Constitutive expression of IGF-binding protein-3 by mammary epithelial cells alters signaling through Akt and p70S6 kinase

C J Grill, U Sivaprasad and W S Cohick

Department of Animal Sciences, Rutgers, The State University of New Jersey, 108 Foran Hall, 59 Dudley Road, New Brunswick, New Jersey 08901-8520, USA

(Requests for offprints should be addressed to W S Cohick; Email: cohick@aesop.rutgers.edu)

Abstract

IGF-binding protein-3 (IGFBP-3) potentiates IGF-I action in the non-transformed mammary epithelial cell line, MAC-T, via a mechanism that is independent of its ability to bind IGF-I. The goal of the present study was to determine if IGFBP-3 might enhance IGF action by influencing intracellular signaling events downstream of the IGF receptor.

IGF-I stimulated a time-dependent activation of Akt in which phosphorylation of Ser⁴⁷³ was detectable by 1 min and maximal at 15 min. In contrast, no activation of extracellular signal-regulated kinase (ERK)1/2 by IGF-I was observed although basal phosphorylation was readily detectable. In MAC-T cells constitutively expressing IGFBP-3 (+BP3), phosphorylation of Akt following stimulation with IGF-I was enhanced relative to mock-transfected cells (Mock). The enhancement was detectable within 1 min of IGF-I treatment and persisted for up to 10 h. The increased phosphorylation observed by Western blotting corresponded to a 1.7-fold increase in Akt kinase activity. The enhanced Akt response was elicited by factors that activate the IGF receptor but exhibit reduced affinity for IGFBP-3, such as Long R³IGF-I, B chain IGF-I and insulin. In contrast, [Leu⁶⁰]IGF-I, which binds IGFBP-3 but has reduced affinity for the IGF receptor, failed to induce comparable activation, suggesting that an association between IGF-I and IGFBP-3 is not required for the effect. The enhanced Akt activation could not be mimicked by addition of exogenous IGFBP-3. Akt phosphorylation was also enhanced by transforming growth factor- α in +BP3 cells, indicating that the effect was not specific to IGF-I. Similar to Akt, phosphorylation of p70S6 kinase (p70^{S6K}) by IGF-I was also enhanced in +BP3 cells relative to Mock cells at both 15 min and 10 h. However, this was largely an effect of lower basal activation of p70^{S6K} in +BP3 cells.

These data indicate that endogenous IGFBP-3 potentiates IGF action in MAC-T cells by enhancing signaling via the phosphatidylinositol 3-kinase pathway at a point that is downstream of IGF receptor activation. Further studies will delineate specific mechanisms by which IGFBP-3 may influence intracellular events that regulate growth in mammary epithelial cells.

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Introduction

The insulin-like growth factors (IGF) regulate multiple processes including growth, differentiation and cell survival (Stewart & Rotwein 1996). Evidence that IGF-I plays a central role in mammary development has accumulated from studies utilizing whole organ mammary cultures (Ruan *et al.* 1992, Plaut *et al.* 1993), *in vivo* IGF-I administration (Ruan *et al.* 1995) and transgenic models (Weber *et al.* 1998). Recently, studies with

IGF-I knockout mice have demonstrated that mammary development does not proceed in the absence of IGF-I, even if growth hormone is present (Ruan & Kleinberg 1999). An autocrine/paracrine mechanism of IGF action in the mammary gland is suggested by studies in mice showing that IGF-I mRNA expression is localized to the mammary epithelia of the rapidly proliferating terminal end bud as well as the surrounding stroma during postpubertal development or early pregnancy (Richert & Wood 1999). Similarly,

IGF-I mRNA expression is detectable in ductal epithelium and stroma of late pregnant heifers (Plath-Gabler *et al.* 2001). Thus, delineating the mechanism by which IGF-I regulates cellular proliferation in the mammary gland is essential to understanding both normal mammary gland development as well as abnormal growth processes involved in breast tumorigenesis and metastasis.

IGF-I regulates intracellular processes including growth by activating the IGF receptor (IGFR), a tyrosine kinase receptor that signals through both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (LeRoith *et al.* 1995). In addition to the interaction between the IGF ligands and the IGFR, a family of high-affinity IGF-binding proteins (IGFBP) regulates IGF biological action (Ferry *et al.* 1999). One of these, IGFBP-3, has been shown to both potentiate as well as to inhibit cellular growth, in addition to exerting IGF-independent effects on apoptosis (Clemmons 1997, Rajah *et al.* 1997, Butt *et al.* 2000). Inhibition of IGF action by IGFBP-3 has largely been attributed to its ability to sequester IGF away from the IGFR that transduces its intracellular effects (Imai *et al.* 2000). However, it has also been reported that IGFBP-3 may inhibit IGF activation of the IGFR via a mechanism that is independent of a physical interaction between IGF-I and IGFBP-3 (Ricort & Binoux 2001). In contrast, IGFBP-3 has been shown to enhance IGF-stimulated cell proliferation in both bovine mammary epithelial cells (Grill & Cohick 2000) as well as MCF-7 breast tumor cells (Chen *et al.* 1994). Our laboratory has reported that the potentiating effect of IGFBP-3 on IGF-stimulated DNA synthesis is mediated by a mechanism that does not appear to involve a direct physical interaction between the IGF ligand and IGFBP-3, but does involve IGFR activation (Grill & Cohick 2000). Therefore, the present study investigated the hypothesis that endogenous IGFBP-3 might enhance IGF action by directly influencing intracellular signaling events downstream of the IGFR.

Materials and methods

Antibodies and materials

Cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA) and chemicals from Sigma (St Louis, MO, USA) unless other-

wise specified. Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ, USA). IGF-I, Long R³IGF-I and [Leu⁶⁰]IGF-I were obtained from GroPep (North Adelaide, Australia). B chain IGF-I analogue was provided by Dr Margaret Cascieri (Merck, Sharp and Dohme, Rahway, NJ, USA). Recombinant glycosylated bovine IGFBP-3 was purified from Chinese hamster ovary cells as previously described (Cohick & Clemmons 1994). Recombinant human transforming growth factor- α (TGF- α) was from Intergen Co. (Purchase, NY, USA). Antibodies directed against phosphorylated Akt/protein kinase B (PKB) (Ser⁴⁷³), phosphorylated p70S6 kinase (p70^{S6K}) (Thr⁴²¹/Ser⁴²⁴) and total p70^{S6K} were purchased from Cell Signaling Technology (Beverly, MA, USA). Phosphospecific antibody for extracellular signal-regulated kinase (ERK)1/2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for total Akt/PKB and ERK1/2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Hygromycin was purchased from Calbiochem (La Jolla, CA, USA).

Mammalian cell culture

The MAC-T bovine mammary epithelial cell line was established from primary bovine mammary alveolar cells by immortalization with the SV40 large-T antigen (Huynh *et al.* 1991). The transfected MAC-T cell lines constitutively expressing bovine IGFBP-3 (+BP3) and mock-transfected control cell lines (Mock) have been previously described (Grill & Cohick 2000). Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin (DMEM)+10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. Transfected cell lines were maintained in selection media consisting of DMEM+10% FBS and 0.1 mg/ml hygromycin. Prior to stimulation with growth factors, cells were incubated in serum-free phenol red-free DMEM (PRF-DMEM) supplemented with 0.2% bovine serum albumin and 30 nM sodium selenite for 24 h. Media were replaced with fresh serum-free PRF-DMEM without additives $\pm$ growth factors for the indicated times. For all experiments, hygromycin was omitted from the culture media of transfected cells.

Western immunoblotting

Cells were lysed by the addition of lysis buffer (1% Triton X-100, 80 mM β -glycerophosphate, 50 mM HEPES, 2 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium orthovanadate (Na_3VO_4), and 10 $\mu\text{g}/\text{ml}$ leupeptin, trypsin inhibitor and aprotinin), scraped and incubated at 4 °C for 40 min. Lysates were cleared by centrifugation at 13 000 *g* for 15 min at 4 °C. Total protein content of the cleared lysates was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of lysate were separated by SDS-PAGE and the resolved proteins were transferred to nitrocellulose membranes (0.45 μm ; Bio-Rad) and blocked in Tris-buffered saline + 0.05% Tween 20 (v/v) and 5% (w/v) non-fat dried milk for 1 to 2 h at room temperature. Membranes were incubated overnight at 4 °C in the presence of specific antibodies. Immunoreactive bands were detected by incubation with horseradish peroxidase-conjugated secondary antibodies followed by ECL+ (Amersham Pharmacia, Piscataway, NJ, USA) and autoradiography. Membranes were stripped (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl) and re-probed with antibodies directed against total protein.

In vitro Akt/PKB kinase activity

Akt1/PKB α kinase activity was measured using a kit from Upstate Biotechnology, Inc. according to the manufacturer's directions. Briefly, confluent cell monolayers were stimulated with 200 ng/ml IGF-I for 15 min and lysed in immunoprecipitation kinase buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4 , 0.1% (v/v) 2-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β -glycerophosphate, 0.1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, aprotinin, pepstatin A and 1 μM microcystin). Whole cell lysates were pre-cleared by centrifugation at 13 000 *g* and pre-adsorbed by incubation with protein G-agarose bead slurry. Prior to the immunoprecipitation reaction, antibody-bead immunocomplexes were formed by incubating antibodies directed against the pleckstrin homology domain of human Akt1/PKB α with 50 μl protein G-agarose bead slurry overnight at 4 °C. Akt1/PKB α was immunoprecipitated by incubating lysates contain-

ing equal amounts of protein (300 μg) with the preformed immunocomplexes for 90 min at 4 °C. For the evaluation of *in vitro* Akt1/PKB α kinase activity, washed immunoprecipitates were resuspended in kinase buffer (20 mM MOPS, pH 7.2, 25 mM β -glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4 and 0.1% 2-mercaptoethanol) and reacted with an Akt1/PKB α -specific substrate and [γ ³²P]ATP (NEN Life Science Products, Boston, MA, USA) for 10 min at 30 °C with continuous agitation. The reaction was terminated by the addition of 40% trichloroacetic acid and the reaction volume was spotted onto P81 phosphocellulose paper. Following washes in 0.75% phosphoric acid and acetone, incorporated radioactivity was measured by liquid scintillation counting.

Results

IGF-I induces a time-dependent activation of Akt/PKB but not ERK1/2

The PI3K and MAPK pathways represent two well-delineated mechanisms by which the activated IGF-R transduces signals to the cell interior. To determine if IGF-I activates one or both of these pathways in MAC-T cells, we utilized phospho-specific antibodies directed against phosphorylated Akt and ERK1/2 (Fig. 1). A time-dependent activation of Akt was observed following exposure to IGF-I, indicating that IGF-I activates the PI3K pathway in this cell line. Phosphorylation of Akt was detectable following stimulation with 200 ng/ml IGF-I for as little as 1 min and was sustained through 60 min of exposure to IGF-I with maximal phosphorylation occurring at 15 min. In contrast, stimulation of MAC-T cells with 200 ng/ml IGF-I for up to 1 h failed to increase ERK1/2 phosphorylation above basal levels (Fig. 1). In addition, Western blotting with phospho-specific antibodies for two other MAPK family members, p38 and c-Jun N-terminal kinase (JNK), also failed to confirm the involvement of MAPK modules in IGF-I signaling (data not shown).

Akt phosphorylation by IGF is enhanced in MAC-T cells expressing IGFBP-3

Having established that IGF-I is likely transmitting signals via the PI3K pathway in the parental

Figure 1 IGF-I stimulation induces a time-dependent activation of Akt, but not ERK1/2 in MAC-T cells. Near-confluent cells were serum-starved for 24 h. Media were replaced with fresh serum-free media±IGF-I (200 ng/ml) for the designated times. Cell lysates (30 µg) were Western blotted using antibodies specific for phosphorylated (P) Akt and ERK1/2. Membranes were stripped and reprobed with antibodies that detect total Akt and ERK1/2.

MAC-T cell line, we wanted to determine if the presence of endogenous IGFBP-3 could influence phosphorylation events in this signaling cascade. IGF-I-treated cell lysates from +BP3 cells were immunoblotted with phosphospecific antibodies to Akt. Enhanced phosphorylation of Akt following stimulation with IGF-I was observed in +BP3 cells relative to Mock cells at each of the time-points examined (Fig. 2A). The enhancement was detectable within 1 min of IGF-I treatment, and persisted for up to 10 h.

To ensure that this was not an inadvertent clonal effect, two additional +BP3 and two additional Mock clones were tested. As shown in Fig. 2B, Akt phosphorylation was enhanced in all three +BP3 clones relative to the three Mock clones following exposure to IGF-I for 15 min. Densitometry analysis indicated that IGF-I-stimulated Akt phosphorylation was, on average, 1.8-fold greater in the +BP3 clones compared with the Mock clones at 15 min ($P<0.02$ by Student's *t*-test). This effect was sustained through 10 h of IGF-I treatment, with a 2.1-fold increase in Akt phosphorylation observed ($P<0.01$ by Student's *t*-test). In addition, activation of Akt by IGF-I in wild-type parental MAC-T cells was similar to that observed in the Mock clones at both time-points. Phosphorylation of ERK1/2 in

Figure 2 IGF-I-stimulated Akt phosphorylation is enhanced in MAC-T cells expressing IGFBP-3. MAC-T cells constitutively expressing IGFBP-3 (+BP3) or mock-transfected control cells (Mock) were grown to near confluence and serum-starved for 24 h. Media were replaced with fresh serum-free media±IGF-I (200 ng/ml) for the indicated times. Cell lysates (30 µg) were Western blotted with antibodies specific for phosphorylated Akt. Membranes were stripped and reprobed with antibodies that detect total Akt. Each panel shows a representative blot of at least four independent experiments that gave similar results. (A) Comparison of one +BP3 and one Mock clone. (B) Comparison of three individual +BP3 clones (+), three individual Mock clones (-) and wild-type MAC-T cells (WT). Levels of phosphorylated Akt in IGF-I-treated cells were significantly greater in +BP3 versus Mock cells ($P<0.02$ at 15 min and $P<0.01$ at 10 h by Student's *t*-test).

cells treated with or without IGF-I was not different between +BP3 and Mock cells (data not shown). To confirm that the increased phosphorylation of Akt following IGF-I stimulation corresponded to

Figure 3 IGF-I-stimulated Akt kinase activity is enhanced in MAC-T cells expressing IGFBP-3. Near-confluent +BP3 or Mock cells were serum-starved for 24 h. Fresh serum-free media $\pm$ IGF-I (200 ng/ml) were added for 15 min. Cell lysates were immunoprecipitated with anti-Akt antibodies and assayed for kinase activity *in vitro* by measuring [γ -³²P]ATP incorporation. The results are expressed as counts per min and are representative of three separate experiments ($P < 0.01$; Student's paired *t*-test).

an increase in enzymatic activity, *in vitro* kinase assays were performed. Exposure of +BP3 cells to IGF-I for 15 min increased Akt enzyme activity in each of three experiments, with increases ranging between 1.4- and 1.9-fold above the enzyme activity observed in IGF-I-stimulated Mock cells ($P < 0.01$; Student's paired *t*-test), confirming the results obtained by Western blotting (Fig. 3).

In order to determine if the ability of IGF-I to enhance Akt activation in +BP3 cells was independent of a physical association between the IGF ligand and IGFBP-3, IGF analogues with varying affinity for IGFBP-3 and the IGFR were tested. As shown in Fig. 4, phosphorylation of Akt was enhanced by Long R³IGF-I in +BP3 cells relative to Mock cells. This IGF analogue activates the IGFR but exhibits reduced affinity for IGFBP-3. Similar results were observed with B-chain IGF-I (data not shown), another analogue that exhibits greatly reduced affinity for bovine IGFBP-3 but that activates the IGFR (Bayne *et al.* 1988). In addition, insulin at concentrations that activate the IGFR (5 μ g/ml) produced a similar effect. In contrast, Akt activation by [Leu⁶⁰]IGF-I, an IGF analogue with normal affinity for IGFBP-3 but reduced affinity for the IGFR, was attenuated

Figure 4 Enhancement of Akt phosphorylation does not require association of IGF-I with IGFBP-3. Near-confluent +BP3 or Mock cells were serum-starved for 24 h. Fresh serum-free media $\pm$ 200 ng/ml Long R³IGF-I (LR³), [Leu⁶⁰]IGF-I (Leu⁶⁰), IGF-I or 5 μ g/ml insulin (Ins) were added for the indicated times. Cell lysates (20 μ g) were Western blotted with antibodies specific for phosphorylated Akt. Membranes were then stripped and reprobed with antibodies that detect total Akt. Each panel shows a representative blot of at least four independent experiments, with the exception of Leu⁶⁰ which was tested twice at all time-points.

relative to the other analogues tested in both +BP3 and Mock cells (Fig. 4). It is interesting that the response to [Leu⁶⁰]IGF-I in terms of Akt phosphorylation was also diminished in Mock cells relative to the Akt activation elicited by the other analogues. This suggests that, despite its markedly reduced affinity for the IGFR, [Leu⁶⁰]IGF-I at 200 ng/ml is still sufficient to activate the receptor to some degree. This is supported by our findings that 20 ng/ml IGF-I is sufficient to maximally activate Akt (data not shown). Collectively, these data suggest that the enhanced activation of the IGFR signaling cascade observed in +BP3 cells does not require a physical association between IGF-I and IGFBP-3.

Figure 5 Akt phosphorylation is also enhanced by TGF- α in MAC-T cells expressing IGFBP-3. +BP3 or Mock cells were grown to near confluence and serum-starved for 24 h. Media were replaced with fresh serum-free media $\pm$ TGF- α (100 ng/ml) for the indicated times. Cell lysates (30 μ g) were separated by SDS-PAGE and Western blotted with antibodies specific for phosphorylated Akt. Membranes were stripped and reprobed with antibodies that detect total Akt. Each panel shows a representative blot of two separate experiments that gave similar results.

Activation of other tyrosine kinase receptors, such as the epidermal growth factor receptor, also stimulates Akt phosphorylation via the PI3K pathway. Therefore, in order to determine if the enhanced activation of Akt in +BP3 cells was specific to the IGFR, we compared the response of +BP3 and Mock cells to TGF- α . Similar to the IGF-I response, Akt phosphorylation was also enhanced in +BP3 cells by TGF- α relative to the Mock cells (Fig. 5). The time-course of this response was different from that observed following IGF-I treatment in that maximal activation was observed following 1 min of exposure to TGF- α , and virtually gone by 15 min. These data suggest that the enhanced activation of Akt observed in +BP3 cells is caused by an event that is downstream of IGFR activation.

Exogenous IGFBP-3 does not alter activation of Akt by IGF-I

To determine if the enhanced activation of Akt by IGF-I observed in +BP3 cells could be mimicked by exogenous IGFBP-3, Mock cells were preincubated for 24 h with 500 ng/ml recombinant glycosylated bovine IGFBP-3. As shown in Fig. 6, phosphorylated Akt following IGF-I stimulation was not different between Mock cells treated with

Figure 6 Exogenous IGFBP-3 does not mimic the effect of constitutive IGFBP-3 expression on IGF-I-stimulated Akt phosphorylation. Cells were grown to near confluence and serum-starved for 24 h. Media were aspirated and replaced with fresh serum-free media $\pm$ recombinant glycosylated bovine IGFBP-3 (500 ng/ml) for 24 h. Media were again aspirated and replaced with fresh serum-free media $\pm$ IGF-I (200 ng/ml) and/or IGFBP-3 (500 ng/ml) for 15 min. Lanes represent (1) +BP3 cells without IGF-I, (2) Mock cells without IGF-I, (3) +BP3 cells+IGF-I, (4) Mock cells+IGF-I, (5 and 7) Mock cells+IGFBP-3 alone, (6 and 8) Mock cells+IGFBP-3+IGF-I. Lanes 5 and 6 represent cells treated with IGFBP-3 during the preincubation alone, while lanes 7 and 8 represent cells treated with IGFBP-3 during both the preincubation as well as the IGF-I treatment interval. Cell lysates (20 μ g) were separated by SDS-PAGE and immunoblotted with antibodies that recognize phosphorylated Akt. Membranes were stripped and reprobed with antibody for total Akt. Similar results were obtained in two experiments.

(lanes 6 and 8) or without (lane 4) IGFBP-3. Preincubation with IGFBP-3 did not affect Akt activation by IGF-I regardless of whether IGFBP-3 was removed prior to addition of IGF-I (lane 6) or included during IGF-I stimulation (lane 8). Addition of exogenous IGFBP-3 alone in the absence of IGF-I also failed to activate Akt (lanes 5 and 7). Lower concentrations of IGFBP-3 that more closely mimicked the levels of IGFBP-3 secreted by +BP3 cells (70 ng/ml) were also tested and found not to affect the ability of IGF-I to activate Akt in Mock cells (data not shown).

Phosphorylation of p70^{S6K} by IGF-I is enhanced in MAC-T cells expressing IGFBP-3

The ribosomal protein kinase p70^{S6K} acts as a key regulator of cell growth through control of the protein translational apparatus (Pullen & Thomas 1997, Avruch 1998). Because p70^{S6K} is a downstream effector of Akt, we next determined whether +BP3 cells would also exhibit enhanced

Figure 7 Activation of p70^{S6K} is enhanced in +BP3 cells due to attenuation of basal p70^{S6K} phosphorylation. Near-confluent cells were serum-starved for 24 h. Media were replaced with fresh serum-free media for the indicated times. Cell lysates (25 µg) were Western blotted with antibodies that recognize phosphorylated Thr⁴²¹/Ser⁴²⁴ sites of p70^{S6K}. Membranes were stripped and reprobed with antibody for total p70^{S6K} protein. (A) Comparison of one +BP3 and one Mock clone treated ± Long R³IGF-I (200 ng/ml) for 15 min or 10 h. The autoradiographs shown are representative of at least three experiments for each time-point. Activation of Akt was greater in +BP3 cells ($P < 0.01$). (B) Comparison of basal levels of phosphorylated p70^{S6K} at 15 min in three individual +BP3 clones (+), three individual Mock clones (-) and wild-type (WT) MAC-T cells. Results are representative of two separate experiments ($P < 0.01$).

signaling through p70^{S6K}. Western blotting was performed with an antibody specific for Thr⁴²¹/Ser⁴²⁴ phosphorylation sites, which detects both the p70 and p85 isoforms of p70^{S6K}. As shown in Fig. 7A, Long R³IGF-I stimulated phosphorylation of p70^{S6K} in both +BP3 and Mock cells at 15 min and 10 h. Similar to Akt activation, the response to either IGF-I or Long R³IGF-I was greater in +BP3 cells relative to Mock cells at both 15 min (3.9 ± 0.6 -fold versus 2.0 ± 0.5 -fold respectively; mean $\pm$ S.E. of four experiments; $P < 0.01$ by paired Student's *t*-test) and 10 h (4.8 ± 0.9 -fold versus 2.3 ± 0.2 -fold respectively; mean $\pm$ S.E. of three

experiments; $P < 0.01$). The additional two +BP3 clones also had a greater response to IGF-I at 10 h relative to the additional two Mock clones (4.0-fold versus 2.6-fold respectively; data not shown).

Since the enhanced response appeared to be due in part to a two- to threefold lower basal phosphorylation of p70^{S6K} in the +BP3 cells relative to the Mock cells, we compared basal phosphorylation of p70^{S6K} at 15 min across the six clones directly. As shown in Fig. 7B, all three +BP3 clones had markedly lower basal phosphorylation of p70^{S6K} relative to the three Mock clones (2.9-fold difference; $P < 0.01$). In addition, basal phosphorylation of p70^{S6K} in the parental wild-type MAC-T cells resembled that of the Mock clones.

Discussion

Intensive investigation into IGFBP-3 action has established it as a complex protein that exerts multiple effects on cellular growth and apoptosis, most likely through diverse mechanisms. The ability of IGFBP-3 to potentiate IGF-I action has been recognized for many years (DeMellow & Baxter 1988, Conover 1992). We recently reported that MAC-T cells transfected to constitutively express IGFBP-3 were both more sensitive and responsive to IGF-I in terms of DNA synthesis. The potentiating effect of IGFBP-3 was not dependent on a physical association between IGF and IGFBP-3 since enhanced DNA synthesis was also observed with agents that activate the IGFR but do not bind IGFBP-3, such as Long R³IGF-I and insulin (Grill & Cohick 2000). The finding that IGFBP-3 could potentiate IGF action without actually binding it agrees with studies in which preincubation of bovine fibroblasts with exogenous IGFBP-3 enhanced their responsiveness to [QAYL]IGF-I, an analogue which also fails to bind IGFBP-3 (Conover 1992). However, a mechanism to explain the potentiating effect has not been definitively established to date.

Our results indicate that endogenous production of IGFBP-3 may potentiate IGF action by enhancing IGF signaling via the PI3K pathway. The serine/threonine protein kinase Akt/PKB is a downstream target of PI3K (Datta *et al.* 1999). In the present study, an enhanced degree of Akt phosphorylation was observed as early as 1 min

and was sustained for up to 10 h in +BP3 cells. *In vitro* kinase assays confirmed that constitutive expression of IGFBP-3 was associated with an increase in Akt enzymatic activity relative to Mock cells.

Both Long R³IGF-I and 5 µg/ml insulin stimulated Akt activation to a similar degree as native IGF-I in +BP3 cells. Long R³IGF-I has been reported to bind IGFBP-3, albeit with a 45- to 60-fold lower potency than native IGF-I (Devi *et al.* 2001). Therefore it is difficult to definitively rule out the influence of an IGFBP-3/IGF-I interaction on the ability of IGF-I to interact with its receptor with this analogue alone. However, similar results were obtained with B chain IGF-I, an analogue in which the first 16 amino acids of IGF-I are replaced with the first 17 amino acids of the B-chain of insulin (Bayne *et al.* 1988). This analogue exhibits a complete loss of affinity for bovine IGFBP-3 (Clemmons *et al.* 1992). In addition, the finding that 5 µg/ml insulin, which does not bind IGFBP-3, elicits a similar response, supports the suggestion that this interaction is not essential to the enhanced signaling through Akt.

Conover *et al.* (2000) have recently reported that inhibition of the PI3K pathway with LY 294002 inhibited the ability of exogenous IGFBP-3 to potentiate IGF-I-stimulated aminoisobutyric acid uptake in bovine fibroblasts. Furthermore, they found that preincubation with IGFBP-3 enhanced IGF-I-stimulated Akt activation. They further postulated that preincubation with IGFBP-3 caused dephosphorylation of basal Akt, resulting in enhanced sensitivity of Akt phosphorylation to IGF-I. It is interesting that their cell culture model as well as that of the present study both represent cell lines that are not only IGF-I responsive but that synthesize IGFBP-3 in response to IGF-I (Bale & Conover 1992, Cohick & Turner 1998). In the present work, Akt activation was also enhanced by TGF-α in +BP3 cells, suggesting that the enhanced Akt activation elicited by IGF-I is mediated at a point downstream of IGFR autophosphorylation. This concurs with the findings of Conover *et al.* (2000) who also reported that the potentiating effect of IGFBP-3 on IGF action was not associated with enhanced phosphorylation of the IGFR or insulin receptor substrate-2 (IRS-2).

One possibility to explain the present findings is that secreted IGFBP-3 may bind to a postulated cell membrane binding site/receptor and trigger

events that influence the intracellular signaling machinery. This mechanism has been proposed to explain the ability of IGFBP-3 to inhibit cellular growth and/or stimulate apoptosis independent of the IGFR system (Oh *et al.* 1993, Rajah *et al.* 1997, Baxter *et al.* 2000). For example, IGFBP-3 has been reported to interact with the TGF-β receptor system to phosphorylate TGF-β receptor I as well as the signaling molecules Smad2 and Smad3 (Fanayan *et al.* 2002). However, in the present study, exposure of Mock cells to exogenous IGFBP-3 alone for 24 h failed to stimulate Akt activation. Similarly, exogenous IGFBP-3 alone had no effect on phosphorylation of the IGFR or IRS-2 in bovine fibroblasts (Conover *et al.* 2000). In addition to the lack of an effect of exogenous IGFBP-3 alone, pretreatment of cells with IGFBP-3 prior to IGF-I stimulation had no effect on the ability of IGF-I to stimulate Akt phosphorylation in Mock cells. Studies by others have shown that exogenous IGFBP-3 inhibits activation of the IGFR signaling cascade by IGF-I, either by directly sequestering IGF-I away from the IGFR (Imai *et al.* 2000) or by a mechanism independent of IGF binding (Ricort & Binoux 2001). The present studies examining the effect of exogenous IGFBP-3 were designed to mimic the situation in +BP3 cells and did not address whether exogenous IGFBP-3 can inhibit IGF-I activation of intracellular signaling cascades. In these experiments, the molar quantities of IGF-I exceeded those of IGFBP-3, so that all free IGF-I would not be sequestered.

Alternatively, the enhanced activation of Akt in response to IGF-I may relate to an intracellular action of IGFBP-3. IGFBP-3 contains a nuclear localization signal, and has been found in the nucleus in a number of cell lines (Jaques *et al.* 1997, Li *et al.* 1997, Schedlich *et al.* 1998, Wraight *et al.* 1998). IGFBP-3 has been shown to bind the nuclear receptor retinoid X receptor-α (RXR-α), a key regulator of gene transcription (Liu *et al.* 2000). The interaction between IGFBP-3 and RXR-α is involved in the ability of these agents to inhibit apoptosis. IGFBP-3 contains consensus phosphorylation sites for several serine/threonine kinases including MAPK, protein kinase A and protein kinase C, and phosphorylation of the secreted protein has been demonstrated (Hoeck & Mukku 1994, Coverley & Baxter 1997, Pattison *et al.* 1999). It is plausible that phosphorylation of IGFBP-3 intracellularly could modulate its ability to interact

with components of the signaling machinery. Further studies will be necessary to determine if intracellular IGFBP-3 acts via a mechanism different from the secreted form of the protein.

The ability of PI3K to stimulate cell growth has been linked to another downstream effector, p70^{S6K}. While p70^{S6K} activation was enhanced in +BP3 cells, this appeared to be related largely to an attenuation of basal p70^{S6K} phosphorylation. These findings are consistent with and may explain the lower basal [³H]thymidine incorporation yet greater IGF-stimulated DNA synthesis that is consistently observed in +BP3 cells relative to Mock cells (Grill & Cohick 2000). One possibility is that IGFBP-3 may activate dephosphorylation of p70^{S6K}, as has been suggested for Akt (Conover *et al.* 2000). However, it is interesting that the higher levels of phosphorylated Akt in IGF-I-treated cells did not appear to result in greater levels of phosphorylated p70^{S6K}. The role of alternative downstream targets of Akt in mediating this effect is presently being investigated.

Results of the present study indicate that IGFBP-3 may potentially bind intracellular molecules involved in the IGF-R signaling cascade or in some way modulate the ability of such molecules to activate essential components of this cascade. Confirmation of such a mechanism would expand the role of IGFBP-3 to include modulation of IGF action via effects on intracellular signaling cascades as has been suggested for its proposed IGF-independent action on apoptosis.

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