newton's Third Law. As many as 10 billion cells per day undergo apoptosis in an adult human, requiring a corresponding level of mitosis to maintain cell populations [141]. During development, apoptosis is critically important in the formation of the nervous system and immune system, both of which are initially over-populated with cells. Following differentiation of these cells, those that fail to either produce synaptic connections or develop antigen specificities, respectively, are destroyed by apoptosis [142, 143]. Apoptosis is also critically important in wound healing [144], prevention of autoimmune disease [145], follicular atresia [146] and mammary gland involution [147].

Because apoptosis is so important to normal physiology, impaired regulation of the apoptotic pathways is implicated in a variety of disease states, including cancer, autoimmune diseases, AIDS, ischemia, and neurodegenerative diseases (Parkinson's,

Alzheimer's, Huntington's and ALS) [66]. In some of these pathologies, there is a shortage of apoptosis, while in others, there is excessive cell death. Cancer can result exclusively from cell proliferation run amok, but generally also features the suppression of apoptosis due to any of a variety of mechanisms [148, 149].

B cell lymphoma patients provided some of the earliest evidence that cancer could develop from impaired apoptosis with the discovery that Bcl-2 was over-expressed in the cancer cells, thus making them resistant to apoptosis [150]. Another particularly sinister means of apoptotic avoidance during cancer development occurs when tumor cells inhibit their own death receptor pathways to avoid destruction by T cells and NK cells. This can occur via down-regulation of Fas, expression of a defective isoform of Fas [151], or secretion of a soluble form of Fas that sequesters the immuno cell-derived FasL [152]. An additional means of apoptotic evasion is known as FasL-mediated counterattack, whereby tumor cells express FasL of their own that attacks and kills lymphocytes that have invaded the tumor [153].

Probably the most well-known cancer-causing apoptotic deficiency involves the tumor suppressor gene p53. p53 can activate DNA repair enzymes, regulate the cell cycle and transcriptionally regulate a variety of pro- and anti-apoptotic genes, including Bax and Bcl-2 [154, 155]. Defects in p53 can therefore cause a wide array of problems. In fact, p53 is the most widely mutated gene in human cancer, with approximately 50% of tumors expressing either a damaged form of the protein, or no form at all [156].

RIBOTOXIC STRESS

Ribotoxic stress is the process whereby cells activate stress-activated protein kinases (SAPK) in response to damage to a specific region of the 28S ribosomal RNA, known as the α-sarcin/ricin loop [157], which, in active ribosomes, is responsible for facilitating the peptidyl transferase reaction [158, 159]. It has been proposed that binding to specific nucleotide sequences of the α-sarcin/ricin loop acts to both inhibit translation and, independent of this function, stimulate activation of SAPK [157]. A variety of ribosome inactivating proteins (RIPs) have been shown to initiate SAPK signaling via interaction with the α-sarcin/ricin loop, including α-sarcin [157, 160], ricin [157, 161], Shiga toxin 1 [162], and onnamide A and theopederin B [163]. Additionally, the antibiotics anisomycin (ANS) [157, 164, 165] and blasticidin S (BCS) [157, 166], as well as ultraviolet radiation have also been found to initiate the ribotoxic stress response [167, 168]. In contrast to genotoxic or hyperosmotic stress, the ribotoxic stress response requires ribosomes to be translationally active at the time of contact, as pre-treatment with other translational inhibitors, such as cyclohexamide, prevent SAPK activation in response to α-sarcin/ricin loop targeting agents [157, 167, 168]. Additionally, some, though not all, ribotoxic stressors activate SAPK very rapidly (usually within 5 to 15 minutes), whereas oxidative and several other forms of cellular stress do so with slower kinetics [168].

STRESS ACTIVATED PROTEIN KINASES

C-jun N-terminal kinases (JNK-1, -2, -3) and p38 (-α, -β, - γ, -δ isoforms) make up the SAPK subgroup of the mitogen activated protein kinase (MAPK) family of serine-

threonine kinases. They are stimulated by a wide variety of extracellular signals, as well as intracellular changes in the metabolic state or homeostatic environment of the cell. The speed and duration of their activation, as well as their subcellular localization and availability of downstream substrates all affect the ultimate cellular consequences of SAPK activation [169].

The JNK family consists of three genes (JNK1-3) that encode at least 10 splice variant isoforms that range in size from 46 to 55 kDa. JNK-1 and JNK-2 are believed to be ubiquitously expressed, while JNK-3 is primarily expressed in neuronal tissues [170, 171]. JNK can be activated by a myriad of stimuli, including cytokines like TNF-α [172, 173], heat stress [171], ribotoxic stress [157], or growth factors such as IGF-I [174-176], that lead to either pro-apoptotic or pro-survival phenotypes. Once activated, JNK can carry out its functions either by phosphorylating transcription factors that regulate expression of genes related to proliferation and apoptosis [177], or by phosphorylating cytosolic proteins and altering their activation status [178-180]. However, the consequences of JNK activation appear to be directly related to the time-course of its activation. Studies by Chen and Tan found an association between longer term JNK activation and apoptotis, while short term, transient JNK activation seemed to promote cell survival [181]. This relationship was more rigorously studied by Ventura et al. utilizing chemical genetic techniques, and it was determined that JNK activation lasting up to one hour led to cell survival while sustained JNK activity lasting one to six hours led to pro-apoptotic signaling [182].

JNK can play an influential role in the intrinsic apoptotic pathway through regulation of Bcl-2 family proteins. Kim et al. found that the alkaloid compound

staurosporine, known as a potent, though relatively non-selective, inhibitor of kinase activity and under investigation as an anti-cancer drug, led to activation of JNK and subsequent phosphorylation of Bax, which then led to translocation of this now active protein to the mitochondria in HepG2 cells. However, pretreatment with a selective JNK inhibitor prevented this phosphorylation from occurring and subsequently prevented mitochondrial translocation [178]. Several years earlier, Lei et al. performed an elegant study that clearly illustrated the relationship between JNK and pro-apoptotic Bax by utilizing gene disruption studies. Murine fibroblasts underwent rapid apoptosis in response to JNK activation, but this apoptosis was prevented in cells deficient in Bax. As further evidence, cells lacking JNK were examined and these cells were also unable to undergo mitochondrial mediated apoptosis because Bax translocation did not occur, indicating that JNK activation was a required event [180].

JNK activity can also influence Bax translocation via its ability to phosphorylate and deactivate anti-apoptotic members of the Bcl-2 family. Evidence suggests that JNK can phosphorylate the Bax translocation inhibiting protein, 14-3-3, which causes a conformational change and abolishes its ability to bind Bax and retain it in the cytosol [179]. Additionally, Maundrell et al. found that JNK could alter Bcl-2 function directly by phosphorylating it both in vitro and in COS-7 cells [183]. JNK can also influence Bcl-2 function through a more convoluted mechanism that results in Bcl-2 being conformationally altered to become a pro-apoptotic molecule. For this to occur, JNK phosphorylates the orphan nuclear receptor, Nur77, facilitating its nuclear export and mitochondrial translocation, where it (Nur77) binds to Bcl-2 and alters its function [184]. This interaction will be discussed in more detail later.

JNK-1 and JNK-2 are often lumped together and referred to simply as JNK, because their ubiquitous nature has led to a belief that they are generally overlapping in function, providing redundancy for each other. However, more and more evidence is emerging for distinct roles for JNK-1 and JNK-2 [185]. Liu et al. reported that JNK-1 was required for TNF-α to induce apoptosis in mouse fibroblasts, and JNK-2 antagonized this function [186]. They proposed that this was due to "futile" activation of JNK-2, which competed with JNK-1 for phosphorylation by upstream kinases. Another report, also using mouse fibroblasts, found that the absence of JNK-1 led to decreased cell proliferation, while the absence of JNK-2 enhanced it, suggesting a stronger pro-apoptotic role for JNK-2 and a cell survival role for JNK-1 [187]. A similar distinction has also been found regarding the regulation of the tumor suppressor protein p53. In primary human fibroblasts, inhibition of JNK-2 caused a substantial decrease in p53 protein levels. This was in direct contrast to the increased levels observed following inhibition of JNK-1, again suggesting that the JNKs are not always redundant, and may in some cases directly oppose each other [188].

Similar to JNK, p38 is often associated with growth inhibition and apoptosis [189-191]. The physiological consequences of p38 activation, similar to JNK activation, vary greatly and depend on cellular context [192]. p38 responds to a variety of stress stimuli, including UV radiation, osmotic shock, hypoxia and inflammatory cytokines [169]. However, in contrast to JNK, which modulates the activity of a variety of other apoptotic factors, p38 appears to affect the cellular stress response via modulation of gene expression, at both the transcriptional and post-transcriptional levels.

Transcriptional regulation by p38 takes two main forms. First, p38 can activate a variety of downstream transcription factors, including Stat1, ATF2, Elk-1 and p53, which lead directly to altered expression of stress responsive genes. Additionally, p38 can modulate changes in chromatin structure. In particular, p38 responds to osmotic stress by binding with a histone deacetylase and recruiting it to osmoresponsive genes and facilitating their transcription [193]. p38 also influences chromatin structure as part of the inflammatory response. TNF-α and IL-1β expression are both upregulated by NF-κB-mediated transcription that requires p38 phosphorylation of histone H3 to expose the otherwise hidden promoters [194]. This particular feature has made p38 a popular target for therapeutic intervention in Crohn's disease and rheumatoid arthritis, both of which show the greatest symptom relief from the combined inhibition of TNF-α and IL-1β [195].

Following transcription, p38 can influence gene expression in much the same way as a microRNA, by regulating translation through both mRNA stability and translational blockade. Through its downstream mediator MAPKAP2, p38 can further regulate expression of TNF-α and IL-1β by controlling their translational access. Inhibition of p38 in human monocyte cells prevented protein expression of either cytokine, but did not diminish mRNA levels of either, indicating that p38 was specifically regulating their translation [196, 197]. This translational control occurs through interaction of MAPKAP2 with specific sequences in the 3'-untranslated region (3'UTR) of the cytokine mRNA. These sequences, called AU-rich elements (ARE) influence translational access through as yet poorly understood mechanisms [198, 199]. Additionally, the control of translation by AREs is often dependent on cellular context. For example, during UV-

induced stress, the ARE-laden 3'UTR of p53 enhanced translation of a reporter gene to which it was fused, but prevented translation in the unstressed state [200].

The more common and well studied role of AREs is in regulation of transcript stability. Through interaction with ARE binding proteins, transcripts can either be specifically targeted for destruction, or protected from the same fate [201, 202]. Two major ARE-dependent degradation pathways have been characterized; the exosome pathway and the processing bodies (P-bodies) pathway. In general, deadenylation precedes the processing of an mRNA by either of these pathways, both in vivo and in vitro [203-205]. The exosome pathway involves a large complex of proteins that interact either directly with AREs or possibly through interaction with certain ARE binding proteins, such as TTP and KSRP, to degrade target transcripts 3' to 5' [205]. P-bodies are subcellular bodies within the cytosol that, along with structures called stress granules, sequester and degrade mRNA transcripts. Both de-capping and degradation factors are maintained within the p-bodies. mRNAs are held in the stress-granules until the decision to translate or degrade them has been made. Several ARE binding proteins, including TTP, TIA-1 and TIAR are found in and around stress granules and/or p-bodies [205, 206]. Interestingly, cyclooxygenase 2 (COX-2) mRNA stability was substantially decreased in several cell systems by p38 inhibitors, and this process was dependent on 3'UTR AREs, and associated with increased TIA-1 expression, suggesting a possible role for the p-body pathway [207-213]. Additionally, IL-6, IL-8 and TNF-α mRNA have all been found to be stabilized by p38 through an ARE dependent mechanism [210, 214-217]. These studies suggest that p38-mediated post-transcriptional regulation may take the form of either mRNA stability or translational control, and that this regulation may be

gene, cell type and/or species specific. Additionally, the close relationship between the regulation of mRNA stability and translational access suggests that they may be governed by similar, but as yet poorly understood mechanisms, including the miRNA pathway. A recent study found that a variety of the proteins involved in ARE-mediated mRNA stability were targets for miRNAs, suggesting yet another potential level of complexity [218].

III. IGFBP-3 AND APOPTOSIS

IGFBP-3 has been implicated in growth inhibition and apoptosis for more than a decade. It was initially believed that the mechanism was simply to sequester IGF-I away from its receptor, thus preventing activation of IGF-I-stimulated signaling [25]. A number of studies supported this hypothesis, as IGFBP-3 was shown to inhibit IGF-I-stimulated DNA synthesis in rat osteoblasts and human placental fibroblasts [219-221]. This was supported by a study showing that mutation of IGFBP-3 to a form that has very weak IGF binding affinity eliminated the ability of IGFBP-3 to inhibit IGF-I-stimulated DNA synthesis in porcine smooth muscle cells [222]. While IGF-dependent inhibition of growth is likely responsible for IGFBP-3 action in some situations, a role for IGFBP-3, independent of IGF binding, has emerged over time, with several important questions garnering the attention of those in the field. There is evidence that IGFBP-3 can influence cell growth/death by both direct induction of apoptosis, as well as through modulation of the apoptotic effects of other agents, and that this can potentially occur via interaction with cell-surface, cytoplasmic, nuclear and mitochondrial molecules [223]. However, it remains controversial as to whether or not IGFBP-3 actions are governed by subcellular location, post-translational modification or if the protein even needs to be inside the cell at all.

IGF-INDEPENDENT ACTIONS OF IGFBP-3 ON CELL DEATH AND GROWTH INHIBITION

Proposed IGF-independent actions of IGFBP-3 were first suggested in 1993 when Oh et al. presented evidence that exogenous, recombinant IGFBP-3 could bind the cell surface of Hs578T breast cancer cells and inhibit cell growth in the presence of IGF analogs with normal receptor affinity, but reduced affinity for IGFBP [224]. These data suggested that, in the absence of its normal ligand, IGFBP-3 could find an alternative binding partner on the cell surface. Another study the same year found that over-expression of human IGFBP-3 in Balb/c fibroblasts inhibited insulin-stimulated cell proliferation, as well as basal growth rates of the cultures [225]. Again, IGFBP-3 was able to exert a significant influence on cell proliferation that was not directly related to IGF-I. However, a major point of contention in early studies that examined the IGF-independent function of IGFBP-3 was the difficulty in establishing a truly IGF-free system, since many cell types in culture produce their own IGF. The discovery that IGFBP-3 could inhibit cell growth in mouse fibroblasts with a targeted deletion of the IGF-IR gene provided strong support for an IGF-independent role. These cells completely lacked the IGF-IR, and were thus unable to initiate IGF signaling. Despite this, IGFBP-3 over-expression led to 10-fold lower growth rates compared to wild-type cells [226]. These findings were lent further credence by reports showing that IGFBP-3 mutants with little to no affinity for IGF-I or IGF-II could still induce apoptosis [227, 228]. In PC-3 human prostate cells, both rhIGFBP-3 and a non-IGF binding mutant of IGFBP-3 were able to induce apoptosis to similar levels [227]. Mutants of IGFBP-3 with reduced or eliminated IGF-I binding affinity were also able to induce apoptosis in mesenchymal chondroprogenitor cells [229].

The relationship between IGFBP-3 and apoptosis is not limited to a specific species or cell type. Spoerri et al. found that IGFBP-3 could inhibit progression of proliferative diabetic retinopathy by inducing apoptosis in human retinal endothelial cells [230]. Another group found that IGFBP-3 expression was time-dependently induced by doxorubicin and led to increased apoptosis in H9c2 cardiac cells [231]. They went on to propose that IGFBP-3 may play a role in cardiac dysfunction. IGFBP-3 has also been implicated in apoptotic progression of multiple cancer cell types, including breast, prostate and non-small cell lung cancer [232].

In addition to reports of direct apoptotic induction by IGFBP-3, there are many reports of IGFBP-3 playing a role as a modulator of the apoptotic actions of other agents. In contrast to the work of others, Perks et al. showed that exogenous IGFBP-3 alone had no direct effect on apoptosis in MCF-7 cells [233]. However, co-incubation with C-2 ceramide potentiated ceramide-induced apoptosis in Hs578T breast epithelial cells [233]. In rat mesangial cells, which are critical for glomerular filtration, IGFBP-3 enhanced apoptosis induced by both high glucose and TNF-α [234]. Exogenous rhIGFBP-3 has also been shown to contribute to cytokine-mediated apoptosis in insulin-secreting cells from both the rat and hamster, although this effect was blocked by co-incubation with IGF-I [235]. IGFBP-3 has long been thought to inhibit growth by sequestering IGF-I away from its receptor. However, this report raises the possibility that IGF-I/IGFBP-3 binding may also serve to inhibit the actions of IGFBP-3 by sequestration from the cell surface.

All of this work led, ultimately, to Silha et al. producing the first in vivo demonstration of IGF-independent actions for IGFBP-3. They found that a non-IGF

binding mutant of IGFBP-3 was equally potent at suppressing prostate tumor growth as wild-type IGFBP-3 at later stages of tumor progression (17 and 21 weeks). This contrasted with early (12 week) examination at which time only wild-type IGFBP-3 inhibited tumor growth [236]. These data strongly suggest that IGFBP-3 can influence apoptosis through both IGF-dependent and IGF-independent mechanisms. It is likely that a complex web of additional factors determines which specific modes of action IGFBP-3 will act through.

PROPOSED MECHANISMS OF IGFBP-3 ACTION IN APOPTOSIS

The mechanisms by which IGFBP-3 participates in apoptosis have not been fully elucidated. While several studies have demonstrated partial mechanisms, a complete pathway has not been determined, with several major questions yet to be definitively answered. First, does IGFBP-3 need to be inside the cell to exert its apoptotic actions, or can it influence apoptotic progression from the cell surface? Second, if it does need to be inside the cell, does it need to be in the nucleus? Lee et. al. showed that IGFBP-3 induced apoptosis in CHO cells expressing the transferrin receptor, and that incubation with a transferrin receptor neutralizing antibody prevented IGFBP-3-induced apoptosis [237]. This report suggested that cellular re-uptake of the secreted protein was important, and that IGFBP-3 was not able to affect apoptosis via interaction with the cell surface. Weinzimer et al. lent support to this hypothesis when they showed that IGFBP-3 could bind to transferrin, opening up the possibility that this could be an important pathway for IGFBP-3 cellular uptake [238]. Interestingly, Baumrucker et al. found that IGFBP-3 could not bind to bovine transferrin, but could bind the similar iron-binding, cell surface

molecule lactoferrin, which also can facilitate IGFBP-3 cellular uptake [239, 240]. However, it remains to be seen if the transferrin/lactoferrin pathways are important IGFBP-3 interacting partners in other cell systems. Further support for a cellular re-uptake model was found in a study where IGFBP-3 mutants that lacked functional cell surface binding retained the ability to induce apoptosis in T47D human breast cancer cells [241], which suggests that a carrier molecule, perhaps transferrin, plays a role in cellular uptake of IGFBP-3 when direct cell surface association is prevented. This does not exclude a possible role for IGFBP-3 at the cell surface, but does suggest that an alternative pathway can be utilized when direct cell surface association is prevented. Another report found that expression of the GGG-IGFBP-3 mutant, which lacks IGF binding ability, led to caspase-dependent apoptosis in MCF-7 cells. The authors suggested that this occurred through a putative IGFBP-3 cell-surface death receptor, since caspase-8 was activated, but caspase-9 was not [242]. However, a putative IGFBP-3 receptor has been a controversial topic since it was first proposed in 1993, and has yet to be independently confirmed. Additionally, Holly and coworkers have reported that the addition of exogenous glycosylated or non-glycosylated IGFBP-3 to MCF-7 cells had no effect on apoptosis. This work raised the as yet unanswered question of why exogenous and endogenous IGFBP-3 play different roles in the same cell system [233, 243].

NUCLEAR LOCALIZATION OF IGFBP-3

In 1994, it was reported that IGFBP-3 contained a five amino acid motif in the c-terminal region that coded for a nuclear localization signal [244]. Several years later Schedlich et al. found that this sequence motif facilitated IGFBP-3 nuclear import via the

importin β pathway, though little functional information was initially reported for IGFBP-3 within the nucleus [245]. Schedlich et al. later found that IGFBP-3 could bind to the retinoid X receptor-α (RXR-α) in the nucleus and affect expression of retinoid responsive genes [246]. Lee et al. presented evidence that IGFBP-3 interacts with RXR-α in the nucleus and facilitates its interaction with the orphan nuclear receptor, Nur77. This complex then translocates, without IGFBP-3, to the mitochondria to induce apoptosis. Loss of either IGFBP-3 or RXR-α prevented this apoptotic induction [247]. A second study from the same group found that IGFBP-3 and RXR-α treatments synergized in the induction of apoptosis in prostate cancer cells [248]. A follow-up study suggested that IGFBP-3 and Nur77 could interact directly in the cytoplasm to facilitate apoptosis, further demonstrating the complexities of this multifunctional protein [249]. However, a distinction between the nuclear and cytosolic relationships between IGFBP-3 and Nur77 has not yet been explained. Apoptotic effects of nuclear IGFBP-3 were also reported by Santer et al. in U2 OS cells, who further described a proteasome-dependent mechanism by which this function may be regulated [250]. Each new layer of regulation strengthens the arguments for biological consequences when IGFBP-3 occupies a specific sub-cellular location.

Differences in the subcellular localization of IGFBP-3 may play a role in the potentially diverse mechanisms by which IGFBP-3 exerts its apoptotic effects. Butt et al. found that cytosolic activity likely played a role in apoptosis in T47D cells when they observed that an IGFBP-3 mutant that lacked the ability to associate with the cell surface or localize to the nucleus was still was able to induce apoptosis. Additional support was provided for this hypothesis by Bhattacharyya et al., who demonstrated that an IGFBP-3

mutant, restricted to the cytoplasm by the lack of a functional nuclear localization signal or signal peptide, was still able to induce apoptosis in PC-3 prostate cancer cells [251]. A possible mechanism of cytosolic action was hinted at by Butt et al., when they demonstrated that IGFBP-3 expression altered the equilibrium of Bax and Bcl-2 expression, favoring the apoptotic Bax and promoting apoptosis [252]. These data suggest that, while IGFBP-3 has been shown to accumulate in the nucleus in some cell systems, nuclear localization may not be a universal mechanism requirement for it to induce apoptosis.

POST-TRANSLATIONAL MODIFICATION OF IGFBP-3

In addition to subcellular localization, post-translational modification has also been studied as a potential modifier of IGFBP-3 action. Metabolic labeling studies found that IGFBP-3 could be phosphorylated, and that a cell membrane bound kinase, transglutaminase 2, might be responsible [253-255]. However, beyond some small alterations in cell surface binding affinity, little functional significance was attributed to IGFBP-3 phosphorylation. It was later found that IGFBP-3 could also be phosphorylated by DNA-dependent protein kinase (DNA-PK), leading to greater nuclear accumulation [256]. Building on these data, Cobb et al. utilized a paired glioblastoma cell system, where one cell line lacked functional DNA-PK, and showed that IGFBP-3 could only induce apoptosis in the presence of active DNA-PK. Chemical inhibition of DNA-PK in prostate cancer cells also abrogated the apoptotic activities of IGFBP-3, as well as inhibited its ability to bind to RXR-α [257]. An additional, unpublished study from the same group found divergent roles for IGFBP-3 following phosphorylation by DNA-PK

or casein kinase 2 (CK2). While DNA-PK phosphorylation promoted apoptosis, CK2 phosphorylation of IGFBP-3 inhibited apoptotic progression (presented at Gordon Research Conference on IGF in physiology and disease, March 2007). CK2 phosphorylation of IGFBP-3 had been previously studied, but at the time, the only functional significance of this modification was found to be diminished cell surface binding and inhibition of ALS binding to the IGFBP-3-IGF-I complex [258]. There was no observed alteration in growth inhibition compared to non-phosphorylated IGFBP-3. Additionally, glycosylation status did not influence phosphorylation status. This agrees with previous studies that found little functional significance for IGFBP-3 glycosylation, with no changes in IGF or ALS binding observed with alterations in glycosylation pattern. Only a minor change in cell surface association was seen [259].

Proteolysis can play an important role in regulation of IGFBP-3 function. In some systems, proteolytic cleavage of IGFBP-3 renders it inactive and allows for either increased cell proliferation [260] or decreased apoptosis [261]. This can occur through inhibition of either the IGF-dependent, or IGF-independent functions of IGFBP-3. In breast cancer, proteolysis of IGFBP-3 by matrix metalloproteinase (MMP)-7 renders it unable to bind IGF-I, thus increasing IGF bioavailability [262, 263]. In keratinocytes, MMP-19 cleaves IGFBP-3, which allows greater local availability of IGF-I, and greater IGF-IR phosphorylation [264]. Additional studies have also found positive associations between IGFBP-3 proteolysis and IGF-I availability in prostate cancer [265, 266].

In contrast to the above mentioned studies, there are several reports of IGFBP-3 proteolytic fragments that have increased bioactivity. Lalou et al. found that a 16 kDa amino terminal fragment of IGFBP-3 could inhibit IGF and insulin stimulated activation

of IGF-IR despite having no detectable affinity for either ligand [267]. Salahifar et al. also found an amino terminal fragment of IGFBP-3, corresponding to amino acids 1-97, that efficiently inhibited DNA synthesis in MCF-7 breast cancer cells [268]. PC-3 prostate cancer cells were also found to be susceptible to growth inhibition by an amino terminal IGFBP-3 fragment, independent of IGF-I binding [269]. This amino terminal fragment was also able to inhibit the mitogenic actions of fibroblast growth factor (FGF) in mouse fibroblasts with or without a functional IGF-IR, further suggesting that it has IGF-independent growth inhibitory activity [270]. These data clearly describe a complex regulatory system that depends on cellular context. The apoptotic functions of IGFBP-3 are likely to be influenced by its localization and post-translational modification, as well as its interactions with other molecules.

Figure 1

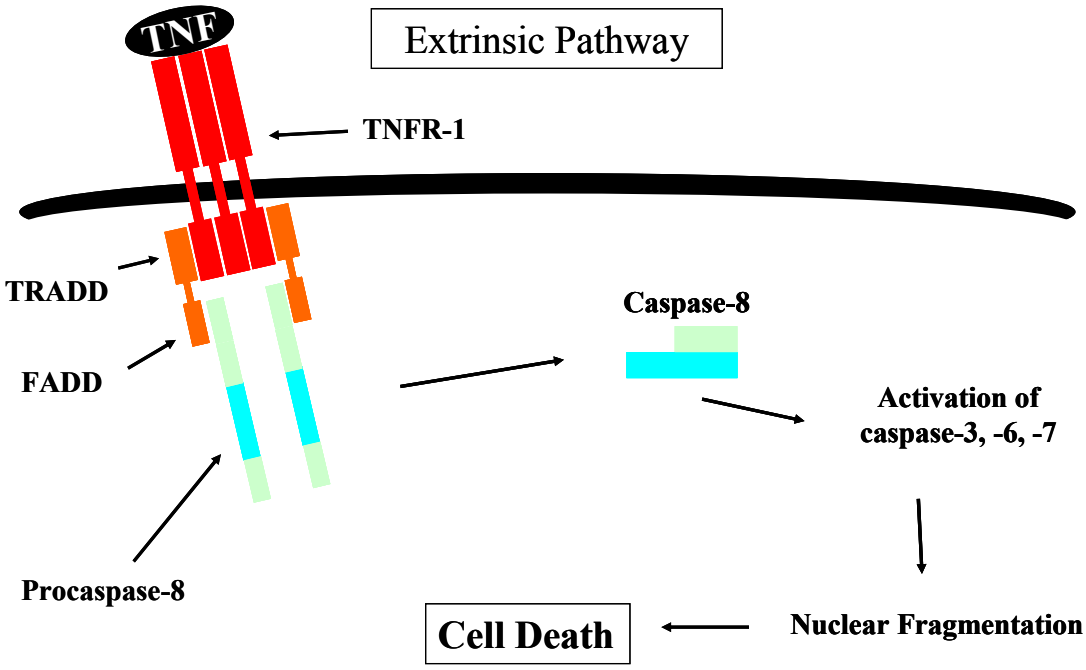


Figure 1. The Extrinsic Apoptotic Pathway. Binding of a pro-apopototic ligand (e.g. TNF-α) causes trimerization of the TNFR-1 and assembly of a complex containing TRADD and FADD. This complex recruits procaspase-8 and facilitates its cleavage and activation. Caspase-8 activation can lead to activation of downstream caspases, nuclear fragmentation and cell death.

Figure 2

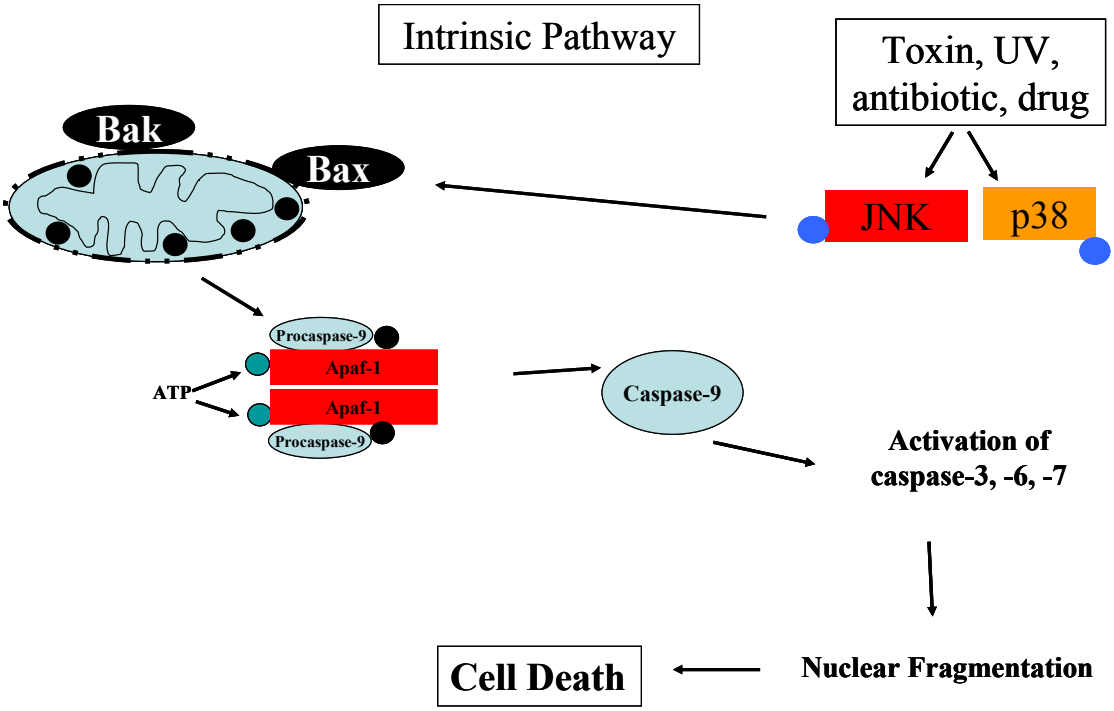


Figure 2. The Intrinsic Apoptotic Pathway. Exposure of the cell to a stress (e.g. toxic, UV, antibiotic or drug) leads to activation of the SAPK. This can facilitate translocation of Bcl-2 family members (e.g. Bax, Bak) to the mitochondria where they are able to induce pore formation and cytochrome c leakage. Cytochrome c, Apaf-1 and procaspase-9 form a complex called the apoptosome, which leads to caspase-9 activation, subsequent activation of downstream caspases, nuclear fragmentation and cell death.

OBJECTIVES

IGFBP-3 is one of six high affinity IGFBP that modulate the biological actions of IGF-I. Interestingly, both growth promoting and growth inhibiting agents have been reported to increase IGFBP-3 expression. Our laboratory has previously reported the signaling pathways involved in growth factor-stimulated IGFBP-3 expression, but have not previously examined how the ribotoxic stressors ANS and ricin induce IGFBP-3. Therefore, we investigated the signaling pathways and transcriptional and post-transcriptional regulatory mechanism of ANS and RTA-induced IGFBP-3 expression (Chapter II).

MAC-T cells are an immortalized, but not transformed, mammary epithelial cell line that retain the features of primary cells. Given how significantly ANS increased IGFBP-3 expression in these cells, we investigated whether or not ANS had a similar effect on IGFBP-3 expression in primary bovine mammary epithelial cells to verify that the response was not an artifact of the immortalized cell line. In addition, we investigated whether IGFBP-3 expression was regulated by ANS in other species (Chapter III).

Emmerging evidence suggests an IGF-independent role for IGFBP-3 in regulation of cell death that may comprise a substantial portion of its biological activity. However, the specific mechanisms by which IGFBP-3 carries out these functions remain unclear. Therefore, we sought to define the specific role played by IGFBP-3 during ribotoxic stress-induced apoptosis (Chapter IV).

CHAPTER II

REGULATION OF PRO-APOPTOTIC IGFBP-3 BY P38 MAPK DURING RIBOTOXIC STRESS IN MAMMARY EPITHELIAL CELLS

ABSTRACT

Ribotoxic stress is the response to damage at the sarcin/ricin loop of the 28S ribosomal subunit of translationally active cells. We have found that the ribotoxic stress inducers anisomycin (ANS) and ricin A-chain (RTA) induce both apoptosis and IGFBP-3 expression in normal epithelial cells. Knockdown of IGFBP-3 with siRNA demonstrated that IGFBP-3 played a critical role in the ability of these agents to induce apoptosis. ANS and RTA strongly activated the stress-activated protein kinase (SAPK) pathways, p38 and JNK-1/2. Additionally, ANS transiently activated ERK-1/2. Chemical inhibitor and RNAi studies determined that p38 was required for both stressors to induce IGFBP-3, and that ERK-1/2 were also involved, at least in part, in ANS-induced IGFBP-3 expression. In contrast, JNK-1/2 were not involved in the ability of either RTA or ANS to induce IGFBP-3, but did play a small but significant regulatory role in basal IGFBP-3 expression. ANS and RTA both increased IGFBP-3 mRNA stability through a mechanism involving the 3' untranslated region (3'UTR). Inhibition or knock-down of p38 prevented this enhanced transcript stability. RTA also induced IGFBP-3 expression by transcriptional activation of the IGFBP-3 promoter. This process also required p38 activation, indicating that the p38 pathway regulated IGFBP-3 expression at both transcriptional and post-transcriptional levels. This report identifies IGFBP-3 as a novel target gene induced during ribotoxic stress, and in addition demonstrates the first evidence of p38 regulation of IGFBP-3 expression.

INTRODUCTION

Ribotoxic stress is the process whereby cells activate stress-activated protein kinases (SAPK) in response to damage to a specific region of the 28S ribosomal RNA, known as the α-sarcin/ricin loop [271]. In active ribosomes, this region is responsible for facilitating the peptidyl transferase reaction [159]. It has been proposed that binding to specific nucleotide sequences of the α-sarcin/ricin loop acts to both inhibit translation and, independent of this function, stimulate activation of SAPK [271]. A variety of ribosome inactivating proteins (RIPs) have been shown to initiate SAPK signaling via interaction with the α-sarcin/ricin loop, including α-sarcin, ricin A chain (RTA), Shiga toxin 1, onnamide A and theopederin B [162, 163, 271]. Additionally, the antibiotics anisomycin (ANS) and blasticidin S, as well as ultraviolet radiation have also been found to initiate the ribotoxic stress response [167, 168, 271]. The SAPK pathways, p38 and JNK1/2, are often thought of together as modulators of apoptosis in cells faced with threats to their homeostatic well-being. A wide variety of cellular stressors, including those from physiological, pharmacological and environmental sources can activate the SAPK, leading to cell death. However, it has not been conclusively determined if ribotoxic stress-induced apoptosis occurs as a consequence of induction of stress-responsive genes, including TNF-α and some interleukins, or via activation of cellular apoptotic pathways independent of de novo protein synthesis [169, 191].

IGF binding protein-3 (IGFBP-3) is a multi-factorial protein that has been proposed to modulate cell death through diverse mechanisms, including IGF-independent effects [272, 273]. Several growth factors and cytokines have been shown to induce

IGFBP-3 expression [274-279]. Furthermore, this increased IGFBP-3 expression has been shown to modulate, at least in part, the growth inhibitory effects of several of these agents[280]. This led us to postulate that IGFBP-3 may play a role in ribotoxic stress-induced apoptosis. We report here that the ribotoxic stressors ANS and RTA each induce apoptosis and IGFBP-3 expression in a normal epithelial cell line. Furthermore, knockdown of IGFBP-3 by RNAi significantly reduced apoptosis in response to each of these individual stressors. Since both ERK-1/2 and the SAPK pathways p38 and JNK are activated by RTA and ANS in a wide variety of cell types and thought to be integral to apoptosis, we sought to determine if they played a role in regulating IGFBP-3 expression at the molecular level. Interestingly, we found that p38 was a key regulator of IGFBP-3 expression in response to both RTA and ANS, while ERK-1/2 played a partial role in ANS-induced IGFBP-3 expression and the JNK pathway played no role in stress-induced IGFBP-3 regulation.

MATERIALS AND METHODS

Chemical reagents

Antibodies against phosphorylated phosphor-ERK-1/2, JNK-1/2, and total JNK-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total ERK-1/2, phosphorylated and total p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The ERK-1/2 inhibitor PD98059, the p38 MAPK inhibitor SB203580 and JNK inhibitor SP600125 were purchased from Calbiochem (La Jolla, CA). Anisomycin and Ricin A-chain were purchased from Sigma (St. Louis, MO). siRNA SmartPool reagents for MEK-1, p38, and JNK-2 were purchased from Dharmacon Inc. (Lafayette, CO). Additionally, a custom SmartPool siRNA for bovine IGFBP-3 was purchased from Dharmacon. siRNA oligos against MEK-2 were purchased from Cell Signaling Technology, Inc.

Cell Culture

The bovine mammary epithelial cell (MEC) line MAC-T [281] was routinely maintained as previously described [282]. For experiments, cells were plated and grown to confluence in phenol-red free DMEM containing 10% FBS and antibiotics unless otherwise stated. Cells were rinsed with phenol-red free serum-free (SF) DMEM, and incubated in SF DMEM with 0.2% BSA and 30 nM sodium selenite overnight prior to exposure to treatments in SF DMEM without additives.

Measurement of Apoptosis

Confluent MAC-T cells were serum starved overnight as described above and exposed to treatments. Apoptosis was measured by the Cell Death Detection ELISA^{PLUS} (Roche, Indianapolis, IN) according to manufacturer's instructions.

Western Immunoblotting and Ligand Blotting

Cells were either lysed or conditioned media was collected as previously described [283]. Total protein content of lysates was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). For signaling experiments, proteins (25 μg) were separated on 10% SDS PAGE gels. For secreted protein, conditioned media were lyophilized and separated on 12.5% SDS PAGE gels. Resolved proteins were transferred to nitrocellulose (0.2 μm; Bio-Rad) or PVDF (0.45 μM; Millipore, Bedford, MA) membranes. Membranes were blocked for 1 h at room temperature in Tris-buffered saline + .05% Tween-20 (v/v) (TBS-T) and 5% non-fat dried milk (w/v), and incubated with primary antisera at 4^{o}C overnight with gentle agitation. Membranes were then washed in TBS-T and incubated for 2 h at room temperature with appropriate HRP-conjugated secondary antibodies. Peroxidase activity was detected with an ECL Western Detection Kit (Pierce, Rockford, IL). Following detection of phosphorylated proteins, membranes were stripped in a buffer containing 62 mM Tris-Cl, pH 6.8, 2% SDS and 0.7% (v/v) β-mercaptoethanol for 30 min at 55^{o}C, and then probed for total proteins as described. For ligand binding assays, conditioned media were prepared and separated as previously described [284]. Membranes were then incubated overnight with [^{125}I]-IGF-I (GE Healthcare, Piscataway, NJ), after which they were washed and autoradiographed for 1-3 days with intensifying screens.

Reverse Transcription and quantitative RT-PCR

Total RNA was isolated and analyzed by qRT-PCR as previously described [285], with minor modifications as follows: Primers for cyclophilin were Forward, 5'-GAGCACTGGAGAGAAAGGATTTGG-3'; Reverse, 5'-TGAAGTCACCACCCTGGCACATAA-3'. For qRT-PCR analysis, samples were diluted 1:4 and 7.5 µl were amplified in a 27.5 µl reaction containing 12.5 µl Platinum SYBR Green qPCR Supermix-UDG with ROX (Invitrogen, Carlsbad, CA), 6.5 µl H_2O and 0.5 µl (~200 µM) of each gene specific primer.

siRNA Experiments

MAC-T cells were grown to ~70% confluence and transfected with 50 nM MEK-1 + 50 nM MEK-2, 50 nM JNK-2, 100 nM p38 or 50 nM IGFBP-3 siRNA, as well as a corresponding concentration of scrambled control siRNA using GeneEraser siRNA Transfection Reagent (Stratagene, La Jolla, CA) according to manufacturer's instructions. After 48 h, cells were washed and incubated overnight in serum-free media, then treated for analysis of gene knockdown and downstream gene expression changes. Gene knockdown was verified by Western immunoblot for both phosphorylated and total forms of MEK-1/2, JNK-2 and p38. Changes in IGFBP-3 gene expression were measured by qRT-PCR and ligand blotting as described above.

Luciferase Reporter Construction

For promoter activity assays, 1028 bp of IGFBP-3 promoter sequence was amplified from bovine genomic DNA (Novagen, EMD, San Diego, CA) and cloned into pBluescript-TOPO (Invitrogen). The following primers were used: Forward 5'-CCTGCATAATGCGAATGTTTC-3'; Reverse 5'-ATAGCTGCTGCGCTCGCATC-3'.

The cloned region corresponded to bp 3328-4356 of IGFBP-3 (GenBank™ accession number AF305712). The construct was analyzed by restriction digest and DNA sequencing, then was excised with Hin*d* III and cloned into pGL3 basic (pGL3 basic-BP3), upstream of firefly luciferase. Restriction digests were performed to verify the sense orientation. For mRNA stability assays, the 3'UTR of bovine IGFBP-3, corresponding to bp 1010-2434 of IGFBP-3 (GenBank™ accession number NM_174556), was amplified by RT-PCR with the following primers: Forward 5'-GGGGTCTAGATAGACTGTGGCCACTTAA-3'; Reverse 5'-GGGGGTCGACTTTCTTTATGGTAAAAAT. Xba I and Sal I restriction sites (underlined) were introduced during amplification on the 5' and 3' ends of the clone, respectively, allowing for digestion and cloning into pGL3 promoter vector (Promega), downstream of firefly luciferase. DNA sequencing was performed to verify the orientation and integrity of the clone.

Transient Transfection and Luciferase Assay

MAC-T cells were transiently transfected using SuperFect (Qiagen, Valencia, CA) according to manufacturer's instructions. Briefly, cells were plated in 96-well plates 24 h prior to transfection and grown to 75% confluence. Plasmid DNA (pGL3 basic or IGFBP-3 promoter construct and pRL-TK for promoter assays; pGL3 promoter vector or pGL3-BP3-UTR and pRL-TK for mRNA stability assays) and SuperFect reagent were diluted in serum-free media, combined, and incubated at room temperature for 10 min. DMEM supplemented with 10% FBS and antibiotics was added to the mixture, gently mixed and immediately added to the cells for 3 h, at which time the transfection media were removed and cells were washed in PBS and incubated an additional 24 h in

complete media. Following this, cells were serum-starved overnight and then treated with for 16 h (promoter assays) or 8 (ANS) to 12 (RTA) h (mRNA stability assays) in the presence or absence of signal transduction inhibitors. Following treatment, luciferase activity was measured with the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions. Luciferase activity was measured on an Lmax Luminometer (Molecular Devices, Sunnyvale, CA). Data are presented as the ratio of Firefly/*Renilla* luciferase activity.

Statistical Analysis

Data from experiments were analyzed by ANOVA with differences considered significant for $p < 0.05$. Tukey and SNK post-hoc tests were utilized for pair-wise comparisons. Analyses were performed with SigmaStat (2.03) software (SPSS Inc., Chicago, IL).

RESULTS

Ribotoxic stressors induce both apoptosis and IGFBP-3 in normal mammary epithelial cells

To determine if ANS or RTA induced apoptosis, cells were treated for 6 h and apoptosis was measured as a function of nucleosome accumulation. ANS induced apoptosis in MAC-T cells in a concentration-dependent manner from 0.025 to 0.1 μM (Figure 1A; $n = 3$, $p < 0.05$). RTA also induced apoptosis in a concentration-dependent manner from 0.1 μg/ml to 1 μg/ml (Figure 1B; $n = 3$, $p < 0.05$).

In order to determine if ANS or RTA induced IGFBP-3 expression, cells were treated with increasing concentrations of either factor and qRT-PCR was performed. Exposure of cells to ANS (0.025 to 0.1 μM) for 3 h stimulated 20- to 50-fold increases in IGFBP-3 mRNA levels compared to serum-free controls (Figure 2A; $n = 3$, $p < 0.05$). Time course analysis indicated that maximal increases were observed at 8 h, with IGFBP-3 mRNA levels declining slightly at 12 h (Figure 2B). The abundance of IGFBP-3 protein present in conditioned media was also increased following exposure to ANS, indicating that the enhanced IGFBP-3 mRNA levels translated into increases in IGFBP-3 protein (Figure 2C). In contrast, ANS had an inhibitory effect on IGFBP-2 accumulation in conditioned media. We have previously shown that MAC-T cells produce small amounts of IGFBP-4 and IGFBP-5 [284], but neither of these were regulated by ANS (Figure 2C). ANS at 1 μM, a concentration that inhibits protein synthesis by greater than 90% in these cells, had little effect on IGFBP-3 mRNA or protein levels.

RTA treatments from 100 ng/ml to 10 μg/ml for 3 h also induced significant increases in IGFBP-3 mRNA levels, ranging from approximately 2.5- to 6-fold (Figure 2D; $n = 3$, $p < 0.05$). Time course analysis demonstrated that a low concentration of RTA (10 ng/ml) significantly increased IGFBP-3 mRNA when cells were treated over longer intervals (8 to 12 h) (Figure 2E; $n = 3$, $p < 0.05$). As seen with ANS, treatment with RTA increased IGFBP-3 protein levels in the conditioned media. A low concentration of RTA (10 ng/ml) was required for long-term accumulation of IGFBP-3 protein, due to the severe deleterious effect of high levels of RTA on protein synthesis. RTA inhibited secretion of other IGFBP over the same timeframe (Figure 2F). These data indicate that the ability of both ANS and RTA to induce IGFBP-3 expression was specific for IGFBP-3, relative to the other forms of IGFBP synthesized by these cells.

Anisomycin and ricin A-chain require IGFBP-3 to induce apoptosis

To determine if IGFBP-3 plays a functional role in ribotoxic stress-induced apoptosis, cells were transfected with siRNA specific for bovine IGFBP-3. The effectiveness and specificity of the siRNA was verified by ligand blotting of conditioned media from untreated and ANS treated cells (Figure 3A). Substantial decreases in IGFBP-3 protein were consistently observed, while other IGFBP forms were not affected. For apoptosis measurements, cells transfected with either scrambled or IGFBP-3 siRNA were treated for 6 h with 0.1 μM ANS or 16 h with 10 ng/ml RTA. Apoptosis was assessed by nucleosome accumulation. Knockdown of IGFBP-3 significantly diminished both basal and ANS-stimulated apoptosis. While ANS was still able to moderately induce apoptosis, levels were not different from those observed under basal conditions in cells exposed to scrambled siRNA (Figure 3B; $n = 4$, $p < 0.05$). Likewise, both basal and

RTA-induced apoptosis were significantly attenuated by knockdown of IGFBP-3 (Figure 3C; $n = 3$, $p < 0.05$).

Anisomycin and ricin A-chain activate the SAPK pathways

A hallmark of ribotoxic stress is activation of ERK-1/2 and the SAPK pathways. To determine if ANS activated ERK-1/2 or the SAPK pathways in MAC-T cells, cells were treated with 0.1 μM ANS for 5 to 180 min. Activation of ERK-1/2 was weakly observed after 5 min, with maximal activation occurring between 15 and 45 min. Weak actiavtoin was still observable after 90 min, but had disappeared by 180 min after treatment (Figure 4A). Activation of p38 was observed within 5 min of treatment, with maximal activation occurring between 15 and 45 min after treatment (Figure 4B). Weak activation of JNK1/2 was observed at 5 min, with strong activation beginning at 15 min that was maintained through at least 90 min (Figure 4B). Weak activation of JNK1/2 was still noticeable 3 h post treatment. Exposure to RTA also stimulated robust activation of the SAPK pathways, however, the temporal pattern of activation differed from that observed with ANS. Activation of either pathway was not observed until 2 to 4 hr after RTA addition, but was maintained through at least 8 hr post treatment (Figure 4C).

Anisomycin and ricin A-chain-induced IGFBP-3 expression is dependent on p38 and independent of JNK activation

To determine whether p38 and/or JNK1/2 played a role in ANS or RTA-induced IGFBP-3 expression, cells were treated with specific inhibitors of p38 (SB203580) or JNK1/2 (SP600125) prior to exposure to 0.1 μM ANS or 0.1 μg/ml RTA for 3 h. Chemical inhibition of p38 completely abolished the ability of either ANS or RTA to

induce IGFBP-3 expression (Figure 5A-B; $n = 3$, $p < 0.05$). In contrast, inhibition of JNK1/2 did not affect ANS or RTA-induced IGFBP-3 expression.

RNAi-induced knockdowns of p38 and JNK-2 were utilized to lend further specificity to the results observed with chemical inhibitors. Western blot analysis verified substantial reductions in both active and total forms of p38 and JNK-2, following transfection of MAC-T cells with siRNA oligos (Figures 6A and 6B). Treatment of siRNA transfected cells with 0.1 μM ANS or 0.1 μg/ml RTA for 3 h produced results very similar to those observed with the inhibitors. p38 depletion significantly abrogated ANS and RTA-induced IGFBP-3 expression (Figures 6C and 6D; $n = 3$, $p < 0.05$). Knock-down of JNK-2 with siRNA had no effect on ANS- or RTA-induced IGFBP-3 mRNA levels.

Anisomycin-induced IGFBP-3 is partially dependent on ERK-1/2

To determine if ERK-1/2 played a role in ANS-induced IGFBP-3 expression, cells were treated with PD98059 (10 μM) for 30 min prior to treatment with 0.1 μM ANS for 3 h. Inhibition of ERK-1/2 activity significantly reduced the ability of ANS to induce IGFBP-3 mRNA levels (Figure 7A; $n = 3$, $p < 0.05$).

siRNA against MEK-1 and MEK-2 was used to support the results observed with the chemical inhibitor. Co-transfection of MEK-1 and MEK-2 siRNA led to substantial decreases in both MEK isoforms (Figure 7B). Treatment of siRNA transfected cells with 0.1 μM ANS for 3 h produced results that closely mimicked those observed with PD98059. Inhibition of the ERK-1/2 pathway by RNAi-mediated knockdown of MEK-1/2 led to a significant decrease in ANS-induced IGFBP-3 expression (Figure 7C; $n = 3$, $p < 0.05$).

JNK-2 regulates basal IGFBP-3 expression via a transcriptional mechanism

To determine if the ERK-1/2 or SAPK pathways played a role in regulating basal IGFBP-3 expression, MAC-T cells were treated with the same concentrations of the chemical inhibitors PD98059, SB203580 and SP600125 described above, but without subsequent ANS or RTA treatment. Inhibition of ERK-1/2 led to a significant decrease in basal IGFBP-3 (Figure 8A; $n = 3$, $p = 0.05$), while p38 inhibition tended to decrease IGFBP-3 mRNA levels (Figure 8A; $n = 3$, $p = 0.07$). These findings are consistent with the previously observed roles for each pathway in the regulation of ANS and/or RTA-induced IGFBP-3 expression. Interestingly, inhibition of JNK with SP600125 led to an approximately 2.5-fold increase in basal IGFBP-3 mRNA levels (Figure 8A; $n = 3$, $p < 0.05$). This result was supported in cells transfected with JNK-2 siRNA, as knockdown of basal JNK-2 expression also led to a significant increase in IGFBP-3 mRNA levels (Figure 8B; $n = 3$, $p < 0.05$).

A luciferase construct containing 1028 bp of the IGFBP-3 promoter was transfected into MAC-T cells 48 h prior to treatment with 0.05 μM ANS, in the presence or absence of 50 μM SP600125 or 30 μM SB203580. While ANS treatment failed to activate the promoter, treatment for 16 h with the JNK inhibitor, SP600125, led to an approximately 2-fold increase in IGFBP-3 promoter activity that was not altered by ANS treatment (Figure 9;, $n = 3$, $p < 0.05$). In contrast, inhibition of p38 with SB203580 had no effect on promoter activity.

Anisomycin and ricin A-chain increase IGFBP-3 mRNA stability through a p38-dependent, 3'UTR mediated mechanism

MAC-T cells, transfected with a luciferase construct containing 1.4 kb of the IGFBP-3 3'UTR were treated with ANS (0.05 μM) for 8 h, or RTA (50 ng/ml) for 12 h. ANS treatment increased firefly luciferase activity approximately 55% above controls (Figure 7A; $n = 4$, $p < 0.05$), while treatment with RTA increased luciferase activity approximately 105% above controls (Figure 7B, $n = 3$, $p < 0.05$). These increases were significantly ablated by pretreatment with the p38 inhibitor SB203580 (Figure 7A; $n = 4$, $p < 0.05$, Figure 7B; $n = 3$, $p < 0.05$), while the JNK inhibitor SP600125 had no effect.

Ricin stimulates IGFBP-3 transcription by a p38 dependent mechanism.

MAC-T cells transfected with a luciferase construct containing 1.1 kb of the bovine IGFBP-3 promoter were treated with ANS (0.05 μM) or RTA (50 ng/ml) for 16 h. ANS treatment failed to stimulate luciferase activity (data not shown). However, RTA treatment induced an approximately 2-fold increase in IGFBP-3 promoter activity (Figure 8; $n = 4$, $p < 0.05$). This increase was significantly decreased by pre-treatment with the p38 inhibitor. Interestingly, inhibition of JNK activity led to a small, but significant, increase in IGFBP-3 promoter activity in response to RTA treatment (Figure 8; $n = 4$, $p < 0.05$).

DISCUSSION

In the present study we found that the stressors RTA and ANS led to apoptosis in normal mammary epithelial cells. This has been reported to occur through the ribotoxic stress response, whereby cellular stressors that target the α-sarcin/ricin loop of the 28S ribosomal subunit trigger apoptosis via a characteristic activation of the SAPK p38 and JNK1/2 pathways [271]. Additionally, apoptotic induction can occur independently of translational inhibition, as it can occur at concentrations of RTA and ANS, 10 ng/ml and 0.025 μM respectively, which inhibit protein synthesis less than 10% (data not shown). In addition to apoptotic induction, we observed large increases in IGFBP-3 mRNA and protein. There have been several studies documenting the ability of anti-proliferative agents like TNF-α [274], retinoic acid [278], and the tumor suppressor p53[275] to stimulate IGFBP-3 expression, however this is the first report implicating the toxic stressors RTA and ANS as inducers of IGFBP-3 expression. A recent report indicates the presence of a retinoic acid response element in the human IGFBP-3 promoter [286], but beyond this there is still little known about how cellular stressors and growth inhibitors induce IGFBP-3 expression.

The observed increases in protein were specific for IGFBP-3, as both RTA and ANS treatment tended to decrease the accumulation of other IGFBP, in a manner consistent with low-level protein synthesis inhibition. This suggests that IGFBP-3 may be selectively translated during this process, possibly because of its role in apoptosis. Such selective translation is not without precedent. As part of the unfolded protein response (UPR), during endoplasmic reticulum stress, a blockade on translation occurs

when PERK phosphorylates eIF2α and prevents translational initiation of most transcripts. However, this process selectively promotes translation of ATF4 mRNA, allowing for increased production of a transcription factor that contributes to the UPR [287].

It has been reported that exposure of cells to RTA can induce expression of cytokines such as TNF-α and IL-1 by both transcriptional and post-transcriptional mechanisms[288]. This represents a substantial stress response, mediated by the SAPK pathways[161]. We report here for the first time that IGFBP-3 is also a target gene of SAPK-mediated, toxin-induced cellular stress. While there is not a great deal known about how IGFBP-3 induction occurs during cellular stress, there have been a number of studies demonstrating a functional role for this protein in apoptosis. The addition of exogenous IGFBP-3 alone has been shown to directly inhibit growth and/or stimulate apoptosis in both prostate and breast tumor cell lines [289-291]. Additionally, IGFBP-3 has been shown to enhance the apoptosis-inducing actions of other agents, including UV light, TNF-α and paclitaxel [292-294]. Increased IGFBP-3 expression has also been shown to modulate the growth inhibitory effects of TGF-β, antiestrogens, retinoic acid and TNF-α in breast cancer cells[280].

Since these studies have generally involved cancer cells, we hypothesized that IGFBP-3 might be part of the inherent apoptotic machinery in normal mammary epithelial cells. MAC-T cells provide a good model, since they produce low, but detectable levels of endogenous IGFBP-3 under basal conditions, allowing for studies that do not rely on engineered over-expression models, or exogenous protein addition. In these cells, we found that we could substantially inhibit both basal and stressor-

induced IGFBP-3 expression via RNAi, and that this depletion significantly inhibited apoptotic induction by both RTA and ANS. This is the first report demonstrating a requirement for IGFBP-3 in ribotoxic stress. Based on these findings we sought to determine the signaling pathways responsible for IGFBP-3 induction during this apoptotic progression.

As mentioned above, a hallmark of ribotoxic stress is the rapid and sustained activation of SAPK pathways. These pathways are often thought of together as modulators of growth inhibition and apoptosis [190, 191]. To study the potential roles for p38 and JNK-1/2 in IGFBP-3 induction, we used a two-pronged approach involving both chemical inhibitors and siRNA to ensure specificity. The chemical inhibitors and RNAi data mirrored each other in demonstrating that p38 was responsible for both RTA- and ANS-induced IGFBP-3 expression, while the JNK pathway appeared to have no role. This indicates a distinction between the roles of the p38 and JNK signaling pathways in the induction of IGFBP-3 during apoptosis. While only JNK-2 was knocked down by RNAi, blocking both JNK-1 and JNK-2 with SP600125 had no effect on the ability of either stressor to induce IGFBP-3 expression, suggesting that JNK-1 is also not required for this regulation. Interestingly, exogenous IGFBP-3 has been shown to enhance signaling through p38 in MCF-10A cells, leading to increased cell proliferation [295]. In contrast, IGFBP-3 suppressed p38 signaling in RINmf5 rat insulinoma cells, leading to increased apoptosis [296]. This raises the possibility that beyond the regulatory role of p38 in IGFBP-3 expression that we have reported here, p38 may also interact with IGFBP-3 in some functional way.

In addition to the SAPK, ERK-1/2 were found to be at least partially involved in ANS-induced IGFBP-3 expression. However, this does not necessarily indicate that ERK activation is required for ANS to induce IGFBP-3. MAC-T cells have high levels of basal ERK phosphorylation, which may lead to the pathway playing a permissive role in IGFBP-3 expression. This hypothesis is well supported by data from our laboratory indicating that IGF-I-stimulation of IGFBP-3 expression is decreased 60% by inhibition of ERK, despite the fact that IGF-I does not activate ERK in MAC-T cells [282]. Additional evidence in favor of ERK as a permissive pathway exists in the response of MAC-T cells to TNF-α. TNF-α treatment also induces IGFBP-3 expression, and also fails to activate the ERK pathway (unpublished data). Yet, as with IGF-I, inhibition of ERK with PD98059 significantly inhibits the observed IGFBP-3 induction.

Regulation of IGFBP-3 expression by traditional IGF-I signaling pathways has been extensively studied in our laboratory and roles for both MAPK and PI3-K have been well established [282]. However, no role for SAPK had been previously determined. p38 influences gene expression through both transcriptional as well as post-transcriptional mechanisms. Transcriptional activation has been shown to occur through activation of downstream transcription factors or through interaction with histone deacetylases to modify chromatin structure [193, 194]. Post-transcriptional gene regulation by p38 can occur through two different mechanisms as well; modulation of translational access [196], and regulation of transcript stability [208]. We ruled out translational access as the mechanism of action for p38 in both RTA- and ANS-induced IGFBP-3 expression, because, with this scenario, inhibition or knockdown of p38 would lead to decreased IGFBP-3 protein, but unchanged IGFBP-3 mRNA levels, which is not

what we observed. We therefore focused our attention on mRNA stability. p38 has been shown to regulate the stability of stress related transcripts, such as TNF-α, through 3'UTR mediated mechanisms [207], so we sought to determine if such regulation occurred in response to RTA or ANS treatment. Using a luciferase construct containing a cloned bovine IGFBP-3 3'UTR, we determined that both RTA and ANS did increase transcript stability, and that these increases required p38 activity.

After ascertaining that both ribotoxic stress treatments increased IGFBP-3 transcript stability, we sought to determine if either treatment stimulated IGFBP-3 promoter activity. Using an approximately 1.1 kb clone of the bovine IGFBP-3 promoter, we found that RTA treatment stimulated a significant increase in promoter activity. This increase in promoter activity was also found to be partially dependent on p38, demonstrating that RTA regulates IGFBP-3 expression by affecting both transcription and mRNA stability via this pathway. Within the same assay, ANS had no effect on IGFBP-3 promoter activity. While it is still possible that ANS activates the promoter through an unknown upstream enhancer element, or through an as yet unidentified coding region element, it is clear that ANS does not use the same mechanism as RTA. It is also possible that ANS does not activate the promoter at all, and accomplishes its up-regulation of IGFBP-3 through a mechanism that is entirely dependent on mRNA stability. We have pursued this further by testing the 5'UTR of bovine IGFBP-3 in luciferase assays with and without the 3'UTR, but found no change in transcript stability (data not shown). In light of the well-characterized role of p38 as a regulator of stress responsive genes, these data suggest that IGFBP-3 may be an integral part of the inherent stress response of normal mammary epithelial cells.

While JNK-1/2 appears to play no role in RTA- and ANS-induced IGFBP-3 expression, JNK inhibition significantly increased basal IGFBP-3 promoter activity approximately 2-fold. This corresponded with a significant increase in IGFBP-3 mRNA. This suggests a potential role for JNK in regulating basal IGFBP-3 production, which may be important given the role IGFBP-3 appears to play in apoptosis. This apparent repression of basal IGFBP-3 expression by JNK is not without precedent. JNK activation was found to repress telomerase gene expression in A2780 ovarian adenocarcinoma cells via transcriptional inhibition [297]. JNK inhibition relieved this repression by allowing the displacement of repressive Sp3 and an increase in permissive Sp1 binding to the promoter. The bovine IGFBP-3 promoter contains several Sp1/Sp3 binding sites within the first few hundred bp upstream of the transcription start site, raising the possibility that a similar mechanism of JNK-mediated transcriptional repression may be occurring in MAC-T cells. Additionally, IGF-I-induced transcriptional activation has been shown to occur with as few as 128 bp of promoter sequence [298], indicating that this region is actively involved in regulation of IGFBP-3 transcription.

The toxins RTA and ANS attack the 28S ribosomal subunit and induce apoptosis via the SAPK pathways as part of the ribotoxic stress response. We report here for the first time that IGFBP-3 is a target gene of toxin-induced ribotoxic stress, and that IGFBP-3 is critical for apoptotic progression. Additionally, we have demonstrated that, while RTA and ANS activate both the p38 and JNK pathways, only p38 is required for IGFBP-3 expression, providing a functional distinction between the two pathways. This is also the first evidence that a SAPK pathway is involved in IGFBP-3 regulation. Further studies are ongoing in our laboratory to determine the specific function of IGFBP-3

during ribotoxic stress, as well as to further elucidate the regulatory mechanisms by which p38 governs its expression.

Figure 1

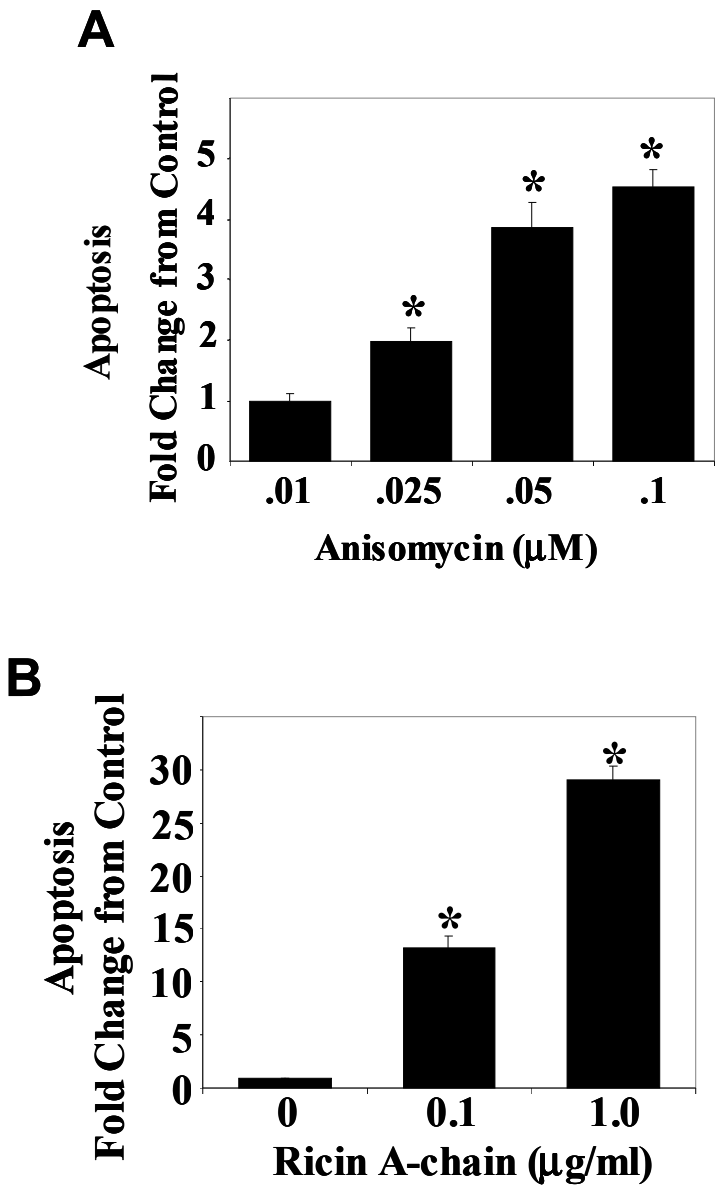
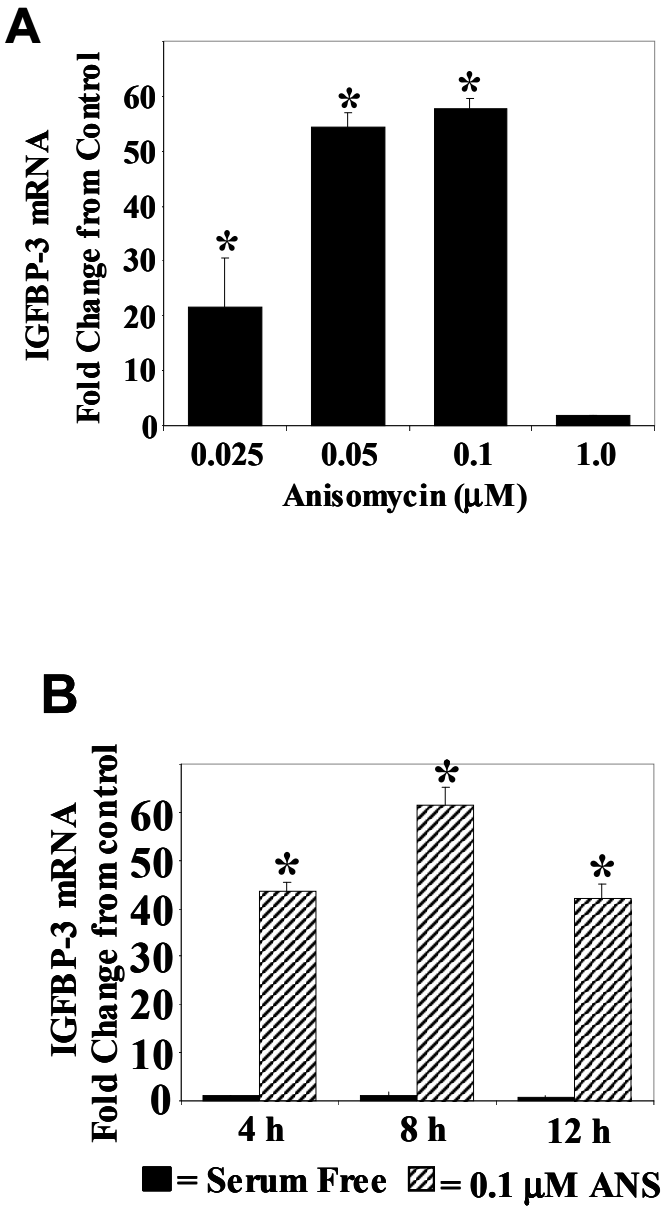


Figure 1. Anisomycin and Ricin A-chain induce apoptosis in MAC-T cells.

Confluent MAC-T cells were serum-starved overnight and treated with ANS (A) or RTA (B) for 6 h. Apoptosis was measured by nucleosome accumulation with the Cell Death

ELISA^{PLUS} (Roche) according to manufacturer's instructions. Bars represent mean +/- SEM of 3 independent experiments, with each treatment performed in triplicate within experiments. * indicates $p < 0.05$

Figure 2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Anisomycin (μM)
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IGFBP-3 mRNA
Fold Change from control
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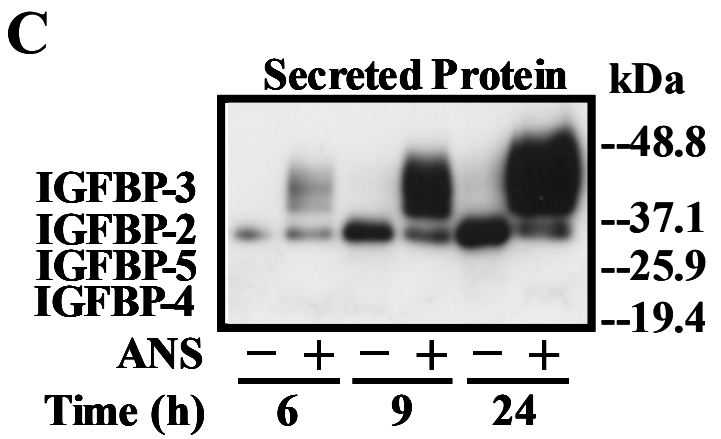
Figure 2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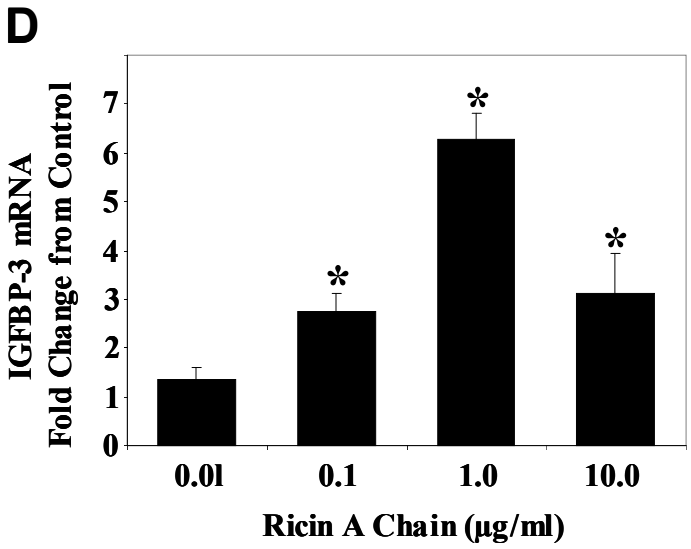
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Ricin A Chain (µg/ml)

Figure 2

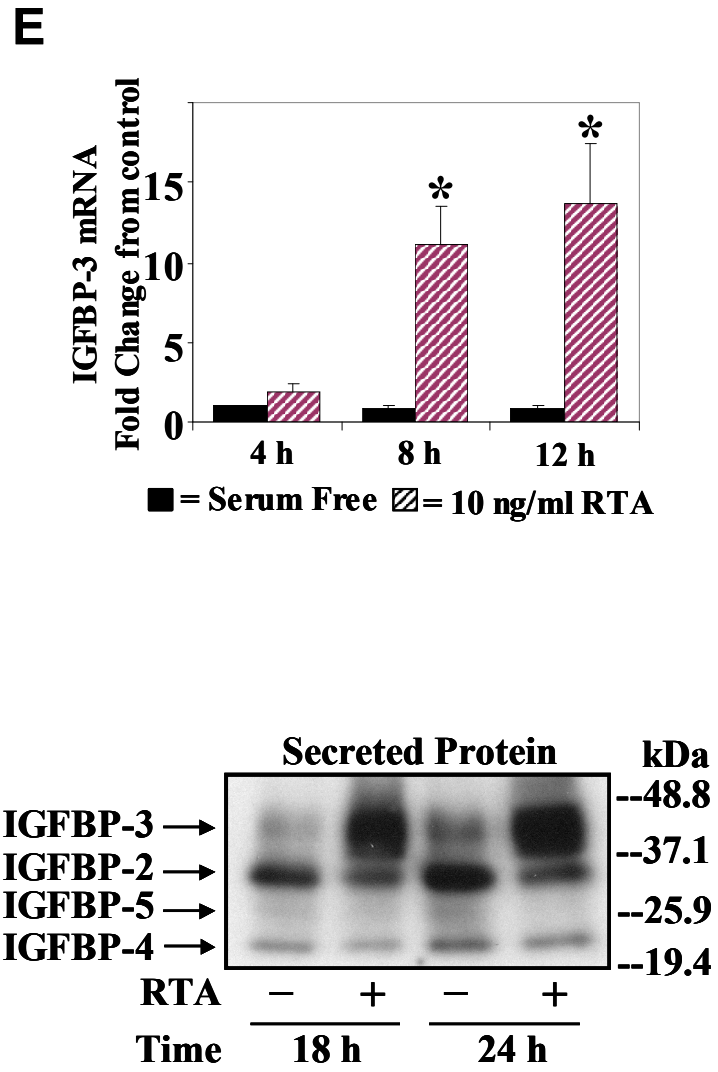
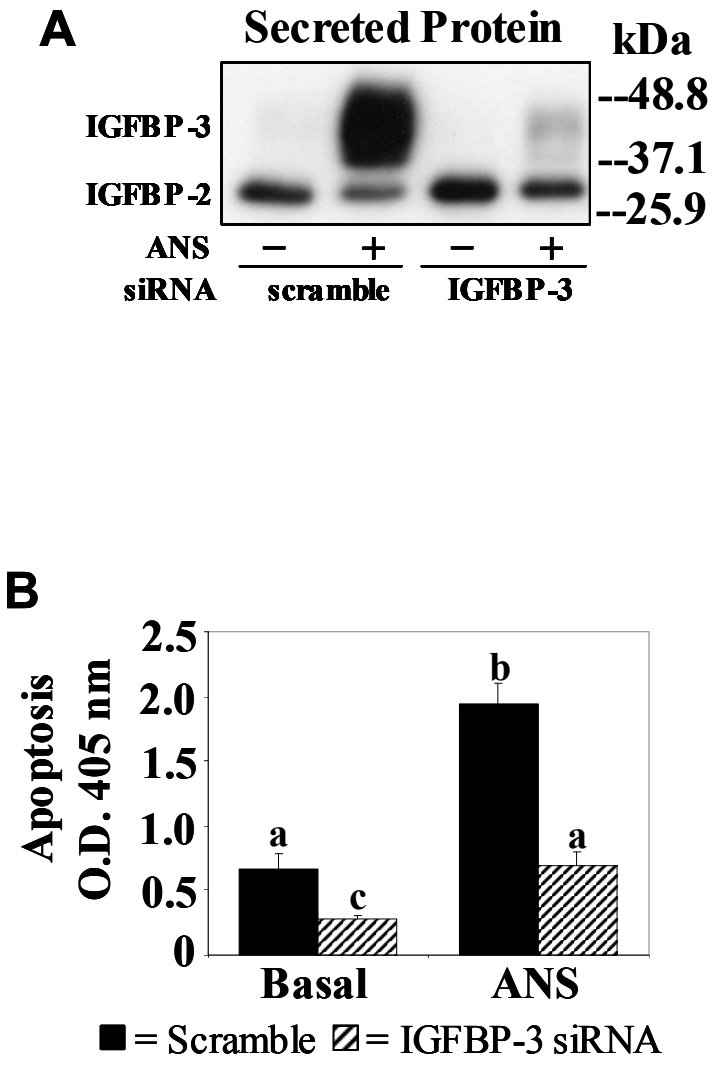


Figure 2. Anisomycin and ricin A-chain induce IGFBP-3 mRNA and protein. (A, B, D, E) Confluent MAC-T cells were serum-starved overnight and treated with ANS or RTA for 3 h (A, D) or the indicated times (B, E). Total RNA was collected and IGFBP-3 mRNA levels were analyzed by qRT-PCR, with data corrected for cyclophilin levels. Bars represent mean +/- SEM of at least 3 independent experiments, with each treatment

measured in triplicate within experiments. * indicates $p < 0.05$. (C, F) Confluent MAC-T cells were serum-starved overnight and treated with ANS (0.1 μM) or RTA (10 ng/ml) for the indicated times. Equal volumes of conditioned media were analyzed by western ligand blotting with [^{125}I]-IGF-I. Data are representative of at least 2 independent experiments.

Figure 3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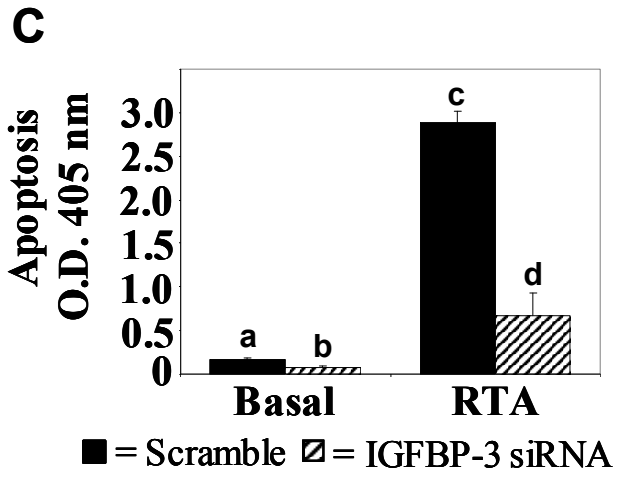

Figure 3. Anisomycin and ricin A-chain-induced apoptosis is IGFBP-3 dependent. (A) MAC-T cells were transfected with 50 nM scrambled or IGFBP-3 siRNA for 48 h, serum-starved overnight, and treated with ANS (0.1 μM) for 24 h. Equal volumes of conditioned media were analyzed by western ligand blotting with [^{125}I]-IGF-I. (B, C) MAC-T cells were transfected with siRNA as in (A), serum-starved overnight, and treated with ANS (0.1 μM) for 6 h or RTA (10 ng/ml) for 16 h. Apoptosis was measured by nucleosome accumulation with the Cell Death ELISA^{PLUS}. Bars represent mean +/- SEM of at least 3 independent experiments. Different superscripts indicate $p < 0.05$.

Figure 4

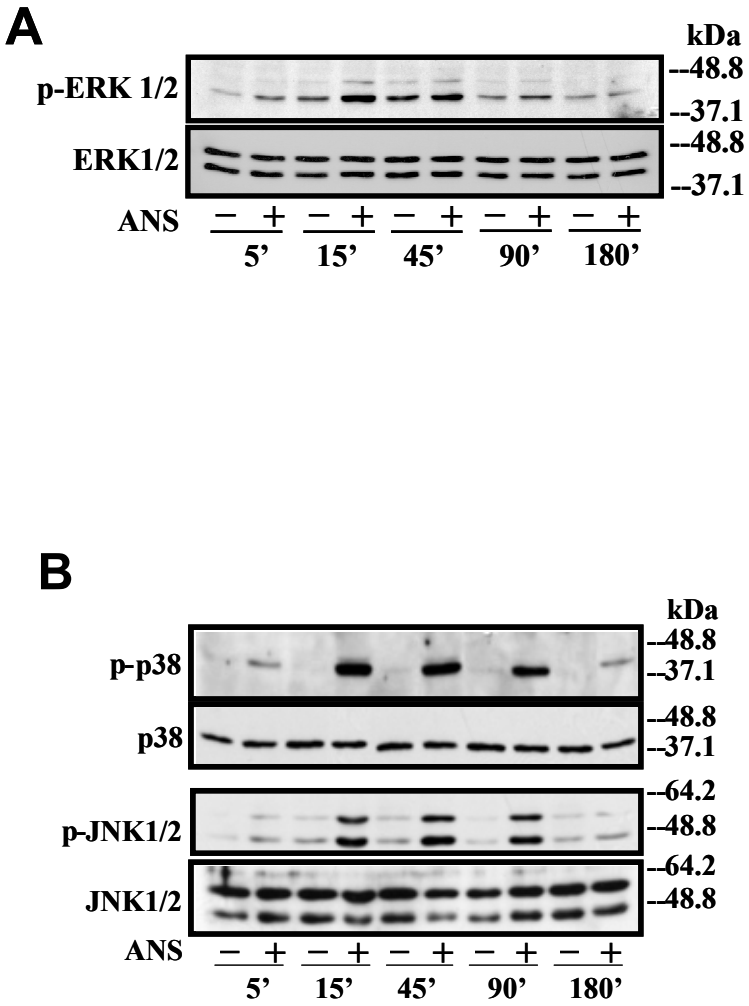
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Figure 4

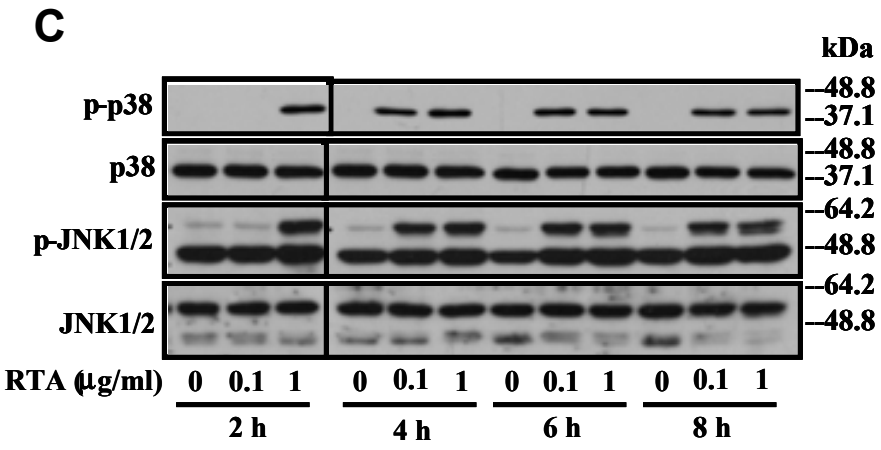


Figure 4. Anisomycin and ricin A-chain activate p38 and JNK-1/2 in MAC-T cells. Confluent MAC-T cells were serum-starved overnight and treated with 0.1 μM ANS (A-B) for 5 to 180 min or 0.1 or 1 μg/ml RTA (C) for 2 to 8 h. Total protein was collected and analyzed by western immunoblotting with antibodies for phosphorylated and total forms of p38 and JNK-1/2. Data are representative of 3 independent experiments.

Figure 5

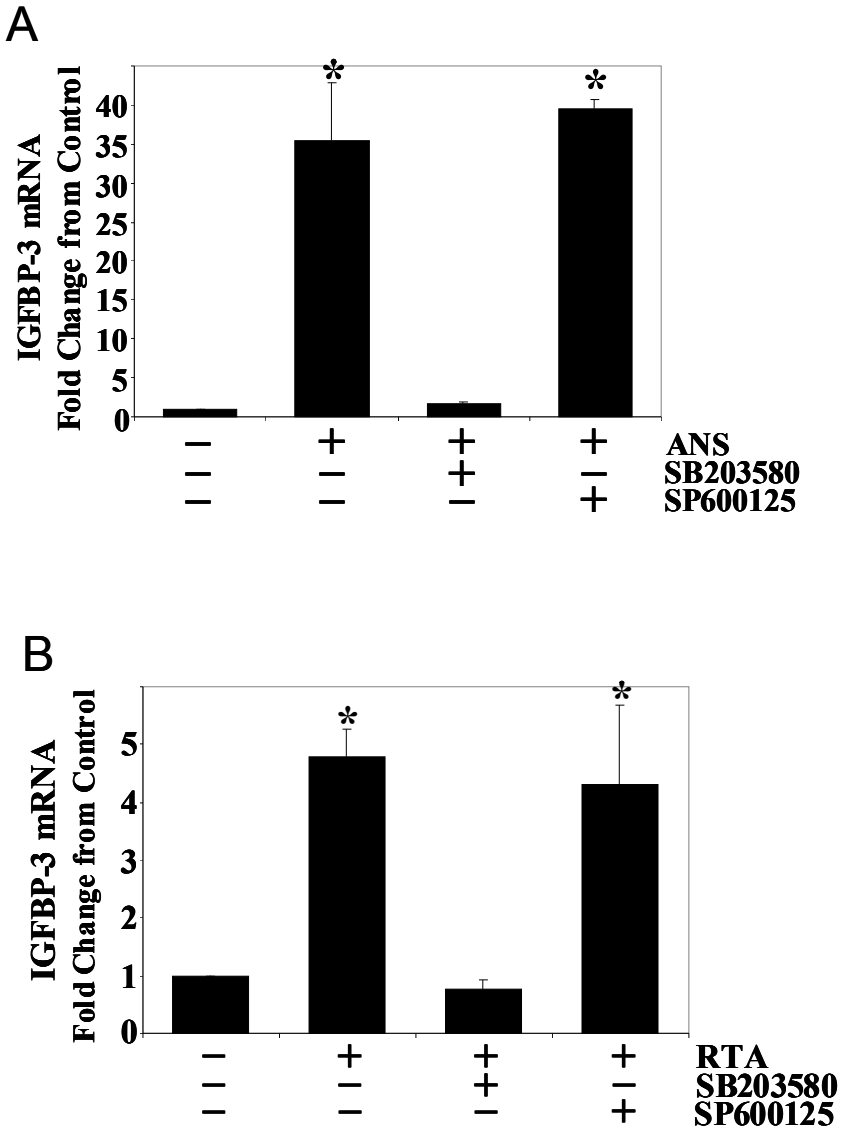
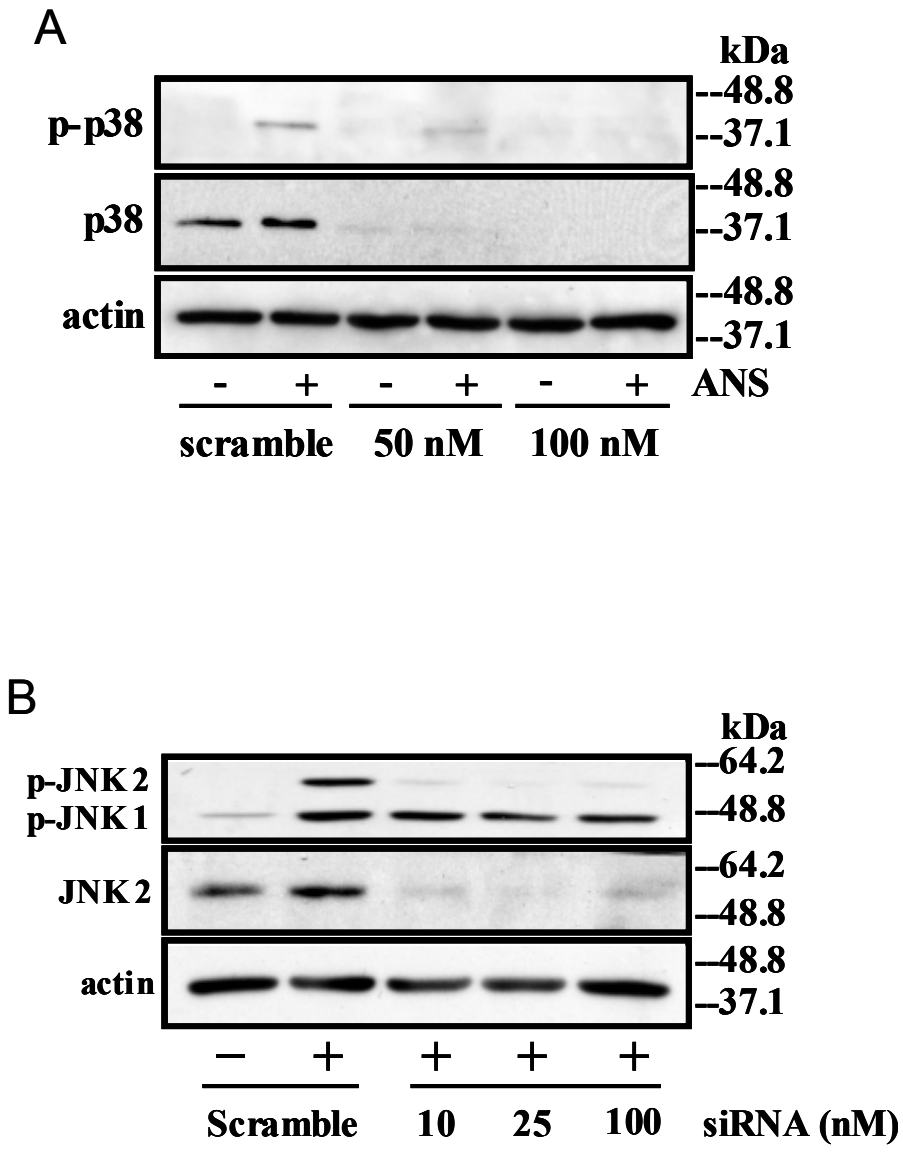


Figure 5. Anisomycin and ricin A-chain induce IGFBP-3 in a p38 dependent manner. Confluent MAC-T cells were serum-starved overnight, pretreated for 30 min with vehicle, 30 μM SB203580 or 50 μM SP600125, and treated with 0.1 μM ANS (A) or 100 ng/ml RTA (B) for 3 h. Total RNA was collected and IGFBP-3 levels were

analyzed by qRT-PCR, with data corrected for cyclophilin levels. Data represent at least 3 independent experiments. * indicates $p < 0.05$ compared to control.

Figure 6


A
kDa
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B
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Figure 6

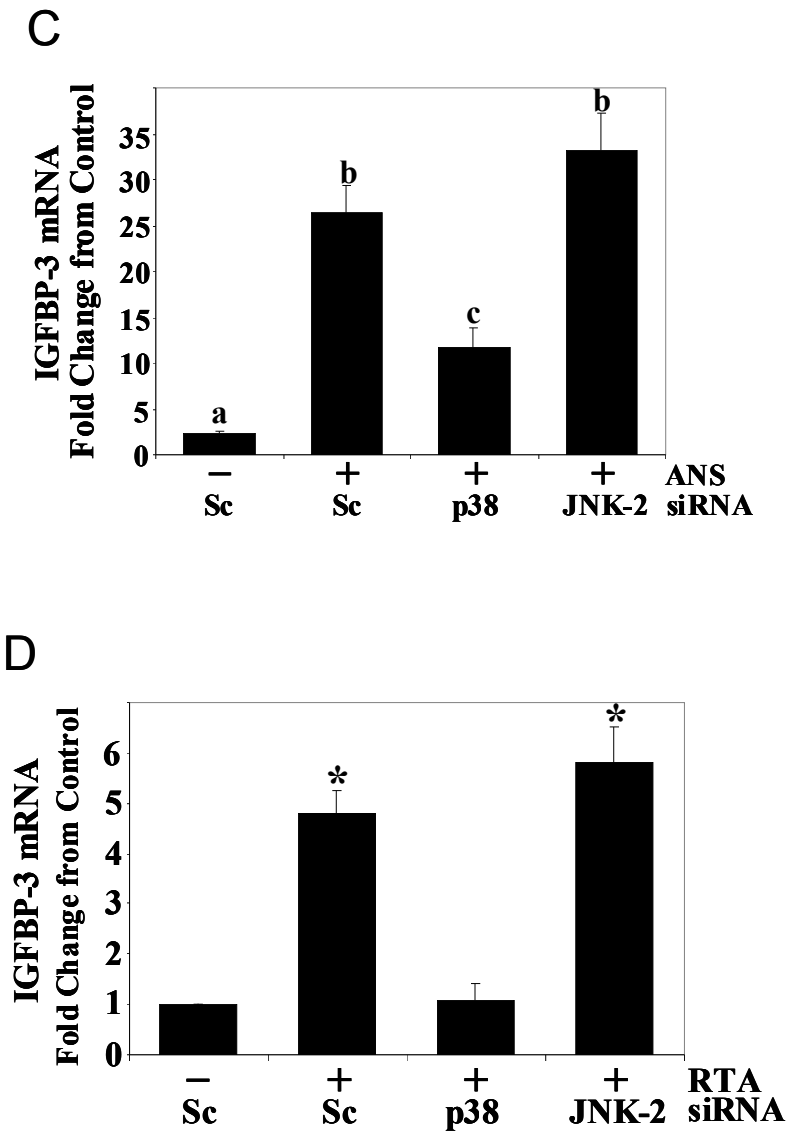
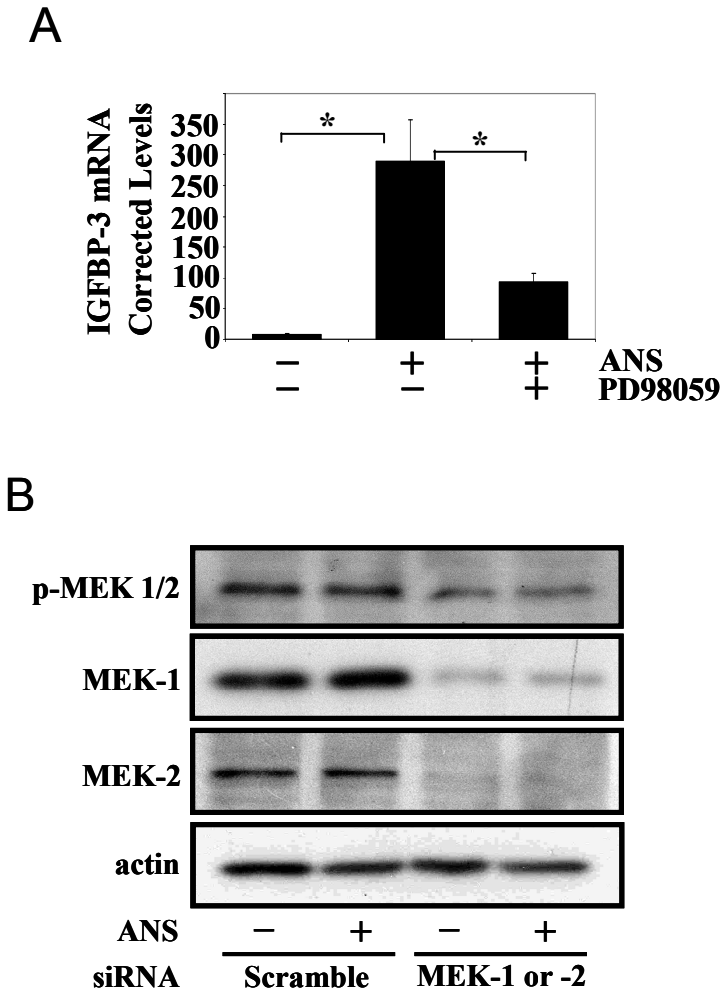


Figure 6. siRNA induced knockdown of p38 and JNK-2 verify p38 dependence of Anisomycin- and ricin A-chain-induced IGFBP-3 mRNA. MAC-T cells were transfected with scrambled, p38 (A) or JNK-2 (B) siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 30 min to activate the p38 and JNK

pathways. Total protein was analyzed by western immunoblotting to verify protein knockdown. Actin was used to verify equal loading of protein across samples. Data are representative of 3 independent experiments. (C, D) MAC-T cells were transfected with 100 nM scrambled, 100 nM p38 or 50 nM JNK-2 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS or 100 ng/ml RTA for 3 h. Total RNA was collected and IGFBP-3 levels were analyzed by qRT-PCR, with data corrected for cyclophilin levels. Bars represent mean +/- SEM of at least 3 independent experiments. * indicates $p < 0.05$ compared to control. (C) Different superscripts indicate $p < 0.05$.

Figure 7


A
IGFBP-3 mRNA Corrected Levels
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300
250
200
150
100
50
0
*
*
−
+
+
ANS
−
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ANS
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+
siRNA
Scramble
MEK-1 or -2

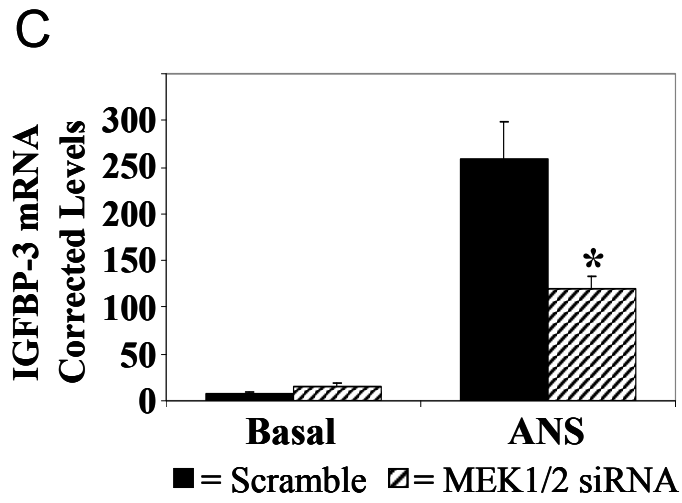


Figure 7. Anisomycin-induced IGFBP-3 expression is partially dependent on ERK-1/2. (A) Confluent MAC-T cells were serum-starved overnight, pretreated for 30 min with vehicle or 10 μM PD98059, then treated with 0.1 μM ANS for 3 h. Total RNA was collected and IGFBP-3 levels were analyzed by qRT-PCR, with data corrected for cyclophilin levels. (B-C) MAC-T cells were co-ntransfected with 100 nM scrambled or 50 nM each MEK-1 and MEK-2 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 30 min (B) or 3 h (C). (B) 25 μg total protein were analyzed by western immunoblot to verify knockdown of phosphorylated MEK-1/2 and total MEK-1 and MEK-2. Equal protein loading was verified by immunoblot with actin. (C) Total RNA was collected and IGFBP-3 levles were analyzed by qRT-PCR, with data corrected for cyclophilin levels. Data represent 3 independent experiments. * $p < 0.05$ for indicated comparisons (A) or compared to ANS treated, scrambled siRNA sample (C).

Figure 8

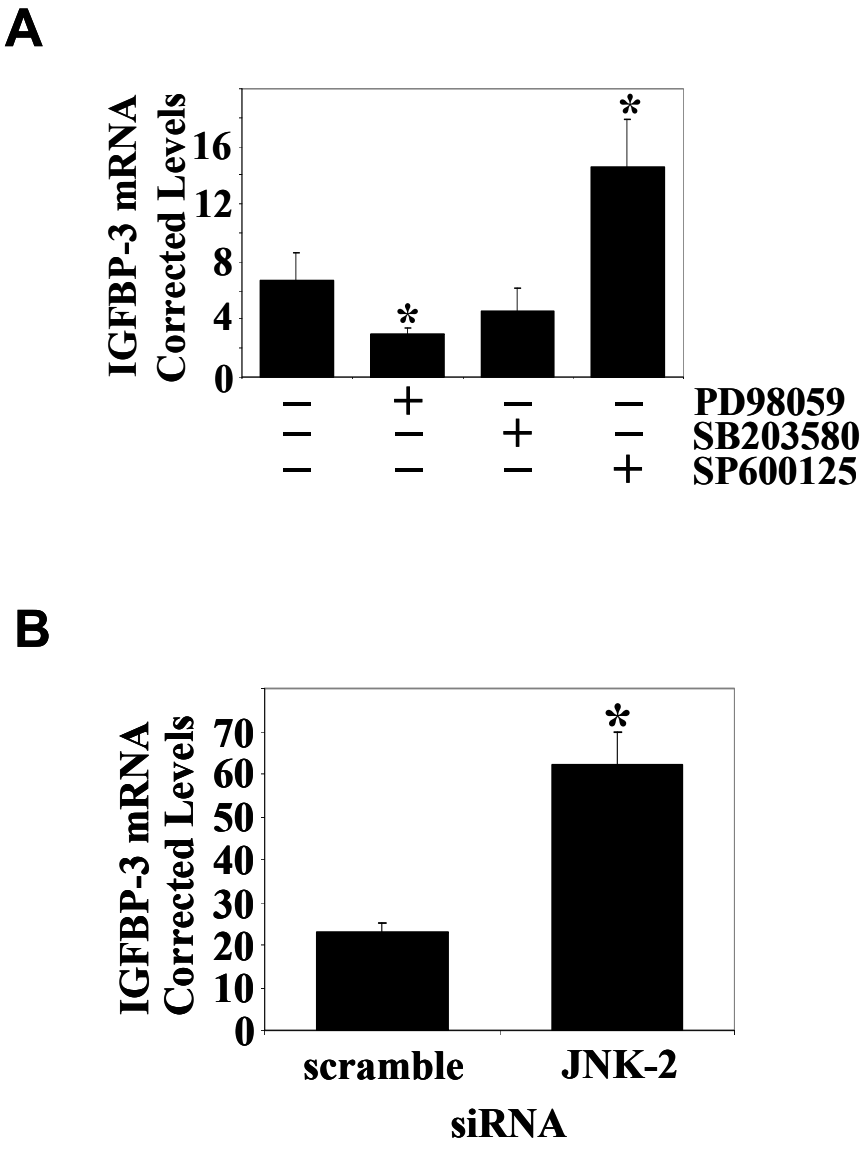


Figure 8. Basal IGFBP-3 mRNA levels are regulated by JNK-2. (A) Confluent MAC-T cells were serum-starved overnight and treated for 3 h with vehicle, PD98059 (10 μM), SB203580 (30 μM) or SP600125 (50 μM). (B) MAC-T cells were transfected for 48 h with 50 μM scrambled or JNK-2 siRNA, serum-starved overnight and treated with fresh serum-free media for 3 h. (A-B) Total RNA was collected and IGFBP-3 levels

were analyzed by qRT-PCR, with data corrected for cyclophilin levels. Data represent 3 independent experiments. * indicates $p < 0.05$ compared to control.

Figure 9

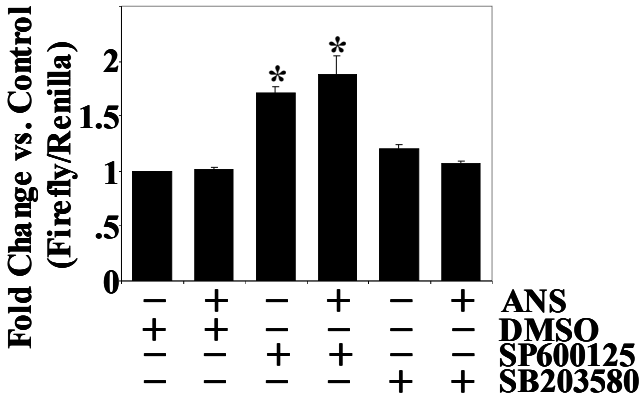


Figure 9. Inhibition of basal JNK activity stimulates the IGFBP-3 promoter. MAC-T cells were co-transfected with a pGL3 firefly luciferase vector containing 1028 bp of the IGFBP-3 promoter and a pRL-TK renilla luciferase vector for 3 h. Following a 24 h recovery in complete medium, transfected cells were serum-starved overnight and treated with vehicle, SB203580 (30 μM) or SP600125 (50 μM) in the presence or absence of 0.05 μM ANS for 16 h. Luciferase activity was measured with the Dual-Glo luciferase kit (Promega). Data are displayed as the ratio of firefly to renilla activity. Data represent 3 independent experiments. * indicates $p < 0.05$ compared to untreated control.

Figure 10

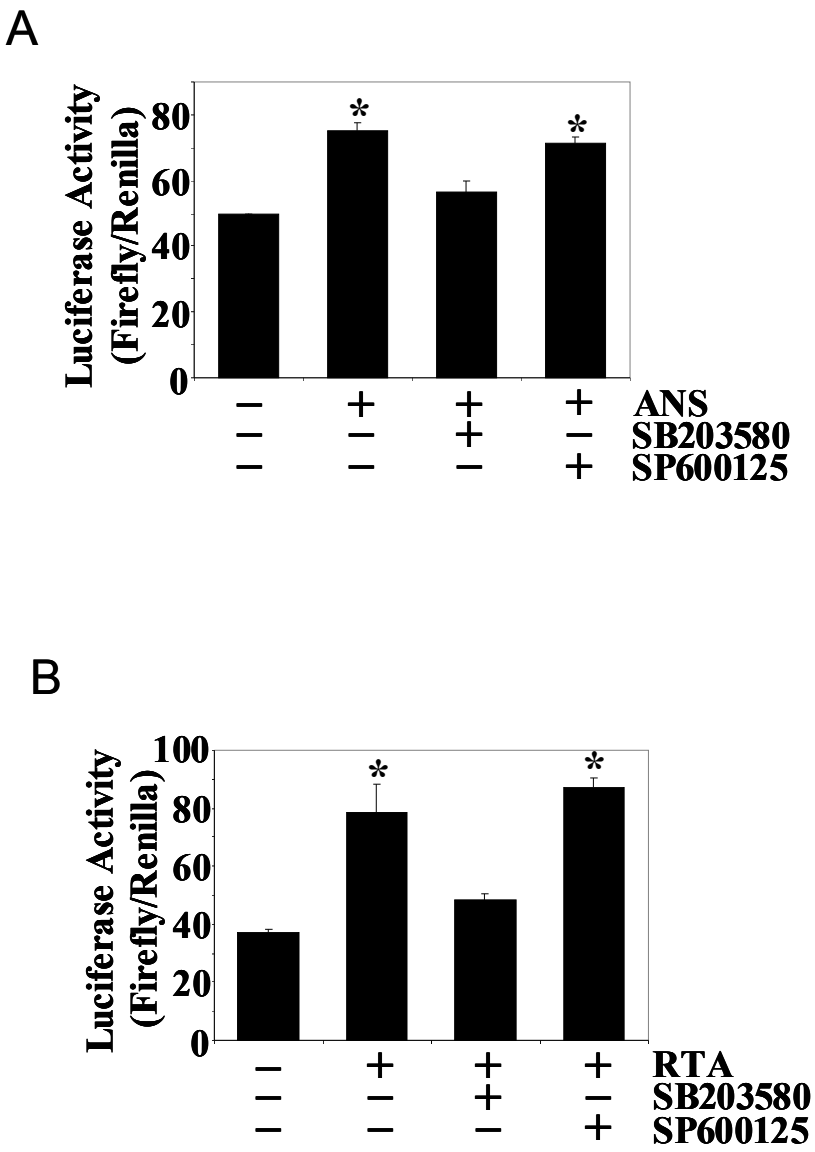


Figure 10. Anisomycin and ricin A-chain enhance IGFBP-3 mRNA stability via a p38-dependent, 3'UTR mediated mechanism. MAC-T cells were transfected with pGL3-BP3-UTR and pRL-TK constructs for 3 h, allowed a 24 h recovery in complete media, and serum-starved overnight. Following 30 min pretreatment with inhibitors (30 μM SB203580 or 50 μM SP600125), cells were treated with 0.05 μM ANS for 8 h (A) or

50 ng/ml RTA for 12 h (B). Luciferase activity was measured with the Dual-Glo Luciferase Asssay (Promega). Bars represent mean +/- SEM of 4 independent experiments, with each treatment performed in triplicate within experiments. * indicated $p < 0.05$.

Figure 11

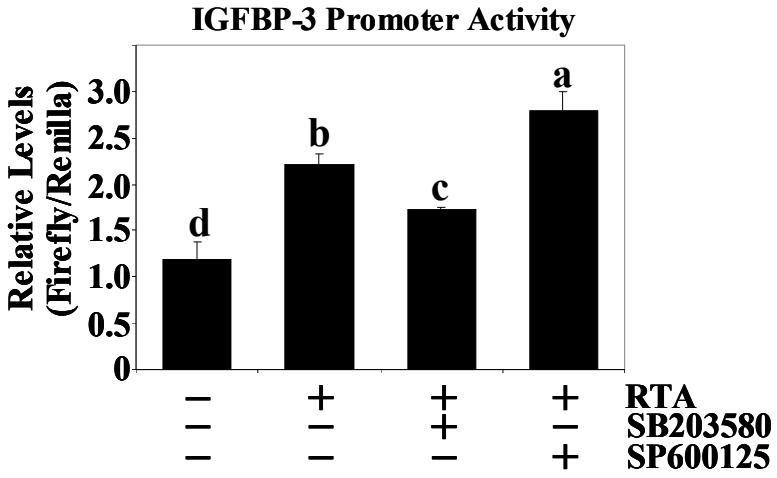


Figure11. Ricin-A chain stimulates the IGFBP-3 promoter via a p38-mediated mechanism. MAC-T cells were transfected with pGL3-BP3 and pRL-TK constructs for 3 h, allowed a 24 h recovery in complete media, and serum-starved overnight. Following pretreatment with inhibitors (30 μM SB203580 or 50 μM SP600125), cells were treated with 50 ng/ml RTA for 16 h. Luciferase activity was measured with the Dual-Glo Luciferase Assay. Bars represent mean +/- SEM of 3 independent experiments, with treatments performed in triplicate within experiments. Superscripts are significantly different from each other, $p < 0.05$.

Chapter III

ANISOMYCIN REGULATES IGFBP-3 EXPRESSION IN BOVINE, HUMAN AND MURINE MAMMARY EPITHELIAL CELLS

ABSTRACT

ANS induced a large, rapid increase in IGFBP-3 expression in the immortalized, non-transformed, bovine mammary epithelial cell line, MAC-T. Similar to MAC-T cells, low concentrations of ANS induced large increases in IGFBP-3 mRNA after 3 h of treatment in primary bovine mammary epithelial (BME) cells, supporting the validity of the MAC-T cell line as a model for normal BME. Human mammary epithelial (MCF-10A) and murine mammary epithelial (NMuMG) cells both responded to ANS treatment with increased IGFBP-3 production, indicating that the increased IGFBP-3 expression in MAC-T cells was not species specific. However, the increases in IGFBP-3 expression in MCF-10A and NMuMG cells were of substantially smaller magnitude than in either MAC-T or BME cells, though there was minimal difference in the relative sensitivity of either cell type in terms of protein synthesis inhibition.

INTRODUCTION

The MAC-T cell line was established from mammary epithelial cells harvested from a lactating dairy cow [281]. They are immortalized but non-transformed, and retain the morphological and biochemical properties of differentiated mammary cells [284]. They have been shown to exhibit a nearly identical IGFBP secretion profile to that of BME cells, both basally and in response to IGF-I [284, 285]. Primary BME cells were isolated from a lactating cow from the University of Vermont herd as described [285, 299]. However, no comparative studies have yet been undertaken comparing the responses of MAC-T cells and BME cells to the ribotoxic stressor ANS.

MCF-10A cells were derived from the adherent fraction of cells from the normal tissue of a female patient with fibrocystic disease, following long-term cultures in serum-free media. They do not form tumors when implanted in nude mice, but will grow in soft agar, thereby displaying some characteristics for transformed cells, while retaining morphological features of luminal ductal cells [300]. The sensitivity of these cells to ANS has not been previously determined.

NMuMG cells were derived from an adult NAMRU mouse and display many structural and biochemical features of differentiated cells, although they will form benign cystadenomas when implanted into nude mice [301]. These cells demonstrated sensitivity to ANS both in terms of morphological changes, as well as the activation of ERK-1/2, p38 and JNK-1/2 signaling pathways (our unpublished data). However, little is known about their regulation of IGFBP production. Therefore, the primary goal of this study was to determine if ANS induced IGFBP-3 expression in primary bovine cells, as

well as human and murine mammary epithelial cell cultures. A second goal was to determine if they exhibited similar dose response curves for protein synthesis inhibition since we were ultimately interested in using concentrations of ANS that induced apoptosis with minimal effects on translation.

MATERIALS AND METHODS

Chemical Reagents

[^{32}P]-dCTP and [^{35}S]-methionine were purchased from GE Healthcare (Piscataway, NJ) and MP Biomedicals (Solon, OH), respectively. Anisomycin was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture plasticware was purchased from Bectin-Dickinson (Franklin Lakes, NJ). Cell culture reagents were from Invitrogen (Carlsbad, CA) except fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), EGF (GroPep, North Adelaide, Australia), and insulin, hydrocortisone, choleratoxin and phenol-red free Dulbecco's Modified Eagle's Medium (Sigma–Aldrich).

Cell Culture

BME and NMuMG cells were routinely maintained in phenol red-containing DMEM supplemented with 4.5 g/L d-glucose, 10% FBS, 5 μg/ml insulin and antibiotics (20 U/ml penicillin, 20 μg/ml streptomycin, 50 μg/ml gentamicin). For experiments, both cell types were cultured in the same medium lacking phenol red and insulin. MCF-10A cells were maintained in phenol red-free DMEM-F12 supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 50 μg/ml hydrocortisone, 100 ng/ml choleratoxin and antibiotics (20 U/ml penicillin, 20 μg/ml streptomycin). Experiments were performed in the same medium. All cell types received overnight washouts in their respective media without serum, but supplemented with 0.2% BSA and 30 nM sodium selenite. All cells were cultured at 37^{o}C in a 5% CO_2, humidified atmosphere.

Northern Blot Analysis

Total RNA was isolated and analyzed by Northern blotting as described [302]. Briefly, 10 µg denatured RNA was separated by electrophoresis, transferred to nylon membranes (Biotrans; ICN, Irvine, CA), then hybridized overnight with [^{32}P]-dCTP-labeled cDNA probes for bovine, mouse or human IGFBP-3, bovine IGFBP-6, bovine IGFBP-2, 18S ribosomal RNA or GAPDH. Membranes were washed and exposed to film with intensifying screens overnight to several days at -80^{o}C. Band intensity was measured by densitometry using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and IGFBP-3 values were normalized to GAPDH (BME, MCF-10A) or 18S (NMuMG).

Protein Synthesis Inhibition Experiments

Cells were grown to confluence in 6-well plates, washed twice in serum-free DMEM lacking methionine, and incubated for 45 min in this same medium. They were then treated for 2 h with ANS in methionine-free, serum free media, with 15 µCi (1.5 µl) [^{35}S]-methionine added to each well during the second hour. Cells were then washed twice in PBS and collected in 1 ml/well ice cold 5% TCA. Collections were repeated two more times (3 ml total). Samples were then centrifuged at 7000 rpm for 15 min at 4^{o}C. Pellets were then washed three times in 1 ml ice cold 5% TCA and centrifuged at 7000 rpm for 10 min at 4^{o}C. Finally, pellets were resuspended in 500 µl 0.1 M NaOH, combined with 5 ml scintillation fluid and radioactivity was measured using a scintillation counter. All treatments were performed in triplicate within an experiment and a minimum of three experiments were performed for each cell line.

Statistical Analysis

Data from experiments were analyzed by ANOVA with differences considered significant for $p < 0.05$. Tukey and SNK post-hoc tests were utilized for pair-wise

comparisons. Analyses were performed with SigmaStat (2.03) software (SPSS Inc., Chicago, IL).

RESULTS/DISCUSSION

Primary BME cells were treated with ANS to support the validity of the MAC-T cell line as a model for normal cells, and verify that the observed induction of IGFBP-3 was not an artifact of the immortalized cell line (Figure 1; $n = 3$, $p < 0.05$). Treatment of BME cells with ANS induced IGFBP-3 mRNA levels between 3- and 20-fold above control levels at concentrations of ANS ranging from 0.025 μM to 0.1 μM, with no increase observed at 1 μM. This pattern was consistent with that observed for MAC-T cells, including the diminished response at 1 μM ANS. This higher concentration of ANS inhibits translation by more than 90% in MAC-T cells, suggesting that IGFBP-3 mRNA induction may be facilitated by synthesis of a new protein product, although this intermediate has yet to be identified.

We next sought to determine if the response was species-specific. ANS treatment of MCF-10A human mammary epithelial cells and NMuMG murine mammary epithelial cells also caused an induction of IGFBP-3 mRNA levels (Figures 2 and 3, respectively). MCF-10A cells exhibited the same pattern of IGFBP-3 mRNA induction, though with substantially smaller magnitude, with a maximum induction of approximately 3-fold compared to untreated controls (Figure 2; $n = 3$, $p < 0.05$). NMuMG cells exhibited an increase in IGFBP-3 mRNA levels that ranged from approximately 2.7- to 6.4-fold (Figure 3; $n = 4$, $p < 0.05$). However, in contrast to the bovine cell types, both MCF-10A and NMuMG cells displayed a significant induction of IGFBP-3 after treatment with 1 μM ANS. This could indicate that these cell types are more resistant to ANS-induced translation inhibition, or that they may have an alternate pathway that does not require a

new protein intermediate to be synthesized. To further elucidate this, we sought to determine the extent of protein synthesis inhibition caused by ANS in both MCF-10A and NMuMG cells.

Our laboratory previously determined the level of protein synthesis inhibition caused by ANS in MAC-T cells to be less than 10% for cells treated with 0.025 μM ANS, approximately 40% for cells treated with 0.1 μM ANS, and greater than 90% for cells treated with 1 μM ANS. Treatment of MCF-10A cells with ANS in the presence of [^{35}S]-methionine resulted in 75% inhibition of protein synthesis with 0.1 μM ANS treatment (compared to ~40% in MAC-T cells) and nearly 95% inhibition of translation with 1 μM ANS treatment. These data indicate that MCF-10A cells are, in fact, at least as sensitive to ANS as MAC-T cells, and may be more sensitive at low concentrations. This suggests that MCF-10A cells may utilize a different pathway than MAC-T cells to induce IGFBP-3 in response to ANS treatment. NMuMG cells were similarly sensitive to ANS, with greater than 50% inhibition of protein synthesis with 0.1 μM ANS and greater than 85% inhibition with 1 μM ANS treatment (Figure 5). Taken together, these data suggest that differences in the degree of protein synthesis inhibition with 1 μM ANS treatment do account for the differences in IGFBP-3 mRNA levels observed across the different cell lines.

Figure 1

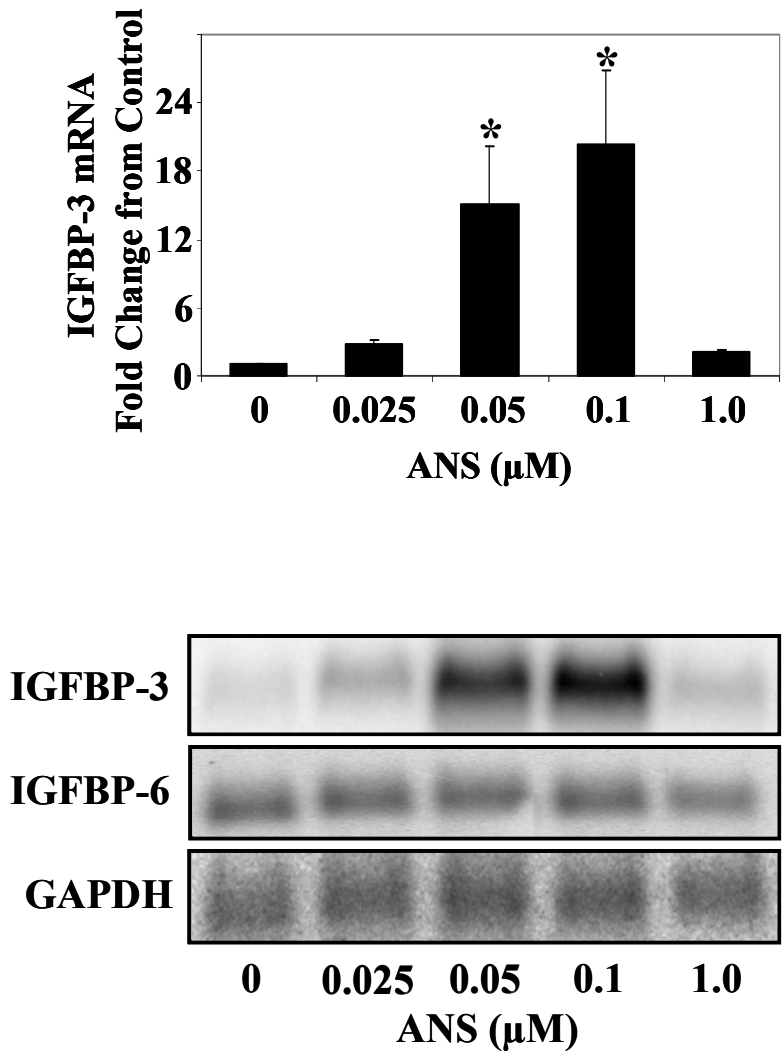


Figure 1. ANS induces IGFBP-3 mRNA levels in primary BME cells. Confluent BME cells were serum-starved overnight and treated for 3 h with ANS. Total RNA was collected and analyzed (10 µg) by Northern blotting. Membranes were probed with [^{32}P]-cDNA for bovine IGFBP-3, IGFBP-6 and GAPDH. Data represent fold change

from untreated control based on densitometry analysis of IGFBP-3 band intensity, normalized for GAPDH. Data represent mean +/- SEM for 3 independent experiments. * indicates $P < 0.05$

Figure 2

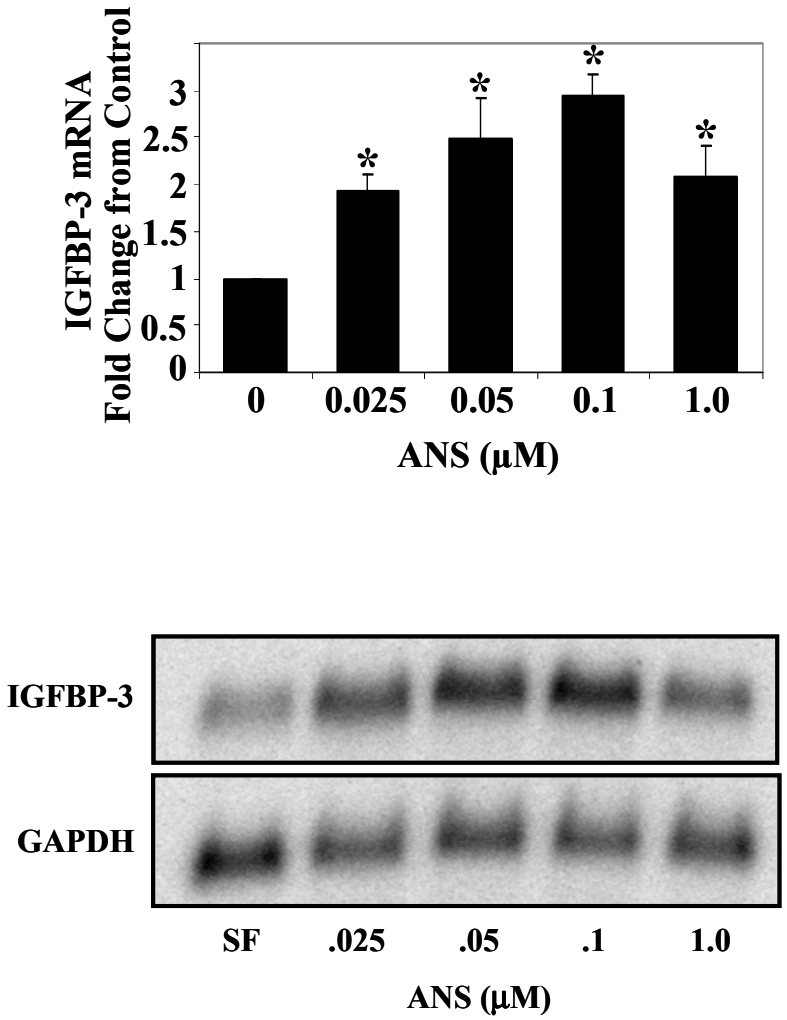


Figure 2. ANS induces IGFBP-3 mRNA levels in MCF-10A cells. Confluent MCF-10A cells were serum-starved overnight and treated for 3 h with ANS. Total RNA was collected and analyzed (10 μg) by Northern blotting. Membranes were probed with [^{32}P]-cDNA for human IGFBP-3 and GAPDH. Data represent fold change from untreated control based on densitometry analysis of IGFBP-3 band intensity, normalized

for GAPDH. Data represent mean +/- SEM for 3 independent experiments. * indicates P < 0.05

Figure 3

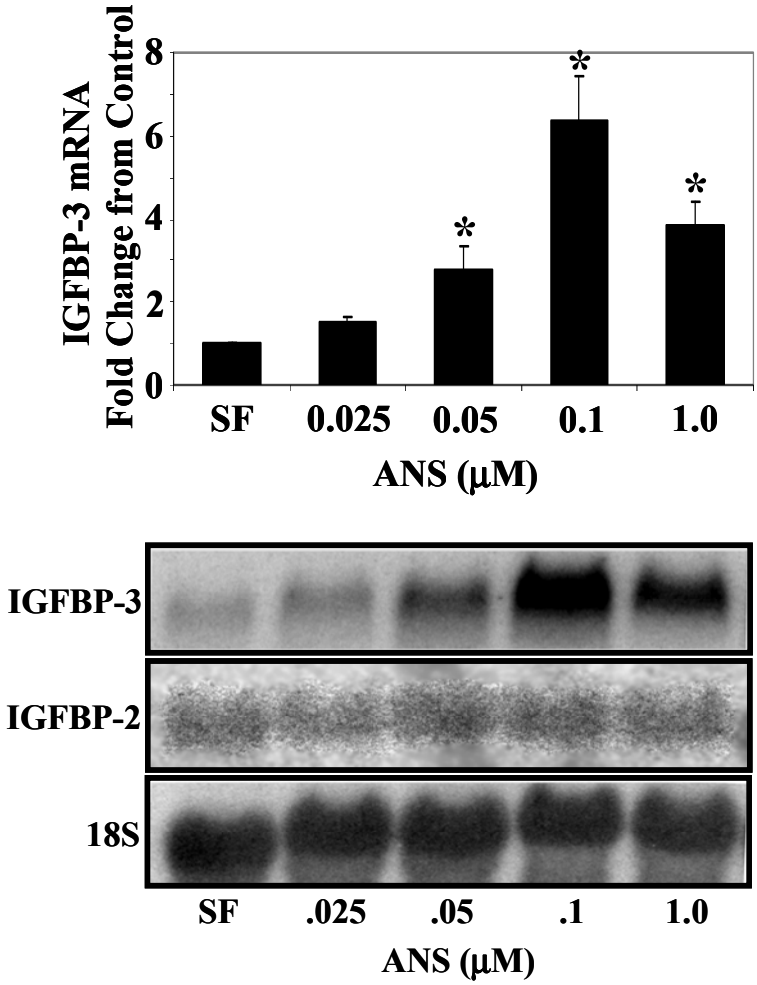


Figure 3. ANS induces IGFBP-3 mRNA levels in NMuMG cells. Confluent NMuMG cells were serum-starved overnight and treated for 6 h with ANS. Total RNA was collected and analyzed (10 μg) by Northern blotting. Membranes were probed with [^{32}P]-cDNA for murine IGFBP-3, bovine IGFBP-2 and 18S ribosomal RNA. Data represent fold change from untreated control based on densitometry analysis of IGFBP-3

band intensity, normalized for 18S. Data represent mean +/- SEM for 4 independent experiments. * indicates $P < 0.05$

Figure 4

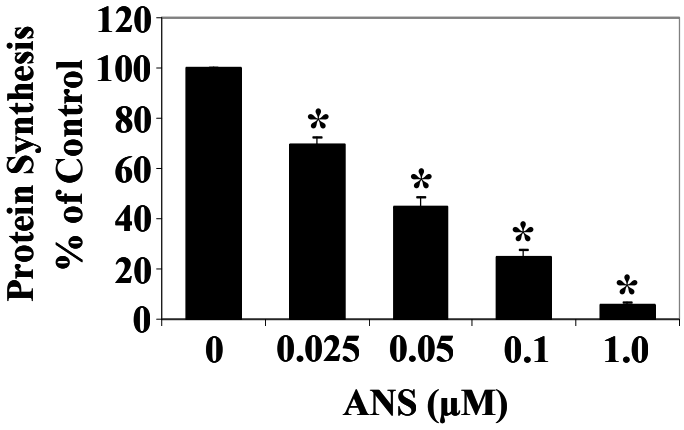


Figure 4. ANS causes dose-dependent inhibition of protein synthesis in MCF-10A cells. Confluent MCF-10A cells were washed twice in methionine-free, serum-free media, and incubated in methionine-free media for 45 min. Cells were then treated for 2 h with ANS, during the second hour of which 15 μCi [^{35}S]-methionine was added to each sample well. Cells were precipitated with TCA and total incorporated cpm were measured with a scintillation counter. Data represent 3 independent experiments and are presented as % of control. * indicates $P < 0.05$

Figure 5

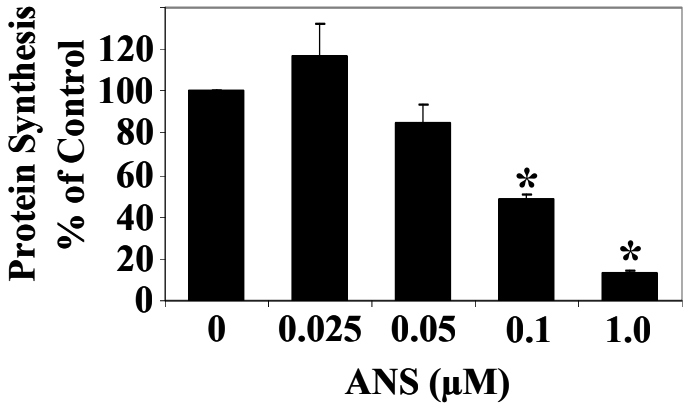


Figure 5. ANS causes dose dependent inhibition of protein synthesis in NMuMG cells. Confluent NMuMG cells were washed twice in methionine-free, serum-free media, and incubated in methionine-free media for 45 min. Cells were then treated for 2 h with ANS, during the second hour of which 15 μCi [^{35}S]-methionine was added to each sample well. Cells were precipitated with TCA and total incorporated cpm were measured with a scintillation counter. Data represent 3 independent experiments and are presented as % of control. * indicates $P < 0.05$

Chapter IV

RIBOTOXIC STRESS-INDUCED APOPTOSIS REQUIRES IGFBP-3 AND JNK-2-DEPENDENT NUCLEO-CYTOSOLIC TRANSLOCATION OF NUR77 IN NORMAL MAMMARY EPITHELIAL CELLS

ABSTRACT

Ribotoxic stress occurs in response to damage to the α-sarcin/ricin loop of the 28S ribosomal subunit in translationally active cells. This damage can be caused by a variety of ribosome inactivating proteins, including α-sarcin, ricin, and Shiga toxin 1. Additionally, the antibiotics anisomycin (ANS) and blasticidin S, as well as UV light can stimulate this stress response. However, concentrations of the ribotoxic stressors ricin and anisomycin (ANS) that only minimally affected translation still induced apoptosis in normal mammary epithelial cells. Since both of these stressors induced IGFBP-3 expression, we examined the role of IGFBP-3 in this apoptotic model. ANS significantly activated caspase-9, and stimulated cytochrome c release from the mitochondria, while only minimally activating caspase-8, suggesting that it activated the intrinsic apoptotic pathway. Knock-down of IGFBP-3 with siRNA substantially inhibited cytochrome c release, downstream caspase activation, and PARP cleavage in response to ANS. Similarly, siRNA-mediated knockdown of JNK-2 also inhibited apoptotic progression through the intrinsic pathway. However, knockdown of IGFBP-3 did not alter the ability of ANS to activate JNK, suggesting that the link between IGFBP-3 and JNK-2 was a third molecule affected directly by both of them. Subsequently, we have found that siRNA-mediated knockdown of either IGFBP-3 or JNK-2 prevented translocation of pro-apoptotic Nur77 out of the nucleus and into the cytosolic compartment, suggesting that it may be the common target molecule for both IGFBP-3 and JNK-2 during ribotoxic stress-induced apoptosis. These data are further supported by the localization of IGFBP-3 to the nucleus in these cells, and its subsequent loss following siRNA transfection.

INTRODUCTION

Ribotoxic stress is the process where stress-activated protein kinases (SAPK) are activated in response to damage to a specific region of the 28S ribosomal RNA, leading to cell death [271]. A variety of toxins belonging to the ribosome inactivating protein (RIP) family have been shown to induce this response, including α-sarcin, ricin A chain (RTA) and Shiga toxin 1 [162, 163, 271]. Additionally, the antibiotics anisomycin (ANS) and blasticidin S, as well as ultraviolet radiation have also been found to initiate the ribotoxic stress response following interaction with the ribosome [167, 168, 271]. In addition, a wide variety of other cellular stressors, including those from physiological, pharmacological and environmental sources, can activate SAPK pathways and cell death.

IGF binding protein-3 (IGFBP-3) is a multi-factorial protein that has been proposed to modulate cell death through diverse mechanisms, including IGF-independent effects [272, 273]. These pro-apoptotic effects have been reported to occur as a results of its association with the cell surface [242], presence in the cytosol [251] and/or localization to the nucleus [247], suggesting that IGFBP-3 actions are both complex and dependent on cellular context. Interestingly, IGFBP-3 expression has been shown to be regulated by both growth-promoting and growth-inhibiting factors [274-279]. In some cell models, IGFBP-3 has been shown to modulate, at least in part, the growth inhibitory effects of several cellular stressors, including TNF-α and doxorubicin [231, 280]. We have previously shown (Chapter II) that the toxins ANS and RTA each induce apoptosis, as well as IGFBP-3 expression in a normal epithelial cell line. This led us to postulate that IGFBP-3 may play a role in apoptosis in mammary epithelial cells exposed to

ribotoxic stress-inducing toxins. Indeed, we found that knockdown of IGFBP-3 with siRNA significantly reduced apoptosis in response to both ANS and RTA. Since both toxins also activate the SAPK pathways, we sought to determine in the present study where in the apoptotic pathway IGFBP-3 was acting, and if it was required for the SAPK pathways to induce apoptosis.

MATERIALS AND METHODS

Chemical reagents

Antibodies against Nur77, tubulin and PARP were purchased from Cell Signaling Technology, Inc. (Danvers, MA). A second antibody against Nur77 was purchased from Active Motif (Carlsbad, CA). Porin, cytochrome c and Hsp60 antibodies were purchased from Mito Sciences (Eugene, OR), BD Pharmingen (San Jose, CA) and Abcam Inc. (Cambridge, MA), respectively. Anisomycin was purchased from Sigma (St. Louis, MO). siRNA SmartPool reagents for p38 and JNK-2, as well as scrambled control siRNA, were purchased from Dharmacon Inc. (Lafayette, CO). Additionally, a custom SmartPool siRNA for bovine IGFBP-3 was also purchased from Dharmacon. Custom siRNA oligos against bovine IGFBP-2 were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture plasticware was purchased from Bectin-Dickinson (Franklin Lakes, NJ). Cell culture reagents were from Invitrogen (Carlsbad, CA), with the exception of fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA) and phenol-red free Dulbecco's Modified Eagle's Medium (Sigma–Aldrich). Leptomycin B was also purchased from Sigma-Aldrich.

Cell Culture

The bovine mammary epithelial cell (MEC) line MAC-T was routinely maintained as previously described [282]. For experiments, cells were plated and grown to confluence in phenol-red free DMEM containing 10% FBS and antibiotics unless otherwise stated. Cells were rinsed with phenol-red free serum-free (SF) DMEM, and incubated in SF

DMEM with 0.2% BSA and 30 nM sodium selenite overnight prior to exposure to treatments in SF DMEM without additives.

Measurements of Caspase Activation

Confluent MAC-T cells were serum starved overnight as described above and exposed to treatments. Caspase activation was measured by the Homogenous Fluorometric Caspase Assay (Roche, Indianapolis, IN), EnzoLyte Caspase-3/7 Assay (AnaSpec) and/or SensoLyte Caspase Sampler Assay (AnaSpec) according to manufacturers' instructions. Fluorescence was measured with background subtracted on a BioTek Synergy HT microplate reader.

TUNEL Assay

TUNEL Assays were performed with the In Situ Cell Death Detection Kit, Fluorescein (Roche) according to manufacturer's instructions. Briefly, MAC-T cells were plated in 8-well chamber slides, transfected with 50 nM scrambled, IGFBP-3 or IGFBP-2 siRNA for 48 h, serum starved overnight and treated with or without ANS for 6 h. Cells were then fixed and permeablized before incubation with TUNEL reagents. Hoechst stain (Sigma-Aldrich) was used to stain nuclei following incubation with TUNEL reagents. Slides were then washed in PBS and treated with Prolong Antifade reagent (Invitrogen) to preserve the fluorescent signal. For a more detailed protocol, see Appendix.

Cell Fractionation

For experiments requiring sub-cellular fractionation, mitochondrial and nuclear fractionation kits were purchased from Active Motif (Carlsbad, CA). Mitochondrial, cytosolic and nuclear protein fractions were isolated according to manufacturer's

instructions. Protein concentrations of the fractions were measured with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) using BSA as a standard.

Western Immunoblotting

Cells were lysed as previously described [283]. Total protein content of lysates was determined using the Bio-Rad Protein Assay. Total or organelle-specific protein (30-100 μg) were separated on SDS PAGE gels. Resolved proteins were transferred to nitrocellulose (0.2 μm; Bio-Rad) membranes. Membranes were blocked for 1 h at room temperature in Tris-buffered saline + 0.05% Tween-20 (v/v) (TBS-T) and 5% non-fat dried milk (w/v), and incubated with primary antisera at 4^{o}C overnight with gentle agitation. Membranes were then washed in TBS-T and incubated for 2 h at room temperature with appropriate HRP-conjugated secondary antibodies. Peroxidase activity was detected with an ECL Western Detection Kit (Pierce, Rockford, IL).

siRNA Experiments

MAC-T cells were grown to ~70% confluence and transfected with 50 nM JNK-2, 100 nM p38, 50 nM IGFBP-3 siRNA or 50 nM IGFBP-3 siRNA, as well as a corresponding concentration of scrambled control siRNA using GeneEraser siRNA Transfection Reagent (Stratagene, La Jolla, CA) according to manufacturer's instructions. Gene knockdown was previously verified by Western immunoblot for both phosphorylated and total forms of JNK-2 and p38 (Chapter II).

Statistical Analysis

Data from experiments were analyzed by ANOVA with differences considered significant for $p < 0.05$. Tukey and SNK post-hoc tests were utilized for pair-wise

comparisons. Analyses were performed with SigmaStat (2.03) software (SPSS Inc., Chicago, IL).

RESULTS

Ribotoxic stress-induced apoptosis occurs through the intrinsic apoptotic pathway.

To determine whether ribotoxic stress-induced apoptosis occurs primarily through the intrinsic or extrinsic apoptotic pathway, activation of caspase-9 and –8, respectively, were assessed. As shown in Figure 1A, MAC-T cells treated for 6 h with 0.1 μM ANS displayed a significant increase in caspase-9 activity ($n = 3$, $p < 0.05$), compared to untreated control cells. In contrast, a non-significant increase was observed in caspase-8 activation. Consistent with caspase-9 activation, ANS treatment led to substantial translocation of cytochrome c from the mitochondria to the cytosol of fractionated cells (Figure 1B).

Activation and progression of the intrinsic apoptotic pathway requires IGFBP-3

To determine the role of IGFBP-3 in the activation of the intrinsic apoptotic pathway during ribotoxic stress, IGFBP-3 was knocked down using siRNA and several markers of apoptosis were assessed. MAC-T cells transfected with siRNA against IGFBP-3 showed significantly reduced total caspase activation (Figure 2A; $n = 3$, $p < 0.05$). This finding was further supported by measurement of caspase-3/7 activation, which was reduced by knockdown of IGFBP-3, but was not diminished at all by knockdown of IGFBP-2, a major form of IGFBP produced by these cells (Figure 2B). Western immunoblotting of fractionated cells demonstrated that cytochrome c translocation from the mitochondria to the cytosol was also substantially inhibited by knockdown of IGFBP-3 (Figure 2C). Finally, knockdown of IGFBP-3 with siRNA led to a marked reduction in the number of ANS-induced TUNEL positive cells, while cells

with IGFBP-2 knocked down looked similar to those transfected with scrambled siRNA (Figure 2D).

JNK-2 and p38 are required for ANS-induced activation and progression of the intrinsic apoptotic pathway

To determine the role of the SAPK pathways in ANS-induced apoptosis, p38 or JNK-2 were knocked down using siRNA. Knockdown of JNK-2 led to a significant reduction in ANS-induced caspase activation (Figure 3A; $n = 5$, $p < 0.05$), as well as a substantial decrease in the cleavage of PARP, a major DNA repair enzyme and downstream target of caspase-3 (Figure 3B). Knockdown of p38 also led to a significant reduction in ANS-induced caspase activation. As shown in Chapter II, knockdown or inhibition of p38 led to a significant reduction in IGFBP-3 expression. This suggests that the impact of p38 knockdown on apoptosis may be due to the concurrent decrease in IGFBP-3. In contrast, the role of JNK-2 in ANS-induced apoptosis appeared to be independent of IGFBP-3 expression, as knockdown or inhibition of JNK-2 did not affect IGFBP-3 levels.

Anisomycin induces Nur77 translocation into the cytosolic compartment

As shown in Chapter II, JNK-2 did not play a role in ANS-induced IGFBP-3 expression. Additionally, we found that IGFBP-3 knockdown did not alter the ability of ANS to activate JNK-2 (Figure 4A). Therefore, we sought to determine if a third, common molecule was required for IGFBP-3 and JNK-2 to regulate the apoptotic pathway. Nur77 has been proposed to play a role in the ability of exogenous IGFBP-3 to influence apoptosis. Therefore, we hypothesized that it might be important in mediating the effect of endogenous IGFBP-3 in MAC-T cells. Western blotting of fractionated cells

treated with ANS for 90 min demonstrated a consistent discrease in nuclear Nur77 coupled with an increase in cytosolic Nur77 (Figure 4B). Intact PARP was used as a marker to ensure purity of the nuclear fraction. PARP cleavage has not occurred yet with ANS treatment due to the early time point examined. To further support the role of Nur77 nucleo-cytosolic translocation, cells were pre-treated for 30 min with 20 nM leptomycin B (LMB) prior to treatment for 90 min with ANS. LMB-treated cells displayed reduced movement of Nur77 to the cytosol in response to ANS (Figure 4B). Additionally, the caspase activation normally observed after 6 h treatment with 0.1 μM ANS was significantly inhibited by LMB pre-treatment (Figure 4C; $n = 3$, $p < 0.05$).

Knockdown of nuclear IGFBP-3 or JNK-2 prevents Nur77 nucleo-cytosolic translocation

Immunoblotting of nuclear and cytosolic protein fractions from MAC-T cells demonstrated that IGFBP-3 was localized to the nucleus (Figure 5A). Interestingly, ANS treatment did not change the amount of IGFBP-3 that was in the nucleus. In consideration that this might have been due to the short time point, 18 and 24 h ANS treatments were examined and no changes in nuclear IGFBP-3 accumulation were observed (data not shown). No cytosolic protein was observed in either control or ANS-treated cells. To determine if IGFBP-3 or JNK-2 were required for Nur77 translocation to the cytosol, cells were transfected with siRNA to knockdown either IGFBP-3 or JNK-2, then treated with ANS for 90 min. Nur77 translocation was measured by western blotting of both cytosolic and nuclear protein fractions. The increase in cytosolic Nur77 observed following ANS treatment was lost in cells with either IGFBP-3 or JNK-2 knockdown (Figure 5B). Interestingly, the presence of Nur77 in the nucleus was strongly

attenuated in siRNA transfected cells, suggesting an unexpected regulatory mechanism where knockdown of IGFBP-3 or JNK-2 led to inhibition of Nur77 expression (Figure 5B). Whole cell lysates were immunoblotted for Nur77 to verify that loss of nuclear Nur77 was due to a loss of expression and not to a change in subcellular localization (Figure 5C).

Anisomycin-induced apoptosis is rescued by IGF-I

AKT has been reported to phosphorylate Nur77 and prevent its nuclear export. We have previously reported that IGF-I causes rapid and sustained activation of Akt in MAC-T cells. Therefore, we sought to determine if this might be a mechanism by which IGF-I rescues ANS-treated cells. IGF-I treatment completely rescued MAC-T cells from ANS-induced apoptosis (Figure 6A; $n = 3$, $p < 0.05$). Activation of caspase-3/7 was completely abolished with co-incubation of 20 ng/ml IGF-I with 0.1 μM ANS for 6 h. To further support this finding, cells co-treated for 90 min with ANS and IGF-I were fractionated and Nur77 localization was assessed. ANS and IGF-I co-treated cells showed enhanced Nur77 nuclear localization, coupled with decreased cytosolic localization, compared to cells treated with ANS alone, indicating that IGF-I was inhibiting Nur77 cytosolic translocation (Figure 6B).

DISCUSSION

In the present study we sought to elucidate the role of IGFBP-3 in mammary epithelial cell apoptosis. As shown in Chapter II, we found that the ability of ANS and ricin A-chain to activate apoptosis was significantly inhibited by RNAi-induced knockdown of IGFBP-3. However, the specific mechanism through which IGFBP-3 functions during ribotoxic stress-induced apoptosis remained unknown. In order to assess the role of IGFBP-3, we first needed to determine whether ribotoxic stress-induced apoptosis occurred through the intrinsic or extrinsic apoptotic pathways. To determine this, we measured the activation of caspase-8 and -9 in response to 6 h treatment with 0.1 μM ANS, and found significant activation of caspase-9, but no activation of caspase-8. This suggested that apoptosis was occurring through the mitochondrial pathway and not via the extrinsic death receptor pathway. This finding differed from a report by Hori et al. that found that caspase-8 activation was required for ANS-induced apoptosis in U937 lymphoma cells [303]. In contrast, another group found that ANS treatment could induce cytochrome c release in ML-1 leukemia cells, suggesting that the pathway of ANS-induced apoptotic progression may be cell type specific [304]. Therefore, to further assess a role for the intrinsic pathway, we measured cytochrome c release following 4 h treatment with ANS. Cells that were separated into mitochondrial and cytosolic protein fractions demonstrated substantial translocation of cytochrome c into the cytosolic compartment. This indicated that mitochondrial membrane integrity had been compromised and the intrinsic cell death pathway had been initiated.

In our previous studies (Chapter II), the finding that IGFBP-3 was required for ANS-induced apoptosis was based on nucleosome accumulation, which is a late marker for apoptotic cell death. Since the intrinsic pathway is initiated with the release of cytochrome c and subsequent activation of caspases, we examined each of these markers to determine if IGFBP-3 was needed at an early time point in apoptotic progression. We found that knockdown of IGFBP-3 prevented caspase activation and cytochrome c release in response to ANS. Previous reports by Kim et al. and Lee et al. found that either over-expression or addition of exogenous IGFBP-3, respectively, could activate caspases in breast and prostate cancer cells [242, 247]. However, this is the first report to show that loss of endogenous IGFBP-3 expression prevents initiation of the intrinsic apoptotic pathway, and the first demonstration that cytochrome c release and subsequent caspase activation can be inhibited by knockdown of endogenous IGFBP-3.

In MAC-T cells, intracellular IGFBP-3 appears to reside exclusively in the nucleus. Immunoblot analysis of fractionated cells failed to detect any protein in cytosolic extracts, while nuclear protein was readily detectable. As reported in Chapter II, ANS stimulates a large increase in secreted IGFBP-3. However, this induction does not appear to substantially alter nuclear accumulation of the protein, suggesting that the basal levels of IGFBP-3 present in MAC-T cell nuclei are necessary and sufficient for IGFBP-3 to participate in apoptosis. A role for nuclear IGFBP-3 in apoptosis has been reported in prostate cancer cells [247, 249], but we provide here the first evidence for IGFBP-3 as a part of the inherent apoptotic machinery in a non-transformed cell model. Our findings suggest that IGFBP-3 may play a similar role to several other apoptotic

proteins in that it is present in the cell under normal conditions, and becomes actively involved in apoptosis upon stimulation of the cell by an apoptosis inducing agent.

We have previously found (Chapter II) that p38 was required for IGFBP-3 induction by ANS. Therefore we were not surprised to find that RNAi-induced knockdown of p38 led to inhibition of caspase activation, since this should result from the loss of IGFBP-3 expression. However, knockdown of JNK-2 also inhibited caspase activation, even though it did not play a role in ANS-induced IGFBP-3 expresion, suggesting that its role in apoptosis was independent of IGFBP-3 expression. Since both IGFBP-3 and JNK-2 were required for ANS-induced apoptosis, we examined whether or not IGFBP-3 regulated the ability of ANS to activate JNK-2. In contrast to a report by Lee et al. suggesting that the addition of IGFBP-3 to prostate cancer cells could stimulate JNK phosphorylation [249], we found that IGFBP-3 played no role in the ability of ANS to activate JNK-2. Therefore, we began to investigate whether a third molecule was affected in some way by both JNK-2 and IGFBP-3.

The JNK pathway has been reported to be involved in the mitochondrial apoptotic pathway via its ability to modulate Bcl-2 family proteins. In particular, the ability of JNK to facilitate Bax translocation to the mitochondria has been well established in several cell systems, including HepG2 [178], murine fibroblasts [180] and COS-1 cells [179]. However, we were not able to detect any changes in the subcellular localization of Bax following ANS treatment (data not shown), despite its ability to stimulate rapid and sustained JNK activation. Therefore, we sought to find an alternative pathway that might explain how ANS-induced apoptosis was dependent on both IGFBP-3 and JNK.

The orphan nuclear receptor Nur77 has been reported to induce the mitochondrial apoptotic pathway in a variety of cell types. These actions are dependent on sub-cellular localization, since nuclear accumulation of Nur77 has been associated with cell survival, while cytosolic/mitochondrial localization induced apoptosis [305]. In MAC-T cells, we found that ANS treatment for 90 min stimulated a small but consistent increase in cytosolic Nur77, suggesting that it may be a necessary player in ribotoxic stress-induced apoptosis. Nur77 has been reported to utilize the nuclear export sequence (NES) contained in its heterodimeric binding partner, RXR, to facilitate its nuclear export, and is therefore likely to be sensitive to inhibitors of the Crm1/exportin-1 nuclear export pathway [306]. Additionally, while bovine Nur77 has not been explicitly characterized, a target gene with 94% identity to human Nur77 has been reported (GeneBank Accession number NP_001069379) and we have found a candidate leucine-rich NES in the carboxy terminus at amino acids 552-565 (LSRLLGKLPELRTL**),** suggesting that Nur77 may also have the potential to utilize the Crm1/exportin-1 pathway without RXR binding.

LMB inhibits the Crm1/exportin-1 pathway, and we found that pretreatment for 30 min with LMB prevented both ANS-induced Nur77 nuclear export, as well caspase activation. This finding suggested an important role for Nur77 in ANS-induced apoptosis. Han et al. presented evidence that Nur77 nuclear export depended on phosphorylation by JNK in several cancer cell lines, and was inhibited by phosphorylation by Akt [184]. In agreement with this finding, we found that treatment with IGF-I, which we have shown strongly activates Akt in MAC-T cells [282], prevented Nur77 nuclear export and ANS-induced apoptosis. Our data, supported by studies from Han et al. and Lee et al. suggest that Nur77 may be a common protein

downstream of JNK-2 and IGFBP-3 in the intrinsic apoptotic pathway. To further support this hypothesis, MAC-T cells with either JNK-2 or IGFBP-3 knocked down by siRNA were treated with ANS and Nur77 nuclear export was measured. Cytosolic accumulation of Nur77 was prevented, indicating that its translocation was both JNK-2 and IGFBP-3 dependent. However, most surprisingly, nuclear localization of Nur77 was also substantially diminished in cells with siRNA-mediated knockdown of either JNK-2 or IGFBP-3. Immunoblotting of whole cell lysates demonstrated that this was not due to a change in subcellular localization, but was in fact due to a loss of expression. This finding is significant because it suggests that IGFBP-3 and JNK-2 may modulate apoptosis at the level of gene expression in addition to influencing protein-protein interactions.

The JNK pathway plays a well-established role in gene regulation via its ability to activate downstream transcription factors [307]. In fact, the JNK pathway has been linked to Nur77 expression in MA-10 murine Leydig tumor cells via transcriptional regulation through Jun binding to AP-1 sites in the Nur77 promoter [308]. In contrast, very little is known about the role of IGFBP-3 in transcriptional regulation. Schedlich et al. showed that IGFBP-3 could attenuate transactivation of RAR response elements by RXR:RAR heterodimers through dimerization with either RXR or RAR, which prevented RXR:RAR dimers from forming [246]. Therefore, the absence of IGFBP-3 would allow transcription of genes containing RAR response elements. However, what we have found suggests that IGFBP-3 may be required for Nur77 gene expression, as opposed to hindering it. Further investigation of this potential transcriptional regulation is ongoing in our laboratory.

Ribotoxic stress leads to apoptosis via activation of the SAPK pathways. We report here that Nur77 nuclear export is required for ANS to induce this apoptotic response, and that Nur77 translocation requires both JNK and IGFBP-3 (for proposed apoptotic pathway, see Chapter V, Figure 1). Our findings suggest that not only are JNK and IGFBP-3 necessary for Nur77 movement, but that they may also be necessary for Nur77 expression. Studies are ongoing in our laboratory to further determine the role of IGFBP-3 in Nur77 action and expression, as we provide further evidence that IGFBP-3 is a part of the intrinsic apoptotic machinery in normal cells.

Figure 1

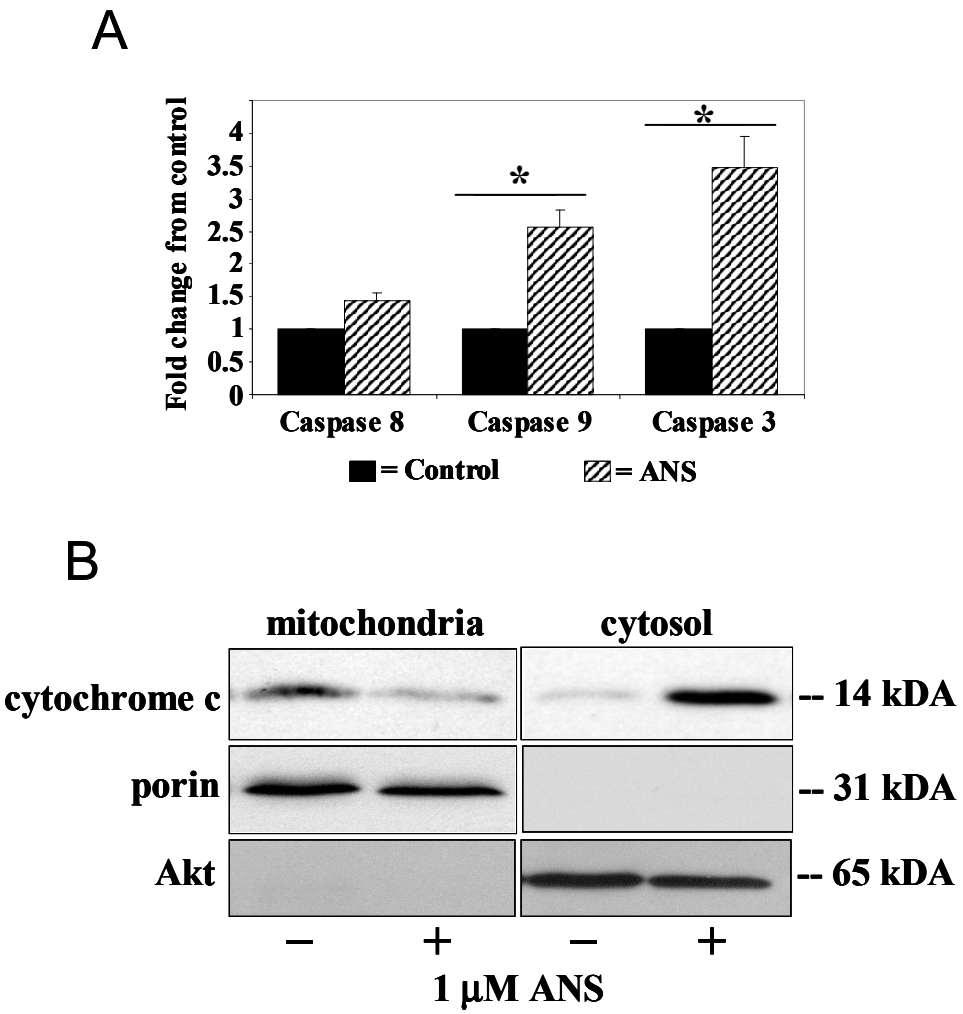
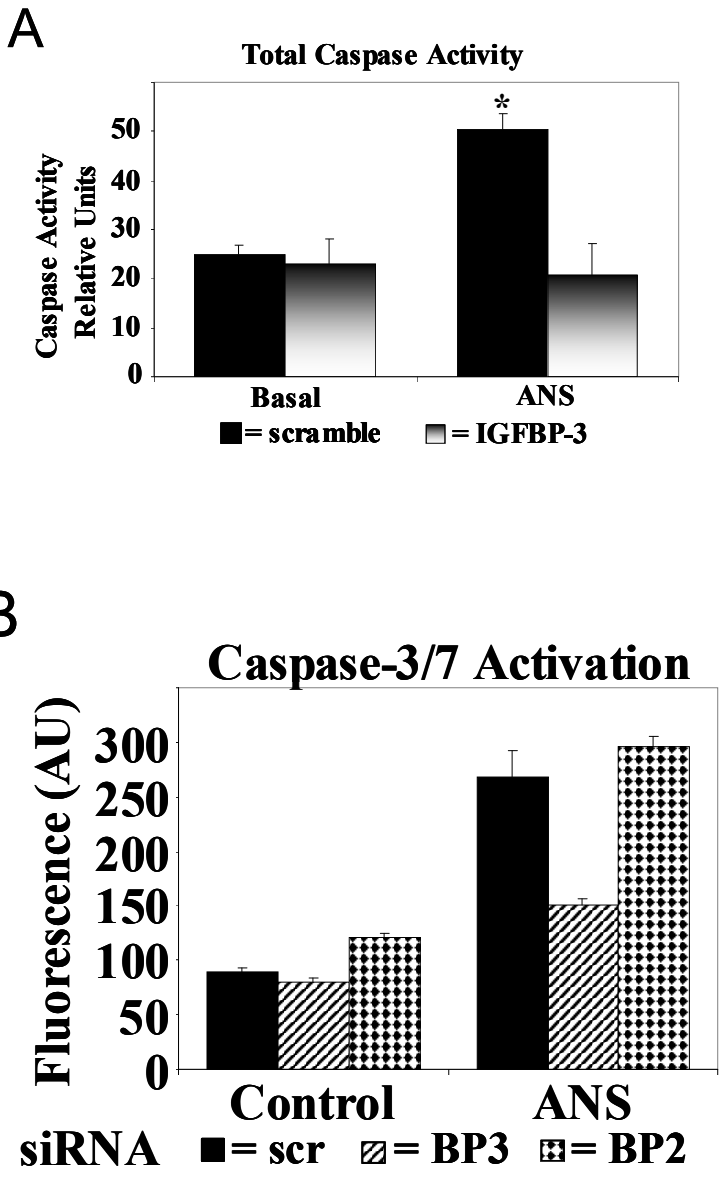


Figure 1. Anisomycin activates the intrinsic apoptotic pathway in MAC-T cells. (A) Confluent MAC-T cells were serum-starved overnight and treated with 0.1 µM ANS for 6 h. Cells were collected in Assay Buffer (supplied in SensoLyte kit) and incubated with substrates for caspase-8, -9 or -3 for 2 h. 100 µl of each sample was then analyzed for caspase activity by measurement of fluorescence. Data represent mean +/- SEM for 3 independent experiments. (B) Confluent MAC-T cells were serum-starved overnight and

treated with 1 μM ANS for 4 h. Cells were separated into mitochondrial and cytosolic protein fractions, separated by SDS-PAGE and immunoblotted for cytochrome c. The integrity of the protein fractions was assessed by immunoblotting for porin and Akt. Data are representative of at least 3 independent experiments. * indicates $p < 0.05$

Figure 2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Caspase Activity
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Figure 2

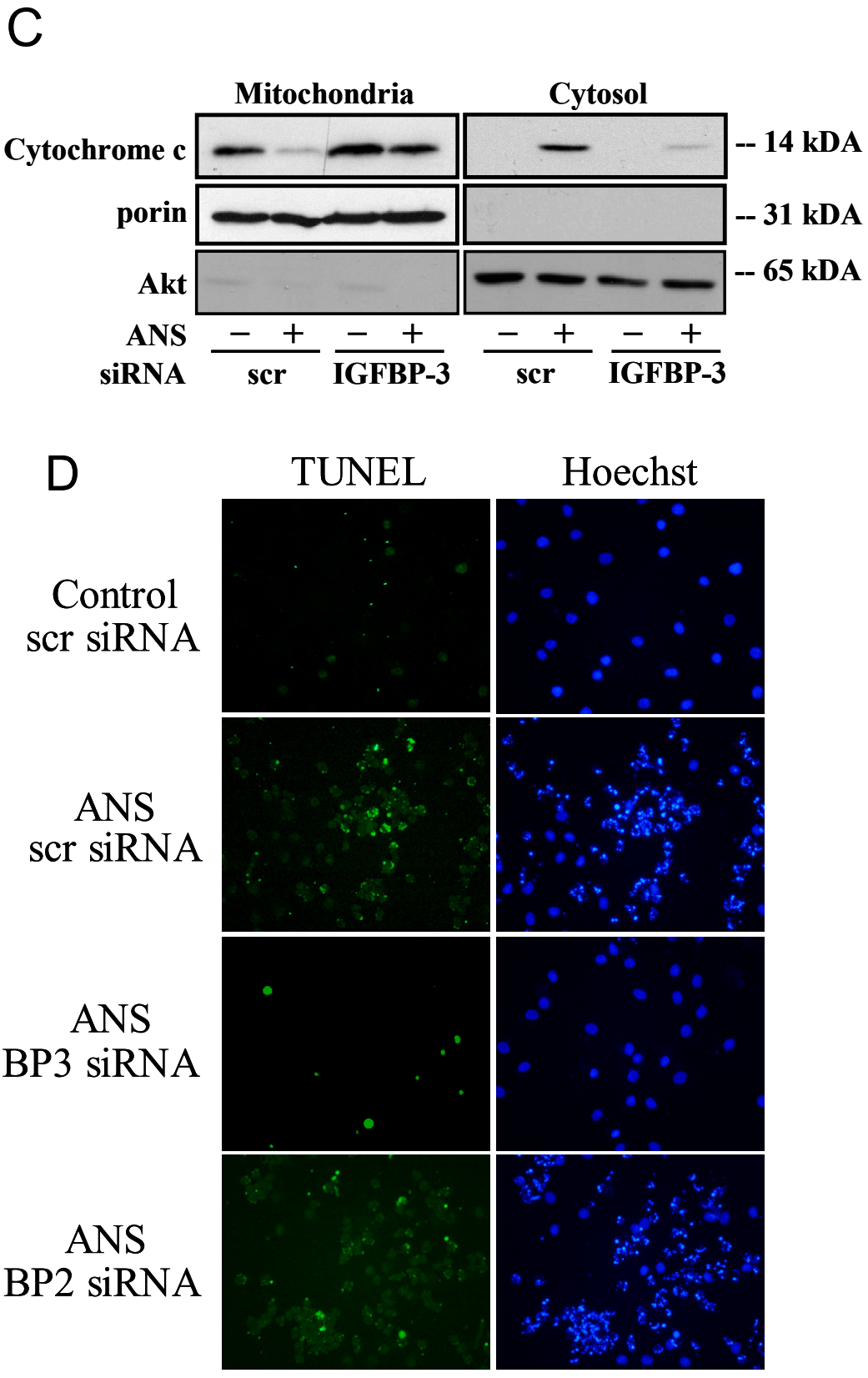
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Figure 2

Figure 2. Anisomycin-induced activation of the intrinsic apoptotic pathway requires IGFBP-3. MAC-T cells were transfected with 50 nM scrambled or IGFBP-3 siRNA (A,C) or 50 nM scrambled, IGFBP-3 or IGFBP-2 siRNA (B, D) for 48 h, serum-starved overnight and treated with ANS for 6h (A,B,D) or 4 h (C). (A) Caspase activation was measured with the Homogenous Fluorometric Caspase Assay (Roche) according to the manufacturer's instructions. Data represent mean +/- SEM of 3 independent experiments. (B) Caspase-3/7 activation was measured with the EnzoLyte Fluorometric Caspase Assay (AnaSpec) according to manufacturer's instructions. Data represent mean +/- SD for two independent experiments. (C) Mitochondrial and cytosolic protein fractions were immunoblotted for cytochrome c, porin and Akt as described. (D) Apoptosis was measured by TUNEL staining with the In Situ Cell Death Detection Kit, Fluorescein (Roche) according to manufacturer's instructions. Data are representative of at least 2 independent experiments (C,D). * indicates $p < 0.05$

Figure 3

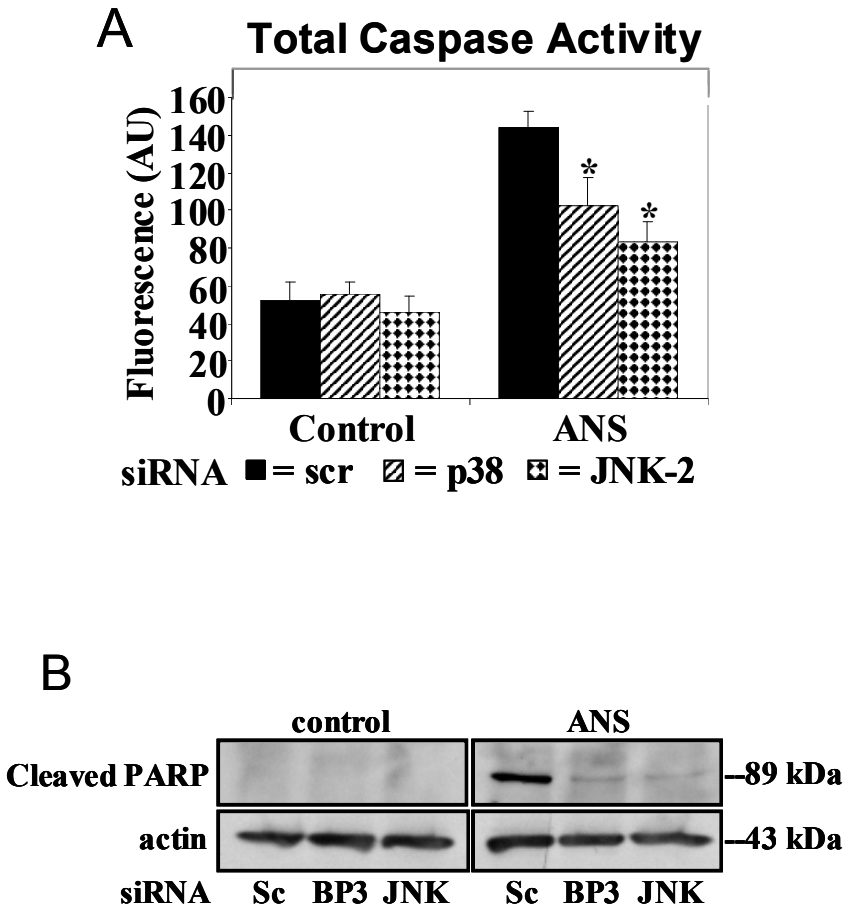

Figure 3. Anisomycin-induced caspase activation requires JNK-2 and p38. (A) MAC-T cells were transfected with 100 nM scrambled (scr), 100 nM p38 or 50 nM JNK-2 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 6 h. Caspase activation was measured with the Homogenous Fluorometric Caspase Assay according to manufacturer's instructions. Data represent the mean +/- SEM of 5 independent experiments. (B) MAC-T cells were transfected with 50 nM scrambled (scr), IGFBP-3 or JNK-2 siRNA for 48 h, serum-starved overnight and treated with 0.1

μM ANS for 6 h. Total protein was collected and immunoblotted for cleaved PARP. Actin served as a loading control. Data are representative of 3 independent experiments (B). * indicates $p < 0.05$ vs. scrambled siRNA control treated with ANS.

Figure 4

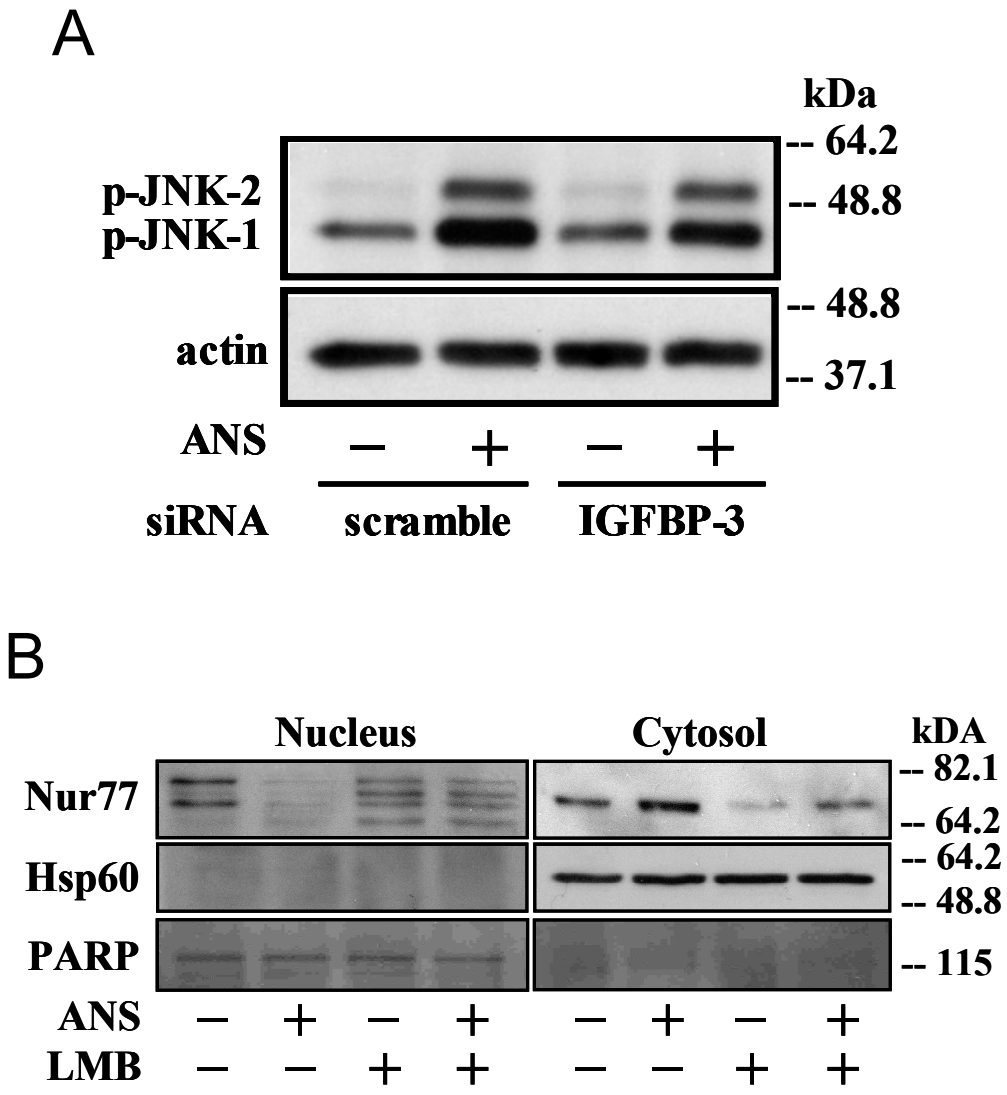
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Figure 4

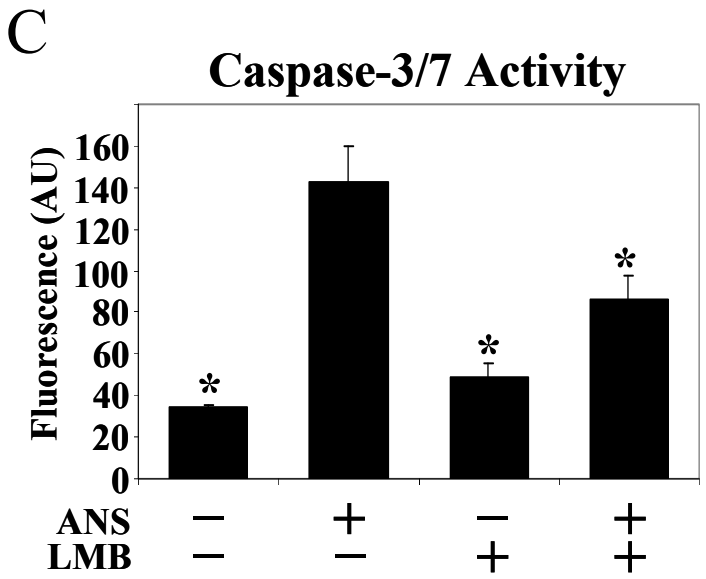

Figure 4. Inhibition of Nur77 nucleo-cytosolic translocation prevents anisomycin-induced caspase-3/7 activation. (A) MAC-T cells were transfected with 50 nM scrambled or IGFBP-3 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 30 min. Total protein was collected and 25 μg were immunoblotted for phosphorylated JNK1-2 and actin. (B) Confluent MAC-T cells were serum-starved overnight and treated with 0.1 μM ANS for 90 min with or without 30 min pre-treatment with 20 nM leptomycin B (LMB). Nuclear and cytosolic protein fractions were immunoblotted for Nur77. PARP and Hsp60 were used to verify the integrity of the protein fractions. (C) Confluent MAC-T cells were serum-starved overnight, pre-treated for 30 min with 20 nM LMB and treated with 0.1 μM ANS for 6 h. Caspase-3/7 activity was measured with the EnzoLyte Caspase-3/7 Assay (AnaSpec) according to

manufacturer's instructions. Data are representative of 3 independent experiments (A,B) and represent mean +/- SEM for 3 independent experiments (C). * indicates $p < 0.5$ compared to ANS treated sample.

Figure 5

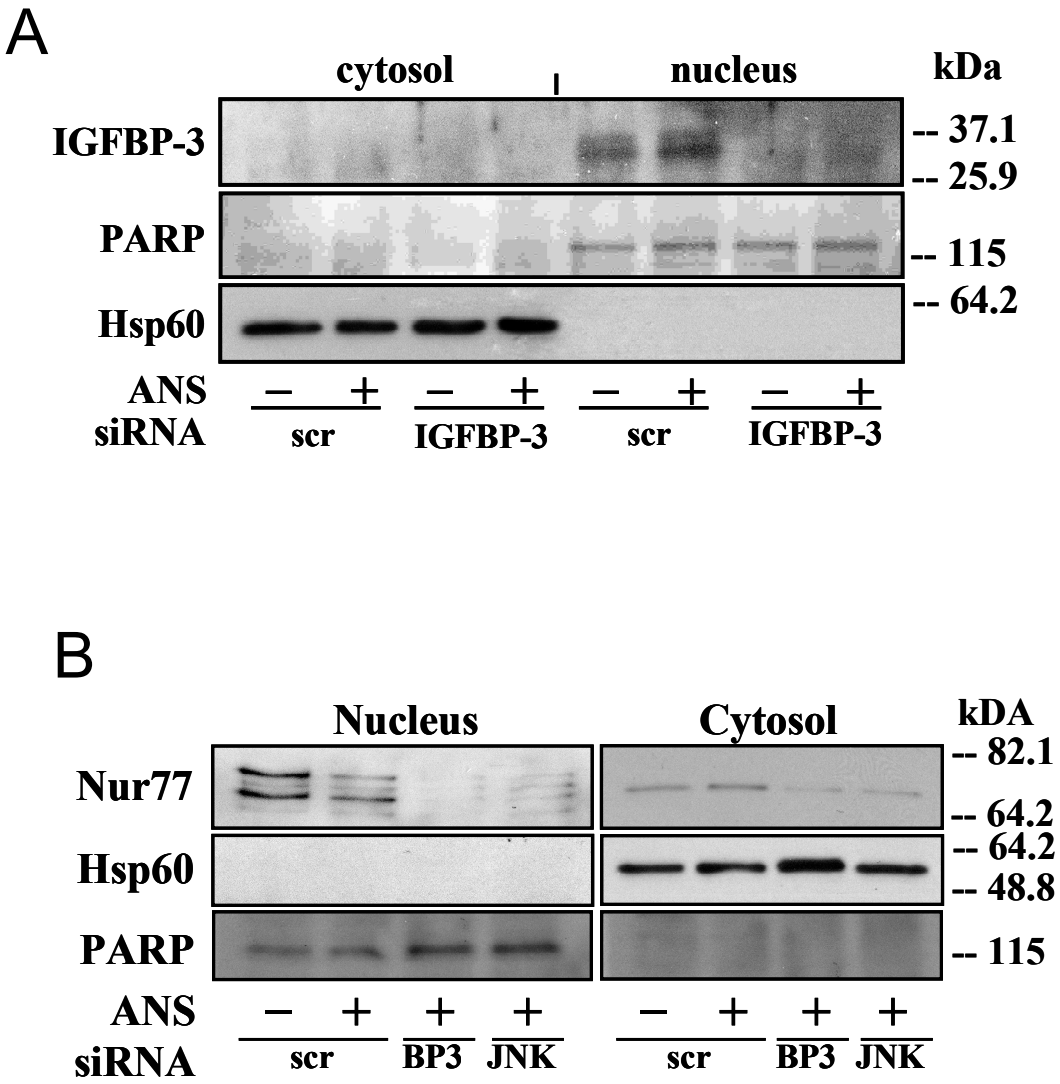
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Figure 5

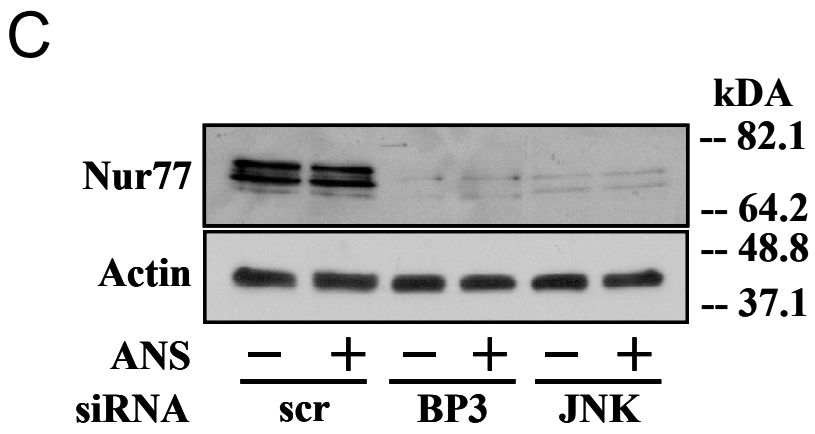


Figure 5. JNK-2 and nuclear IGFBP-3 are required for anisomyin-induced Nur77 translocation. (A) MAC-T cells were transfected with 50 nM scrambled or IGFBP-3 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 6 h. Nuclear and cytosolic protein fractions were immunoblotted for IGFBP-3, PARP and Hsp60. (B-C) MAC-T cells were transfected with 50 nM scrambled, JNK-2 or IGFBP-3 siRNA for 48 h, serum-starved overnight and treated with 0.1 μM ANS for 90 min. (B) Nuclear and cytosolic fractions were immunoblotted for Nur77, PARP and Hsp60. (C) Total cell lysates were immunoblotted for Nur77 and actin. Data are representative of at least 3 independent experiments.

Figure 6

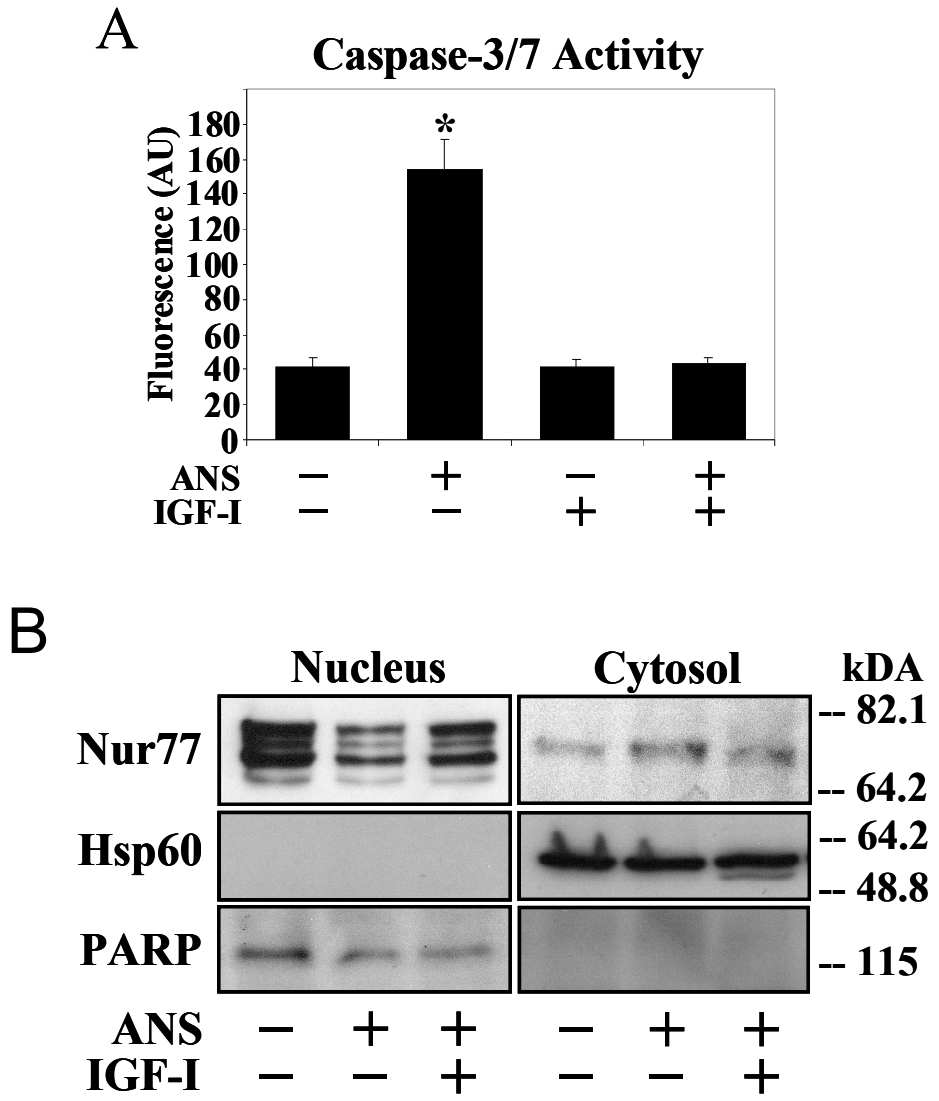


Figure 6. IGF-I rescues anisomycin treated cells from apoptosis and inhibits Nur77 translocation. Confluent MAC-T cells were serum-starved overnight and treated with 0.1 μM ANS +/- 100 ng/ml IGF-I for 6 h (A) or 90 min (B). (A) Caspase-3/7 activity was measured with the EnzoLyte Caspase-3/7 Assay according to manufacturer's

instructions. (B) Nuclear and cytosolic protein fractions were immunoblotted for Nur77, PARP and Hsp60. (A) Data represent means +/- SEM for 3 independent experiments. (B) Data are representative of 2 independent experiments. * indicates $p < 0.05$

Chapter V

CONCLUSION & REFERENCES

CONCLUSION

This project was initiated because our laboratory was interested in the role of IGF-I and IGFBP in normal mammary gland physiology. Our previous data showed that growth factors (IGF-I, EGF, TGF-α) could induce IGFBP-3 expression, and that IGFBP-3 overexpression led to increased IGF-I-stimulated cell proliferation. However, we also found that ANS could stimulate IGFBP-3 expression while inducing apoptosis, suggesting that IGFBP-3 might also play a role in cell death.

The first aim addressed in the thesis was to identify the mechanisms by which ANS induces IGFBP-3 expression. Our laboratory had previously found that IGF-I induced IGFBP-3 expression through the MAPK and PI3-K pathways. In the present work, it was found that ANS induced IGFBP-3 primarily through the p38 pathway, though the MAPK pathway also contributed. In contrast, while both the p38 and JNK pathways were activated by ANS, JNK did not play a role in ANS-induced IGFBP-3 expression. Subsequent experiments determined that ANS (as well as RTA) induced IGFBP-3 through increases in mRNA stability and, in the case of RTA, transcriptional activation. We were not able to show that ANS activated the IGFBP-3 promoter using a 1.1 kb promoter fragment fused to luciferase. However, given the magnitude and rapidity of the IGFBP-3 mRNA induction, it remains highly probable that promoter activation may occur through elements that are upstream of the 1.1 kb promoter region that we cloned.

A second aim of the thesis was to determine mechanistically how IGFBP-3 plays a role in apoptosis. At the time this work was initiated, there were a few published

reports showing that IGFBP-3 could localize to the nucleus in some cells. We therefore hypothesized that subcellular localization may govern IGFBP-3 actions in response to diverse stimuli. We knew that both IGF-I and ANS treatment led to substantial increases in secreted IGFBP-3, but we did not know where, or if, IGFBP-3 acted inside the cell. Subsequent studies described in this thesis showed that IGFBP-3 was readily detectable in the nucleus of MAC-T cells, but was absent from the cytosol. This finding led us to suggest that ANS treatment might increase IGFBP-3 nuclear accumulation and that this might underlie the mechanism of how IGFBP-3 is involved in apoptosis. However, much to our surprise, IGFBP-3 levels in the nucleus did not change in response to ANS treatment.

To address what role IGFBP-3 was playing in ANS-induced apoptosis, an RNAi approach was used. It was determined that IGFBP-3 was needed for ANS-induced apoptosis, as knockdown of IGFBP-3 all but eliminated nucleosome accumulation, while IGFBP-2 siRNA failed to have an effect. A role in the intrinsic apoptotic pathway was identified by showing a requirement for IGFBP-3 in ANS-induced cytochrome c release, caspase activation and PARP cleavage. These findings suggested that IGFBP-3 functioned early in apoptotic initiation, as it was operating upstream of the mitochondria in an apoptotic pathway that did not utilize the death receptor/caspase-8 apoptotic program.

An additional goal was to identify a link between increased IGFBP-3 expression in response to ANS and its ability to induce apoptosis. Therefore, we sought to determine which of the SAPK were involved in each of these actions. We anticipated that one of the SAPK pathways would regulate IGFBP-3 expression, and that the same

pathway would be required for ANS-induced apoptosis by virtue of its regulation of IGFBP-3. This proved to be the case for the p38 pathway. However, while JNK-2 played no role in IGFBP-3 expression as described above, it was necessary for ANS-induced apoptosis. We therefore sought to find a common molecule that both IGFBP-3 and JNK might interact with to initiate apoptosis. The orphan nuclear receptor Nur77 appears to be one such molecule. It is known to induce apoptosis at the mitochondria upon translocation out of the nucleus. It has also been shown to require phosphorylation by JNK and an as yet unclear nuclear interaction with IGFBP-3 for this translocation to occur. These factors made it a potentially viable candidate as the convergence point for JNK-2 and IGFBP-3 in ANS-induced apoptosis. We were able to show that ANS stimulated the migration of Nur77 out of the nucleus, and that this movement was prevented by knockdown of either IGFBP-3 or JNK-2. Surprisingly, the interesting observation was made that knockdown of IGFBP-3 or JNK-2 affected not just Nur77 translocation, but its expression as well. Nur77 protein levels declined dramatically in cells transfected with either IGFBP-3 or JNK-2 siRNA. While these results suggest a mechanism for how IGFBP-3 functions in ANS-induced apoptosis, several questions remain for future investigators. What is the specific interaction, if any, between IGFBP-3 and Nur77? How do IGFBP-3 and JNK-2 affect Nur77 expression? The answers to these questions may shed even more light on the complex and ever-expanding biological role of IGFBP-3.

In the past few years, our laboratory has become interested in the effects of ricin and Shiga toxin on mammalian cells. These agents belong to a family of ribosome inactivating proteins (RIPs) that attack cells at the 28S ribosomal subunit and induce

apoptosis through the ribotoxic stress response. ANS also acts at the level of the ribosome to initiate apoptosis via this response. An organism (human or otherwise) may come into contact with one or more of the above stressors unintentionally, through the environment, or possible intentionally, through nefarious actions. For example, the Department of Homeland Security lists ricin as a potential bioterror weapon, and Shiga toxin belongs to the family of toxins that induce food poisoning by E. Coli 0157. Cells that encounter these stressors have a biological imperative to respond in a manner aimed at preserving the overall organism, meaning that apoptotic cell death is the outcome if survival without damage is not possible. We found that RTA has many similarities to ANS. Not only does it induce IGFBP-3 expression through the same pathways, but it too requires IGFBP-3 to cause apoptosis. We began this project looking at the role of IGFBP-3 in normal mammary gland physiology, but we have gone on to find that IGFBP-3 may play a significant role not only in healthy cells, but in those under attack as well.

Figure 1

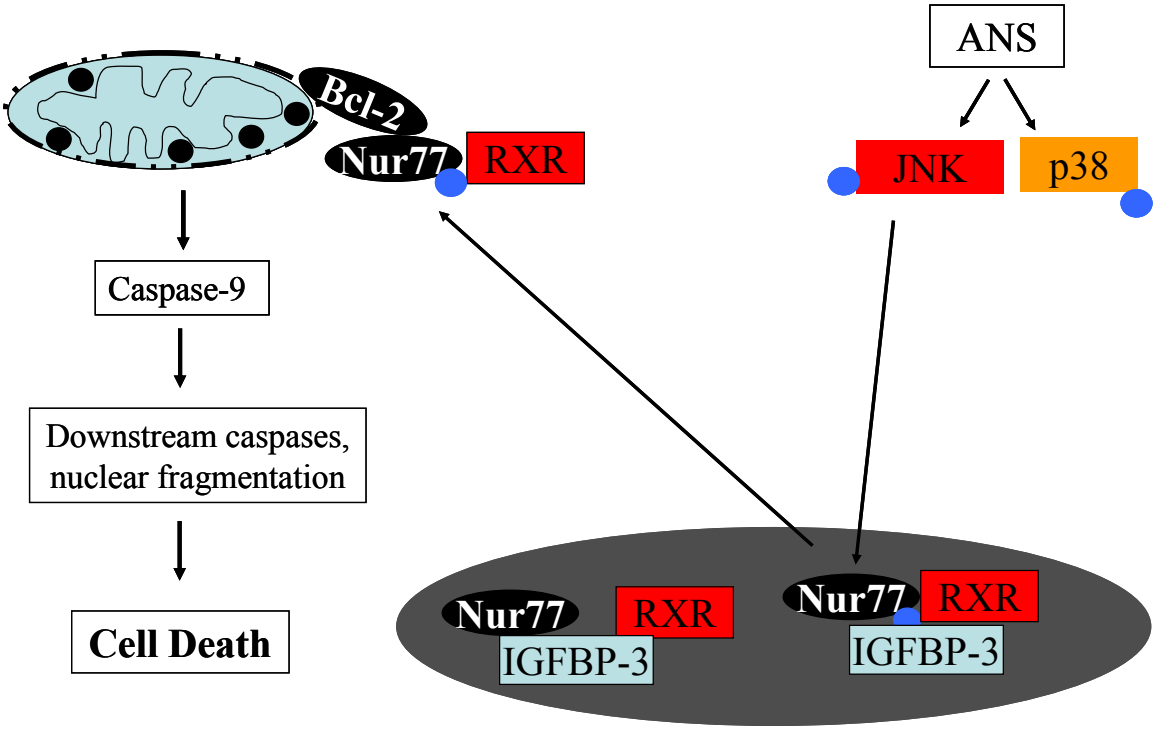


Figure 1. Proposed pathway for ANS-induced apoptosis in MAC-T cells. ANS treatment induces phosphorylation of the SAPK pathways. JNK-2 might then facilitate phosphorylation of Nur77, causing it and RXR-α to separate from IGFBP-3 within the nucleus and translocate to the mitochondria. At the mitochondria, Nur77 may interact with Bcl-2 to induce the intrinsic pathway.

REFERENCES

1 LeRoith, D. and Roberts, C. T., Jr. (2003) The insulin-like growth factor system and cancer. Cancer Lett. **195**, 127-137

2 Ciampolillo, A., De Tullio, C., Perlino, E. and Maiorano, E. (2007) The IGF-I axis in thyroid carcinoma. Curr Pharm Des. **13**, 729-735

3 Monti, S., Proietti-Pannunzi, L., Sciarra, A., Lolli, F., Falasca, P., Poggi, M., Celi, F. S. and Toscano, V. (2007) The IGF axis in prostate cancer. Curr Pharm Des. **13**, 719-727

4 Helle, S. I. and Lonning, P. E. (1996) Insulin-like growth factors in breast cancer. Acta Oncol. **35 Suppl 5**, 19-22

5 Cohick, W. S. and Clemmons, D. R. (1993) The insulin-like growth factors. Annual Review of Physiology. **55**, 131-153

6 Daughaday, W. H. and Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev. **10**, 68-91

7 Phillips, L. S., Pao, C. I. and Villafuerte, B. C. (1998) Molecular regulation of insulin-like growth factor-I and its principal binding protein, IGFBP-3. Progress in Nucleic Acid Research and Molecular Biology. **60**, 195-265

8 Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C. and Forbes, B. E. (2005) Molecular interactions of the IGF system. Cytokine Growth Factor Rev. **16**, 421-439

9 Liu, J.-P., Baker, J., Perkins, A. S., Robertson, E. J. and Efstratiadis, A. (1993) Mice carrying null mutations of the genes encoding insulin- like growth factor I (*Igf-1*) and type 1 IGF receptor (*Igf1r*). Cell. **75**, 59-72

10 Nakae, J., Kido, Y. and Accili, D. (2001) Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev. **22**, 818-835

11 LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C. T., Jr. (1995) Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocrine Reviews. **16**, 143-163

12 Nishimoto, I., Hata, Y., Ogata, E. and Kojima, I. (1987) Insulin-like growth factor II stimulates calcium influx in competent BALB/c 3T3 cells primed with epidermal

growth factor. Characteristics of calcium influx and involvement of GTP-binding protein. J Biol Chem. **262**, 12120-12126

13 Ikezu, T., Okamoto, T., Giambarella, U., Yokota, T. and Nishimoto, I. (1995) In vivo coupling of insulin-like growth factor II/mannose 6-phosphate receptor to heteromeric G proteins. Distinct roles of cytoplasmic domains and signal sequestration by the receptor. J Biol Chem. **270**, 29224-29228

14 Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S. and Chen, E. (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. The EMBO Journal. **5**, 2503-2512

15 Stewart, C. E. and Rotwein, P. (1996) Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. Physiological Reviews. **76**, 1005-1026

16 Gross, J. M. and Yee, D. (2003) The type-1 insulin-like growth factor receptor tyrosine kinase and breast cancer: biology and therapeutic relevance. Cancer Metastasis Rev. **22**, 327-336

17 Jones, J. I. and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocrine Reviews. **16**, 3-34

18 Duan, C. and Xu, Q. (2005) Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. Gen Comp Endocrinol. **142**, 44-52

19 Clemmons, D. R. (2001) Use of mutagenesis to probe IGF-binding protein structure/function relationships. Endocr Rev. **22**, 800-817

20 Baxter, R. C. (2000) Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab. **278**, E967-976.

21 Twigg, S. M. and Baxter, R. C. (1998) Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. J Biol Chem. **273**, 6074-6079

22 Cohick, W. S. (1998) Role of the insulin-like growth factors and their binding proteins in lactation. Journal of Dairy Science. **81**, 1769-1777

23 Rajaram, S., Baylink, D. J. and Mohan, S. (1997) Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev. **18**, 801-831

24 Bunn, R. C. and Fowlkes, J. L. (2003) Insulin-like growth factor binding protein proteolysis. Trends in Endocrinology and Metabolism. **14**, 176-181

25 Firth, S. M. and Baxter, R. C. (2002) Cellular actions of the insulin-like growth factor binding proteins. Endocrine Reviews. **23**, 824-854

26 Burrows, C., Holly, J. M., Laurence, N. J., Vernon, E. G., Carter, J. V., Clark, M. A., McIntosh, J., McCaig, C., Winters, Z. E. and Perks, C. M. (2006) Insulin-like growth factor binding protein 3 has opposing actions on malignant and nonmalignant breast epithelial cells that are each reversible and dependent upon cholesterol-stabilized integrin receptor complexes. Endocrinology. **147**, 3484-3500

27 Wang, G. K., Hu, L., Fuller, G. N. and Zhang, W. (2006) An interaction between insulin-like growth factor-binding protein 2 (IGFBP2) and integrin alpha5 is essential for IGFBP2-induced cell mobility. J Biol Chem. **281**, 14085-14091

28 Baker, J., Liu, J.-P., Robertson, E. J. and Efstratiadis, A. (1993) Role of insulin-like growth factors in embryonic and postnatal growth. Cell. **75**, 73-82

29 Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A. J., Davenport, M. L. and Efstratiadis, A. (1996) Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev Biol. **177**, 517-535

30 Salmon, A. N. and Daughaday, W. J. (1957) A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J Lab Clin Med. **49**, 825

31 Sjogren, K., Liu, J. L., Blad, K., Skrtic, S., Vidal, O., Wallenius, V., LeRoith, D., Tornell, J., Isaksson, O. G., Jansson, J. O. and Ohlsson, C. (1999) Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A. **96**, 7088-7092

32 Yakar, S., Liu, J. L., Stannard, B., Butler, A., Accili, D., Sauer, B. and LeRoith, D. (1999) Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A. **96**, 7324-7329

33 Ueki, I., Ooi, G. T., Tremblay, M. L., Hurst, K. R., Bach, L. A. and Boisclair, Y. R. (2000) Inactivation of the acid labile subunit gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system. Proc Natl Acad Sci U S A. **97**, 6868-6873

34 Yakar, S., Rosen, C. J., Beamer, W. G., Ackert-Bicknell, C. L., Wu, Y., Liu, J. L., Ooi, G. T., Setser, J., Frystyk, J., Boisclair, Y. R. and LeRoith, D. (2002) Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest. **110**, 771-781

35 Ning, Y., Schuller, A. G., Bradshaw, S., Rotwein, P., Ludwig, T., Frystyk, J. and Pintar, J. E. (2006) Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. Mol Endocrinol. **20**, 2173-2186

36 Wood, T. L., Rogler, L. E., Czick, M. E., Schuller, A. G. and Pintar, J. E. (2000) Selective alterations in organ sizes in mice with a targeted disruption of the insulin-like growth factor binding protein-2 gene. Mol Endocrinol. **14**, 1472-1482

37 Silha, J. V. and Murphy, L. J. (2002) Insights from insulin-like growth factor binding protein transgenic mice. Endocrinology. **143**, 3711-3714

38 Salih, D. A., Tripathi, G., Holding, C., Szestak, T. A., Gonzalez, M. I., Carter, E. J., Cobb, L. J., Eisemann, J. E. and Pell, J. M. (2004) Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice. Proc Natl Acad Sci U S A. **101**, 4314-4319

39 Dai, Z., Xing, Y., Boney, C. M., Clemmons, D. R. and D'Ercole, A. J. (1994) Human insulin-like growth factor-binding protein-1 (hIGFBP-1) in transgenic mice: characterization and insights into the regulation of IGFBP-1 expression. Endocrinology. **135**, 1316-1327

40 Rajkumar, K., Barron, D., Lewitt, M. S. and Murphy, L. J. (1995) Growth retardation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. Endocrinology. **136**, 4029-4034

41 Gay, E., Seurin, D., Babajko, S., Doublier, S., Cazillis, M. and Binoux, M. (1997) Liver-specific expression of human insulin-like growth factor binding protein-1 in transgenic mice: repercussions on reproduction, ante- and perinatal mortality and postnatal growth. Endocrinology. **138**, 2937-2947

42 Crossey, P. A., Jones, J. S. and Miell, J. P. (2000) Dysregulation of the insulin/IGF binding protein-1 axis in transgenic mice is associated with hyperinsulinemia and glucose intolerance. Diabetes. **49**, 457-465

43 Modric, T., Silha, J. V., Shi, Z., Gui, Y., Suwanichkul, A., Durham, S. K., Powell, D. R. and Murphy, L. J. (2001) Phenotypic manifestations of insulin-like growth factor-binding protein-3 overexpression in transgenic mice. Endocrinology. **142**, 1958-1967

44 Murphy, L. J., Molnar, P., Lu, X. and Huang, H. (1995) Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. J Mol Endocrinol. **15**, 293-303

45 Wang, J., Niu, W., Witte, D. P., Chernausek, S. D., Nikiforov, Y. E., Clemens, T. L., Sharifi, B., Strauch, A. R. and Fagin, J. A. (1998) Overexpression of insulin-like growth factor-binding protein-4 (IGFBP-4) in smooth muscle cells of transgenic mice

through a smooth muscle alpha-actin-IGFBP-4 fusion gene induces smooth muscle hypoplasia. Endocrinology. **139**, 2605-2614

46 Salih, D. A., Mohan, S., Kasukawa, Y., Tripathi, G., Lovett, F. A., Anderson, N. F., Carter, E. J., Wergedal, J. E., Baylink, D. J. and Pell, J. M. (2005) Insulin-like growth factor-binding protein-5 induces a gender-related decrease in bone mineral density in transgenic mice. Endocrinology. **146**, 931-940

47 Wood, T. L., Richert, M. M., Stull, M. A. and Allar, M. A. (2000) The insulin-like growth factors (IGFs) and IGF binding proteins in postnatal development of murine mammary glands. J Mammary Gland Biol Neoplasia. **5**, 31-42

48 Stull, M. A. and Wood, T. L. (2003) Expression of the IGFs, IGF-IR and IGFBPs in the normal mammary gland and breast. Breast Dis. **17**, 15-26

49 Richert, M. M., Schwertfeger, K. L., Ryder, J. W. and Anderson, S. M. (2000) An atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia. **5**, 227-241

50 Ruan, W. and Kleinberg, D. L. (1999) Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology. **140**, 5075-5081

51 Sinowatz, F., Schams, D., Kolle, S., Plath, A., Lincoln, D. and Waters, M. J. (2000) Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution. J Endocrinol. **166**, 503-510

52 Ruan, W., Catanese, V., Wieczorek, R., Feldman, M. and Kleinberg, D. L. (1995) Estradiol enhances the stimulatory effect of insulin-like growth factor-I (IGF-I) on mammary development and growth hormone-induced IGF-I messenger ribonucleic acid. Endocrinology. **136**, 1296-1302

53 Walden, P. D., Ruan, W., Feldman, M. and Kleinberg, D. L. (1998) Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. Endocrinology. **139**, 659-662

54 Richards, R. G., Klotz, D. M., Walker, M. P. and Diaugustine, R. P. (2004) Mammary gland branching morphogenesis is diminished in mice with a deficiency of insulin-like growth factor-I (IGF-I), but not in mice with a liver-specific deletion of IGF-I. Endocrinology. **145**, 3106-3110

55 Bauman, D. E., Mather, I. H., Wall, R. J. and Lock, A. L. (2006) Major advances associated with the biosynthesis of milk. J Dairy Sci. **89**, 1235-1243

56 Eppler, E., Zapf, J., Bailer, N., Falkmer, U. G., Falkmer, S. and Reinecke, M. (2002) IGF-I in human breast cancer: low differentiation stage is associated with decreased IGF-I content. Eur J Endocrinol. **146**, 813-821

57 Neuenschwander, S., Schwartz, A., Wood, T. L., Roberts, C. T., Jr., Hennighausen, L. and LeRoith, D. (1996) Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. Journal of Clinical Investigation. **97**, 2225-2232

58 Prosser, C. G., Sankaran, L., Henninghausen, L. and Topper, Y. J. (1987) Comparison of the roles of insulin and insulin-like growth factor I in casein gene expression and in the development of alpha-lactalbumin and glucose transport activities in the mouse mammary epithelial cell. Endocrinology. **120**, 1411-1416

59 Duclos, M., Houdebine, L.-M. and Djiane, J. (1989) Comparison of insulin-like growth factor 1 and insulin effects on prolactin-induced lactogenesis in the rabbit mammary gland in vitro. Mol.Cell.Endocrinol. **65**, 129-134

60 Fielder, P. J., Thordarson, G., English, A., Rosenfeld, R. G. and Talamantes, F. (1992) Expression of a lactogen-dependent insulin-like growth factor- binding protein in cultured mouse mammary epithelial cells. Endocrinology. **131**, 261-267

61 Boutinaud, M., Shand, J. H., Park, M. A., Phillips, K., Beattie, J., Flint, D. J. and Allan, G. J. (2004) A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. J Mol Endocrinol. **33**, 195-207

62 Kuhl, N. M., Hoekstra, D., De Vries, H. and De Keyser, J. (2003) Insulin-like growth factor-binding protein 6 inhibits survival and differentiation of rat oligodendrocyte precursor cells. Glia. **44**, 91-101

63 Green, D. R. (1998) Apoptotic Pathways: The Roads to Ruin. Cell. **94**, 695-698

64 Norbury, C. J. and Hickson, I. D. (2001) Cellular responses to DNA damage. Annu Rev Pharmacol Toxicol. **41**, 367-401

65 Hacker, G. (2000) The morphology of apoptosis. Cell Tissue Res. **301**, 5-17

66 Elmore, S. (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol. **35**, 495-516

67 Savill, J. and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. Nature. **407**, 784-788

68 Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. **104**, 487-501

69 Ashkenazi, A. and Dixit, V. M. (1998) Death receptors: signaling and modulation. Science. **281**, 1305-1308

70 Rupinder, S. K., Gurpreet, A. K. and Manjeet, S. (2007) Cell suicide and caspases. Vascul Pharmacol. **46**, 383-393

71 Hsu, H., Xiong, J. and Goeddel, D. V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell. **81**, 495-504

72 Philchenkov, A. (2004) Caspases: potential targets for regulating cell death. J Cell Mol Med. **8**, 432-444

73 Thorburn, A. (2004) Death receptor-induced cell killing. Cell Signal. **16**, 139-144

74 Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H. and Peter, M. E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo J. **14**, 5579-5588

75 Shearwin-Whyatt, L. M., Harvey, N. L. and Kumar, S. (2000) Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. Cell Death Differ. **7**, 155-165

76 Jin, Z. and El-Deiry, W. S. (2006) Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis. Mol Cell Biol. **26**, 8136-8148

77 Gupta, S. (2003) Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). Int J Oncol. **22**, 15-20

78 Wolf, B. B. and Green, D. R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem. **274**, 20049-20052

79 Aggarwal, B. B., Bhardwaj, U. and Takada, Y. (2004) Regulation of TRAIL-induced apoptosis by ectopic expression of antiapoptotic factors. Vitam Horm. **67**, 453-483

80 Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G. and Vandenabeele, P. (2004) Toxic proteins released from mitochondria in cell death. Oncogene. **23**, 2861-2874

81 Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. **102**, 33-42

82 Cai, J., Yang, J. and Jones, D. P. (1998) Mitochondrial control of apoptosis: the role of cytochrome c. Biochim Biophys Acta. **1366**, 139-149

83 van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S. M., Rodriguez, I., Alnemri, E. S., Gevaert, K., Vandekerckhove, J., Declercq, W. and Vandenabeele, P. (2002) The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. Cell Death Differ. **9**, 20-26

84 Garrido, C., Galluzzi, L., Brunet, M., Puig, P. E., Didelot, C. and Kroemer, G. (2006) Mechanisms of cytochrome c release from mitochondria. Cell Death Differ. **13**, 1423-1433

85 Chinnaiyan, A. M. (1999) The apoptosome: heart and soul of the cell death machine. Neoplasia. **1**, 5-15

86 Schimmer, A. D. (2004) Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. Cancer Res. **64**, 7183-7190

87 Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G. and Penninger, J. M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature. **410**, 549-554

88 Li, L. Y., Luo, X. and Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. **412**, 95-99

89 Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. **391**, 43-50

90 Cory, S. a. A., JM. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nature Reviews. Cancer. **2**, 647-656

91 Youle, R. J. and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol. **9**, 47-59

92 Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell. **87**, 619-628

93 Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell. **80**, 285-291

94 Lin, B., Kolluri, S. K., Lin, F., Liu, W., Han, Y. H., Cao, X., Dawson, M. I., Reed, J. C. and Zhang, X. K. (2004) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. Cell. **116**, 527-540

95 Hsu, Y. T., Wolter, K. G. and Youle, R. J. (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A. **94**, 3668-3672

96 Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G. and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. **139**, 1281-1292

97 Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J. and Shore, G. C. (1998) Regulated targeting of BAX to mitochondria. J Cell Biol. **143**, 207-215

98 Tan, Y. J., Beerheide, W. and Ting, A. E. (1999) Biophysical characterization of the oligomeric state of Bax and its complex formation with Bcl-XL. Biochem Biophys Res Commun. **255**, 334-339

99 Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J. C. (2000) Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. Biochem J. **345 Pt 2**, 271-278

100 Li, H., Zhu, H., Xu, C. J. and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. **94**, 491-501

101 Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. **94**, 481-490

102 Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. and Reed, J. C. (1998) Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A. **95**, 4997-5002

103 Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A. and Martinou, J. C. (1998) Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J Cell Biol. **143**, 217-224

104 Kim, T. H., Zhao, Y., Barber, M. J., Kuharsky, D. K. and Yin, X. M. (2000) Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. J Biol Chem. **275**, 39474-39481

105 Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R. and Newmeyer, D. D. (2002) Bid, Bax, and lipids cooperate

to form supramolecular openings in the outer mitochondrial membrane. Cell. **111**, 331-342

106 Eskes, R., Desagher, S., Antonsson, B. and Martinou, J. C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol. **20**, 929-935

107 Martinou, J. C. and Youle, R. J. (2006) Which came first, the cytochrome c release or the mitochondrial fission? Cell Death Differ. **13**, 1291-1295

108 Chipuk, J. E., Bouchier-Hayes, L. and Green, D. R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ. **13**, 1396-1402

109 Slee, E. A., Adrain, C. and Martin, S. J. (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem. **276**, 7320-7326

110 Sakahira, H., Enari, M. and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature. **391**, 96-99

111 Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J. and Williams, L. T. (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science. **278**, 294-298

112 Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr., Booth, C. J., Mehal, W. Z., Inayat, I. and Flavell, R. A. (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science. **311**, 847-851

113 Kuribayashi, K., Mayes, P. A. and El-Deiry, W. S. (2006) What are caspases 3 and 7 doing upstream of the mitochondria? Cancer Biol Ther. **5**, 763-765

114 Stegh, A. H. and Peter, M. E. (2001) Apoptosis and caspases. Cardiol Clin. **19**, 13-29

115 Bergeron, L., Perez, G. I., Macdonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K. E., Flaws, J. A., Salter, J. C., Hara, H., Moskowitz, M. A., Li, E., Greenberg, A., Tilly, J. L. and Yuan, J. (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev. **12**, 1304-1314

116 Fernandes-Alnemri, T., Armstrong, R. C., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwack, G. and Alnemri, E. S. (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc Natl Acad Sci U S A. **93**, 7464-7469

117 Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W. W. and Dixit, V. M. (1996) ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. J Biol Chem. **271**, 16720-16724

118 Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A. and et al. (1992) Molecular cloning of the interleukin-1 beta converting enzyme. Science. **256**, 97-100

119 Faucheu, C., Diu, A., Chan, A. W., Blanchet, A. M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A. and et al. (1995) A novel human protease similar to the interleukin-1 beta converting enzyme induces apoptosis in transfected cells. Embo J. **14**, 1914-1922

120 Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T. T., Yu, V. L. and Nicholson, D. W. (1995) Molecular cloning and pro-apoptotic activity of ICErelII and ICErelIII, members of the ICE/CED-3 family of cysteine proteases. J Biol Chem. **270**, 15870-15876

121 Chang, H. Y. and Yang, X. (2000) Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol Biol Rev. **64**, 821-846

122 Cohen, G. M. (1997) Caspases: the executioners of apoptosis. Biochem J. **326 (Pt 1)**, 1-16

123 Fuentes-Prior, P. and Salvesen, G. S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J. **384**, 201-232

124 Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H. and Wallach, D. (1995) A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J Biol Chem. **270**, 7795-7798

125 Boldin, M. P., Goncharov, T. M., Goltsev, Y. V. and Wallach, D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell. **85**, 803-815

126 Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S. and Dixit, V. M. (1998) An induced proximity model for caspase-8 activation. J Biol Chem. **273**, 2926-2930

127 Slee, E. A., Adrain, C. and Martin, S. J. (1999) Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ. **6**, 1067-1074

128 Shi, Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell. **9**, 459-470

129 Orth, K., O'Rourke, K., Salvesen, G. S. and Dixit, V. M. (1996) Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. J Biol Chem. **271**, 20977-20980

130 Yang, X., Chang, H. Y. and Baltimore, D. (1998) Autoproteolytic activation of pro-caspases by oligomerization. Mol Cell. **1**, 319-325

131 Muzio, M., Salvesen, G. S. and Dixit, V. M. (1997) FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J Biol Chem. **272**, 2952-2956

132 Sloviter, R. S. (2002) Apoptosis: a guide for the perplexed. Trends Pharmacol Sci. **23**, 19-24

133 Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M. and Salvesen, G. S. (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. J Biol Chem. **272**, 7797-7800

134 Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M. and Mak, T. W. (1998) Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell. **94**, 339-352

135 Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P. and Flavell, R. A. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell. **94**, 325-337

136 Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P. and Wallach, D. (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity. **9**, 267-276

137 Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature. **384**, 368-372

138 Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W. and Mak, T. W. (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. **12**, 806-819

139 Korfali, N., Ruchaud, S., Loegering, D., Bernard, D., Dingwall, C., Kaufmann, S. H. and Earnshaw, W. C. (2004) Caspase-7 gene disruption reveals an involvement of the enzyme during the early stages of apoptosis. J Biol Chem. **279**, 1030-1039

140 Ruchaud, S., Korfali, N., Villa, P., Kottke, T. J., Dingwall, C., Kaufmann, S. H. and Earnshaw, W. C. (2002) Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. Embo J. **21**, 1967-1977

141 Renehan, A. G., Booth, C. and Potten, C. S. (2001) What is apoptosis, and why is it important? Bmj. **322**, 1536-1538

142 Nijhawan, D., Honarpour, N. and Wang, X. (2000) Apoptosis in neural development and disease. Annu Rev Neurosci. **23**, 73-87

143 Opferman, J. T. and Korsmeyer, S. J. (2003) Apoptosis in the development and maintenance of the immune system. Nat Immunol. **4**, 410-415

144 Greenhalgh, D. G. (1998) The role of apoptosis in wound healing. Int J Biochem Cell Biol. **30**, 1019-1030

145 Osborne, B. A. (1996) Apoptosis and the maintenance of homoeostasis in the immune system. Curr Opin Immunol. **8**, 245-254

146 Tilly, J. L., Kowalski, K. I., Johnson, A. L. and Hsueh, A. J. (1991) Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology. **129**, 2799-2801

147 Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996) Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development. **122**, 181-193

148 King, K. L. and Cidlowski, J. A. (1998) Cell cycle regulation and apoptosis. Annu Rev Physiol. **60**, 601-617

149 Kerr, J. F., Winterford, C. M. and Harmon, B. V. (1994) Apoptosis. Its significance in cancer and cancer therapy. Cancer. **73**, 2013-2026

150 Vaux, D. L., Cory, S. and Adams, J. M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature. **335**, 440-442

151 Elnemr, A., Ohta, T., Yachie, A., Kayahara, M., Kitagawa, H., Ninomiya, I., Fushida, S., Fujimura, T., Nishimura, G., Shimizu, K. and Miwa, K. (2001) Human pancreatic cancer cells express non-functional Fas receptors and counterattack lymphocytes by expressing Fas ligand; a potential mechanism for immune escape. Int J Oncol. **18**, 33-39

152 Cheng, J., Zhou, T., Liu, C., Shapiro, J. P., Brauer, M. J., Kiefer, M. C., Barr, P. J. and Mountz, J. D. (1994) Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science. **263**, 1759-1762

153 Koyama, S., Koike, N. and Adachi, S. (2001) Fas receptor counterattack against tumor-infiltrating lymphocytes in vivo as a mechanism of immune escape in gastric carcinoma. J Cancer Res Clin Oncol. **127**, 20-26

154 Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B. and Reed, J. C. (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. **9**, 1799-1805

155 Pietenpol, J. A. and Stewart, Z. A. (2002) Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology. **181-182**, 475-481

156 Wang, X. W. and Harris, C. C. (1997) p53 tumor-suppressor gene: clues to molecular carcinogenesis. J Cell Physiol. **173**, 247-255

157 Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L. and Magun, B. E. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Molecular and Cellular Biology. **17**, 3373-3381

158 Brimacombe, R. (1995) The structure of ribosomal RNA: a three-dimensional jigsaw puzzle. Eur J Biochem. **230**, 365-383

159 Rodriguez-Fonseca, C., Amils, R. and Garrett, R. A. (1995) Fine structure of the peptidyl transferase centre on 23 S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. J Mol Biol. **247**, 224-235

160 Wool, I. G., Gluck, A. and Endo, Y. (1992) Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. Trends Biochem Sci. **17**, 266-269

161 Korcheva, V., Wong, J., Corless, C., Iordanov, M. and Magun, B. (2005) Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. Am J Pathol. **166**, 323-339

162 Smith, W. E., Kane, A. V., Campbell, S. T., Acheson, D. W., Cochran, B. H. and Thorpe, C. M. (2003) Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect Immun. **71**, 1497-1504

163 Lee, K. H., Nishimura, S., Matsunaga, S., Fusetani, N., Horinouchi, S. and Yoshida, M. (2005) Inhibition of protein synthesis and activation of stress-activated protein kinases by onnamide A and theopederin B, antitumor marine natural products. Cancer Sci. **96**, 357-364

164 Cano, E., Hazzalin, C. A. and Mahadevan, L. C. (1994) Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. Molecular and Cellular Biology. **14**, 7352-7362

165 Zinck, R., Cahill, M. A., Kracht, M., Sachsenmaier, C., Hipskind, R. A. and Nordheim, A. (1995) Protein synthesis inhibitors reveal differential regulation of mitogen-activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. Molecular and Cellular Biology. **15**, 4930-4938

166 Johnson, C. R., Jiffar, T., Fischer, U. M., Ruvolo, P. P. and Jarvis, W. D. (2003) Requirement for SAPK-JNK signaling in the induction of apoptosis by ribosomal stress in REH lymphoid leukemia cells. Leukemia. **17**, 2140-2148

167 Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A. and Magun, B. E. (1998) Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. J Biol Chem. **273**, 15794-15803

168 Iordanov, M. S. and Magun, B. E. (1999) Different mechanisms of c-Jun NH(2)-terminal kinase-1 (JNK1) activation by ultraviolet-B radiation and by oxidative stressors. J Biol Chem. **274**, 25801-25806

169 Raman, M., Chen, W. and Cobb, M. H. (2007) Differential regulation and properties of MAPKs. Oncogene. **26**, 3100-3112

170 Johnson, G. L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. **298**, 1911-1912

171 Kyriakis, J. M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. **81**, 807-869

172 Wajant, H., Pfizenmaier, K. and Scheurich, P. (2003) Tumor necrosis factor signaling. Cell Death Differ. **10**, 45-65

173 Baud, V. and Karin, M. (2001) Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol. **11**, 372-377

174 Monno, S., Newman, M. V., Cook, M. and Lowe, W. L., Jr. (2000) Insulin-like growth factor I activates c-Jun N-terminal kinase in MCF-7 breast cancer cells. Endocrinology. **141**, 544-550

175 Krause, D., Lyons, A., Fennelly, C. and O'Connor, R. (2001) Transient activation of Jun N-terminal kinases and protection from apoptosis by the insulin-like growth factor I receptor can be suppressed by dicumarol. J Biol Chem. **276**, 19244-19252

176 Walsh, P. T., Smith, L. M. and O'Connor, R. (2002) Insulin-like growth factor-1 activates Akt and Jun N-terminal kinases (JNKs) in promoting the survival of T lymphocytes. Immunology. **107**, 461-471

177 Chen, Z., Gibson, T. B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C. and Cobb, M. H. (2001) MAP kinases. Chem Rev. **101**, 2449-2476

178 Kim, B. J., Ryu, S. W. and Song, B. J. (2006) JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells. J Biol Chem. **281**, 21256-21265

179 Tsuruta, F., Sunayama, J., Mori, Y., Hattori, S., Shimizu, S., Tsujimoto, Y., Yoshioka, K., Masuyama, N. and Gotoh, Y. (2004) JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. Embo J. **23**, 1889-1899

180 Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D. and Davis, R. J. (2002) The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Mol Cell Biol. **22**, 4929-4942

181 Chen, Y. R. and Tan, T. H. (2000) The c-Jun N-terminal kinase pathway and apoptotic signaling (review). Int J Oncol. **16**, 651-662

182 Ventura, J. J., Hubner, A., Zhang, C., Flavell, R. A., Shokat, K. M. and Davis, R. J. (2006) Chemical genetic analysis of the time course of signal transduction by JNK. Mol Cell. **21**, 701-710

183 Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschert, U., Vial-Knecht, E., Martinou, J. C. and Arkinstall, S. (1997) Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem. **272**, 25238-25242

184 Han, Y. H., Cao, X., Lin, B., Lin, F., Kolluri, S. K., Stebbins, J., Reed, J. C., Dawson, M. I. and Zhang, X. K. (2006) Regulation of Nur77 nuclear export by c-Jun N-terminal kinase and Akt. Oncogene. **25**, 2974-2986

185 Bode, A. M. and Dong, Z. (2007) The functional contrariety of JNK. Mol Carcinog. **46**, 591-598

186 Liu, J., Minemoto, Y. and Lin, A. (2004) c-Jun N-terminal protein kinase 1 (JNK1), but not JNK2, is essential for tumor necrosis factor alpha-induced c-Jun kinase activation and apoptosis. Mol Cell Biol. **24**, 10844-10856

187 Sabapathy, K. and Wagner, E. F. (2004) JNK2: a negative regulator of cellular proliferation. Cell Cycle. **3**, 1520-1523

188 Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Pincus, M. R., Gaarde, W. A., Holbrook, N. J., Shiloh, Y. and Ronai, Z. (2001) Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol Cell Biol. **21**, 2743-2754

189 Hyman, K. M., Seghezzi, G., Pintucci, G., Stellari, G., Kim, J. H., Grossi, E. A., Galloway, A. C. and Mignatti, P. (2002) Transforming growth factor-beta1 induces apoptosis in vascular endothelial cells by activation of mitogen-activated protein kinase. Surgery. **132**, 173-179

190 Kim, J. Y., Choi, J. A., Kim, T. H., Yoo, Y. D., Kim, J. I., Lee, Y. J., Yoo, S. Y., Cho, C. K., Lee, Y. S. and Lee, S. J. (2002) Involvement of p38 mitogen-activated protein kinase in the cell growth inhibition by sodium arsenite. J Cell Physiol. **190**, 29-37

191 Qi, X., Pramanik, R., Wang, J., Schultz, R. M., Maitra, R. K., Han, J., DeLuca, H. F. and Chen, G. (2002) The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D(3)-induced growth inhibition. J Biol Chem. **277**, 25884-25892

192 Bradham, C. and McClay, D. R. (2006) p38 MAPK in development and cancer. Cell Cycle. **5**, 824-828

193 De Nadal, E., Zapater, M., Alepuz, P. M., Sumoy, L., Mas, G. and Posas, F. (2004) The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature. **427**, 370-374

194 Saccani, S., Pantano, S. and Natoli, G. (2002) p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. Nat Immunol. **3**, 69-75

195 Kumar, S., Boehm, J. and Lee, J. C. (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov. **2**, 717-726

196 Prichett, W., Hand, A., Sheilds, J. and Dunnington, D. (1995) Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor alpha. J Inflamm. **45**, 97-105

197 Young, P., McDonnell, P., Dunnington, D., Hand, A., Laydon, J. and Lee, J. (1993) Pyridinyl imidazoles inhibit IL-1 and TNF production at the protein level. Agents Actions. **39 Spec No**, C67-69

198 Kontoyiannis, D., Kotlyarov, A., Carballo, E., Alexopoulou, L., Blackshear, P. J., Gaestel, M., Davis, R., Flavell, R. and Kollias, G. (2001) Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. Embo J. **20**, 3760-3770

199 Neininger, A., Kontoyiannis, D., Kotlyarov, A., Winzen, R., Eckert, R., Volk, H. D., Holtmann, H., Kollias, G. and Gaestel, M. (2002) MK2 targets AU-rich elements and regulates biosynthesis of tumor necrosis factor and interleukin-6 independently at different post-transcriptional levels. J Biol Chem. **277**, 3065-3068

200 Mazan-Mamczarz, K., Galban, S., Lopez de Silanes, I., Martindale, J. L., Atasoy, U., Keene, J. D. and Gorospe, M. (2003) RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. Proc Natl Acad Sci U S A. **100**, 8354-8359

201 Mukherjee, D., Gao, M., O'Connor, J. P., Raijmakers, R., Pruijn, G., Lutz, C. S. and Wilusz, J. (2002) The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. Embo J. **21**, 165-174

202 Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J., Stoecklin, G., Moroni, C., Mann, M. and Karin, M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell. **107**, 451-464

203 Brewer, G. and Ross, J. (1988) Poly(A) shortening and degradation of the 3' A+U-rich sequences of human c-myc mRNA in a cell-free system. Mol Cell Biol. **8**, 1697-1708

204 Lieberman, A. P., Pitha, P. M. and Shin, M. L. (1992) Poly(A) removal is the kinase-regulated step in tumor necrosis factor mRNA decay. J Biol Chem. **267**, 2123-2126

205 Barreau, C., Paillard, L. and Osborne, H. B. (2005) AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res. **33**, 7138-7150

206 Kedersha, N. and Anderson, P. (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans. **30**, 963-969

207 Bachelor, M. A., Silvers, A. L. and Bowden, G. T. (2002) The role of p38 in UVA-induced cyclooxygenase-2 expression in the human keratinocyte cell line, HaCaT. Oncogene. **21**, 7092-7099

208 Dean, J. L., Brook, M., Clark, A. R. and Saklatvala, J. (1999) p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. J Biol Chem. **274**, 264-269

209 Jang, B. C., Sanchez, T., Schaefers, H. J., Trifan, O. C., Liu, C. H., Creminon, C., Huang, C. K. and Hla, T. (2000) Serum withdrawal-induced post-transcriptional stabilization of cyclooxygenase-2 mRNA in MDA-MB-231 mammary carcinoma cells requires the activity of the p38 stress-activated protein kinase. J Biol Chem. **275**, 39507-39515

210 Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J. and Clark, A. R. (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. Mol Cell Biol. **20**, 4265-4274

211 Ridley, S. H., Dean, J. L., Sarsfield, S. J., Brook, M., Clark, A. R. and Saklatvala, J. (1998) A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. FEBS Lett. **439**, 75-80

212 Said, F. A., Werts, C., Elalamy, I., Couetil, J. P., Jacquemin, C. and Hatmi, M. (2002) TNF-alpha, inefficient by itself, potentiates IL-1beta-induced PGHS-2 expression in human pulmonary microvascular endothelial cells: requirement of NF-kappaB and p38 MAPK pathways. Br J Pharmacol. **136**, 1005-1014

213 Dixon, D. A., Balch, G. C., Kedersha, N., Anderson, P., Zimmerman, G. A., Beauchamp, R. D. and Prescott, S. M. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. J Exp Med. **198**, 475-481

214 Brook, M., Sully, G., Clark, A. R. and Saklatvala, J. (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. FEBS Lett. **483**, 57-61

215 Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K. and Holtmann, H. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. Embo J. **18**, 4969-4980

216 Han, Q., Leng, J., Bian, D., Mahanivong, C., Carpenter, K. A., Pan, Z. K., Han, J. and Huang, S. (2002) Rac1-MKK3-p38-MAPKAPK2 pathway promotes urokinase plasminogen activator mRNA stability in invasive breast cancer cells. J Biol Chem. **277**, 48379-48385

217 Zhao, W., Liu, M. and Kirkwood, K. L. (2007) p38alpha stabilizes interleukin-6 mRNA via multiple AU-rich elements. J Biol Chem

218 Asirvatham, A. J., Gregorie, C. J., Hu, Z., Magner, W. J. and Tomasi, T. B. (2007) MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. Mol Immunol

219 Schmid, C., Rutishauser, J., Schlapfer, I., Froesch, E. R. and Zapf, J. (1991) Intact but not truncated insulin-like growth factor binding protein-3 (IGFBP-3) blocks IGF-I induced stimulation of osteobalsts: control of IGF signalling to bone cells by IGFBP-3 specific proteolysis? Biochemical and Biophysical Research Communications. **179**, 579-585

220 Schmid, C., Keller, C., Schlapfer, I., Veldman, C. and Zapf, J. (1998) Calcium and insulin-like growth factor I stimulation of sodium-dependent phosphate transport and proliferation of cultured rat osteoblasts. Biochem Biophys Res Commun. **245**, 220-225

221 Rogers, J., Wiltrout, L., Nanu, L. and Fant, M. E. (1996) Developmentally regulated expression of IGF binding protein-3 (IGFBP-3) in human placental fibroblasts: effect of exogenous IGFBP-3 on IGF-1 action. Regul Pept. **61**, 189-195

222 Imai, Y., Moralez, A., Andag, U., Clarke, J. B., Busby, W. H., Jr. and Clemmons, D. R. (2000) Substitutions for hydrophobic amino acids in the N-terminal domains of IGFBP-3 and -5 markedly reduce IGF-I binding and alter their biologic actions. The Journal of Biological Chemistry. **275**, 18188-18194

223 Cohen, P. (2006) Insulin-like growth factor binding protein-3: insulin-like growth factor independence comes of age. Endocrinology. **147**, 2109-2111

224 Oh, Y., Muller, H. L., Lamson, G. and Rosenfeld, R. G. (1993) Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. Cell surface binding and growth inhibition. J Biol Chem. **268**, 14964-14971

225 Cohen, P., Lamson, G., Okajima, T. and Rosenfeld, R. G. (1993) Transfection of the human insulin-like growth factor binding protein-3 gene into Balb/c fibroblasts inhibits cellular growth. Mol Endocrinol. **7**, 380-386

226 Valentinis, B., Bhala, A., DeAngelis, T., Baserga, R. and Cohen, P. (1995) The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol Endocrinol. **9**, 361-367

227 Hong, J., Zhang, G., Dong, F. and Rechler, M. M. (2002) Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. The Journal of Biological Chemistry. **277**, 10489-10497

228 Spagnoli, A., Torello, M., Nagalla, S. R., Horton, W. A., Pattee, P., Hwa, V., Chiarelli, F., Roberts, C. T., Jr. and Rosenfeld, R. G. (2002) Identification of STAT-1 as a molecular target of IGFBP-3 in the process of chondrogenesis. J Biol Chem. **277**, 18860-18867

229 Longobardi, L., Torello, M., Buckway, C., O'Rear, L., Horton, W. A., Hwa, V., Roberts, C. T., Jr., Chiarelli, F., Rosenfeld, R. G. and Spagnoli, A. (2003) A novel insulin-like growth factor (IGF)-independent role for IGF binding protein-3 in mesenchymal chondroprogenitor cell apoptosis. Endocrinology. **144**, 1695-1702

230 Spoerri, P. E., Caballero, S., Wilson, S. H., Shaw, L. C. and Grant, M. B. (2003) Expression of IGFBP-3 by human retinal endothelial cell cultures: IGFBP-3 involvement in growth inhibition and apoptosis. Invest Ophthalmol Vis Sci. **44**, 365-369

231 Granata, R., De Petrini, M., Trovato, L., Ponti, R., Pons, N., Ghe, C., Graziani, A., Ferry, R. J., Jr., Muccioli, G. and Ghigo, E. (2003) Insulin-like growth factor binding protein-3 mediates serum starvation- and doxorubicin-induced apoptosis in H9c2 cardiac cells. J Endocrinol Invest. **26**, 1231-1241

232 Lee, H. Y., Chun, K. H., Liu, B., Wiehle, S. A., Cristiano, R. J., Hong, W. K., Cohen, P. and Kurie, J. M. (2002) Insulin-like growth factor binding protein-3 inhibits the growth of non-small cell lung cancer. Cancer Res. **62**, 3530-3537.

233 Perks, C. M., McCaig, C., Clarke, J. B., Clemmons, D. R. and Holly, J. M. (2002) A non-IGF binding mutant of IGFBP-3 modulates cell function in breast epithelial cells. Biochemistry and Biophysics Research Communications. **294**, 988-994.

234 Vasylyeva, T. L., Chen, X. and Ferry, R. J., Jr. (2005) Insulin-like growth factor binding protein-3 mediates cytokine-induced mesangial cell apoptosis. Growth Horm IGF Res. **15**, 207-214

235 Shim, M. L., Levitt Katz, L. E., Davis, J., Dotzler, W. C., Cohen, P. and Ferry, R. J., Jr. (2004) Insulin-like growth factor binding protein-3 is a novel mediator of apoptosis in insulin-secreting cells. Growth Horm IGF Res. **14**, 216-225

236 Silha, J. V., Sheppard, P. C., Mishra, S., Gui, Y., Schwartz, J., Dodd, J. G. and Murphy, L. J. (2006) Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. Endocrinology. **147**, 2112-2121

237 Lee, K. W., Liu, B., Ma, L., Li, H., Bang, P., Koeffler, H. P. and Cohen, P. (2004) Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. J Biol Chem. **279**, 469-476

238 Weinzimer, S. A., Gibson, T. B., Collett-Solberg, P. F., Khare, A., Liu, B. and Cohen, P. (2001) Transferrin is an insulin-like growth factor-binding protein-3 binding protein. J Clin Endocrinol Metab. **86**, 1806-1813.

239 Baumrucker, C. R., Gibson, C. A. and Schanbacher, F. L. (2003) Bovine lactoferrin binds to insulin-like growth factor-binding protein-3. Domest Anim Endocrinol. **24**, 287-303

240 Gibson, C. A., Staley, M. D. and Baumrucker, C. R. (1999) Identification of IGF binding proteins in bovine milk and the demonstration of IGFBP-3 synthesis and release by bovine mammary epithelial cells. J Anim Sci. **77**, 1547-1557

241 Butt, A. J., Fraley, K. A., Firth, S. M. and Baxter, R. C. (2002) IGF-binding protein-3-induced growth inhibition and apoptosis do not require cell surface binding and nuclear translocation in human breast cancer cells. Endocrinology. **143**, 2693-2699

242 Kim, H. S., Ingermann, A. R., Tsubaki, J., Twigg, S. M., Walker, G. E. and Oh, Y. (2004) Insulin-like growth factor-binding protein 3 induces caspase-dependent apoptosis through a death receptor-mediated pathway in MCF-7 human breast cancer cells. Cancer Res. **64**, 2229-2237

243 Gill, Z. P., Perks, C. M., Newcomb, P. V. and Holly, J. M. (1997) Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. The Journal of Biological Chemistry. **272(41)**, 25602-25607

244 Radulescu, R. T. (1994) Nuclear localization signal in insulin-like growth factor-binding protein type 3. Trends Biochem Sci. **19**, 278

245 Schedlich, L. J., Le Page, S. L., Firth, S. M., Briggs, L. J., Jans, D. A. and Baxter, R. C. (2000) Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. J Biol Chem. **275**, 23462-23470

246 Schedlich, L. J., Graham, L. D., O'Han, M. K., Muthukaruppan, A., Yan, X., Firth, S. M. and Baxter, R. C. (2007) Molecular basis of the interaction between IGFBP-3 and retinoid X receptor: role in modulation of RAR-signaling. Arch Biochem Biophys. **465**, 359-369

247 Lee, K. W., Ma, L., Yan, X., Liu, B., Zhang, X. K. and Cohen, P. (2005) Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRalpha/Nur77. J Biol Chem. **280**, 16942-16948

248 Liu, B., Lee, K. W., Li, H., Ma, L., Lin, G. L., Chandraratna, R. A. and Cohen, P. (2005) Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands synergize on prostate cancer cell apoptosis in vitro and in vivo. Clin Cancer Res. **11**, 4851-4856

249 Lee, K. W., Cobb, L. J., Paharkova-Vatchkova, V., Liu, B., Milbrandt, J. and Cohen, P. (2007) Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3. Carcinogenesis. **28**, 1653-1658

250 Santer, F. R., Bacher, N., Moser, B., Morandell, D., Ressler, S., Firth, S. M., Spoden, G. A., Sergi, C., Baxter, R. C., Jansen-Durr, P. and Zwerschke, W. (2006) Nuclear insulin-like growth factor binding protein-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis. Cancer Res. **66**, 3024-3033

251 Bhattacharyya, N., Pechhold, K., Shahjee, H., Zappala, G., Elbi, C., Raaka, B., Wiench, M., Hong, J. and Rechler, M. M. (2006) Nonsecreted insulin-like growth factor

binding protein-3 (IGFBP-3) can induce apoptosis in human prostate cancer cells by IGF-independent mechanisms without being concentrated in the nucleus. J Biol Chem. **281**, 24588-24601

252 Butt, A. J., Firth, S. M., King, M. A. and Baxter, R. C. (2000) Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. The Journal of Biological Chemistry. **275**, 39174-39181

253 Coverley, J. A. and Baxter, R. C. (1995) Regulation of insulin-like growth factor (IGF) binding protein-3 phosphorylation by IGF-I. Endocrinology. **136**, 5778-5781

254 Mishra, S. and Murphy, L. J. (2003) Phosphorylation of insulin-like growth factor (IGF) binding protein-3 by breast cancer cell membranes enhances IGF-I binding. Endocrinology. **144**, 4042-4050

255 Mishra, S. and Murphy, L. J. (2004) Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. J Biol Chem. **279**, 23863-23868

256 Schedlich, L. J., Nilsen, T., John, A. P., Jans, D. A. and Baxter, R. C. (2003) Phosphorylation of insulin-like growth factor binding protein-3 by deoxyribonucleic acid-dependent protein kinase reduces ligand binding and enhances nuclear accumulation. Endocrinology. **144**, 1984-1993

257 Cobb, L. J., Liu, B., Lee, K. W. and Cohen, P. (2006) Phosphorylation by DNA-dependent protein kinase is critical for apoptosis induction by insulin-like growth factor binding protein-3. Cancer Res. **66**, 10878-10884

258 Coverley, J. A., Martin, J. L. and Baxter, R. C. (2000) The effect of phosphorylation by casein kinase 2 on the activity of insulin-like growth factor-binding protein-3. Endocrinology. **141**, 564-570

259 Firth, S. M. and Baxter, R. C. (1999) Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. J Endocrinol. **160**, 379-387

260 Mitsui, Y., Mochizuki, S., Kodama, T., Shimoda, M., Ohtsuka, T., Shiomi, T., Chijiiwa, M., Ikeda, T., Kitajima, M. and Okada, Y. (2006) ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. Cancer Res. **66**, 9913-9920

261 Santer, F. R., Moser, B., Spoden, G. A., Jansen-Durr, P. and Zwerschke, W. (2007) Human papillomavirus type 16 E7 oncoprotein inhibits apoptosis mediated by nuclear insulin-like growth factor binding protein-3 by inducing its ubiquitin/proteasome-dependent degradation. Carcinogenesis

262 Nakamura, M., Miyamoto, S., Maeda, H., Ishii, G., Hasebe, T., Chiba, T., Asaka, M. and Ochiai, A. (2005) Matrix metalloproteinase-7 degrades all insulin-like growth factor binding proteins and facilitates insulin-like growth factor bioavailability. Biochem Biophys Res Commun. **333**, 1011-1016

263 Miyamoto, S., Yano, K., Sugimoto, S., Ishii, G., Hasebe, T., Endoh, Y., Kodama, K., Goya, M., Chiba, T. and Ochiai, A. (2004) Matrix metalloproteinase-7 facilitates insulin-like growth factor bioavailability through its proteinase activity on insulin-like growth factor binding protein 3. Cancer Res. **64**, 665-671

264 Sadowski, T., Dietrich, S., Koschinsky, F. and Sedlacek, R. (2003) Matrix metalloproteinase 19 regulates insulin-like growth factor-mediated proliferation, migration, and adhesion in human keratinocytes through proteolysis of insulin-like growth factor binding protein-3. Mol Biol Cell. **14**, 4569-4580

265 Cohen, P., Peehl, D. M., Graves, H. C. and Rosenfeld, R. G. (1994) Biological effects of prostate specific antigen as an insulin-like growth factor binding protein-3 protease. J Endocrinol. **142**, 407-415

266 Angelloz-Nicoud, P. and Binoux, M. (1995) Autocrine regulation of cell proliferation by the insulin-like growth factor (IGF) and IGF binding protein-3 protease system in a human prostate carcinoma cell line (PC-3). Endocrinology. **136**, 5485-5492

267 Lalou, C., Lassarre, C. and Binoux, M. (1996) A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinology. **137**, 3206-3212

268 Salahifar, H., Firth, S. M., Baxter, R. C. and Martin, J. L. (2000) Characterization of an amino-terminal fragment of insulin-like growth factor binding protein-3 and its effects in MCF-7 breast cancer cells. Growth Horm IGF Res. **10**, 367-377

269 Angelloz-Nicoud, P., Lalou, C. and Binoux, M. (1998) Prostate carcinoma (PC-3) cell proliferation is stimulated by the 22-25-kDa proteolytic fragment (1-160) and inhibited by the 16-kDa fragment (1-95) of recombinant human insulin-like growth factor binding protein-3. Growth Horm IGF Res. **8**, 71-75

270 Zadeh, S. M. and Binoux, M. (1997) The 16-kDa proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 inhibits the mitogenic action of fibroblast growth factor on mouse fibroblasts with a targeted disruption of the type 1 IGF receptor gene. Endocrinology. **138**, 3069-3072

271 Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L. and Magun, B. E. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol Cell Biol. **17**, 3373-3381

272 Baxter, R. C., Butt, A. J., Schedlich, L. J. and Martin, J. L. (2000) Antiproliferative and pro-apoptotic activities of insulin-like growth factor-binding protein-3. Growth Horm IGF Res. **10 Suppl A**, S10-11

273 Firth, S. M. and Baxter, R. C. (2002) Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. **23**, 824-854

274 Rozen, F., Zhang, J. and Pollak, M. (1998) Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breastcancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. Int J Oncol. **13**, 865-869

275 Buckbinder, L., Talbott, R., Velasco-Miguel, S., Takenaka, I., Faha, B., Seizinger, B. R. and Kley, N. (1995) Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature. **377**, 646-649

276 Huynh, H., Yang, X. and Pollak, M. (1996) Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. J Biol Chem. **271**, 1016-1021

277 Erondu, N. E., Dake, B. L., Moser, D. R., Lin, M., Boes, M. and Bar, R. S. (1996) Regulation of endothelial IGFBP-3 synthesis and secretion by IGF-I and TGF-beta. Growth Regul. **6**, 1-9

278 Martin, J. L., Coverley, J. A., Pattison, S. T. and Baxter, R. C. (1995) Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. Endocrinology. **136**, 1219-1226

279 Anwar, A., Zahid, A. A., Scheidegger, K. J., Brink, M. and Delafontaine, P. (2002) Tumor necrosis factor-alpha regulates insulin-like growth factor-1 and insulin-like growth factor binding protein-3 expression in vascular smooth muscle. Circulation. **105**, 1220-1225

280 Ali, O., Cohen, P. and Lee, K. W. (2003) Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. Horm Metab Res. **35**, 726-733

281 Huynh, H. T., Robitaille, G. and Turner, J. D. (1991) Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res. **197**, 191-199

282 Sivaprasad, U., Fleming, J., Verma, P. S., Hogan, K. A., Desury, G. and Cohick, W. S. (2004) Stimulation of insulin-like growth factor (IGF) binding protein-3 synthesis by IGF-I and transforming growth factor-alpha is mediated by both phosphatidylinositol-3 kinase and mitogen-activated protein kinase pathways in mammary epithelial cells. Endocrinology. **145**, 4213-4221

283 Grill, C. J., Sivaprasad, U. and Cohick, W. S. (2002) Constitutive expression of IGF-binding protein-3 by mammary epithelial cells alters signaling through Akt and p70S6 kinase. J Mol Endocrinol. **29**, 153-162

284 Cohick, W. S. and Turner, J. D. (1998) Regulation of IGF binding protein synthesis by a bovine mammary epithelial cell line. J Endocrinol. **157**, 327-336

285 Fleming, J. M., Leibowitz, B. J., Kerr, D. E. and Cohick, W. S. (2005) IGF-I differentially regulates IGF-binding protein expression in primary mammary fibroblasts and epithelial cells. J Endocrinol. **186**, 165-178

286 Chang, Y. S., Cho, J. Y., Cho, H. A., Kim, H. J., Chang, J., Ahn, C. M., Kim, S. K. and Kim, S. K. (2006) 9-cis retinoic acid induces insulin-like growth factor binding protein-3 through DR-8 retinoic acid responsive elements. Cancer Biol Ther. **5**, 586-592

287 Shen, X., Zhang, K. and Kaufman, R. J. (2004) The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. J Chem Neuroanat. **28**, 79-92

288 Korcheva, V., Wong, J., Lindauer, M., Jacoby, D. B., Iordanov, M. S. and Magun, B. (2007) Role of apoptotic signaling pathways in regulation of inflammatory responses to ricin in primary murine macrophages. Mol Immunol. **44**, 2761-2771

289 Rajah, R., Valentinis, B. and Cohen, P. (1997) Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem. **272**, 12181-12188

290 Hong, J., Zhang, G., Dong, F. and Rechler, M. M. (2002) Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. J Biol Chem. **277**, 10489-10497

291 Butt, A. J., Firth, S. M., King, M. A. and Baxter, R. C. (2000) Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. J Biol Chem. **275**, 39174-39181

292 Fowler, C. A., Perks, C. M., Newcomb, P. V., Savage, P. B., Farndon, J. R. and Holly, J. M. (2000) Insulin-like growth factor binding protein-3 (IGFBP-3) potentiates paclitaxel-induced apoptosis in human breast cancer cells. Int J Cancer. **88**, 448-453

293 Hollowood, A. D., Lai, T., Perks, C. M., Newcomb, P. V., Alderson, D. and Holly, J. M. (2000) IGFBP-3 prolongs the p53 response and enhances apoptosis following UV irradiation. Int J Cancer. **88**, 336-341

294 Rajah, R., Lee, K. W. and Cohen, P. (2002) Insulin-like growth factor binding protein-3 mediates tumor necrosis factor-alpha-induced apoptosis: role of Bcl-2 phosphorylation. Cell Growth Differ. **13**, 163-171

295 Martin, J. L., Weenink, S. M. and Baxter, R. C. (2003) Insulin-like growth factor-binding protein-3 potentiates epidermal growth factor action in MCF-10A mammary epithelial cells. Involvement of p44/42 and p38 mitogen-activated protein kinases. J Biol Chem. **278**, 2969-2976

296 Chen, X. and Ferry, R. J., Jr. (2006) Novel actions of IGFBP-3 on intracellular signaling pathways of insulin-secreting cells. Growth Horm IGF Res. **16**, 41-48

297 Bilsland, A. E., Stevenson, K., Atkinson, S., Kolch, W. and Keith, W. N. (2006) Transcriptional repression of telomerase RNA gene expression by c-Jun-NH2-kinase and Sp1/Sp3. Cancer Res. **66**, 1363-1370

298 Erondu, N. E., Toland, B., Boes, M., Dake, B., Moser, D. R. and Bar, R. S. (1997) Bovine insulin-like growth factor binding protein-3: organization of the chromosomal gene and functional analysis of its promoter. Endocrinology. **138**, 2856-2862

299 Wellnitz, O. and Kerr, D. E. (2004) Cryopreserved bovine mammary cells to model epithelial response to infection. Vet Immunol Immunopathol. **101**, 191-202

300 Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F. and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. **50**, 6075-6086

301 Owens, R. B. (1974) Glandular epithelial cells from mice: a method for selective cultivation. J Natl Cancer Inst. **52**, 1375-1378

302 Cohick, W. S., Wang, B., Verma, P. and Boisclair, Y. R. (2000) Insulin-Like growth factor I (IGF-I) and cyclic adenosine 3',5'-monophosphate regulate IGF-binding protein-3 gene expression by transcriptional and posttranscriptional mechanisms in mammary epithelial cells. Endocrinology. **141**, 4583-4591

303 Hori, T., Kondo, T., Tabuchi, Y., Takasaki, I., Zhao, Q. L., Kanamori, M., Yasuda, T. and Kimura, T. (2007) Molecular mechanism of apoptosis and gene expressions in human lymphoma U937 cells treated with anisomycin. Chem Biol Interact

304 Ganju, N. and Eastman, A. (2003) Zinc inhibits Bax and Bak activation and cytochrome c release induced by chemical inducers of apoptosis but not by death-receptor-initiated pathways. Cell Death Differ. **10**, 652-661

305 Zhang, X. K. (2007) Targeting Nur77 translocation. Expert Opin Ther Targets. **11**, 69-79

306 Cao, X., Liu, W., Lin, F., Li, H., Kolluri, S. K., Lin, B., Han, Y. H., Dawson, M. I. and Zhang, X. K. (2004) Retinoid X receptor regulates Nur77/TR3-dependent apoptosis [corrected] by modulating its nuclear export and mitochondrial targeting. Mol Cell Biol. **24**, 9705-9725

307 Bogoyevitch, M. A. and Kobe, B. (2006) Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev. **70**, 1061-1095

308 Inaoka, Y., Yazawa, T., Uesaka, M., Mizutani, T., Yamada, K. and Miyamoto, K. (2007) Regulation of NGFI-B/Nur77 gene expression in the rat ovary and in leydig tumor cells MA-10. Mol Reprod Dev

APPENDIX

Luciferase Constructs

The 3'UTR of IGFBP-3 (1424 bp) was cloned into the pGL3-promoter vector (Promega) to produce pGL3-BP3-3'UTR (Figure 1) according to the following scheme:

1. Preparation of the plasmid
    a. Digested pGL3-promoter vector with Xba I and Sal I to remove SV-40 late poly(A) sequence.
    b. Purified the plasmid DNA by agarose gel electrophoresis and gel extraction.
2. Preparation of IGFBP-3 3'UTR cDNA (bp 1010-2434 of GenBank NM_174556)
    a. Isolated total RNA from serum treated MAC-T cells
    b. Performed RT reaction using 2 μM gene specific primer (5'-GGGGGTCGACTTTCTTTATGGTAAAAAT-3')
        i. 65°C, 5 min (RNA, Primer, dNTP, H_2O)
        ii. Ice 1 min
        iii. Add 5X buffer, DTT
            1. (42°C, 2 min)
        iv. Add SuperScript II RT
            1. 42°C, 50 min
            2. 70°C 15 min
    c. Amplified 3'UTR by PCR using touchdown protocol
        i. F primer: 5'-GGGGTCTAGATAGACTGTGGCCACTTAA-3'

ii. R primer: 5'-GGGGGTCGACTTTCTTTATGGTAAAAAT-3'

1. Underlined bases are Xba I and Sal I restriction sites

iii. Cycling Parameters

1. 95°C 2 min
2. 95°C 30 sec
3. 60°C 30 sec*
4. 72°C 90 sec
5. 72°C 10 min
6. 4°C ∞
7. *annealing temperature decrease by 0.5°C every cycle for 30 cycles, then 15 cycles are run at lowest temperature.

d. Purified amplified DNA by agarose gel electrophoresis and gel extraction

e. Digested PCR product with BamH1 to verify product

3. Cloning of IGFBP-3 3'UTR

a. TOPO cloning

i. Combined 2 µl purified PCR product, 1 µl salt solution, 2 µl H_2O, 1 µl TOPO vector

ii. Incubated 15 min at room temperature

iii. Added 2 µl reaction to 50 µl TOP10 cells

iv. Incubated on ice 25 min

v. Heat shocked cells 45 s at 42°C

vi. Incubated on ice 2 min

vii. Added 250 µl S.O.C. medium, incubated 1 h at 37°C, 225 rpm

viii. Spread 50 ul of cells on LB+amp plate

ix. Incubated overnight at 37^{o}C

x. Picked six colonies and grew 5 ml LB+amp cultures for 12 h

xi. Concurrently performed colony PCR on same six colonies to verify positive clones (used T7 and T3 primers)

xii. Inoculated 100 ml LB+amp culture with 500 µl of colony 5 small culture, grew overnight at 37^{o}C, 225 rpm

xiii. Purified plasmid DNA with Qiagen maxi-prep kit

xiv. Verified DNA by sequencing

b. pGL3 cloning

i. Digested 2 µg DNA with Xba I and Sal I for 2 h at 37^{o}C

ii. Purified DNA by gel extraction

iii. Ligated 100 ng of Xba I and Sal I digested pGL3-promoter vector with 84 ng Xba I and Sal I digested 3'UTR DNA for 15 min at room temperature with T4 DNA ligase

iv. Added 2 µl ligation product to 50 µl TOP10 cells and transformed as before

v. Picked 10 colonies and performed colony PCR as before with gene specific primers

vi. Inoculated 100 ml LB+amp culture with 500 µl of colony 9, grew overnight at 37^{o}C, 225 rpm

vii. Purified plasmid DNA with maxi-prep kit

viii. Restriction digested DNA to verify insert and orientation

The pGL3-BP3-5'UTR (Figure 2) and pGL3-BP3-UTRs (Figure 3) plasmids were constructed as follows:

1. Preparation of plasmid
    a. Digested pGL3-promoter vector and pGL3-BP3-3'UTR vector with Hind III and Nco I to remove to produce correct sticky ends
    b. Purified the plasmid DNA by agarose gel electrophoresis and gel extraction.
2. 5"UTR sequence (137 bp) is highly GC rich (87%)
    a. Synthesized by GenScript Corp. to contain Hind III and Nco I restriction sites in 5' and 3' ends, respectively
    b. Provided in storage vector
3. Cloning into pGL3-promoter vector
    a. Digested 2 µg storage plasmid with Hind III and Nco I to excise 5'UTR
    b. Purified DNA by gel extraction
    c. Ligated 100 ng Hind III and Nco I digested pGL3 plasmids with 9 ng insert overnight at 17^{o}C with T4 DNA ligase
    d. Transformed 2 µl ligation product into 50 µl TOP10 cells as described
    e. Verified positive clones by restriction digest
    f. Grew and purified maxi-prep cultures as described

A pair of AU-rich elements (AREs) was deleted from the 3' end of the IGFBP-3 3'UTR according to the following protocol:

1. Primer design

a. Primers were designed to span the region to be deleted

b. F: 5'-GAAAAGTTTGTCTTGCAATGTATTTA—
AAAATTTTTACCATAAAGAAAGTCGAC-3'

c. R:5'-GTCGACTTTCTTTATGGTAAAAATTTT—
TAAATACATTGCAAGACAAACTTTTC-3'

2. Cycling parameters

a. 95^{o}C, 30 s

b. 18 cycles

i. 95^{o}C 30 s

ii. 55^{o}C 1 min

iii. 68^{o}C 6 min 15 s

3. Mutant selection and transformation

a. Cooled PCR reaction to room temp

b. Added 1 µl (10 U) Dpn I

c. Incubated 37^{o}C, 1 h

d. Added 1 µl Dpn I digested DNA to 50 µl XL1-Blue competent cells on ice for 30 min

e. Heat shocked cells at 42^{o}C for 45 s

f. Cooled on ice for 2 min

g. Added 500 µl 42^{o}C NZY^{+} broth

h. Incubated 37^{o}C, 1h, 225 rpm

i. Spread 250 µl cells on each of two LB+amp plates

j. Incubated plates 37^{o}C overnight

k. Picked 4 colonies, grew 5 ml LB+amp cultures for 12 h

l. Purified plasmid DNA by mini-prep

m. Verified mutation by sequencing

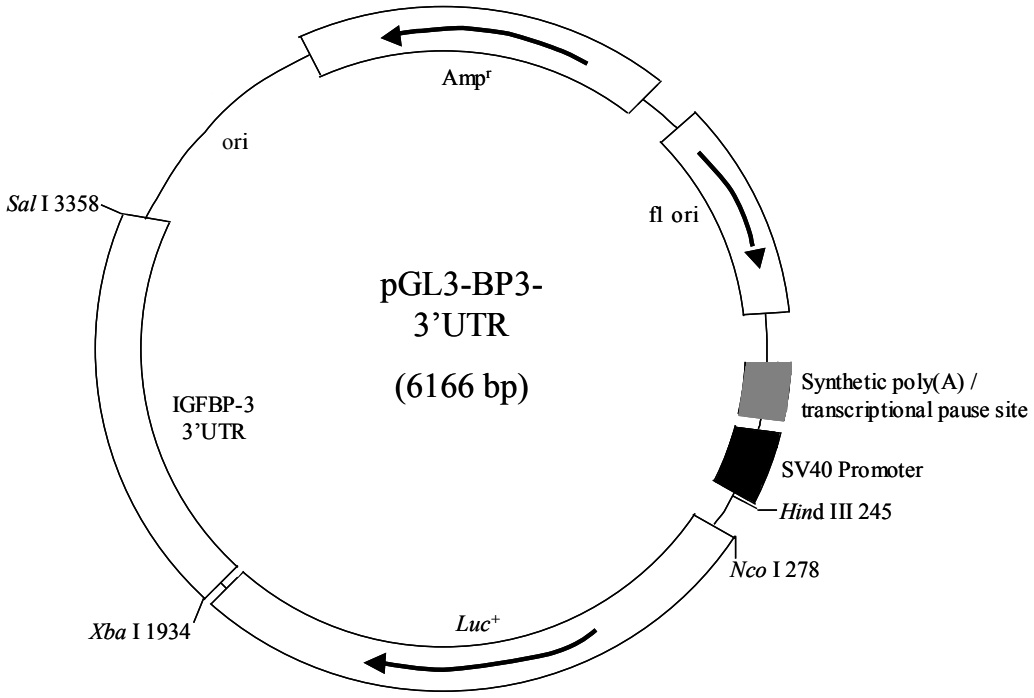
TUNEL Assay

TUNEL assays were performed on ANS-treated cells transfected with scrambled, IGFBP-3 or IGFBP-2 siRNA according to the following protocol:

1. Preparation of cells
    a. MAC-T cells were plated at 3.5 x 10^4 cells/cm^2 in chamber slides (0.69 cm^2 surface area) in phenol red-free DMEM-H + 10% FBS (no antibiotics)
2. Transfection
    a. 24 h after plating, cells were transfected with siRNA
        i. 50 nM scramble, IGFBP-3 or IGFBP-2
    b. combined 8 µl GeneEraser transfection reagent (Stratagene) with 100 µl serum-free media for 15 min at room temperature
    c. added siRNA oligos to mixture for 15 min at room temperature
    d. removed media from cells, added 200 µl fresh DMEM-H + 10% FBS
    e. added 55 µl transfection mixture to each well
3. Treatment of cells
    a. 48 h after transfection, cells washed twice in serum-free media
    b. incubated overnight in serum-free media supplemented with 0.2% BSA, 30 nM sodium selenite, 1 µg/ml gentamycin.
    c. Cells were treated for 6 h in fresh media +/- 0.1 µM ANS

4. TUNEL assay
    a. Treatments were removed and cells were washed once in 300 µl PBS
    b. Cells were fixed 1 h at room temperature with 400 µl 4% paraformaldehyde
    c. Cells were washed with 400 µl PBS
    d. Cells were permeablized for 2 min on ice with 400 µl 0.1% Triton X-100 in 0.1% sodium citrate
    e. Washed cells in PBS
    f. DNase treated positive control 10 min at room temperature
        i. 244 µl PBS + 40 µl DNase I (2.7 U/µl, Qiagen)
    g. washed control well 5 X in PBS
    h. removed chambers from slides by blotting edges with 70% ethanol and incubating at room temp for 5 min
    i. combined 50 µl Enzyme solution with 450 µl Labeling solution
    j. added 17 µl TUNEL reaction solution to each sample
    k. added cover slips and incubated 1 h at 37^{o}C, 5% CO_2
    l. rinsed slides in PBS
    m. added 20 µl/sample Hoechst 33342 stain (1µg/ml in H_2O)
        i. incubated 10 min at room temperature in the dark
    n. rinsed slides 3 x in PBS
    o. added 30 µl Prolong Antifade reagent to each group of 4 wells
    p. covered with cover slips and let dry overnight
    q. took TUNEL and Hoechst images with fluorescent microscope

Figure 1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3'UTR
(6166 bp)
IGFBP-3
3'UTR
Synthetic poly(A) /
transcriptional pause site
SV40 Promoter
Hind III 245
Nco I 278
Luc+
Xba I 1934

Figure 2

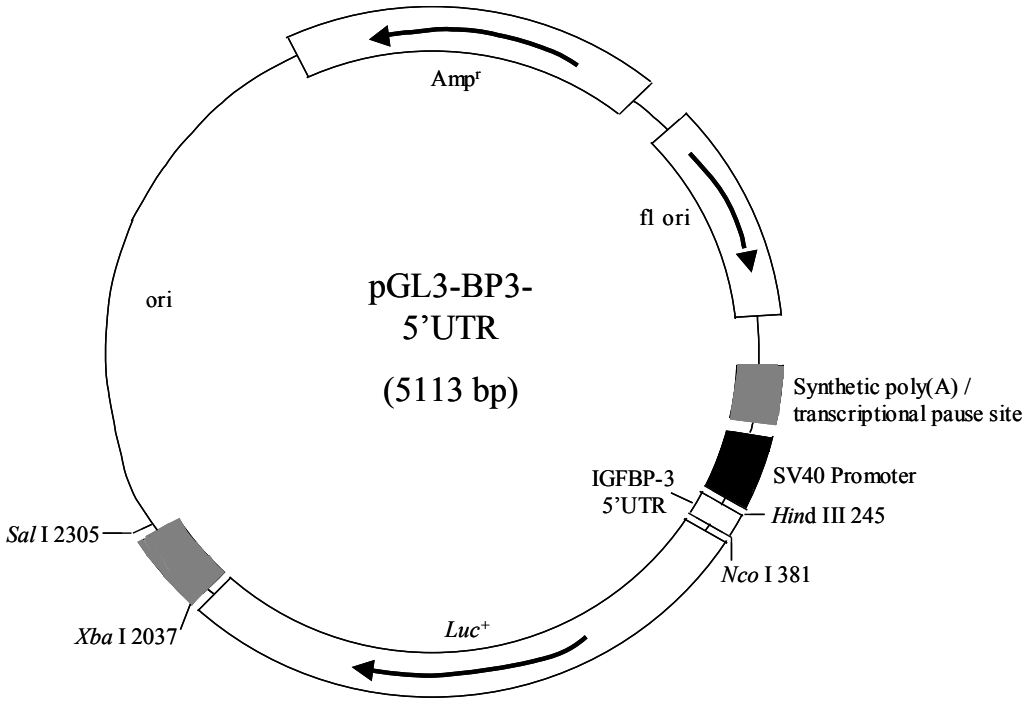
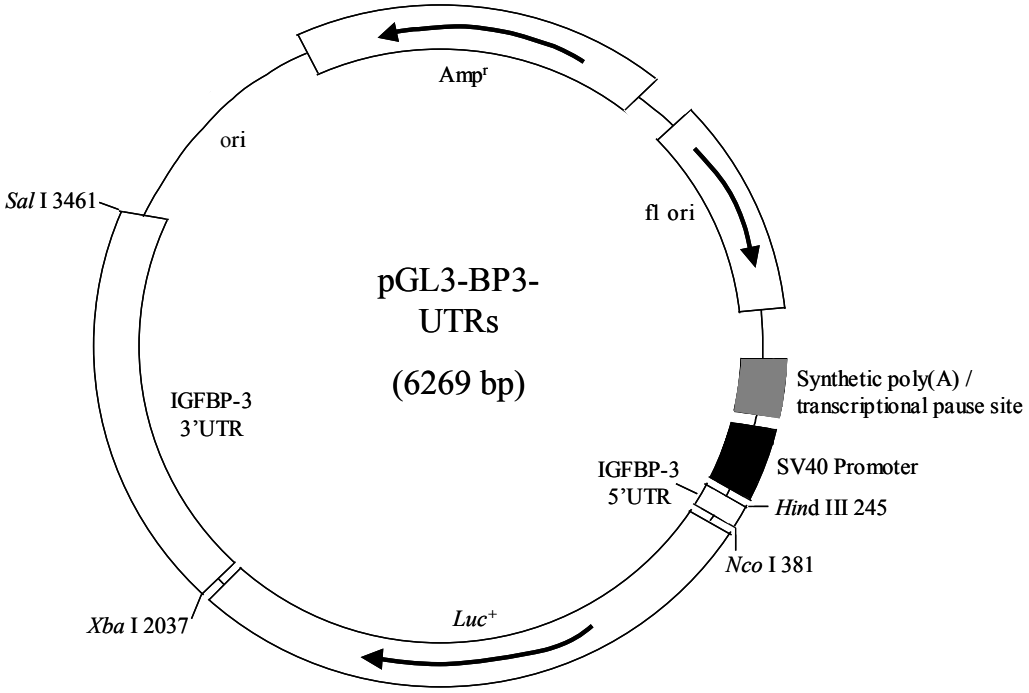

Amp^r
fl ori
ori
pGL3-BP3-
5'UTR
(5113 bp)
Synthetic poly(A) /
transcriptional pause site
SV40 Promoter
IGFBP-3
5'UTR
Hind III 245
Nco I 381
Sal I 2305
Xba I 2037
Luc+

Figure 3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(6269 bp)
Synthetic poly(A) /
transcriptional pause site
IGFBP-3
3'UTR
IGFBP-3
5'UTR
SV40 Promoter
Hind III 245
Nco I 381
Luc+
Xba I 2037

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B.S. May 2002. Genetics and Development, Cornell University, Ithaca, NY

Work Experience:

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September 2002-May 2003: General Biology Laboratory Instructor
- Responsible for two weekly laboratory sections covering a variety of introductory biology topics

September-December 2003: Introductory Animal Science,
- Administrative responsibilities

January-May 2004, 2005, 2008: Animal Microtechniques and Tissue Culture
- Twice weekly laboratory sections covering principles of mammalian tissue culture techniques including differentiation and transfection

September-December 2004, 2005: Animal Nutrition Laboratory
- Weekly laboratory sections covering animal nutrition topics including ruminant and non-ruminant nutrition and digestive tract physiology

January-May 2006: Laboratory Animal Science: Management and Techniques
- Weekly laboratory sections covering proper animal handling, as well as simple surgical techniques including ovarectomy and castration

September-December 2007: Endocrinology
- Administrative responsibilities

Publications:

Leibowitz, BJ and Cohick, WS. IGFBP-3 and JNK-2 are required for Nur77-mediated apoptosis during ribotoxic stress-induced activation of the intrinsic apoptotic pathway in mammary epithelial cells. (in preparation)

Leibowitz, BJ, Sivaprasad, S, Jetzt, A, Cohick, WS. Regulation of pro-apoptotic IGFBP-3 by p38 MAPK during ribotoxic stress in mammary epithelial cells. (submitted for review)

Fleming, JM, **Leibowitz, BJ**, Kerr, DE, Cohick, WS. IGF-1 differentially regulates IGF binding protein expression in primary mammary fibroblasts and epithelial cells. J Endocrinol. 2005 Jul;186(1):165-78