Insulin-like growth factor-binding proteins (IGFBPs) play an integral role in modifying insulin-like growth factor actions in a wide variety of cell types. Recent evidence suggests that IGFBP-3 and IGFBP-5 also have effects on cell growth that are insulin-like growth factor-independent. In investigating possible mechanisms for this effect, the intracellular trafficking of IGFBP-3 and IGFBP-5, both of which contain sequences with the potential for nuclear localization, was studied in T47D cells. Nuclear uptake of fluorescently labeled IGFBP-3 and IGFBP-5 was observed in a proportion of T47D cells that appeared to be rapidly dividing. IGFBP-1 and IGFBP-2, which do not possess the putative domain for nuclear translocation, were not transported to the nuclei of T47D cells. When T47D cells were preincubated with excess unlabeled IGFBP-3, nuclear localization of labeled IGFBP-3 or IGFBP-5 was not detected, indicating that their nuclear translocation involves a common pathway. Inhibition of receptor-mediated endocytosis did not affect nuclear uptake of IGFBP-3, suggesting that it uses an alternative non-classical import pathway for transport across the plasma membrane. In addition, a variant form of IGFBP-3 with a mutation in the putative nuclear localization sequence was unable to translocate to the nuclei of T47D cells, suggesting that nuclear translocation of IGFBP-3 was dependent on these carboxyl-terminal basic residues.

The insulin-like growth factors (IGF-I and IGF-II) are potent mitogens, which stimulate proliferation in many normal and malignant cell types (1). They bind to specific receptors, designated the type I and II IGF receptors (2), although the mitogenic effects of the IGFs are mediated through the type I IGF receptor. The IGFs also have high affinity for a family of tent mitogens, which stimulate proliferation in many normal and malignant cell types, also induce the expression and secretion of IGFBP-3 (5, 9). Several reports have now shown that the growth inhibitory effects of TGFβ and retinoic acid (10, 11) and the TGFβ induction of apoptosis (12) are mediated, at least in part, by IGFBP-3. There are fewer reports on IGF-independent action of IGFBP-5. These include the stimulation of bone cell growth by IGFBP-5 in the absence of increased IGF-I binding to its receptor (13).

Although the precise mechanism of this growth regulation is unknown, one plausible explanation is that IGFBP-3 is transported to the nucleus where it directly or indirectly modulates gene transcription. The basic carboxyl-terminal domain of IGFBP-3 contains a region (amino acids 215–232) with strong sequence homology to previously identified bipartite nuclear localization signals (NLS) (14). In addition, this basic region shares homology with the DNA-binding domains of several transcription factors (15). Nuclear translocation of IGFBP-3 has now been reported in a renal proximal tubule-like cell line (16), and IGFBP-3 has also been detected in the nuclei of lung cancer cells (17) and human keratinocytes (18). Based on sequence homology between the basic carboxyl-terminal regions of IGFBP-3 and IGFBP-5 (IGFBP-3, amino acids 215–232; IGFBP-5, amino acids 201–218), IGFBP-5 also has the potential to be targeted to the nuclei of cells and to interact directly with DNA. The sequences in IGFBP-1, -2, -4, and -6 that correspond to the putative NLS in IGFBP-3 and IGFBP-5 do not contain this basic motif, nor is it represented in any other part of their sequences.

As part of our investigations into the mechanisms that regulate breast cancer cell growth, we have studied the nuclear localization of IGFBP-3 and IGFBP-5 in the T47D cell line, an estrogen receptor-positive human breast cancer cell line that is reported to express IGFBP-2, -4, and -5 (19). Here, we report the novel observation that IGFBP-5 is translocated to the nucleus, confirming the prediction based on sequence homology with IGFBP-3. In addition, we have examined two aspects of the mechanism for nuclear transport of IGFBP-3 and IGFBP-5. We demonstrate a shared and saturable pathway for their nuclear uptake and show that IGFBP-3 does not enter the cell by receptor-mediated endocytosis prior to its nuclear localiza-
tion. Furthermore, we have defined a sequence within the basic motif of IGFBP-3 that is necessary for its nuclear translocation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Natural, glycosylated IGFBP-3 was isolated from Cohn fraction IV of human plasma (20), IGFBP-1 was purified from human amniotic fluid (21), and recombinant human IGFBP-2 was provided by Sandoz, Basel, Switzerland. Recombinant human IGFBP-5 was the generous gift of J. Zapf, Zürich, Switzerland. Expression and purification of recombinant human IGFBP-3 and IGFBP-5(KGRKR→MDGEA) was as described previously (22). Antiseria against IGFBP-1, IGFBP-2, and IGFBP-3 were prepared in this laboratory following immunization of rabbits with purified antigens. Rabbit anti-human IGFBP-5 antiserum was purchased from Upstate Biotechnology, Inc., Lake Placid, NY. Protein A was purchased from Sigma. Chloroquine (Boehringer Mannheim GmbH, Mannheim, Germany) was prepared as a 20 mM stock solution in cell culture medium, colchicine and nocodazole (Sigma) were prepared at 125 mM and 10 mM stock solutions, respectively. Transferrin (Sigma) was similarly labeled without the addition of bovine serum albumin, and the protein labeled control (data not shown). Transferrin (Sigma) was similarly labeled without the addition of bovine serum albumin, and the protein labeled control (data not shown). Transferrin (Sigma) was similarly labeled without the addition of bovine serum albumin, and the protein labeled control (data not shown).

**Fluorescent Labeling of IGFBPs**—The binding proteins were labeled with the fluorophore Cy3 (Amersham Pharmacia Biotech), in 50 mM sodium phosphate buffer, pH 7.0, for 30 min at 22 °C. Following labeling, the fluorophore was removed with 125 mM Tris⋅HCl, pH 7.0, and the labeled bovine serum albumin was added to a final concentration of 10 glit. Labeled binding proteins were separated from free Cy3 dye by size exclusion chromatography on a 1 × 50-cm column of Sephadex G-100 (Amersham Pharmacia Biotech) in 50 mM sodium phosphate buffer, pH 6.8, 0.5 M NaCl, and 1 gliter bovine serum albumin. The fractions were concentrated and desalted by ultrafiltration through Microcon-10 microconcentrators (Amicon, Beverley, MA) and stored in aliquots at −80 °C.

The concentrations of the IGFBP-1, -2, and -3 were determined by Western immunoblotting of labeled IGFBPs following size-exclusion chromatography. Fig. 1 shows unlabeled and labeled IGFBP-1 (lanes 1 and 2), IGFBP-2 (lanes 3 and 4), IGFBP-3 (lanes 5 and 6), and IGFBP-5 (lanes 7 and 8). Following labeling, all the binding proteins showed a slight increase in molecular mass consistent with the addition of multiple Cy3 molecules (766 Da). Importantly, there was no evidence of oligomerization or proteolysis of the binding proteins.

**Cell Culture and Fluorescent Studies**—T47D human breast cancer cells (American Type Culture Collection, HTB 133) were maintained in RPMI medium supplemented with 25 mM NaHCO₃, 2 mM glutamine, 10 μg/ml bovine insulin, and 10% fetal calf serum (Cytosystems, New South Wales, Australia). For fluorescent studies, T47D cells were cultured on glass multwell slides (Nunc Inc., Naperville, IL) treated with 12% Cell-Tak (Becton Dickinson, Bedford, MA) to examine the effect of monolayer wounding, the cells were grown to confluence before the monolayer was scored with a pointed instrument. For other studies, the cells were grown to subconfluence before the medium was changed to serum-free RPMI medium supplemented with 20 mM HEPEs, 2 mM glutamine, 0.06 gliter penicillin, 0.1 gliter streptomycin, and 1 gliter bovine serum albumin. Cells were then treated directly or were treated with inhibitors of endocytosis for 4 h at 37 °C. Labeled binding proteins (0.5 μg/ml) were added and the cells incubated for 60 min at 22 °C. To determine cell viability, the cells were washed and fixed with Histochoice (Amresco, Solon, OH), mounted in antifade mounting medium, and examined using fluorescent microscopy. Similar results were obtained when the cells were incubated at 37 °C.

**Antibody Binding Assay**—Cell surface binding of IGFBP-1, -2, -3, and -5 was performed as described previously for IGFBP-3 (9). Briefly, T47D cells were grown to confluence in 96-well plates and the cells made serum-free for 24 h. The confluent monolayer was incubated with 0.5 μg/ml IGF binding proteins for 4 h at 37 °C. After washing the monolayer, antiserum was added and the cells incubated for 16 h at 22 °C. IGFBP-1, -2, -3, and -5 antisera were used at a dilution of 1:5,000 and IGFBP-5 antiserum at a dilution of 1:1,000. After washing the cells, radiiodinated protein A was added at 40,000 cpm/ml and the cells incubated for 2 h at 22 °C. The cells were then washed and solubilized with 0.5% sodium dodecyl sulfate for 16 h at 22 °C and the lysates counted in a γ-counter.

**Statistical Analysis**—Data were analyzed by analysis of variance followed by Fisher’s protected least significant difference test, using Statview 4.02 (Abacus Concepts, Inc., Berkeley, CA).

**RESULTS**

**Nuclear Transport of IGFBP-3 and IGFBP-5 in T47D Cells**—The putative NLS in the carboxyl-terminal region of IGFBP-3 and IGFBP-5 and the corresponding sequences in IGFBP-1 and IGFBP-2 are shown in Table I. The intracellular movement of these binding proteins was studied by directly labeling them with the fluorescent dye, Cy3. The size and integrity of the labeled binding proteins were determined by Western immunoblotting following size-exclusion chromatography. Fig. 1 shows unlabeled and labeled IGFBP-1 (lanes 1 and 2), IGFBP-2 (lanes 3 and 4), IGFBP-3 (lanes 5 and 6), and IGFBP-5 (lanes 7 and 8). Following labeling, all the binding proteins showed a slight increase in molecular mass consistent with the addition of multiple Cy3 molecules (766 Da). Importantly, there was no evidence of oligomerization or proteolysis of the binding proteins.

When T47D cells were incubated with labeled IGFBP-1 (Fig. 2A) or IGFBP-2 (Fig. 2B), nuclear localization was not observed. In contrast, when cells were incubated with Cy3-labeled IGFBP-3 (Fig. 2C), nuclear localization was observed within 90 min in a small percentage of the cells. In addition to a strong nuclear signal, the cytoplasm showed generalized labeling, suggesting that the IGFBP-3 was not confined to vesicular structures but was free in the cytoplasm. Nuclear localization of IGFBP-3 was most commonly observed at the edge of a monolayer or at a wound edge, which are both regions where cells are likely to be actively dividing. In addition, it was common to observe nuclear uptake in daughter cells within the monolayer. The presence of IGF-I had little effect on the nuclear uptake of IGFBP-3 (data not shown). Fluorescently labeled IGFBP-5 was also found localized to the nucleus of T47D cells (Fig. 2D). With respect to the kinetics of uptake, the percentage of cells with nuclear localization, and the intensity of labeling (both nuclear and cytoplasmic), IGFBP-5 nuclear translocation was indistinguishable from that seen for IGFBP-3. Apart from cells that are actively transporting the labeled binding proteins, cells that have a compromised plasma membrane would also internalize the fluorescent proteins. To distinguish between live and dead cells, calcein AM was added in all fluorescent cell studies. Calcein AM is a cell-permeant, non-fluorescing compound, which, when cleaved by cyclolysin.

![Table I: Comparison of the carboxyl-terminal basic residues of human IGFBP-3 and IGFBP-5 and the corresponding sequence in IGFBP-1 and human IGFBP-2](image-url)
T47D cells were incubated for 90 min with 0.5 μg/ml markers, run in adjacent lanes, are indicated in kilodaltons. Comparing under “Experimental Procedures.” The positions of molecular mass markers, run in adjacent lanes, are indicated in kilodaltons. Comparison between unlabeled and labeled IGFBP-1 (lanes 1 and 2), IGFBP-2 (lanes 3 and 4), IGFBP-3 (lanes 5 and 6), and IGFBP-5 (lanes 7 and 8) showed that there was an increase in molecular mass for each labeled IGFBP compared with its unlabeled counterpart and that the labeling procedure had not compromised the integrity of the binding proteins.

Fig. 1. Western immunoblot of labeled binding proteins. The unlabeled and Cy3-labeled binding proteins were separated on 12% SDS-PAGE and subjected to Western immunoblotting as described under “Experimental Procedures.” The positions of molecular mass markers, run in adjacent lanes, are indicated in kilodaltons.

Fig. 2. Nuclear uptake of IGFBP-3 and IGFBP-5 in T47D cells. T47D cells were incubated for 90 min with 0.5 μg/ml Cy3-labeled IGFBP-1 (A), IGFBP-2 (B), IGFBP-3 (C), and IGFBP-5 (D). Nuclear localization of the labeled binding proteins was observed only for IGFBP-3 and IGFBP-5. Bar = 50 μm.

Cell Surface Association of the Binding Proteins—As binding to the plasma membrane may be the first step in nuclear localization of exogenous IGFBP-3 and IGFBP-5, we have examined the potential for cell surface association by the labeled binding proteins. Fig. 3 shows that IGFBP-1 (B), IGFBP-2 (C), IGFBP-3 (D) and IGFBP-5 (E) all displayed significant cell surface labeling on the T47D cell monolayer compared with control cells incubated with free dye only (A). In the case of IGFBP-3 and IGFBP-5, the images have been selected from areas where nuclear localization was absent to allow for the longer exposures necessary to illustrate cell surface labeling as opposed to nuclear labeling.

To confirm and quantify these findings, we used an antibody binding assay to detect cell surface/extracellular matrix (ECM) association of IGFBP-1, -2, -3 and -5. Exogenously added IGFBPs showed specific binding to the T47D monolayer (Fig. 4) at a concentration identical to that used for cell fluorescent studies. In addition cell surface/ECM binding of endogenous IGFBP-2 and -5 was detected, consistent with their known expression in this cell line (19). At the concentration of IGFBP-1 antiserum used in this assay, a small amount of binding in the absence of added IGFBP-1 was observed. As these cells are not known to express IGFBP-1 this binding is likely to represent cross reactivity with an unknown antigen. However, when increasing amounts of IGFBP-1 (up to 5 μg/ml) were added, there was a corresponding increase in detectable cell surface/ECM binding (data not shown), indicating that IGFBP-1 does indeed bind the cell surface/ECM of T47D cells.

IGFBP-3 and IGFBP-5 Share a Common Nuclear Transport Pathway—To determine whether nuclear localization of IGFBP-3 (Fig. 5A) and IGFBP-5 (Fig. 5C) is specific and saturable, we competed the labeled binding proteins with a 10-fold excess (5 μg/ml) of unlabeled plasma IGFBP-3. Under these conditions, nuclear localization of Cy3-labeled IGFBP-3 (Fig. 5B) and IGFBP-5 (Fig. 5D) was not detected and the labeling was equivalent to that seen when free Cy3 dye (Fig. 3A) was added to cells. Insufficient IGFBP-5 was available to perform a similar study with excess IGFBP-5. These results suggest that nuclear localization of IGFBP-3 and IGFBP-5 in T47D cells is specific and that they share at least some part of their nuclear transport pathway.

As the pathways to nuclear localization of IGFBP-3 and -5 may converge at the point of cell surface binding, the ability of IGFBP-3 to compete with cell surface-bound IGFBP-5 for cell binding sites was examined using an antibody binding assay in cells preincubated with IGFBP-5. When T47D cells were incubated with exogenously added IGFBP-5 (0.5 μg/ml), there was a significant increase (p < 0.0001) in IGFBP-5 cell surface/ECM binding compared with untreated control cells (Fig. 6). When this binding was competed with a 10-fold excess of IGFBP-3, there was a significant decrease (p < 0.0001) in bound IGFBP-5. However, as the amount of cell associated IGFBP-5 at this concentration of IGFBP-3 remained significantly greater than the untreated controls (p < 0.0001), it suggested that cell surface/ECM binding sites are unlikely to be the limiting factor in nuclear uptake of IGFBP-5. Displacement of cell surface/ECM-bound endogenous IGFBP-5 by a 10-fold excess of IGFBP-3 was also observed (data not shown). In addition, excess IGFBP-3 was used to compete for cell surface-
Nuclear Import of IGFBP-3 and IGFBP-5

FIG. 5. IGFBP-3 and IGFBP-5 share a common pathway for nuclear localization in T47D cells. T47D cells were incubated with 0.5 μg/ml Cy3-labeled IGFBP-3 in the absence or presence of excess unlabeled IGFBP-3 (A and B, respectively). Nuclear uptake of both IGFBP-3 and -5 was not detected in the presence of a 10-fold excess of IGFBP-3. Bar = 50 μm.

FIG. 6. Excess IGFBP-3 competes for IGFBP-5 cell surface/ECM binding sites on T47D cells. T47D cells were incubated without or with IGFBP-3 and increasing amounts of IGFBP-5. Competition of exogenously added IGFBP-5 with 5 μg/ml IGFBP-3 led to a significant decrease in cell surface-bound IGFBP-5 (p < 0.0001) compared with control cells with no added IGFBP-3. Results are expressed as mean ± S.E. of 1274-labeled protein A bound as a percent of control from three independent experiments (by analysis of variance and Fisher’s protected least significant difference test).

Table II

Effect of inhibitors of endocytosis on nuclear uptake of IGFBP-3 in T47D cells

| Treatment   | Action                  | Nuclear localization |
|-------------|-------------------------|----------------------|
| Controls    |                        | +                    |
| 200 μM chloroquine | Lysosomotropic agents | +                    |
| 1 μM monensin    |                        | +                    |
| 10 μM colchicine  | Disrupts microtubules  | +                    |
| 10 μM nocodazole |                        | +                    |

T47D cells were incubated with inhibitors of endocytosis as described under “Experimental Procedures.” In treated cells nuclear uptake of Cy3-labeled IGFBP-3 was identical to control cells incubated in serum free media without or with Me2SO or EtOH at concentrations equivalent to that used as solvent for the inhibitors. The agents were tested in at least three independent experiments with similar results.

Mutation in the Basic Region of IGFBP-3 Prevents Its Nuclear Uptake—To examine the sequences involved in nuclear transport of IGFBP-3, we have expressed the wild-type and a mutant form of human IGFBP-3 in Chinese hamster ovary cells. The mutation, 229KGKRKR → MDGEA, is located in the carboxyl-terminal region of the putative bipartite NLS (Table I). This mutant was derived by exchanging part of the putative NLS of IGFBP-3 for the corresponding sequence in IGFBP-1, a binding protein that is not transported to the nucleus (Fig. 2A). Following fluorescent labeling of the wild-type and mutant form of IGFBP-3, their uptake was studied in T47D cells. As shown for plasma IGFBP-3 (Fig. 2C), recombinant wild-type IGFBP-3 was also found localized to the nucleus (Fig. 7A). In contrast, nuclear uptake of the basic mutant by T47D cells was absent (Fig. 7B). We conclude that sequences within the basic region of IGFBP-3 are necessary for the nuclear translocation of exogenous IGFBP-3 in T47D cells.

DISCUSSION

The observation that IGFBP-3 can have effects on cell growth that are independent of the activation of the type I IGF receptor has focused attention on the possible mechanism(s) for this effect. This has led to the recent identification of nuclear IGFBP-3 in several cell types (16–18). Based on a common putative nuclear localization signal in the carboxyl-terminal domains of IGFBP-3 and IGFBP-5, we hypothesized that IGFBP-5 would also translocate to cell nuclei. The present study confirms this hypothesis by describing the nuclear localization of both IGFBP-3 and -5 in the T47D human breast cancer cell line. We have shown previously that IGF-I is capable of releasing a proportion of bound IGFBP-3 from the plasma membrane of neonatal fibroblasts (9), an effect that has the potential to prevent internalization and nuclear uptake of IGFBP-3. However, nuclear uptake of IGFBP-3 was unaffected by the presence of IGF-I. In a recent study, where IGFBP-3 was shown to transport IGF-I to the nucleus, Cy3-labeled IGF-I did not prevent nuclear uptake of fluorescently labeled IGFBP-3 (16). In contrast, the two proteins were co-localized to the same subset of cells. This would suggest that different mechanisms operate at the cell surface depending on whether the IGFBP-3 is destined for nuclear uptake or not.

Within the family of IGFBPs, IGFBP-3 and -5 are closely related both structurally and functionally. The basic motif within their carboxyl-terminal regions confers binding to the cell surface/ECM (22, 28, 29), and we have now shown that it is required for the nuclear uptake of IGFBP-3, and potentially that of IGFBP-5. In addition, we have recently demonstrated that, like IGFBP-3, IGFBP-5 participates in ternary complex formation with the IGFs and the acid-labile subunit (30), a

bound IGFBP-2 (data not shown). This control experiment demonstrated that IGFBP-3 was incapable of displacing IGFBP-2 (p = 0.186) from the T47D cell surface under identical conditions used for IGFBP-3 displacement of bound IGFBP-5.

Nuclear Uptake of Exogenously Added IGFBP-3 Does Not Require Receptor-mediated Endocytosis—Recent evidence suggests that, in cells actively transporting IGF-I to the nucleus, this growth factor crosses the plasma membrane by a pathway that is independent of receptor-mediated endocytosis via clathrin-coated pits (16). To determine whether transport of IGFBP-3 across the plasma membrane utilizes the endocytotic pathway, we treated T47D cells with inhibitors of endocytosis and examined their effect on nuclear uptake of Cy3-labeled IGFBP-3 (Table II). Neither the lysosomotropic agents, chloroquine and monensin (26), nor the microtubule disrupting agents, colchicine and nocodazole (27), prevented nuclear uptake of IGFBP-3 in T47D cells. These results suggest that, like IGF-I, IGFBP-3 internalizes by a mechanism other than classical endocytosis. In parallel with these studies, we examined the internalization of a control protein, Cy3-labeled transferrin, to ensure that all endocytosis was blocked. Colchicine and nocodazole prevented the accumulation of Cy3-transferrin within the perinuclear region, and treatment with chloroquine and monensin led to the appearance of morphological changes consistent with an increase in the rate of fusion of endocytic vesicles caused by an increase in endosomal pH (data not shown).
function we previously reported to involve residues 228–232 of the basic carboxyl-terminal domain of IGFBP-3 (22). IGFBP-1 and -2, which do not contain sequences with homology to known NLS, did not localize to the nucleus. However, labeling at the edge of the monolayer was observed for both Cy3:IGFBP-1 and -2 and we also found evidence for cell surface/ECM association using an antibody binding assay. These findings are consistent with the known interaction between the RGD motif in IGFBP-1 (31) and α,β integrin known to be expressed on T47D cells (32). Although IGFBP-2 also contains an RGD motif, its association with integrins has not been documented. Recently, association between IGFBP-2 and cell surface proteoglycans in the rat olfactory bulb has been reported (33).

The pathway(s) taken by IGFBP-3 and -5 to effect their nuclear uptake are currently unknown. Some secretory proteins destined for nuclear import are diverted away from the secretory pathway (34, 35), but most are initially secreted from the cell prior to internalization and transport to the nucleus (36, 37). The nuclear isoform of IGFBP-3 isolated from human lung cancer cells was reported to be of similar size to the glycosylated form secreted from the cell (17), suggesting that IGFBP-3 was released through the Golgi system prior to uptake and nuclear localization. Therefore, binding to the cell surface and transport across the plasma membrane are likely to be the first steps in nuclear uptake of IGFBP-3. Although the cell surface association proteins for IGFBP-3 and -5 on T47D cells are unknown, there is considerable evidence that IGFBP-3 and -5 interact with the cell surface or ECM in a variety of cell types (6, 9, 29, 38–40). To determine whether IGFBP-3 and -5 shared a common internalization and/or nuclear uptake pathway, T47D cells were preincubated with an excess of unlabeled IGFBP-3. This prevented nuclear transport of both Cy3:IGFBP-3 and -5, either as a result of limitation in cell surface binding sites or by saturation of the soluble cytoplasmic importins (41). In an antibody binding assay, we observed partial displacement of cell surface-bound exogenously added IGFBP-5 in the presence of excess IGFBP-3. From this we conclude that the availability of cell surface binding sites is unlikely to be the limiting factor in nuclear uptake of IGFBP-5 (and presumably also IGFBP-3) but rather that saturation is occurring during cytosolic transport.

Targeting of extracellular proteins to the nucleus requires a means of cytoplasmic access. During the degradation and recycling processes, which occur during receptor-mediated endocytosis, some of the internalized material may escape into the cytoplasm where it becomes biologically active (42). In the present study, nuclear uptake of IGFBP-3 was unaffected in T47D cells treated with the inhibitors of endocytosis and receptor recycling, chloroquine and monensin (26). In addition, when microtubules, which have a role in endocytosis by participating in intracellular movement of endocytotic vesicles (26, 27), were disrupted by treatment with colchicine and nocodazole, there was no effect on nuclear uptake of IGFBP-3. Furthermore, in cells which displayed nuclear uptake of IGFBP-3 and -5, we observed generalized cytoplasmic labeling consistent with an unconstrained distribution of the binding proteins within the cytoplasm. For these reasons, we suggest that the cellular uptake of IGFBP-3, like IGF-I, is independent of receptor-mediated endocytosis. Other potential mechanisms available to polypeptides for transport across the plasma membrane include the use of specialized structures for internalization such as caveolae (43) or by direct transport across the plasma membrane by nonclassical import mechanism(s) (44).

There is now considerable evidence that many hormones and growth factors are capable of modulating cellular responses both indirectly by activation of classical signal transduction pathways and directly by nuclear targeting. These include insulin (45), platelet-derived growth factor (46), and ﬁbroblast growth factor (47). This duality of function may now encompass not only ligands, but also serum binding proteins such as IGFBP-3 and -5 and the androgen-binding protein/sex hormone-binding globulin (48), which also localizes to the nucleus. These binding proteins may not only regulate the bioavailability of their respective hormone or growth factor in the extracellular environment, but may also participate in intracellular events. The intracellular actions of IGFBP-3 and IGFBP-5 may be analogous to that observed for thyroid/steroid hormone receptors (49). These receptors interact with high afﬁnity DNA-binding sites, in a ligand-dependent fashion, to regulate gene expression. Given the ability of IGFBP-3 and IGFBP-5 to bind the ligands IGF-I and IGF-II, together with a demonstrated capacity for nuclear uptake and a potential for speciﬁc DNA interactions, these binding proteins may represent another class of ligand-regulated transcription factors.

The functional signiﬁcance of two separate pathways for the regulation of gene expression by a number of polypeptide hormones is unclear. However, the choice between activation of transmembrane receptors and signal transduction pathways as opposed to internalization and direct nuclear effects appears, in some instances, to be related to progression through the cell cycle (50, 51). Although there is no evidence that IGFBP-3 or -5 activates classical cell signaling pathways, they do have an indirect effect on these pathways by their ability to sequester IGFs and prevent their interaction with the type I IGF receptor. It is therefore conceivable that the choice between indirect and direct effects of IGFBP-3 and -5 may be related to cell cycle factors. We have observed that IGFBP-3 and -5 are translocated to the nuclei of T47D cells at positions within the monolayer where the cells are likely to be actively dividing. In addition, we frequently observe the labeling of nuclei of daughter cells, suggesting that nuclear uptake of these binding proteins may be related to cell cycle events. These observation have also been reported for IGFBP-3 in a renal proximal tubule-like cell line (16) and human keratinocytes (18).

Because the movement of IGFBP-3 from the extracellular microenvironment to an intracellular position could modulate its role in normal and tumor cell growth, we have examined the sequences in IGFBP-3 that are necessary for its nuclear localization. We found that mutation of basic residues within the putative NLS of IGFBP-3 prevented its nuclear uptake. The mutated basic residues are common to IGFBP-3 and IGFBP-5, but no other IGFBP, emphasizing the likelihood of a common mechanism accounting for the nuclear uptake of IGFBP-3 and IGFBP-5. The basic region mutant of IGFBP-3 lacks the cell surface binding determinant (22) and potentially lacks the ability to bind importin subunits (41). As cell surface binding may be a prerequisite for nuclear transport, the use of a cell-free system may help to distinguish between cell surface binding and nuclear translocation events.

Although the function of intracellular IGFBP-3 is unknown,
a general theme that is emerging is that IGFBP-3 mediates, at least in part, the inhibition of cell growth induced by TGFβ (10), retinoic acid (11), and p53 (52), which may involve an increase in apoptosis (12). These effects are potentially related to the actions of IGFBP-3 that are independent of the type I IGF receptor. In the nucleus, IGFBP-3 may bind directly to DNA and act as a transcription factor regulating the expression of apoptotic or other genes. Alternatively, the action of nuclear IGFBP-3 may be indirect, regulating gene expression through interaction with other nuclear proteins. Thus, despite current uncertainties regarding the role of nuclear IGFBP-3, understanding the events that lead to its nuclear localization may provide insight into its role as a cancer cell growth inhibitor.

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