Journal of Cellular Biochemistry 86:583–589 (2002)

Evidence Implicating a Mid-Region Sequence of IGFBP-3 in Its Specific IGF-Independent Actions

A.D. Hollowood,[1] C.E.H. Stewart,[1] C.M. Perks,[1] J.M. Pell,[2] T. Lai,[1] D. Alderson,[1] and J.M.P. Holly[1]*

[1]University Department of Surgery, Bristol Royal Infirmary, Bristol, BS2 8HW, United Kingdom
[2]Babraham Institute, Cambridge, CB2 4AT, United Kingdom

Abstract Insulin-like growth factor binding protein-3 (IGFBP-3) is one of six high affinity-binding proteins that share a common function in regulating the bioavailability of the insulin-like growth factors. The six binding proteins have highly conserved C- and N-terminals that are essential to this function. Additionally, they all have specific functions on cellular homeostasis independent to the regulation of the insulin-like growth factors. It has previously been shown that insulin-like growth factor binding protein-3 can accentuate UV-induced apoptosis in a human carcinoma cell line. Using the KYSE 190 oesophageal carcinoma cell line we have demonstrated that a 15 amino acid (aa) peptide that lies within the mid-region of the protein can mimic the effect of the intact protein. This region contains the serine residues Ser^{111} and Ser^{113}. Using two protocols, we modified these serine residues and have shown that both phosphorylation and derivatization of IGFBP-3 can negate the accentuation of UV-induced cell death. These three independent pieces of evidence support the hypothesis that the variable mid-region is responsible for the specific pro-apoptotic functions of IGFBP-3, and suggest that phosphorylation may provide a mechanism for regulation of this action. J. Cell. Biochem. 86: 583–589, 2002.

Key words: IGFBP-3; apoptosis; structure/function

Insulin-like growth factor binding protein-3 (IGFBP-3) is one of the six high affinity-binding proteins that regulate the bioavailability of the insulin-like growth factors-I and -II (IGF-I and -II), and can thus exhibit growth regulatory effects on cells. However, in addition, it has been shown that IGF-independent inhibition of cell growth can be induced by IGFBP-3 in various cell lines, for example, in the Hs587T breast carcinoma cell line via an interaction with possible cell surface binding sites [Oh et al., 1993]. Recent evidence also indicates that apoptosis can occur following direct exposure to IGFBP-3 in the PC-3 prostate carcinoma cell line [Rajah et al., 1997]. While these are direct growth inhibitory effects, evidence exists to suggest that IGFBP-3 can accentuate ceramide [Gill et al., 1997], paclitaxel [Fowler et al., 1999], and UV-induced apoptosis [Hollowood et al., 2000].

Comprised of three distinct domains with highly conserved N- and C-terminals and a variable mid-region, the six high affinity-binding proteins are structurally and functionally similar. Yet, despite this, it has been demonstrated that the IGF-independent actions of the binding proteins on apoptosis are binding protein specific [Perks et al., 1999]. Phosphorylation is known to occur in IGFBP-1, -3, and -5 and all three have potential serine phosphorylation sites in the variable mid-region [Coverley and Baxter, 1997]. Mutagenesis of the two major phosphorylation sites within the variable mid-region of IGFBP-3 indicated that they account for more than 80% of its phosphorylation [Hoeck and Mukku, 1994] and are putative casein kinase II phosphorylation sites. The heavily phosphorylated form of IGFBP-1 has a much greater affinity for IGF-I, thereby inhibiting IGF action [Frost and Tseng, 1991; Jones et al., 1991]. Phosphorylation of IGFBP-3 has been reported, but little is known about its regulation or consequence. Recent evidence has shown that CKII phosphorylation of IGFBP-3

Grant sponsor: Royal College of Surgeons of England; Grant sponsor: NHS Executive South West Research and Development Directorate; Grant sponsor: BBSRC.

*Correspondence to: Prof. J.M.P. Holly, Division of Surgery, Level 7, Bristol Royal Infirmary, Bristol, UK. E-mail: jeff.holly@bristol.ac.uk

Received 8 October 2001; Accepted 4 April 2002
DOI 10.1002/jcb.10223

does not affect IGF-I or -II binding, but does reduce ALS binding [Coverley et al., 2000]. Phosphorylation could, therefore, inhibit the ternary complex formation although this might only be of importance within the circulation.

Proteolysis of IGFBP-3 has long been recognized as an important way of regulating IGF bioavailability. More recently, fragments of IGFBP-3 that do not bind IGF-I have been shown to have growth inhibitory effects in PC-3 cells [Angelloz-Nicoud et al., 1998] and addition of the non-cytotoxic serine protease inhibitor, AESBF, could block the IGFBP-3 accentuation of ceramide-induced cell death [Maile et al., 1999]. Although, this would suggest that inhibition of a serine protease prevented the generation of bioactive fragments of IGFBP-3, it was also shown that this inhibitor could block the action of pre-cleaved IGFBP-3 leaving uncertainty with regard to the potential mechanism of action.

The six binding proteins have highly conserved N- and C-terminal domains, modifications specific to individual binding sites, such as the major phosphorylation sites and the proteolytic cleavage sites reside within the variable mid-region. We hypothesized that similarly the variable mid-region of IGFBP-3 would be responsible for its very specific IGF-independent apoptotic actions. In order to identify the structural features responsible for this action, we investigated the effects of 17 synthetic peptides (15–20 amino acids (aa)), spanning putative regulatory motifs within IGFBP-3. Only one of those examined, which corresponded to the putative CKII phosphorylation sites within the variable mid-region, could accentuate cell death comparable with that stimulated by intact protein. We utilized specific mechanisms to modify the serines within this region to determine the potential effects.

MATERIALS AND METHODS

Materials

All chemicals, unless stated otherwise were obtained from Sigma (Dorset, UK). Dr. C.A. Maack (Celtrix Pharmaceuticals, Santa Clara, CA Clara) provided recombinant non-glycosylated IGFBP-3 (ngIGFBP-3) and recombinant IGF-I was purchased from GroPep. Casein kinase II was purchased from BioLabs (New England). Synthetic peptides were manufactured by the Microchemical Facility (Babraham Institute) using the Fmoc polyamide mode of peptide synthesis on a Milligan/Biosearch 9500 peptide synthesiser (Hertford, Herts, UK). The sequences of N-, C-terminal, and mid-region peptides corresponded to to aa 1–20, 235–255, and 105–119, respectively.

Cell Culture

The human oesophageal carcinoma cell line KYSE 190 (a gift from Y Shimada, Kyoto University, Japan) was grown in a humidified 5% CO_2 atmosphere at 37°C. Cells were cultured in a mixture of Ham's F12/ RPMI 1640 (1:1) medium supplemented with 2% fetal calf serum (FCS) [Shimada and Imamura, 1993]. Experiments were performed on cells that had been transferred to serum-free media (SFM) Ham's F12/RPMI 1640 (1:1) buffered with HEPES and supplemented with sodium bicarbonate (0.12%) for 24 h. As the cells had to be UV irradiated without media, variation in the absolute amount of cell death occurred between experiments presumably dependent upon passage, cell density, and period of time without media. All the changes in cell death reported were consistent between experiments, although the absolute levels of death varied. The effects of IGFBP-3 on cell death were entirely consistent in all experiments regardless of the variation in absolute death induced by manipulating the cells and exposing to UV. To account for this variation between experiments, a representative sample of three separate experiments has been shown.

Derivatization of IGFBP-3

One microgram of IGFBP-3 was incubated with 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), the serine protease inhibitor, in 0.05 M phosphate buffer at 30°C for 20 min. The AEBSF was then heat inactivated by heating the mixture to 68°C for 30 min.

Phosphorylation of IGFBP-3

Phosphorylation of IGFBP-3 was carried out according to the methods described by Coverley et al. [2000]. Briefly, 1 μg of recombinant IGFBP-3 was dissolved in CKII reaction buffer (10 × concentration), non-radioactive 5′-adenosine-triphosphate (ATP) at a final concentration of 200 μM was added. One microliter of CKII was added to initiate the reaction and made up to 50 μl with dH_2O. The reaction mixture was incubated at 30°C for 2 h. Heating for 20 min at

68°C terminated the reactions. To monitor the incorporation of phosphate groups into IGFBP-3, 1 μCi [^{32}P]γ-ATP was used. The reaction was performed according to the same protocol, but replacing non-radioactive ATP with [^{32}P]γ-ATP. Briefly, trichloroacetic acid (TCA) precipitation was used to monitor the incorporation of [^{32}P]γ-ATP into the protein. Reaction mixtures containing 1 μg of IGFBP-3 were prepared according to the above protocol. Duplicate 5-μl aliquots were removed from the mixture and added to 1 ml of ice cold TCA (100 g/L). Tubes were vortexed, 100 μl of 10 g/L BSA added, vortexed again, and centrifuged for 5 min at 10,000 rpm. The precipitated pellet was washed twice with ice-cold TCA solution and the pellet counted directly for 1 min using a β-counter. Incorporation of phosphate groups was analyzed by 10% non-reducing SDS–PAGE, this also demonstrated that no degradation of the protein occurred during the modification procedure. Non-specific incorporation of phosphate into either CKII or IGFBP-3 after 60 min accounted for 6–12.5%. The control for both derivatization protocols; 1 μg of IGFBP-3, was incubated at 30°C for 2 h in phosphate buffer (without CKII or AEBSF) and then at 68°C for 30 min.

Dialysis of IGFBP-3

Aliquots of IGFBP-3 were dialyzed using the Slide-A-Lyze MINI dialysis units (Perbio, Cheshire, UK) with a 7,000 Dalton molecular weight cut off. Briefly 1 μg of IGFBP-3, phosphorylated IGFBP-3, or derivatized IGFBP-3 in 100 μl was placed in the filters. The filter units were placed in a rack and suspended in 1 L of 0.05% phosphate buffer for 1 h.

Cell Counting

The conditioned media from each well of the six-well plate was removed and centrifuged at 1,200 rpm for 5 min and stored at −20°C. The cells were enzymatically detached from the plates with the addition of 400 μl of trypsin per well. Following detachment, 400 μl of growth medium was added to neutralize the enzyme. The detached cells were added to the pellet obtained from the conditioned medium and centrifuged for 5 min as above. The supernatant was removed and the cells resuspended in 1 ml of phosphate-buffered saline (PBS). A 50 μl aliquot was removed, added to 50 μl of Trypan Blue solution, and mixed. The mixture was placed on a hemocytometer, and the number of living and dead (blue) cells were counted separately. The percentage of dead cells was then calculated.

Flow Cytometry

The mode of apoptotic cell death was assessed by flow cytometry. Cells were seeded at 0.5×10^6 cells/well in a six-well plate and cultured for 24 h before being transferred to SFM for a further 24 h. For each experiment, cells were dosed in triplicate. Both floating cells in the supernatant and the PBS wash were collected from each well prior to trypsinization. Following trypsinization, attached cells were also added to the previously harvested cells. Each sample was fixed in 70% ethanol for a minimum of 24 h at 4°C prior to flow cytometry. Fixed cells were pelleted (6,000 rpm; 5 min) and washed three times with PBS (6,000 rpm; 5 min), the supernatant removed and the cells resuspended in reaction buffer (propidium iodide, 0.05 mg/ml; sodium citrate, 0.1%; RNase A, 0.02 mg/ml; NP-40, 0.3%; pH 8.3). Cells were kept at 4°C for 30 min prior to measurement on a FACSCalibur flow cytometer (Beckton Dickinson) by an argo laser at 488 nM. Data were analyzed using the cell Quest software package (Beckton Dickinson).

Statistical Analysis

Data were analysed using SPSS 10.0 for Windows. Significant effects were determined using ANOVA and the Bonferroni post hoc test. A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS

The KYSE 190-cell line was initially characterized with respect to the IGF system [Hollowood et al., 2000]. In brief, the cells do not produce IGF-I or -II, and addition of either does not produce any mitogenic or survival response. In addition, we have previously demonstrated in this cell line that IGFBP-3 alone has no effect on cell death. However, when the cells are triggered to undergo cell death with 10 J/m^2 of UV irradiation, the addition of exogenous non-glycosylated IGFBP-3 (ngIGFBP-3) results in a significant increase in cell death (Fig. 1, panel A) compared with UV treatment alone at 24 and 48 h ($P = 0.04$ and 0.001, respectively). Apoptosis was confirmed by a 67%

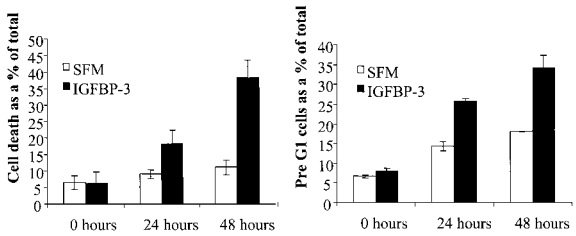

Fig. 1. Effect of UV irradiation on KYSE 190 cells. (**A**) Cell death as a percentage of total as measured by trypan blue exclusion counts. Cells were maintained in six-well plates, allowed to settle for 24 h prior to transferring to SFM for 24 h. Cells were either maintained in SFM or pre-treated with 100 ng/ml IGFBP-3 and then irradiated with 10 J/m^2 UV irradiation after 24 h. Cells were collected and counted at the times indicated. (**B**) Confirmation of apoptotic cell death as measured by PreG1 cells as a percentage of total as described in Materials and Methods section. The data shown is the mean and standard errors of triplicates from a representative experiment from three separate experiments.

increase in PreG1 positive cells as a percentage of the total at 24 h ($P < 0.001$) (Fig. 1, panel B).

Synthetic Peptide Enhances UV-Induced Cell Death

In order to ascertain structural/functional aspects of IGFBP-3, we had previously generated 17 peptides each spanning 15–20 aa of the intact IGFBP-3 protein. One of the mid-region peptides is a 15 aa structure (aa 105–119) spanning the region containing Ser^{111} and Ser^{113} that lie within the variable mid-region of IGFBP-3 and correspond to the putative phosphorylation sites of CKII. The mid-region peptide enhanced UV-induced cell death in a dose-dependent manner with a significant increase in cell death noted at 10 and 50 ng/ml ($P = 0.04$ and 0.014, respectively) (Fig. 2).

This effect was specific to mid-region peptide ($P = 0.023$) as none of the other peptides produced the same response (Fig. 3).

Confirmation of Phosphorylation of IGFBP-3 by CKII

Phosphorylation of IGFBP-3 has previously been proposed to occur at the serine 111 and 113 sites within the variable mid-region of IGFBP-3. TCA acid precipitation was used to monitor incorporation of [^{32}P]-γ-ATP into IGFBP-3. In Figure 4, the autoradiograph of IGFBP-3 (panel A) shows the incorporation of [^{32}P]-γ-ATP into the ngIGFBP-3 reaches a plateau between 30 and 60 min and demonstrates that the phosphorylated IGFBP-3 remains in the intact form during this process. Figure 4 (panel B) shows the TCA precipitable radioactive protein (10^4 cpm) against time and confirms that the specific incorporation of ^{32}P-γ- ATP is dependent on the presence of CKII and ngIGFBP-3.

Phosphorylation and Derivatization of IGFBP-3 Negates Its Effect

Since ngIGFBP-3 accentuates the apoptotic effect of UV irradiation on KYSE190 cells,

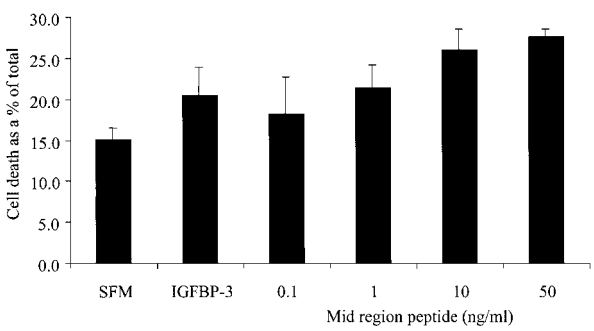

Fig. 2. Dose response to mid-region peptide. Cells were maintained in six-well plates and transferred to SFM 24 h prior to dosing with IGFBP-3 or mid-region peptides at the doses shown. Cells were irradiated and collected and counted after 24 h. Mid-region peptide-induced cell death in a dosed response with a significant increase in cell death noted with 10 ng/ml. The data shown is mean and standard errors of triplicates from a representative experiment from three separate experiments.

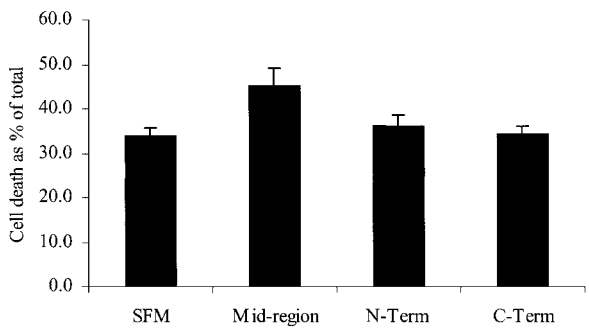

Fig. 3. Mid-region peptide, but not N- or C-terminal peptide enhances UV-induced cell death. Cells were maintained in six-well plates and transferred to SFM 24 h prior to dosing with mid-region peptide or the N- or C-terminal peptide at 10 ng/ml. Cells were irradiated and collected and counted after 24 h. The data shown is mean and standard errors of triplicates from a representative experiment from three separate experiments. The mid-region peptide significantly enhanced cell death ($P = 0.023$). This was completely consistently in all experiments, whereas there was no consistent effect for the other peptides tested.

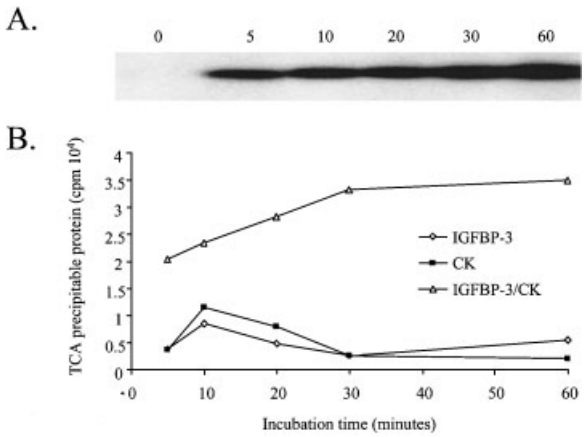

Fig. 4. Phosphorylation of recombinant IGFBP-3. Time course of incorporation of ^{32}P into TCA precipitable protein. One microgram of recombinant IGFBP-3 was incubated at 30°C with 500 U CKII and samples taken at the times shown. Autoradiograph of TCA precipitated protein and corresponding counts.

we determined whether phosphorylation of IGFBP-3 would alter these pro-apoptotic effects. IGFBP-3 (200 ng/ml) accentuated cell death as previously demonstrated ($P = 0.017$), however, addition of phosIGFBP-3 was without effect compared to UV treatment alone. (Fig. 5, panel A). It would have been advantageous to confirm that phosphorylation of these serines in the short synthetic mid-region peptide also negated its activity. However, following several attempts to phosphorylate the peptide with CKII, we were unable to confirm that there had been any incorporation of the phosphate into this short synthetic peptide.

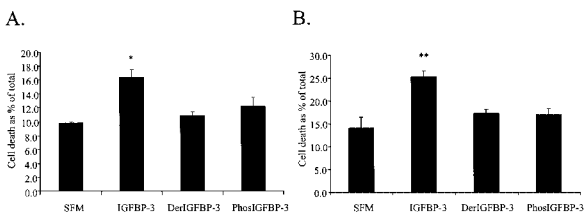

Fig. 5. Effect of phosphorylation or derivatization of IGFBP-3 on IGFBP-3-enhanced cell death. IGFBP-3 was either phosphorylated with CKII or derivatized with AEBSF and aliquots of the reaction mixture dialyzed as described in the Materials and Methods. Cells were plated in six-well plates, transferred to SFM for 24 h prior to dosing with either IGFBP-3, derivatized IGFBP-3 (derIGFBP-3) or CKII phosphorylated IGFBP-3 (phosIGFBP-3) in **panel A** or the dialyzed equivalents in **panel B**. The control IGFBP-3 used in these experiments had similarly been incubated initially at 30°C and then at 68°C. Cells were collected and counted 24 h following treatment with 10 J/m^2 UV irradiation. The data shown is mean and standard error of triplicates from a representative experiment from three separate experiments.

To determine if AEBSF had a direct effect on the ability of IGFBP-3 to enhance cell death, cells were preincubated with either IGFBP-3 or derivatized IGFBP-3. AEBSF had no direct effect on UV-induced cell death. IGFBP-3 derivatized with AEBSF negated the effect of IGFBP-3 enhanced cell death. (Fig. 5, panel A). Dialysis of the derivatization mixture confirmed that it was the derivatized IGFBP-3 and not residual AEBSF that removed the effect of IGFBP-3 ($P < 0.01$) (Fig. 5, panel B).

DISCUSSION

IGFBP-3 has been shown to enhance cell death in a number of epithelial cell models including the KYSE 190 oesophageal carcinoma cells. The effect observed is IGF independent as these cells do not produce or respond to exogenous IGF-I or -II. In addition to this, the cells showed no mitogenic or survival effect in response to IGF-I. [Hollowood et al., 2000]. The IGFBPs have highly conserved N- and C-terminals, which contain the main binding sites for IGFs, suggesting a common action with respect to IGF binding, and hence all function to regulate IGF bioavailability. All six have a variable mid-region and all have specific IGF-independent actions [Perks et al., 1999]. The use of the short peptides led us to hypothesize that the variable mid-region was responsible for the pro-apoptotic actions of IGFBP-3.

The role of IGFBP-3 in cellular homeostasis is a balance between IGF-dependent and -independent actions. IGFBP-3 is secreted in phosphorylated forms [Coverley and Baxter, 1995; Pattison et al., 1999], but alteration in phosphorylation status does not account for any differential IGF-I binding [Hoeck and Mukku, 1994]. However, we show that phosphorylation of IGFBP-3 with casein kinase II negated the enhancement of UV-induced cell death, further confirming an important IGF-independent role for IGFBP-3. The two serine sites Ser^{111} and Ser^{113} within the variable mid-region are putative phosphorylation sites for casein kinase II, and these data suggest that the two sites are important in the pro-apoptotic function of the protein.

It has previously been shown that AEBSF could block the accentuation of ceramide-induced cell death, and it was postulated to block a serine protease on the cell surface [Maile et al., 1999]. Since AEBSF is a serine protease

inhibitor, one would expect a reduction in the IGFBP-3 fragmentation pattern, which was not observed. To dissect this finding further, we determined that pre-incubation of IGFBP-3 with AEBSF negated the effect of IGFBP-3 in the KYSE 190 cells. As AEBSF alone had no effect on cell death in the cell model, this indicated that AEBSF was directly modifying the activity of IGFBP-3. AEBSF inactivates the active serines within the catalytic sites of serine proteases. It is feasible that, as we have suggested before [Whellams et al., 1999] IGFBP-3 itself may have serine protease activity and as such derivatization of the serines within the variable mid-region of IGFBP-3 could explain its loss of bioactivity following pre-incubation with AEBSF. The involvement of serines within the mid-region supports a recent report showing that mid-region fragments of IGFBP-3 inhibited ^{125}I-IGFBP-3 binding with equipotential activity as intact IGFBP-3, and hence retained its IGF-independent growth inhibitory action [Yamanaka et al., 1999].

Phosphorylation and dephosphorylation are major mechanisms for the regulation of protein function, providing rapid and reversible responses to changing environments. It is known that IGFBP-3 exists in different phosphorylated states in vivo, but no function has yet been ascribed for these. Proteolysis is an important way of regulating IGFBP-3 function in terms of regulating IGF bioavailability. Phosphorylated IGFBP-3 has been shown to be relatively resistant to proteolysis, thereby indirectly controlling delivery of ligand without directly affecting the ligand binding.

Our studies imply that the IGF-independent action of IGFBP-3 is due to an active site within the variable mid-region of IGFBP-3, and that this activity is modulated by phosphorylation of serines at positions 111 and 113. This region also contains one of the *N*-linked glycosylative sites at Asn^{109}, raising the possibility that glycosylation may also affect this activity. While the current study and most of our previous work has used a non-glycosylated recombinant IGFBP-3 preparation, we have also compared native glycosylated IGFBP-3 purified from human serum in this model and obtained identical results (unpublished observations). We have also recently compared recombinant glycosylated and non-glycosylated forms of IGFBP-3 in a breast cell line, and again found identical effects on apoptosis [Perks et al., 2001]. The significance of this proximal glycosylation site, therefore, remains to be determined.

These experiments provide three pieces of formal evidence to suggest that the variable mid-region is important in providing some of the differential intrinsic actions of IGFBP-3 and that the role of phosphorylation of IGFBP-3 may be to regulate its IGF-independent pro-apoptotic function. Further evaluation of the mid-region and the phosphorylation status of IGFBP-3 may lead to an increased understanding the mechanisms of IGF-independent actions.

ACKNOWLEDGMENTS

This work was funded by the Royal College of Surgeons of England and in part by the NHS Executive South West Research and Development Directorate. IGFBP-3 synthetic peptides (15–20 amino acids) were generated as a part of a BBSRC grant to JMPH and JMP.

REFERENCES

Angelloz-Nicoud P, Laluo C, 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 8:71–76.

Coverley JA, Baxter RC. 1995. Regulation of insulin-like growth factor (IGF) binding protein-3 phosphorylation by IGF-I. Endocrinology 136:5778–5781.

Coverley JA, Baxter RC. 1997. Phosphorylation of insulin-like growth factor binding proteins. Mol Cell Endocrinol 128:1–5.

Coverley JA, Martin JL, Baxter RC. 2000. The effect of phosphorylation by casein kinase 2 on the activity of insulin-like growth factor-binding protein-3. Endocrinology 141:564–570.

Fowler C, Perks CM, Farndon J, Holly JMP. 1999. IGFBP-3 and -5 have opposing actions on paclitaxel induced apoptosis of human breast cancer cells, Hs578T. Growth Horm IGF Res 9:368.

Frost RA, Tseng L. 1991. Insulin-like growth factor-binding protein-1 is phosphorylated by cultured human endometrial stromal cells and multiple protein kinases in vitro. J Biol Chem 266:18082–18088.

Gill Z, Perks CM, Newcomb P, Holly JMP. 1997. Insulin-like growth factor binding protein 3 (IGFBP-3) predisposes breast cancer cell lines to programmed cell death in a non-IGF-dependent manner. J Biol Chem 272:25602–25607.

Hoeck WG, Mukku VR. 1994. Identification of the major sites of phosphorylation in IGF binding protein-3. J Cell Biochem 56:262–273.

Hollowood A, Lai T, Perks CM, Newcomb P, Alderson D, Holly JMP. 2000. IGFBP-3 prolongs the p53 response

and enhances apoptosis following UV irradiation. Int J Cancer 88:336–341.

Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR. 1991. Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. Proc Natl Acad Sci USA 88:7481–7485.

Maile LA, Gill ZP, Perks CM, Holly JMP. 1999. The role of cell surface attachment and proteolysis in the insulin-like growth factor (IGF)-independent effects of IGF-binding protein-3 on apoptosis in breast epithelial cells. Endocrinology 140:4040–4045.

Oh Y, Muller H, Pham H, Rosenfeld R. 1993. Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human cancer cells. J Biol Chem 268:26045–26048.

Pattison ST, Fanayan S, Martin JL. 1999. Insulin-like growth factor binding protein-3 is secreted as a phosphoprotein by human breast cancer cells. Mol Cell Endocrinol 156:131–139.

Perks CM, Bowen S, Gill ZP, Newcomb PV, Holly JMP. 1999. Differential IGF-independent effects of insulin-like growth factor binding proteins (1–6) on apoptosis of breast epithelial cells. J Cell Biochem 75:652–664.

Perks CM, Clarke JB, McCaig C, Clemmons DR, Holly JMP. 2001. Non-IGF binding mutants of IGFBP-3 and -5 retain their IGF-independent effects on apoptosis in Hs578T human breast cancer cells. The Endocrine Society's 83rd Annual Meeting, Denver, USA: Abstract, pp 2–260, 348.

Rajah R, Valentinis B, Cohen P. 1997. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-β1 on programmed cell death through a p53- and IGF-independent Mechanism. J Biol Chem 272:12181–12188.

Shimada Y, Imamura M. 1993. Prognostic significance of cell culture in carcinoma of the oesophagus. Br J Surg 80:605–607.

Whellams E, Maile L, Holly JMP. 1999. Does IGFBP-3 have intrinsic autoproteolytic activity?: The Endocrine Society. San Diego: The Endocrine Society Press. 402 p.

Yamanaka Y, Fowlkes JL, Wilson EM, Rosenfeld RG, Oh Y. 1999. Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells: Kinetics of IGFBP-3 binding and identification of receptor binding domain on the IGFBP-3 molecule. Endocrinology 140:1319–1328.