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Characterization of the Inhibition of DNA Synthesis in Proliferating Mink Lung Epithelial Cells by Insulin-Like Growth Factor Binding Protein-3

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Abstract Insulin-like growth factor binding protein-3 (IGFBP-3) can inhibit cell growth by directly interacting with cells, as well as by forming complexes with IGF-I and IGF-II that prevent their growth-promoting activity. The present study examines the mechanism of inhibition of DNA synthesis by IGFBP-3 in CCL64 mink lung epithelial cells. DNA synthesis was measured by the incorporation of 5-bromo-2'-deoxyuridine, using an immunocolorimetric assay. Recombinant human IGFBP-3 (rh[N109D,N172D]IGFBP-3) inhibited DNA synthesis in proliferating and quiescent CCL64 cells. Inhibition was abolished by co-incubation of IGFBP-3 with a 20% molar excess of Leu^{60}-IGF-I, a biologically inactive IGF-I analogue that binds to IGFBP-3 but not to IGF-I receptors. DNA synthesis was not inhibited by incubation with a preformed 1:1 molar complex of Leu^{60}-IGF-I and IGFBP-3, indicating that only free IGFBP-3 inhibits CCL64 DNA synthesis. Inhibition by IGFBP-3 is not due to the formation of biologically inactive complexes with free IGF, since endogenous IGFs could not be detected in CCL64 conditioned media; any IGFs that might have been present could only have existed in inactive complexes, since endogenous IGFBPs were present in excess; and biologically active IGFs were not displaced from endogenous IGFBP complexes by Leu^{60}-IGF-I. After incubation with CCL64 cells, ^{125}I-IGFBP-3 was covalently cross-linked to a major ~400-kDa complex. This complex co-migrated with a complex formed after incubation with ^{125}I-labeled transforming growth factor-β (TGF-β) that has been designated the type V TGF-β receptor. ^{125}I-IGFBP-3 binding to the ~400-kDa receptor was inhibited by co-incubation with unlabeled IGF-I or Leu^{60}-IGF-I. The ability of Leu^{60}-IGF-I to decrease both the inhibition of DNA synthesis by IGFBP-3 and IGFBP-3 binding to the ~400-kDa receptor is consistent with the hypothesis that the ~400-kDa IGFBP-3 receptor mediates the inhibition of CCL64 DNA synthesis by IGFBP-3. J. Cell. Biochem. 77:288–297, 2000.

Key words: IGF-I; Leu^{60}-IGF-I; BrdU cell proliferation assay; covalent cross-linking; EGFs; type V TGF-β receptor

Insulin-like growth factor binding protein-3 (IGFBP-3), the most abundant IGFBP in human plasma, binds insulin-like growth factor I and II (IGF-I and IGF-II) with high affinity [Heding et al., 1996; Rechler, 1993]. IGFBP-3 is synthesized in most tissues [Albiston and Herington, 1992]. It can inhibit IGF-I biological activity by forming a complex with IGF-I that prevents the growth factor from binding to and activating IGF-I receptors on target cells [reviewed in Rechler, 1993]. Under certain specific circumstances, IGFBP-3 can potentiate IGF-I actions by mechanisms that are not fully understood [Conover et al., 1996; Jones and Clemmons, 1995; Rechler and Clemmons, 1998].

Over the past few years, several studies have suggested that IGFBP-3 also can inhibit cell growth by a mechanism other than preventing IGFs from activating IGF-I receptors [Cohen et al., 1993; Gucev et al., 1996; Liu et al., 1992; Oh et al., 1993a; Rechler and Clemmons, 1998; Valentinis et al., 1995]. Balb c/3T3 fibroblasts [Cohen et al., 1993] and mouse fibroblasts containing targeted deletions of the IGF-I receptor grew slowly after stable transfection with

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IGFBP-3 [Valentinis et al., 1995], suggesting that IGF-I receptors were not required for growth inhibition by IGFBP-3. IGFBP-3 also inhibited the growth of Hs578T human breast cancer cells by mechanisms that appear not to involve the formation of inactive complexes with IGFs [Oh et al., 1993a]. These cells do not synthesize IGF-I mRNA [Yee et al., 1989], and IGFBP-1 (which also forms biologically inactive complexes with IGF-I and IGF-II) did not inhibit DNA synthesis [Oh et al., 1993a]. Antisense oligonucleotides to IGFBP-3 decreased growth inhibition by transforming growth factor-β (TGF-β) or retinoic acid in MDA-MB-231 breast cancer cells [Gucev et al., 1996], which also do not synthesize IGF-I [Yee et al., 1989], suggesting that IGFBP-3 plays an essential role in growth inhibition by these agents and may act by mechanisms that do not involve the sequestration of IGF.

Direct inhibition of cell growth by IGFBP-3 raised the possibility that this inhibition was mediated by an IGFBP-3 receptor. Association of IGFBP-3 with cell monolayers has been described in many cell types [Booth et al., 1995; Conover et al., 1996; Delbe et al., 1991; Martin et al., 1992; Oh et al., 1992, 1993a; Smith et al., 1992; Yang et al., 1996]. In some cases, IGFBP-3 was internalized [Conover et al., 1996; Delbe et al., 1991; Smith et al., 1992; Yang et al., 1996], cleared from the media, and presumably degraded [Smith et al., 1992], or localized to the nucleus [Li et al., 1997; Schedlich et al., 1998]. Binding was inhibited by heparin [Booth et al., 1995; Martin et al., 1992; Smith et al., 1994; Yang et al., 1996] and by IGF-I [Booth et al., 1995; Martin et al., 1992; Oh et al., 1992, 1993a; Smith et al., 1994; Yang et al., 1996]. Small proteins (20, 26, and 50 kDa) were cross-linked to IGFBP-3 in Hs578T cell monolayers and membrane preparations [Oh et al., 1993b]. The relationship of these observations to a putative growth inhibitory IGFBP-3 receptor, however, is unclear.

Recently, Leal et al. [1997] described an IGFBP-3 receptor in CCL64 mink lung epithelial cells whose properties suggested that it might be a growth inhibitory IGFBP-3 receptor. IGFBP-3 inhibited DNA synthesis in quiescent CCL64 cells, and radiolabeled IGFBP-3 could be cross-linked to an ~400-kDa receptor. TGF-β formed a complex of similar size, designated the type V TGF-β receptor [O'Grady et al., 1991], which has been partially purified [O'Grady et al., 1991] and shown to possess intrinsic serine/threonine protein kinase activity [Liu et al., 1994; O'Grady et al., 1992]. A peptide antagonist (corresponding to amino acids 41–65 of TGF-β1) [Huang et al., 1997; Leal et al., 1997] inhibited the cross-linking of radiolabeled IGFBP-3 or TGF-β to the ~400-kDa receptor and blocked the inhibition of DNA synthesis by either protein, leading the authors to propose that the type V TGF-β receptor was the putative growth inhibitory IGFBP-3 receptor [Leal et al., 1997].

Although this provocative study demonstrated that IGFBP-3 binds to an ~400-kDa receptor on CCL64 cells and inhibits DNA synthesis, it did not address the question of whether IGFBP-3 acted directly on CCL64 cells to inhibit growth, rather than by forming inactive complexes with IGF. Direct inhibition is a prerequisite before assigning a specific role to the ~400-kDa receptor in mediating IGFBP-3 growth inhibition. We now show that free IGFBP-3 acts directly on proliferating CCL64 cells to inhibit DNA synthesis. Co-incubation with a biologically inactive IGF-I analogue, Leu^{60}-IGF-I, that binds to IGFBP-3 but has reduced affinity for the IGF-I receptor [Bayne et al., 1990], reduced the inhibition of DNA synthesis by IGFBP-3 and decreased IGFBP-3 binding to the ~400 kDa receptor, consistent with the hypothesis that this receptor is the growth inhibitory IGFBP-3 receptor.

MATERIALS AND METHODS

Materials

Recombinant human IGFBP-3 containing substitutions at two of three possible N-glycosylation sites (rh[N109D,N172D]IGFBP-3) was expressed in *Escherichia coli* as previously described [Zhang et al., 1998]. This preparation was used throughout this study and is referred to as hIGFBP-3 for simplicity. Leu^{60}-IGF-I and 1:1 molar complexes of Leu^{60}-IGF-I:IGFBP-3 were synthesized as previously described [Bagi et al., 1994]. Human recombinant TGF-β1 (R&D Systems, Minneapolis, MN) was kindly provided by Anita Roberts (National Cancer Institute). Epidermal growth factor (EGF), IGF-I, and chemical reagents were purchased from Sigma Chemical Company (St. Louis, MO). ^{125}I-IGF-I and ^{125}I-IGF-II (2,000 Ci/mmol), and carrier-free Na^{125}I-Iodide, were purchased from Amersham (Arlington Heights, IL). Fetal calf serum (FCS) (40 nm

filtered) was obtained from Hyclone Laboratories (Logan, UT), and Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, pyridoxine hydrochloride, and sodium pyruvate from Life Technologies (Gaithersburg, MD). The BrdU Cell Proliferation ELISA kit was purchased from Boehringer-Mannheim (Indianapolis, IN), and the water-soluble covalent cross-linking agent bis(sulfosuccinimidyl)suberate (BS^3) from Pierce Chemicals (Rockford, IL).

Propagation of Mink Lung Epithelial Cells

Stock cultures of Mv1Lu mink lung epithelial cells (American Type Cell Culture [ATCC], CCL64) were propagated in growth medium (DMEM containing 10% FCS) in 5% CO_2 at 37°C. Cells were split at a 1:20 ratio twice per week. Fresh cells were thawed every 2 months.

5-Bromo-2'-Deoxyuridine (BrdU) Cell Proliferation Assay

Incorporation of BrdU into the DNA of proliferating and quiescent cells was determined according to the manufacturer's instructions. For assays under proliferating conditions, CCL64 cells were plated in 96-well microtiter plates (50,000 cells/well) in serum-free DMEM supplemented with 0.5% bovine serum albumin (BSA) (Sigma, RIA grade) and incubated at 37°C for 3 h. EGF (20 ng/ml) and IGFBP-3 (1 μg/ml, unless otherwise indicated), IGF-I or Leu^{60}-IGF-I (0.3 μg/ml, an ~ 20% molar excess), or 1:1 molar complexes of IGFBP-3 and Leu^{60}-IGF-I (1.25 μg) were added, and the incubation continued overnight. EGF stimulated DNA synthesis about threefold (F. Dong, unpublished results) as previously reported [Kelley et al., 1992]. For assays under quiescent conditions, CCL64 cells were plated in growth medium in 96-well plates (30,000 cells/well) and incubated overnight at 37°C, after which the medium was changed to medium containing 0.1% FCS for 24 h. IGFBP-3, IGF-I and Leu^{60}-IGF-I were added as indicated above, and the incubation continued overnight.

To measure DNA synthesis, BrdU (10 μM) was added for 2–3 h. The cells were lysed and BrdU incorporated into newly synthesized DNA was quantitated by an immunocolorimetric assay using monoclonal antibody to BrdU conjugated to peroxidase. After incubation with tetramethylbenzidine substrate, the reaction product was quantified by measuring the absorbance at 450 nm (reference wavelength, 690 nm) using a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay [ELISA] reader, Tecan Rainbow shell, TECAN U.S., Research Triangle Park, NC).

Charcoal-Separation Binding Assay for IGFBPs

Binding of IGFs to IGFBPs in solution was examined using a charcoal-separation assay in which free IGFBP is adsorbed by activated charcoal, leaving IGF:IGFBP complexes remaining in the charcoal supernate [Yang and Rechler, 1993]. Recombinant human IGFBP-3 or the endogenous IGFBP present in CCL64 conditioned media was used. [Conditioned media were obtained by incubation of confluent CCL64 cells with serum-free DMEM supplemented with 0.5% BSA (RIA grade) for 24 h, after which fresh serum-free DMEM/0.5% BSA containing 20 ng/ml EGF was added and the media collected after 16 h.] To determine whether unoccupied IGFBPs were present in conditioned media, different amounts of media were incubated with ^{125}I-IGF-II (15–20,000 cpm) overnight at 4°C (saturation assay). Competitive inhibition of ^{125}I-IGF-I binding to hIGFBP-3 by unlabeled Leu^{60}IGF-I was determined by incubating hIGFBP-3 (25 ng/ml) with ^{125}I-IGF-I (~40,000 cpm) in the presence of increasing concentrations of Leu^{60}-IGF-I in 0.4 ml of phosphate-buffered saline (PBS) supplemented with 0.2% fatty acid-free BSA (Sigma). Specifically, bound radioactivity was calculated by subtracting the radioactivity in the charcoal supernate in the absence of added IGFBP (5–10% of input radioactivity) from the total bound radioactivity.

Quantitation of IGFs by Competitive Charcoal-Separation Assay

Conditioned media were collected from CCL64 cells incubated for 40 h in serum-free medium plus EGF and were fractionated by gel filtration on a Sephadex G-50 column in 1 M acetic acid. Fractions in the IGF peak were pooled, dried, resuspended, and assayed in a charcoal-separation competitive binding assay, using acid-stripped normal rat serum and ^{125}I-IGF-I. Inhibition by dilutions of the media samples was compared with inhibition by an unlabeled recombinant human IGF-I standard. This assay measures IGF-I and IGF-II with

comparable affinity [Lee and Rechler, 1995]. No IGFs were detected in the media samples at the limit of detection, 1 ng/ml.

Iodination of ^{125}I-IGFBP-3 and ^{125}I-TGF-β

TGF-β1 was iodinated to a specific activity of 78 μCi/μg, using the chloramine T method as previously described [Frolik et al., 1984]. Recombinant hIGFBP-3 (5 μg/5 μl; 50 mM sodium acetate, 105 mM NaCl) was added to 45 μl of 0.6 M potassium phosphate (pH 7.4). Na^{125}I (1 mCi, 10 μl) was added, followed by three additions of 250 ng (5 μl) of freshly dissolved chloramine-T for 2, 1.5, and 1 min, respectively. The reaction was stopped by the addition of 50 mM N-acetyl-tyrosine (20 μl) and, two min later, 60 mM KI (200 μl) and 8 M urea (200 μl). Radiolabeled peptides were immediately purified by gel filtration on a Sephadex G-25M column (PD-10, Pharmacia, Piscataway, NJ) equilibrated with pH 7.4 PBS (Life Technologies) containing 0.1% fatty acid-free BSA and 0.1% Tween 20 (Boehringer-Mannheim). The specific activity of ^{125}I-IGFBP-3 was typically 95 μCi/μg; > 90% of the radioactivity was precipitable with 10% trichloroacetic acid (TCA) (in the presence of 0.2% BSA carrier).

Covalent Cross-Linking of ^{125}I-Labeled IGFBP-3 and TGF-β1 to CCL64 Cells

CCL64 cells (200,000 cells/well) were plated in six-well, 3.5-cm plates in 2 ml DMEM containing 10% FCS, and grown for 2 days. Affinity labeling of cell surface receptors was performed as previously described [Massague, 1987]. In brief, ^{125}I-labeled IGFBP-3 or TGF-β1 were preincubated in binding buffer (50 mM Hepes, pH 7.4; 128 mM NaCl; 5 mM KCl; 5 mM $MgSO_4$; 1.2 mM $CaCl_2$, and 0.2% fatty acid-free BSA) with the indicated amounts of IGFBP-3, TGF-β1, IGF-I or Leu^{60}-IGF-I (30 min, 4°C). Radiolabeled IGFBP-3 (10 million cpm/ml/well) or TGF-β1 (4 million cpm/ml/well) was incubated with the cell monolayer on ice for 4 h. The cells were washed 5 times with cold binding buffer to remove unbound radioactivity, and bound peptide was cross-linked covalently by the addition of BS^3 (100 ng/ml/well) in binding buffer without BSA (20 min, 4°C). Cells were scraped in 1.5 ml 10 mM Tris-HCl, pH 7.4; 0.25 M sucrose; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged (14,000*g*; 4°C). The cell pellet was solubilized with Triton X-100 (60 μl), centrifuged for 10 min, and the Triton-soluble fraction analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using Tris-acetate gradient gels (3–8% acrylamide, Novex, San Diego, CA) under reducing conditions, followed by autoradiography. Autoradiographs were scanned and the images analyzed using the software program NIH Image 1.61/ppc (NIH, Bethesda, MD).

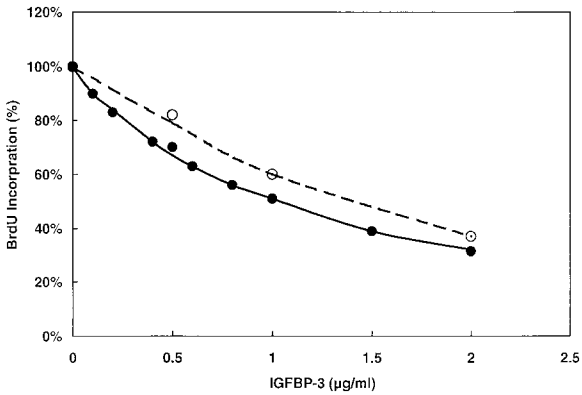

Fig. 1. Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits BrdU incorporation into DNA of proliferating and quiescent mink lung epithelial cells. Proliferating (serum-free medium plus 20 ng/ml epidermal growth factor [EGF]; solid circles, solid lines) or quiescent (0.1% fetal calf serum [FCS]; open circles, dashed lines) CCL64 cells were incubated overnight with the indicated concentrations of IGFBP-3. BrdU was added for 2–3 h, and BrdU incorporation into newly synthesized DNA measured by an immunocolorimetric assay. Incorporation is expressed relative to the incorporation in the absence of IGFBP-3, taken as 100%. The points shown for 0, 0.2, 0.5, and 1 μg/ml are the mean of six experiments (SD 8–20%); the 0.8 μg/ml point is from 3 experiments, the other points from one or two experiments.

RESULTS

IGFBP-3 Inhibits DNA Synthesis in CCL64 Cells by Mechanisms That Do Not Involve the Formation of Biologically Inactive IGF:IGFBP-3 Complexes

IGFBP-3 inhibited DNA synthesis in quiescent CCL64 cells maintained in 0.1% FCS [Leal et al., 1997]. To study growth inhibition within the more appropriate context of cell proliferation, while avoiding adding even small amounts of IGF in serum, we stimulated CCL64 cell DNA synthesis with EGF in serum-free medium [Kelley et al., 1992]. Recombinant hIGFBP-3 inhibited BrdU incorporation into newly synthesized DNA in proliferating CCL64 cells (Fig. 1). Inhibition was dose dependent: 60%

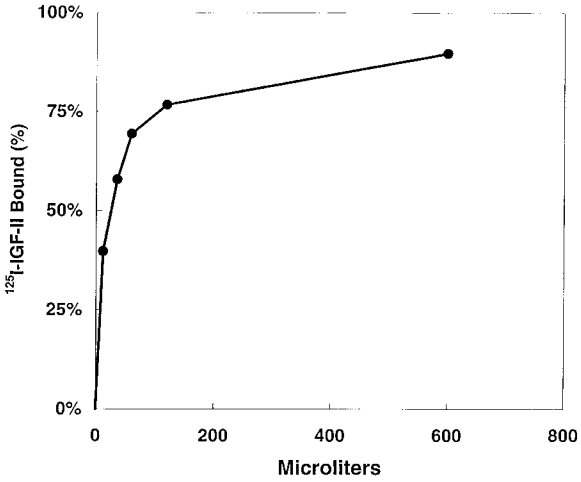

Fig. 2. ^{125}I-labeled insulin-like growth factor II (IGF-II) binds to unoccupied insulin-like growth factor binding proteins (IGFBPs) in conditioned media from CCL64 cells. ^{125}I-IGF-II was incubated with the indicated volumes of CCL64 media (collected in Dulbecco's modified Eagle's medium [DMEM]/0.5% bovine serum albumin [BSA]/20 ng/ml EGF) overnight at 4°C. Bound and unbound radioactivity were separated with activated charcoal. Bound radioactivity (expressed as the percentage of input radioactivity) is plotted against microliters of media.

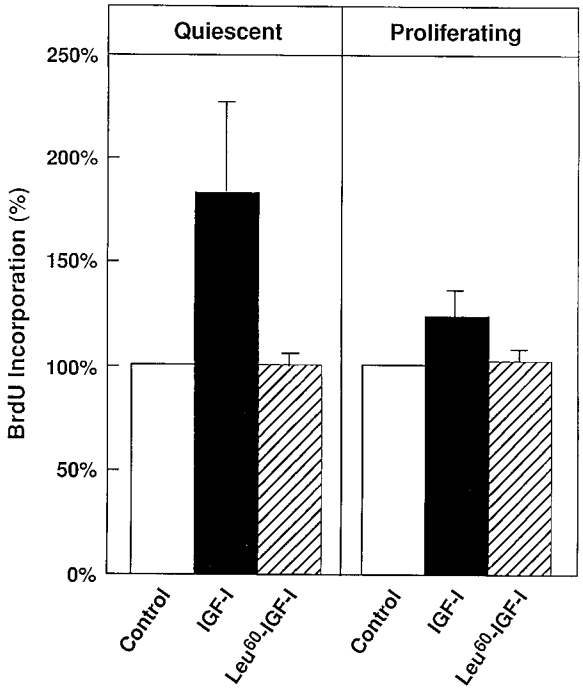

Fig. 3. Ability of insulin-like growth factor I (IGF-I) and Leu^{60}-IGF-I to stimulate BrdU incorporation in proliferating and quiescent CCL64 cells. IGF-I or Leu^{60}-IGF-I (0.3 μg/ml) were incubated with quiescent or proliferating CCL64 cells (in media containing 0.1% fetal calf serum [FCS], or serum-free medium plus 20 ng/ml epidermal growth factor [EGF], respectively) at 37°C overnight. BrdU incorporation is expressed relative to control cells that did not receive IGF-I or Leu^{60}-IGF-I. The mean ±SD of 4–9 experiments is plotted.

at 2 μg/ml and ~40% at 1 μg/ml, the concentration used in subsequent experiments unless otherwise specified. Similar dose-dependent inhibition was observed in quiescent cells incubated in 0.1% FCS (Fig. 1).

The inhibition of DNA synthesis by IGFBP-3 does not result from sequestration of endogenous IGFs in biologically inactive complexes. Functionally significant levels of IGFs could not be detected in CCL64-conditioned media: total combined IGF-I and IGF-II measured by competitive binding assay was <1 ng/ml (results not shown). Any IGF that might have been synthesized would have been present in inactive complexes with endogenous IGFBPs, since unoccupied IGFBPs were present in excess, indicated by the fact that >80% of input radioactivity was bound to IGFBPs after incubation of CCL64 cell conditioned media with ^{125}I-IGF-II (Fig. 2). [A single ~34-kDa protein that binds radiolabeled IGF was identified in conditioned media by ligand blotting (unpublished results).] Incubation with IGF-I (0.3 μg/ml) did not stimulate BrdU incorporation into the DNA of proliferating CCL64 cells (Fig. 3). Since IGFs were not detectable in CCL64 cell conditioned media, and high concentrations of IGF-I did not stimulate DNA synthesis in proliferating CCL64 cells, these results strongly suggest that the inhibition of DNA synthesis by IGFBP-3 results from a direct interaction with CCL64 cells.

DNA Synthesis in CCL64 Cells Is Inhibited by Free IGFBP-3, But Not by IGF:IGFBP-3 Complexes

We next determined whether IGFBP-3 also could inhibit CCL64 DNA synthesis when it was complexed to IGF. To exclude the possibility of independent activation of the IGF-I receptor by added IGF-I, we used an IGF-I analogue, Leu^{60}-IGF-I, that binds to IGFBP-3 with nearly the same affinity as IGF-I but has greatly reduced affinity for IGF-I receptors [Bayne et al., 1990]. Although IGF-I stimulated BrdU incorporation into the DNA of quiescent CCL64 cells by approximately twofold (Fig. 3), Leu^{60}-IGF-I did not stimulate BrdU incorporation in either proliferating or quiescent cells, consistent with its low affinity for

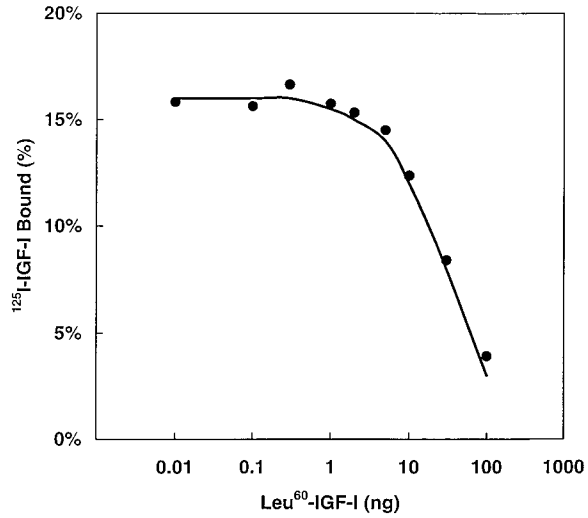

Fig. 4. Leu^{60}-insulin-like growth factor I (IGF-I) competitively inhibits the binding of ^{125}I-labeled IGF-I to insulin-like growth factor binding protein-3 (IGFBP-3). Recombinant hIGFBP-3 (2.5 ng) was incubated with ^{125}I-IGF-I in the presence of different amounts of Leu^{60}-IGF-I. The percentage of input radioactivity bound to IGFBP-3 is plotted against the amount of Leu^{60}-IGF-I added.

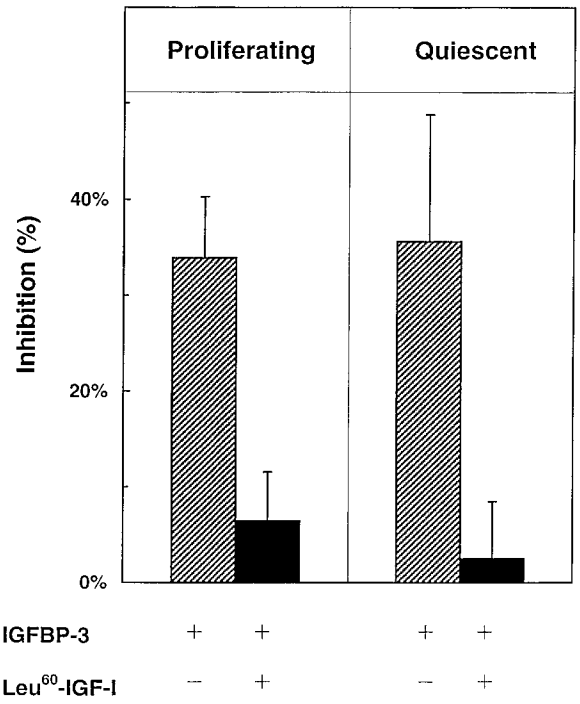

Fig. 5. Effect of Leu^{60}-insulin-like growth factor I (IGF-I) on the inhibition of BrdU incorporation by insulin-like growth factor binding protein-3 (IGFBP-3) in proliferating and quiescent mink lung epithelial cells. Proliferating (left) and quiescent (right) CCL64 cells were incubated overnight with IGFBP-3 in the absence (hatched bar) or presence (solid bar) of a 20% molar excess of Leu^{60}-IGF-I, and BrdU incorporation was determined. The percentage inhibition of BrdU incorporation (relative to cells that received no addition) is plotted. The mean ±SD of seven experiments is plotted.

the signaling IGF-I receptor. Leu^{60}-IGF-I does bind to IGFBP-3 with high affinity, however, as indicated by its ability to competitively inhibit the binding of ^{125}I-IGF-I to hIGFBP-3 (Fig. 4). Thus, Leu^{60}-IGF-I binds to IGFBP-3 but does not independently stimulate DNA synthesis in CCL64 cells, so that any effects it might have on IGFBP-3 inhibition of DNA synthesis should be due to its interaction with IGFBP-3 and not due to activation of the IGF-I receptor.

Leu^{60}-IGF-I markedly decreased the ability of IGFBP-3 to inhibit CCL64 cell DNA synthesis (Fig. 5). IGFBP-3 (1 μg/ml) inhibited DNA synthesis in proliferating and quiescent CCL64 cells by approximately 35%. Co-incubation with a 20% molar excess of Leu^{60}-IGF-I almost completely abolished this inhibition. Similarly, no significant inhibition of DNA synthesis was observed when proliferating and quiescent CCL64 cells were incubated with preformed 1:1 molar complexes of IGFBP-3 and Leu^{60}-IGF-I, compared with ~40% inhibition by an equimolar concentration (1 μg/ml) of free IGFBP-3 (Fig. 6). These results indicate that free IGFBP-3, but not IGFBP-3 complexed to IGFs, inhibits DNA synthesis in CCL64 cells.

IGF-I and Leu^{60}-IGF-I Inhibit the Binding of ^{125}I-IGFBP-3 to an ~400-kDa IGFBP-3 Receptor

Radiolabeled IGFBP-3 binds to ~400-kDa receptors on CCL64 cells [Leal et al., 1997]. If this receptor mediated growth inhibition induced by IGFBP-3 [Leal et al., 1997], one would predict that Leu^{60}-IGF-I would inhibit the binding of IGFBP-3 to the ~400 kDa IGFBP-3 receptor, since Leu^{60}-IGF-I:IGFBP-3 complexes do not inhibit growth.

CCL64 cells were incubated with ^{125}I-IGFBP-3, proteins covalently cross-linked, solubilized with Triton, and the extracts examined by SDS-PAGE (3–8% acrylamide gradient gels). A predominant ~400-kDa complex was observed (Fig. 7). ^{125}I-TGF-β formed a complex of similar size, as well as a more rapidly migrating complex that corresponds to the type III TGF-β receptor [Leal et al., 1997]. Unlabeled TGF-β (1 μg/ml) decreased the amount of ^{125}I-TGF-β cross-linked

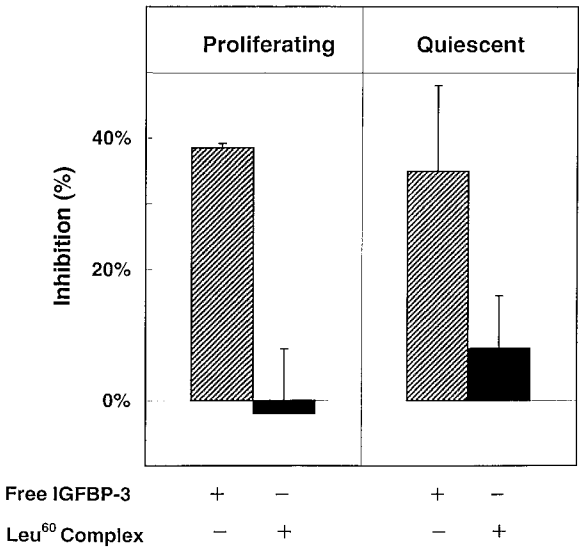

Fig. 6. Inhibition of BrdU incorporation in proliferating and quiescent CCL64 cells after incubation with free insulin-like growth factor binding protein-3 (IGFBP-3) and preformed Leu^{60}IGF-I complexes. Proliferating (left) or quiescent (right) CCL64 cells were incubated overnight with IGFBP-3 (1 μg/ml; hatched bar) or Leu^{60}-IGF-I:IGFBP-3 complex (1.25 μg/ml; solid bar), and BrdU incorporation was determined. The percentage inhibition of BrdU incorporation (relative to cells that were not treated with IGFBP-3 or complex) is plotted. The mean ±SD of two (quiescent) or three (proliferating) experiments is shown.

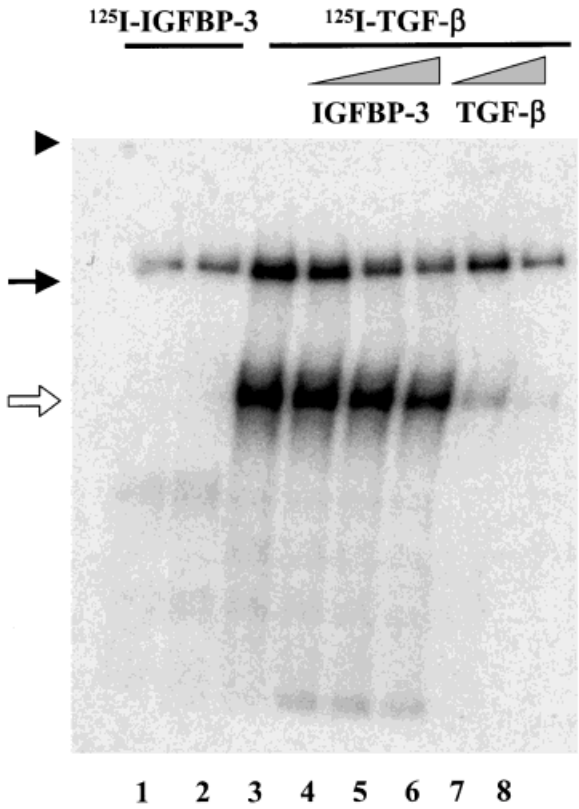

Fig. 7. Affinity cross-linking of ^{125}I-insulin-like growth factor binding protein-3 (IGFBP-3) and ^{125}I-transforming growth factor-β (TGF-β) to CCL64 cells. CCL64 cells were incubated with ^{125}I-IGFBP-3 (**lanes 1,2**) or ^{125}I-TGF-β (**lanes 3–8**) in the absence (**lanes 1–3**) or presence of unlabeled IGFBP-3 (5, 15, or 25 μg, **lanes 4–6**, respectively) or TGF-β (0.1 and 1 μg, **lanes 7** and **8**, respectively). After cross-linking with BS^3, cells were lysed with Triton X-100, and the Triton-solubilized proteins separated on 3–8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The faster-migrating complex with ^{125}I-TGF-β (open arrow) corresponds to the type III TGF-β receptor, the more slowly migrating complex (solid arrow) to the type V TGF-β receptor [Leal et al., 1997]. The top of the gel is indicated by an arrowhead. A representative gel is presented.

to the ∼400-kDa receptors and the type III TGF-β receptors by ∼40% and ∼90%, respectively. Unlabeled IGFBP-3 (25 μg/ml) decreased the amount of ^{125}I-TGF-β cross-linked to the ∼400-kDa receptors to a similar extent (32%), but only minimally decreased cross-linking to type III receptors (14%). These results indicate that IGFBP-3 binds predominantly to the ∼400-kDa receptors, whereas TGF-β also binds to type III TGF-β receptors (as well as smaller type I and type II TGF-β receptors) [Leal et al., 1997].

^{125}I-IGFBP-3 binding to the ∼400-kDa IGFBP-3 receptor was inhibited in a dose-dependent manner by co-incubation with unlabeled IGF-I or Leu^{60}-IGF-I (Fig. 8). Binding was inhibited >40% by 500 ng of either polypeptide. [Co-incubation with IGF-I inhibited IGFBP-3 binding to Triton extracts of CCL64 cells [Leal et al., 1997], but it was not specifically determined whether this represented binding to the ∼400-kDa receptor.] Since Leu^{60}-IGF-I inhibits the binding of IGFBP-3 to the predominant IGFBP-3 binding site, the ∼400-kDa receptor, and reduces growth inhibition by IGFBP-3, these results are consistent with the possibility that the ∼400-kDa IGFBP-3 receptor may mediate direct growth inhibition by IGFBP-3.

DISCUSSION

Recombinant human IGFBP-3 inhibits DNA synthesis in CCL64 cells stimulated to proliferate in serum-free medium with EGF and, as previously reported, in quiescent cells [Leal et al., 1997]. This is a direct effect of IGFBP-3, not the result of IGFBP-3 forming biologically inactive complexes with endogenous IGF. No IGF-I or IGF-II could be detected in CCL64 conditioned media using a sensitive competitive binding assay (limit of detection <1 ng/ml), and even if low

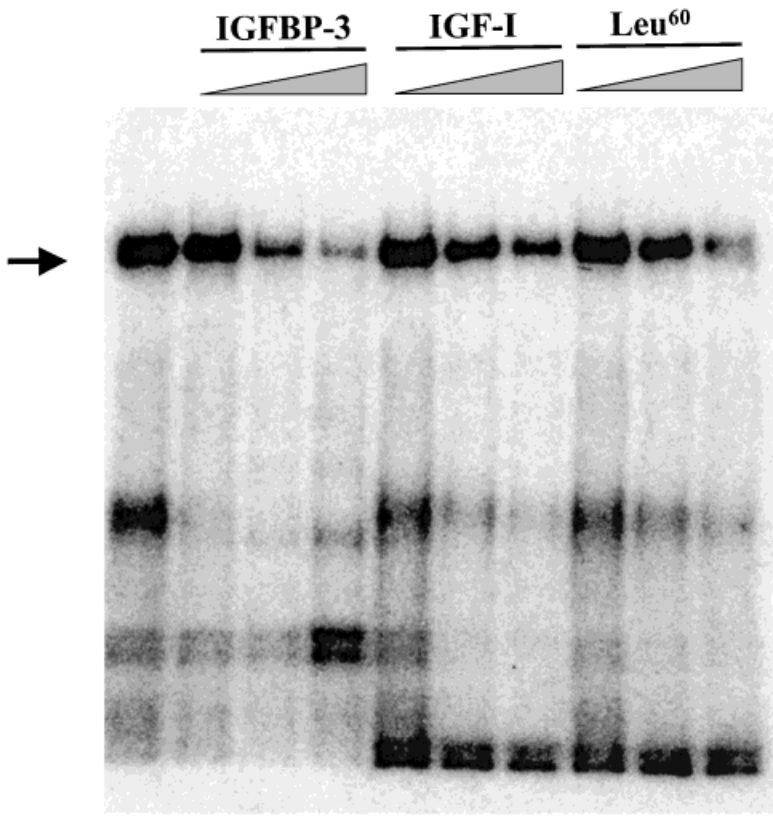

Fig. 8. Competitive inhibition of ^{125}I-insulin-like growth factor binding protein-3 (IGFBP-3) binding to the ~400-kDa receptor complex by unlabeled IGFBP-3, IGF-I, and Leu^{60}-IGF-I. ^{125}I-IGFBP-3 was incubated with CCL64 cells alone (**lane 1**), or in the presence of unlabeled IGFBP-3 (5, 15, and 25 μg, **lanes 2–4**, respectively), IGF-I (5, 50, or 500 ng, **lanes 5–7**, respectively) or Leu^{60}-IGF-I (5, 50 or 500 ng, **lanes 8–10**, respectively). After cross-linking with BS^3, the Triton-soluble proteins were fractionated on 3–8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels and examined by autoradiography. The ~400-kDa receptor is indicated by a solid arrow.

amounts of IGF were synthesized, they would only exist in biologically inactive complexes with endogenous IGFBPs which were present in excess. Moreover, addition of a high concentration of IGF-I (0.3 μg/ml) did not stimulate DNA synthesis in proliferating cells. These results indicate that IGFBP-3 acts directly on CCL64 cells to inhibit DNA synthesis.

The inhibition seen with free IGFBP-3 was not observed when the IGFBP-3 was complexed with Leu^{60}-IGF-I, an IGF-I analogue that binds to IGFBP-3 but does not activate the IGF-I receptor. Inhibition of DNA synthesis was not observed when CCL64 cells were co-incubated with IGFBP-3 and a 20% molar excess of Leu^{60}-IGF-I, or when cells were incubated with preformed 1:1 molar complexes of Leu^{60}-IGF-I:IGFBP-3. The possibility that biologically inactive Leu^{60}-IGF-I might act as an indirect agonist by displacing biologically active IGF-I from complexes with endogenous IGFBP [Loddick et al., 1998; Lowman et al., 1998] was excluded by the fact that incubation with Leu^{60}-IGF-I in the absence of IGFBP-3 did not stimulate DNA synthesis in proliferating or quiescent CCL64 cells. Thus, binding of Leu^{60}-IGF-I to IGFBP-3 blocked its ability to inhibit DNA synthesis in CCL64 cells directly, possibly by inducing a conformational change that prevented IGFBP-3 from interacting with a putative growth inhibitory receptor. These results are consistent with the observation that IGF analogues that do not bind to IGFBP-3 were unable to reverse IGFBP-3-induced growth inhibition in Hs578T breast cancer cells [Oh et al., 1993a].

A candidate growth inhibitory IGFBP-3 receptor on CCL64 cells was recently described [Leal et al., 1997]. Radiolabeled IGFBP-3 was cross-linked predominantly to an ~400-kDa receptor on CCL64 cells. A peptide antagonist [Huang et al., 1997] inhibited IGFBP-3 binding to the ~400-kDa receptor and blocked the inhibition of DNA synthesis by IGFBP-3 [Leal et al., 1997]. If the ~400-kDa receptor were the growth inhibitory IGFBP-3 receptor, one would predict that co-incubation with Leu^{60}-IGF-I, which decreased IGFBP-3 inhibition of DNA synthesis, also would decrease IGFBP-3 binding to the ~400-kDa receptor. Our results confirm this prediction.

Leal et al. [1997] further suggested that the ~400-kDa IGFBP-3 receptor is identical to the

type V TGF-β receptor. Although the two receptors share common properties, the available evidence is not sufficient to determine whether they are identical. They may be members of a family of closely related receptors, which might be difficult to resolve during purification. This could account for the comigration of cross-linked radiolabeled complexes of the two ligands, inhibition of TGF-β binding by unlabeled IGFBP-3, and immunoprecipitation of 400-kDa ^{125}I-IGFBP-3 receptor complexes by antibodies to the type V TGF-β receptor. In addition, an IGFBP-5 receptor described in mouse osteoblasts, which is similar in size to the type V TGF-β receptor and also has intrinsic serine-threonine kinase activity, may be another member of the same receptor family [Andress, 1995, 1998]. The IGFBP-5 receptor appears to differ from the type V TGF-β receptor, however, in that kinase activity is not stimulated by TGF-β [Andress, 1998]. Sequencing and cloning the different receptors will be necessary to establish definitively the relationships between them.

Since the relationship between the ~400-kDa IGFBP-3 receptor and the type V TGF-β receptor is uncertain, whether the type V TGF-β receptor has a critical role in TGF-β-induced growth inhibition [Leal et al., 1997] has no direct bearing on whether the IGFBP-3 receptor mediates IGFBP-3-induced growth inhibition. The ~400-kDa receptor is the only IGFBP-3 receptor identified in CCL64 cells in which IGFBP-3 directly inhibits DNA synthesis, making it a bona fide candidate to be the growth inhibitory IGFBP-3 receptor. The role of the type V TGF-β receptor in growth inhibition by TGF-β is more problematic because TGF-β also binds to three other TGF-β receptors on CCL64 cells, including the receptors mainly responsible for TGF-β signaling, the type I and type II TGF-β receptors [Massague, 1998]. In fact, TGF-β-induced growth inhibition is considerably impaired in CCL64 cells in which either the type I or type II TGF-β receptor is mutated, suggesting that these receptors are involved in growth inhibition [Liu et al., 1997].

In conclusion, the present study provides strong evidence that free IGFBP-3 inhibits DNA synthesis in proliferating CCL64 mink lung epithelial cells by direct cell interaction, rather than by preventing the access of IGF-I to signaling IGF-I receptors. The demonstration of direct action is an essential prerequisite before postulating that an IGFBP-3 receptor might mediate growth inhibition. We have shown that IGFBP-3 binds to an ~400-kDa receptor on CCL64 cells, and demonstrated that Leu^{60}-IGF-I causes a parallel decrease in IGFBP-3 binding to this receptor and IGFBP-3-induced inhibition of DNA synthesis. These results are consistent with the possibility that the ~400-kDa receptor might be the putative growth inhibitory IGFBP-3 receptor. Purification and molecular sequencing of this receptor will be required to understand how it signals, and to demonstrate definitively that it mediates growth inhibition by IGFBP-3.

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