DISTINCT ENDOCYTIC PATHWAYS FACILITATE RE-UPTAKE AND NUCLEAR LOCALIZATION*

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Insulin-like growth factor binding protein-3 (IGFBP-3) is well established as a growth-inhibitory, apoptosis-inducing secreted molecule that acts via insulin-like growth factor (IGF)-independent as well as IGF-dependent pathways. Nuclear localization of IGFBP-3 has been observed and nuclear binding partners for IGFBP-3 demonstrated. However, little is known about the mechanism of IGFBP-3 internalization. We hypothesized that IGFBP-3 is first secreted then taken up again into cells and that its internalization could occur via binding to transferrin or caveolin. Incubation of cells with an IGFBP-3-neutralizing antibody demonstrated that nuclear translocation of endogenous IGFBP-3 requires IGFBP-3 secretion and re-uptake. Nuclear localization of exogenously added IGFBP-3 was rapid, occurring within 15 min, inhibited by co-incubation and extracellular sequestration with IGF-I, and dependent on the transferrin-binding C-terminal peptide region of IGFBP-3. Co-immunoprecipitation assays confirmed that IGFBP-3 binds transferrin but not directly to the transferrin receptor (TFR1); however, transferrin binds TFR1 and a ternary complex is formed. Specific binding to caveolin scaffolding domain was confirmed utilizing radiolabeled IGFBP-3. Blocking TFR1-mediated endocytosis prevents both endogenous and exogenous IGFBP-3 uptake. Suppression of IGFBP-3 internalization by TFR1 blockade inhibited IGFBP-3-induced apoptosis. Together, these data indicate that the actions of IGFBP-3 are mediated by internalization via distinct endocytic pathways.

Substantial progress over the past decade has validated the insulin-like growth factor binding proteins (IGFBPs) as unique intrinsic effectors of cellular growth and apoptosis, properties that can be unrelated to their ability to bind IGFs (for review see Ref. 1). Several lines of evidence support insulin-like growth factor binding protein-3 (IGFBP-3) as a biological mediator of cancer cell apoptosis (2). Previously, we have described IGFBP-3 as the mediator of both TGF-β- and TNF-α-induced apoptosis using antisense oligonucleotides or neutralizing antibodies to IGFBP-3 in PC-3 prostate cancer cells (3, 4).

Nuclear localization of IGFBP-3 is a well described phenomenon and has been demonstrated in a variety of cellular models (5–8). IGFBP-3 possesses a consensus bipartite nuclear localization sequence (9), and nuclear transport is facilitated by importin-β factor (10).

Although advances have been made in our understanding of these IGF-independent effects, including novel nuclear effects on retinoid X receptor-mediated signaling (8), the mechanisms and pathways by which IGFBP-3 is internalized into the cell are poorly understood. The long-standing search for IGFBP-cell surface receptors has not yet provided definitive results.

It is unclear at this time whether IGFBP-3 can act as an intracrine molecule or whether it needs to be secreted and re-uptaken (in an autocrine/paracrine fashion) back into cells. Furthermore, it is unknown how IGFBP-3 enters the cells. Current understanding of macromolecular entry into cells includes two major endocytic pathways, caveolin- and clathrin-mediated uptake.

IGFBP-3 binding to transferrin has been shown by us (11) to be dependent on a region in the C-terminal domain shown in Fig. 1. Analysis of the IGFBP-3 amino acid sequence also demonstrated that a caveolin-scaffolding domain consensus sequence (CSD, ØXØXXØXØ, where Ø is a hydrophobic residue and X is any amino acid residue) (12), FCWCVDKY, also resides near the C-terminal end of IGFBP-3 (Fig. 1). This offered caveolin-1 as an attractive membrane protein partner for IGFBP-3 as well as a mediator of IGFBP-3 internalization.

To resolve some of the issues related to the process of IGFBP-3 internalization, we examined the effect of membrane cholesterol depletion (as a method of blocking caveolin-dependent uptake) and blockade of the transferrin receptor (to arrest domain; Hsp60, heat shock protein 60; IB, immunoblot; IGF, insulin-like growth factor; IP, immunoprecipitation; MTRA, methyl-J2-cyclodextrin; NLS, nuclear localization sequence; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β; TFR1, transferrin receptor; TNF-α, tumor necrosis factor-α; IGFBP-3, insulin-like growth factor binding protein-3; TBS, Tris-buffered saline; rh, recombinant human; DAPI, 4,6-diamidino-2-phenylindole; PVDF, polyvinylidene difluoride; Ab, antibody.
Endogenous Pathways Mediate IGFBP-3 Internalization

**Experimental Procedures**

**Materials**—Recombinant human IGFBP-3 and NLS mutant K228E, R230G were generously provided by Dr. Despouk Macerhanas of Protegion Inc. (Mountain View, CA). The IGFBP-3 polyclonal antibody and 125I-rhIGFBP-3 were purchased from DSL (Webster, TX). Human recombinant IGF-I, and analogues (Leu-60) were from Gropex (Australia). DAPI, PARP antibody, Hsp60 antibody, rhodamine/Texas Red labeled IgG, methyl-β-cyclodextrin, nystatin, progesterone, TNF-α, and CellLyteᵀᴹ NuCLEARᵀᴹ nuclear fractionation kit were from Sigma. Recombinant human TGF-β1 was from R&D Systems (Minneapolis, MN). Human transferrin and G418 was purchased from Invitrogen. Transferrin antibody was purchased from Chemicon (Temecula, CA). Transferrin receptor-1 antibody was from Research Diagnostics (Flanders, NJ). SDS-PAGE reagents, Tween, and fat-free milk were purchased from Bio-Rad. The caveolin scaffolding docking peptide (DGGKRAISPTFTYTVKFWYRR) and non-binding peptide (NNRPDKHAVIGKDPFVIKAESPGFTHSSP) were synthesized and purchased from Genemed (South San Francisco, CA). A caspase-3/7 specific fluorometric assay (ApoONEᵀᴹ, Promega, Madison, WI) was used as a measure of apoptosis induction.

**Co-immunoprecipitation and Western Immunoblots**—Chinese hamster ovary (CHO) cells, which normally do not express the TfR1, have been stably transfected to overexpress the TfR1 (13). Whole cell lysates were pre-purified (if indicated) with anti-transferrin antibodies. Briefly, 250 μl of protein A-agarose was incubated overnight at 4 °C with 5 μl of anti-human transferrin antibodies. 125I of each antibody-treated protein A-agarose was added to 10 μg of CHO-TfR1 cell lysates and incubated for 3 h at 4 °C with shaking. Immunoprecipitated protein A-agarose was then separated from the immunoprecipitated complexes by centrifugation. The supernatants were saved, and the cycle was repeated if indicated with another antibody, and the immunoprecipitated proteins were separated by nonreducing SDS-PAGE (8%) at constant voltage overnight and then transferred to nitrocellulose for 4 h at 170 mA. The nitrocellulose was immersed in blocking solution (5% nonfat milk/TBS) for 45 min with TfR1 antibody (BPS, Burlingame, CA) that has impaired transferrin binding (11) is shown. B, comparison of the corresponding regions of the six known IGFBPs. The number of consensus CSD aromatic amino acids is enumerated on the right column.

**Transferrin and Caveolin binding regions of IGFBP-3**

**Endocytic Pathways Mediate IGFBP-3 Internalization**

Transferrin and caveolin binding regions of IGFBP-3. A, the C-terminal domain of IGFBP-3 including the nuclear localization region, the transferrin-binding region, and the caveolin scaffolding-docking domain (OXXXXXXO), where O is an aromatic residue (12) are outlined. The IGFBP-3 mutant (BPSm) (K228E, R230G) that has impaired transferrin binding (11) is shown. B, comparison of the corresponding regions of the six known IGFBPs. The number of consensus CSD aromatic amino acids is enumerated on the right column.

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Next, cell pellets were washed twice with PBS, 1% Triton X-100 in PBS for 15 min on ice, and cells were used as a measure of apoptosis induction. A caspase-3/7 specific fluorometric assay (ApoONEᵀᴹ, Promega, Madison, WI) was used. Whole cell lysates were prepared (if indicated) with anti-transferrin antibodies. Briefly, 250 μl of protein A-agarose was incubated overnight at 4 °C with 5 μl of anti-human transferrin antibodies. 125I of each antibody-treated protein A-agarose was added to 10 μg of CHO-TfR1 cell lysates and incubated for 3 h at 4 °C with shaking. Immunoprecipitated protein A-agarose was then separated from the immunoprecipitated complexes by centrifugation. The supernatants were saved, and the cycle was repeated if indicated with another antibody, and the immunoprecipitated proteins were separated by nonreducing SDS-PAGE (8%) at constant voltage overnight and then transferred to nitrocellulose for 4 h at 170 mA. The nitrocellulose was immersed in blocking solution (5% nonfat milk/TBS) for 45 min with TfR1 antibody (BPS, Burlingame, CA) that has impaired transferrin binding (11) is shown. B, comparison of the corresponding regions of the six known IGFBPs. The number of consensus CSD aromatic amino acids is enumerated on the right column.

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fetal bovine serum and antibiotics. Cells were cultured overnight and changed to serum-free media for the experiments. rhIGFBP-3 was used at the indicated concentrations. A caspase-3-7-specific fluorometric assay (Apo-ONE, Promega) was used according to the manufacturer’s instructions to measure the degree of apoptosis induction by IGFBP-3.

**Statistical Analyses**—Experiments are means of triplicates, and each experiment was performed 3 times. Data are expressed as mean ± S.E. Statistical analyses were performed using an unpaired t test or by analysis of variance utilizing InStat (GraphPad, San Diego, CA). Differences were considered statistically significant when p < 0.05.

**RESULTS**

**Nuclear Translocation of IGFBP-3 Involves IGFBP-3 Secretion and Re-uptake**—Because TGF-β1 is a potent growth inhibitor of epithelial cells and has been shown to induce apoptosis via an IGFBP-3-dependent pathway in PC-3 cells (3), we investigated the presence of nuclear IGFBP-3 in TGF-β1-treated cells. Consistent with our previously published findings of a 10-fold increase in the presence of IGFBP-3 mRNA and protein in conditioned media of TGF-β1-treated cells, treatment of PC-3 cells with 1 ng/ml TGF-β1 for 48 h also resulted in a 10-fold increase in IGFBP-3 secreted into conditioned media, as well as of IGFBP-3 found in the nuclear fractions and a 5-fold increase of cytoplasmic IGFBP-3 as compared with control serum-free conditions (Fig. 2A). When visualized by confocal indirect immunofluorescence microscopy (Fig. 2B), the presence of IGFBP-3 (shown in red) is clearly increased by TGF-β1 in the 2nd panel both in the nucleus (identified by the DAPI staining in blue) and in the cytoplasm. Addition of a non-internalizable neutralizing IGFBP-3 polyclonal antibody together with TGF-β1 dramatically reduced the amount of intracellular IGFBP-3, abolished the presence of nuclear IGFBP-3 shown by confocal microscopy, and markedly reduced the presence of nuclear IGFBP-3 as shown by Western immunoblot (Fig. 2C).

Cytoplasmic staining of IGFBP-3 may represent newly translated IGFBP-3 in the cytoplasm before secretion. We demonstrated previously that this antibody tightly binds IGFBP-3 secreted from cells as a large molecular weight non-internalized complex (data not shown). These results demonstrate that IGFBP-3 must be secreted before re-uptake and targeting to the nucleus.

**Nuclear Localization of IGFBP-3 Is Rapid, IGF-inhibitable, and C-terminal Sequence-dependent**—22RV1 prostate cancer cells were pulsed with 500 ng of IGFBP-3 alone or in combination with IGF-I or IGF analogue for 15 min; nuclear fractions were isolated and resolved on SDS-PAGE and immunoblotted for IGFBP-3. Fig. 3 shows a definite, intense IGFBP-3 nuclear band at 15 min with a lesser amount in the cytoplasm. The C-terminal mutant (shown in Fig. 1) has greatly reduced ability (~10% of wild type) to localize to the nucleus, and no cytoplasmic presence is detected. The polyclonal IGFBP-3 antibody used recognizes both mutant and wild-type IGFBP-3 with similar affinities (data not shown). Co-incubation with IGF-I resulted in a dramatic retardation of nuclear entry, consistent with an expected extracellular sequestration. Co-treatment with the IGF analogue (long R3-IGF-I) that does not bind to IGFBPs did not impair cellular entry, whereas treatment with the IGF analogue (Leu-60) that has greatly reduced affinity for the type I IGF receptor with normal binding affinity for the IGFBPs did not impair cellular entry, whereas treatment with the IGF analogue (Leu-60) that has greatly reduced affinity for the type I IGF receptor with normal binding affinity for the IGFBPs did not impair cellular entry. These observed effects of IGF and IGF analogues on IGFBP-3 nuclear entry recapitulate previous effects of IGF-I and its analogues on IGFBP-3-induced apoptosis (3). Therefore, the predominant destination of the re-uptaken free IGFBP-3 is the nucleus and is dependent on the C-terminal region that binds transferrin. Of note, the residues mutated are also involved in cell surface (15, 16) and importin-β binding (10).
IGFBP-3 binds transferrin but not directly to transferrin receptor-1 (TfR1); however, a ternary complex may be formed—We and others (11, 17) have described transferrin as an IGFBP-3 binding partner. A series of co-immunoprecipitation experiments was employed to investigate whether IGFBP-3 interacts with the TfR1 either directly or as a complex bound to transferrin. CHO cells, which normally do not express the TfR1, have been stably transfected to overexpress the TfR1 (13). In Fig. 4A, these cells were immunoprecipitated with various antibodies bound to protein A-agarose, and subsequent nonreducing SDS-PAGE was immunoblotted with anti-IGFBP-3 and anti-TfR1 antibodies. In lane 2 of both Fig. 4, A and B, lysates were depleted of transferrin with pre-precipitation by a transferrin antibody. Fig. 4A shows that TfR1 associates with transferrin but not IGFBP-3. In Fig. 4B, note that IGFBP-3 is co-immunoprecipitated by transferrin as well as by the transferrin receptor. However, pre-depleting the lysates with a transferrin immunoprecipitation results in a loss of most of the IGFBP-3 brought down by the TiR indicating that this interaction most likely involves a ternary complex of IGFBP-3, transferrin, and TfR1.

IGFBP-3 binds CSD—We identified a caveolin scaffolding docking domain consensus sequence near the transferrin-binding C-terminal region of IGFBP-3 (Fig. 1A). IGFBP-3 is the only binding protein that contains three consensus aromatic amino acids (Fig. 1B). To verify the interaction between CSD and IGFBP-3, we used ligand dot blot experiments (Fig. 5, A and B). CSD peptide, caveolin-1 residues 82–101 (12), along with a peptide synthesized from an adjacent region of caveolin-1 (residues 53–81) used as a negative control, were immobilized to PVDF membrane. CSD peptide showed saturable binding to 125I-labeled rhIGFBP-3 (0.5 μCi/100 μl) (Fig. 5, A and B), as a 100-fold excess of cold IGFBP-3 (1 μM) co-incubated with radiolabeled IGFBP-3 displaced binding effectively. IGF-I was used as a positive control for IGFBP-3 binding. Specificity of binding with respect to the other IGFs was not tested.

TfR1 Blockade Prevents Exogenous and Endogenous IGFBP-3 Uptake—We hypothesized that IGFBP-3 might internalize via a pathway that involves a classical clathrin-dependent endocytic pathway, of which the transferrin/transferrin receptor is the best studied (18). To test whether blocking the TfR1 prevents IGFBP-3 uptake, we treated LAPC-4 cells with IGFBP-3 for 24 h after pretreatment with and without anti-TfR1 antibody for 1 h. As shown in Fig. 6A, treatment with exogenous recombinant IGFBP-3 results in marked nuclear accumulation of IGFBP-3. Co-treatment with anti-TfR1 antibody shows exclusion of IGFBP-3 from the nucleus and also from cell extracts (data not shown).

To further validate this observation on endogenous IGFBP-3 production and re-uptake, CHO-TfR1 cells were treated with TNF-α alone or in combination with TfR1 antibody for 12 h. We have shown previously (4) that TNF-α treatment induces TFGR-3 mRNA and protein production 5–10-fold. The anti-human IGFBP-3 Ab, purchased from DSL, also detects CHO IGFBP-3 (immunoblot and ELISA data not shown). An antibody to a related receptor (TfR2), serving as a control, did not block IGFBP-3 internalization (data not shown).

Fig. 6B shows that after treatment with TNF-α, these cells demonstrated a large amount of nuclear IGFBP-3 in serum-free conditions presumably secondary to increased re-uptake via increased amounts of TfR1. Blockade of the TfR1 with a specific antibody resulted in a marked decrease in both nuclear
and total intracellular IGFBP-3 as shown by confocal microscopy. Thus, the transferrin internalization pathway participates in the internalization of IGFBP-3.

Inhibition of Caveolae Formation Also Inhibits IGFBP-3 Nuclear Entry—Although caveolae have been described recently (19) as an internalization pathway for several pathogens, including non-enveloped viruses with a classical NLS (e.g. SV40), their role as an internalization pathway for IGFBPs has not been addressed. To examine the role of this pathway, cells were treated with caveolae-inhibiting drugs. Nystatin and methyl-β-cyclodextrin are sterol-binding drugs that sequester cholesterol and are not believed to affect clathrin-mediated endocytosis (20, 21). As shown in Fig. 7A, 22RV1 prostate cancer cells were treated with 25 ng/ml nystatin or 10 nmol MβCD for 2 h in serum-free conditions and then pulsed with 500 ng of IGFBP-3 for 5, 10, and 15 min. Nuclear fractions were run on SDS-PAGE and immunoblotted with anti-IGFBP-3 antibody. Pretreatment with these classical cholesterol depletion agents significantly impaired IGFBP-3 nuclear entry. Nystatin inhibited nuclear entry of IGFBP-3 by over 50% at 5 and 10 min, and cyclodextrin impaired IGFBP-3 nuclear entry by >50% at 5, 10, and 15 min. Quantitation of three separate experiments done in triplicate show a >40% reduction by nystatin in nuclear IGFBP-3 transport associated with a >50% reduction in cytoplasmic IGFBP-3 (Fig. 7B). As a positive control, treatment with nystatin and cyclodextrin also impaired nuclear and cytoplasmic transport of cholera toxin, known to internalize via caveolar pathways (22). Thus, caveolae disruption by cholesterol depletion agents

Fig. 5. IGFBP-3 binds CSD (caveolin 1 residues 82–101). A, ligand dot blot experiment showing IGFBP-3 binding to CSD. Caveolin-1 residues 53–81 (negative control), caveolin-1 residues 82–101, and IGF-1 at 6.25–25 nmol were immobilized on PVDF membrane and probed with 125I-labeled IGFBP-3. B, displacement of IGFBP-3 from caveolin-1 residues 82–101 with 100× molar excess of unlabeled IGFBP-3; caveolin-1 residues 53–81 (negative control).

Fig. 6. Transferrin receptor blockade inhibits endogenous and exogenous IGFBP-3 uptake. A, confocal microscopy using similar staining of LAPC-4 cells treated with IGFBP-3 and/or anti-TfR1 antibody for 3 h. Addition of an anti-TfR1 antibody completely inhibits IGFBP-3 entry. B, confocal microscopy of TfR1 overexpressing CHO cells treated with TNF-α with and without anti-TfR1 antibody for 12 h. Nuclei are stained with DAPI (blue). IGFBP-3 protein was detected using hIGFBP-3 antibody followed by fluorescein-labeled IgG (green). TNF-α treatment increased IGFBP-3 expression, and this effect was blocked by co-treatment with anti-TfR1 antibody treatment.
significantly impairs IGFBP-3 cellular internalization, suggesting that caveolae-mediated endocytosis also participates in IGFBP-3 internalization. Interestingly, this inhibition was lessened at 15 min, suggesting that caveolin blockade is compensated for by entry via an alternate pathway.

Disruption of Caveolin- and Transferrin-mediated Endocytosis in Combination Blocks Endogenous and Exogenous IGFBP-3 Nuclear Entry—Our present data suggest that IGFBP-3 internalization is mediated by both classical TfR/clathrin- and caveolin-mediated pathways. Fig. 5 illustrates blockade of both pathways on both endogenous and exogenous IGFBP-3 internalization. 22RV1 cells were treated in serum-free conditions with MβCD or TfR1 Ab alone or in combination for 1 h and then pulsed with 500 ng IGFBP-3. Nuclear fractions were isolated and resolved by SDS-PAGE under reducing conditions and immunoblotted with anti-IGFBP-3. There is a clear dose-dependent decrease in both endogenous and exogenous IGFBP-3 internalization with MβCD. There is also a decrease in internalization as effected by the TfR1-blocking antibody. However, treatment with a combination of both agents completely abolishes endogenous IGFBP-3 re-uptake, as well as significantly diminishing exogenous IGFBP-3 re-uptake, indicating a contribution from both caveolin- and TfR/clathrin-dependent pathways in the process of IGFBP-3 internalization.

Transferrin Receptor Blockade Inhibits IGFBP-3-induced Apoptosis—As a measure of internalization blockade on IGFBP-3 function, we investigated the effect of TfR1 blockade on IGFBP-3-induced apoptosis in 22RV1 prostate cancer cells. Fig. 6 shows that as expected, IGFBP-3 induced significant rapid apoptosis as measured by fluorometric evaluation of caspase-3/-7 activity in 3 h. Pretreatment with the anti-TfR1 antibody for 2 h reduced apoptosis significantly compared with IGFBP-3 treatment alone (p < 0.005), suggesting that inhibition of IGFBP-3-transferrin-TfR1 association and subsequent internalization blockade inhibits the biological activity of this molecule, and internalization is required for the apoptotic effect of IGFBP-3 in this system. Control treatment with the antibody alone had no effect.

DISCUSSION

The interaction of IGFBP-3 with cells independently of IGFs has been studied from a number of angles. First, it is well recognized that IGFBP-3 interacts with multiple matrix proteins including heparin, proteoglycans, and collagen. IGFBP-3 has also been proposed to bind a cell surface receptor (23), but it is unlikely that this interaction facilitates internalization. The recognition of an NLS in IGFBP-3 led to further study of its
nuclear roles; however, the NLS does not explain the mechanism by which IGFBP-3 enters cells. Here we explore two mechanisms that facilitate the rapid internalization of IGFBP-3.

The precursor forms of all six IGFBPs have secretory signal peptides of between 20 and 30 amino acids, and the mature proteins are all found extracellularly (1). The possibility that IGFBP-3 might re-enter the cell via the TIR1 was raised after we described its binding to serum transferrin (11), and others described transferrin binding to IGF-IGFBP-3 complexes (17). Our current model involves IGFBP-3 binding to transferrin, and this binary complex binds the TIR1 with subsequent internalization of the ternary complex. Transferrin is involved and this binary complex binds the TfR1 with subsequent interplay between IGFBP-3 and caveolin suggest a functional interplay between the caveolin-scaffolding signal complex and IGFBP-3. Interaction between IGFBP-3 and this complex may provide a role for the internalization of signals from clathrin-mediated endocytosis compartments. The observation that IGFBP-3 internalization occurs via two distinct pathways is not unique. GLUT4 uptake has been described via a non-caveolae-mediated process as well as peripheral association with large caveolin-containing domains (43). In addition, the cellular uptake of influenza virus (44), interleukin-2 (45), cholera toxin (22), Shiga toxin (46), epidermal growth factor receptor (47), and insulin receptor (48) has been described to involve both internalization pathways.

Recent evidence uncovers a role for transduction of signals from clathrin-mediated endocytosis. The observation that the activated epidermal growth factor receptor can be detected on endosomes where it can activate Ras indicates that it is the receptor itself that needs to be internalized to initiate signaling (49, 50). Furthermore, nerve growth factor-mediated signaling illustrates how one molecule can mediate dichotomous functions; the activated Trk receptor induces survival when at the plasma membrane, whereas it elicits differentiation of neurite outgrowth if internalized (51). Finally, the internalization of IGFBP-3-transferrin-TIR1 complex may possibly result in the eventual dissociation of IGFBP-3 from the transferrin-TIR1 complex in the acidic luminal endosomal compartment. This may liberate IGFBP-3 to interact with downstream effectors after sorting to endosomes that are permissive to signaling, whereas transferrin-TIR1 is targeted to lysosomes for degradation, similar to that described for Smo-Shh-Ptc complexes (52).

We describe IGFBP-3 internalization via caveolin- and transferrin-dependent pathways. Internalization, and subsequent IGFBP-3 function, may be determined by cell type differences in the relative action or abundance of the endocytic pathways discussed here. Future detailed microscopy work will be needed to determine which endocytic vesicles IGFBP-3 is trafficked through when it enters cells by clathrin-mediated (transferrin-dependent) and caveolae-mediated endocytosis. As yet, it is not clear why and how IGFBP-3 has the capacity to enter cells through multiple pathways. One obvious possibility is that IGFBP-3 binds proteoglycans (53), heparin, and glycosaminoglycans (54) nonspecifically, which places it in proximity to forming endosomes of various pathways. Alternatively, distinct compartmental segregation during endocytosis might determine cytoplasmic signal transduction versus nuclear localization and differential effects on cellular growth and apo-
ptosis, as recently reported (55) for the TGF-β receptor. Taken together, our data indicate that IGFBP-3 enters cells by dual, distinct mechanisms: first, a caveolae-mediated endocytic pathway; and second, entry via a classical clathrin-mediated endocytic pathway via binding to transferrin-TfR1 complexes. Reminiscent of its role as a member of the 150-kDa endocytic pathway via binding to transferrin-TfR1 complexes. Internalization and nuclear localization appear important for the apoptotic actions of growth hormone, we have now described a cell surface ternary complex formation that is conducive to internalization.

It is possible that other IGFBPs may have multiple entry pathways that could be dependent on receptor binding or cell type. After entry into cells, IGFBP-3 rapidly enters the nucleus within minutes, possibly through residue targeting or by its use of the nuclear localization sequence. Internalization and nuclear localization appear important for the apoptotic actions of IGFBP-3.

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