Nuclear Import of Insulin-like Growth Factor-binding Protein-3 and -5 Is Mediated by the Importin β Subunit*

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Although insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 are known to modulate cell growth by reversibly sequestering extracellular insulin-like growth factors, several reports have suggested that IGFBP-3, and possibly also IGFBP-5, have important insulin-like growth factor-independent effects on cell growth. These effects may be related to the putative nuclear actions of IGFBP-3 and IGFBP-5, which we have recently shown are transported to the nuclei of T47D breast cancer cells. We now describe the mechanism for nuclear import of IGFBP-3 and IGFBP-5. In digitonin-permeabilized cells, where the nuclear envelope remained intact, nuclear translocation of wild-type IGFBP-3 appears to occur by a nuclear localization sequence (NLS)-dependent pathway mediated principally by the importin β nuclear transport factor and requiring both ATP and GTP hydrolysis. Under identical conditions, an NLS mutant form of IGFBP-3, IGFBP-3[228KGRKR → MDGEA], was unable to translocate to the nucleus. In cells where both the plasma membrane and nuclear envelope were permeabilized, wild-type IGFBP-3, but not the mutant form, accumulated in the nucleus, implying that the NLS was also involved in mediating binding to nuclear components. By fusing wild-type and mutant forms of NLS sequences (IGFBP-3 [215-232] and IGFBP-5 [201-218]) to the green fluorescent protein, we identified the critical residues of the NLS necessary and sufficient for nuclear accumulation. Using a Western ligand binding assay, wild-type IGFBP-3 and IGFBP-5, but not an NLS mutant form of IGFBP-3, were shown to be recognized by importin β and the αβ heterodimer but only poorly by importin α. Together these results suggest that the NLSAs within the C-terminal domain of IGFBP-3 and IGFBP-5 are required for importin-β-dependent nuclear uptake and probably also accumulation through mediating binding to nuclear components.

The mitogenic effects of insulin-like growth factors (IGFs)1

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§ The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; NLS, nuclear localization signal; T-ag, SV40 large tumor antigen; N1N2 NLS: β-gal, β-galactosidase fused to the NLS derived from N1N2; RRL, rabbit reticulocyte lysate; CLSM, confocal laser scanning microscopy; EGF, enhanced green fluorescent protein; hnRNP, heterogeneous nuclear ribonucleoprotein; FITC, fluorescein isothiocyanate; GTPγS, guanosine 5′-O-[3-thiotriphosphate]; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; BSA, bovine serum albumin; CHO, Chinese hamster ovary.

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Nuclear Import of IGFBP-3 and IGFBP-5

pore complex. The importin α/β heterodimer recognizes three different classes of NLS: those that contain basic residues arranged as a single stretch (e.g., the NLS of the SV40 large tumor antigen, T-ag) (9, 19, 20) or as two clusters of basic residues separated by a spacer region (bipartite NLS) (9, 21) or those resembling the NLS of the yeast homeodomain protein Mata2 (22). Other signal-dependent pathways have been described that include the transport of proteins that bind directly to and are transported by members of the importin β family (in this pathway the adapter, importin α, is not required to effect nuclear transport) (23–25) and those that do not require soluble cytosolic receptors at all but appear to require ATP (26–28).

Significantly, in the context of IGF-independent nuclear action, the C-terminal regions of IGFBP-3 and IGFBP-5 contain a domain with strong sequence homology to the bipartite NLS consensus motif (29). This basic domain is highly conserved in IGFBP-3 and IGFBP-5 from different species, suggesting that it has functional significance. Similar basic sequences have been identified in a number of other secreted proteins and shown to be important for their respective signaling roles. These include platelet-derived growth factor A (30), acidic fibroblast growth factor (31), and parathyroid hormone-related protein (32). We and others have described the nuclear transport of IGFBP-3 and IGFBP-5 in a number of cell lines (33–36).

As part of our investigation into the role of nuclear IGFBP-3 and IGFBP-5, the present study examines the mechanisms for their nuclear import. We report that previously identified NLS-like sequences within IGFBP-3 and IGFBP-5 are necessary and sufficient for their nuclear accumulation. IGFBP-3 nuclear import is an energy-dependent process requiring ATP and GTP hydrolysis and mediated by importin β. In addition, IGFBP-3 and IGFBP-5 are both recognized specifically by importin β and the importin α/β heterodimer but not by importin α. Thus, nuclear import of IGFBP-3, and by analogy IGFBP-5, appears to occur by a signal-dependent importin β-mediated pathway. In addition, we show that, possibly mediated by its NLS, IGFBP-3 is capable of interaction with nuclear binding sites, which may play an important role in its nuclear accumulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IGFBP-3 and IGFBP-5 [5(2KGRKR → MDGEA)] were produced by a replication-deficient adenovirus-mediated expression system, as described previously (37). IGFBP-3 was purified from conditioned media by IGF-I affinity chromatography and reverse-phase high pressure liquid chromatography (38). In studies requiring fluorescently labeled IGFBP-3, the protein was conjugated to dichlorotrizainylaminofluorescein I HCI as described previously for Cy3 (35). The fusion proteins generated by linking β-galactosidase to the bipartite NLS derived from the Xenopus laevis phosphoprotein, NIN2 (NIN2 NLS-β-gal), or the T-ag NLS were expressed, purified, and, where appropriate, fluorescently labeled with 5-iodoacetamidofluorescein as described previously (39). Recombinant human IGFBP-5 was a generous gift from J. Zapf (Zurich, Switzerland). IGFBP-1 was purified from human amniotic fluid (40), recombinant human IGFBP-2 was provided by Sandoz (Basel, Switzerland), and IGF-I was provided by Genentech (San Francisco, CA). Antiserum against IGFBP-3 was prepared in this laboratory following immunization of rabbits with a purified antigen, and the monoclonal antibody specific for importin b was generated from hybridoma (Amresco), the nuclear envelope was permeabilized with 0.25% Triton X-100, and the cells were blocked with 1% BSA in phosphate-buffered saline for 1 h at 22 °C. The cells were then incubated with antiserum against IGFBP-3 or nonimmune rabbit serum (1:5000) diluted in blocking buffer for 1 h at 22 °C. Cells were then washed and incubated with goat anti-rabbit IgG conjugated with rhodamine (Immunotech) diluted 1:200 in blocking buffer for 1 h at 22 °C. Cells were mounted in an antifade medium and examined using a confocal laser scanning microscopic (CLSM) system (Optiscan F900e Personal Confocal System, Victoria, Australia) fitted with a krypton-argon laser and dual channel detection optics. Individual cells were optically sectioned in the xy plane with multiple scan averaging. All images were collected under identical, nonsaturating conditions. The intensity of fluorescent labeling within cells was analyzed using the program NIH Image version 1.61. Pixel intensity, as a measure of fluorescence intensity, was measured within specific regions of the cell (cytoplasmic and nuclear) as well as in regions outside the cell (background). The pixel intensity from each subcellular region was averaged over at least 100 cells. After correction for background fluorescence, the results were expressed as the ratio of nuclear to cytoplasmic fluorescence. The intensity of fluorescent labeling within cells was analyzed using the program NIH Image version 1.61.
amplified from pSF601 using primers 4 and 6. The mutant 215KK → N and 228KGRKR was amplified from pSF170 using primers 2 and 9, and 220RK → A was amplified from pSF601 using primers 4 and 8. The mutant 220KK → HSRBP-3 was amplified from pSF170 using primers 2 and 9, and 215KK → HSRBP-3 was amplified from overlapping oligonucleotides 10 and 11. The mutant 220KGRKR → MDGEA(BP-3) was amplified from pSF110 using primers 1 and 12, and 215KGRKR → MDGEA(BP-3) was amplified from pSF601 using primers 3 and 13. All mutations within the 18-amino acid basic region of IGFBP-3 and IGFBP-5 (except the N-terminal double alanine mutants) are based on the corresponding sequences in IGFBP-1.

The IGFBP-3 double mutant 215K → N and 228KGRKR → HSRBP-3 was amplified from pSF170 using primers 2 and 5, and the double mutant 215K → N and 228KGRKR → MDGEA(BP-3) was amplified from pSF110 using primers 5 and 12. Following amplification, all polymerase chain reaction products were cloned inframe into the KpnI and BamHI restriction sites of pEGFP-C1 and checked by sequencing.

Cell Culture and Transient Transfection—CHO cells were maintained in α-modification of Eagle’s medium supplemented with 10% fetal calf serum (Cytosystems). For transient transfection, CHO cells were cultured on glass coverslips in 6-well dishes. At 70–80% confluence, 2 μg of EGF fusion plasmid was transfected into cells using LipofectAMINE (Life Technologies) according to the manufacturer’s instructions. At 24 h after transfection, cells were fixed with Histochoice (Applied Science) and permeabilized with the weak nonionic detergent digitonin, leaving the nuclear envelope intact (41). Nuclear transport of fluorescently labeled IGFBP-3 was examined in the presence of a transport solution containing RRL (a source of cytosolic proteins), an ATP-regenerating system (to provide energy for translocation), and fluorescently labeled-dextran (to control for membrane integrity). The subcellular distribution of the fluorescent signal was determined using CLSM. In all cells where the plasma membrane had been permeabilized but where the nuclear envelope remained intact (fluorescently labeled dextran being specifically excluded from the nucleus), IGFBP-3 was localized to the cell nuclei (Fig. 1A).

Quantitation using NIH Image version 1.61 (see “Experimental Procedures”) showed that IGFBP-3 accumulated in the nuclei at levels 4.7-fold greater than in the cytoplasm (Fig. 1D).

To demonstrate that nuclear import of IGFBP-3 was a specific and saturable process, we compared fluorescently labeled IGFBP-3 with unlabeled IGFBP-3. Cytosol was preincubated with a 10- or 20-fold excess of unlabeled IGFBP-3 prior to the addition of fluorescently labeled IGFBP-3. Results of the in vitro nuclear transport assay showed that the nuclear to cytoplasmic fluorescence ratio was reduced to 2.6-fold in the presence of a 10-fold excess of unlabeled IGFBP-3 (Fig. 1, B and D). Following preincubation with a 20-fold excess of unlabeled IGFBP-3 (Fig. 1C), this was further reduced to 1.5-fold (Fig. 1D), close to an Fnc value of 1.0 representing equal fluorescence in the nucleus and cytoplasm. Therefore, an Fnc value of 1.5 suggests that little nuclear fluorescence was detectable following the addition of a 20-fold excess of unlabeled IGFBP-3.

RESULTS

Nuclear Import of IGFBP-3 Is a Specific and Saturable Process—NLS-dependent nuclear protein import is an active process requiring cytosolic factors including importin α/β, Ran, and interacting factors (44). Because IGFBP-3 (40–45-kDa glycosylated doublet) is close to the theoretical limit for diffusion into the nucleus, we investigated whether nuclear import could occur by a conventional NLS-mediated pathway. An in vitro nuclear transport assay was used in which the plasma membrane of CHO cells was permeabilized with the weak nonionic detergent digitonin, leaving the nuclear envelope intact (41). Nuclear transport of fluorescently labeled IGFBP-3 was examined in the presence of a transport solution containing RRL (a source of cytosolic proteins), an ATP-regenerating system (to provide energy for translocation), and fluorescently labeled-dextran (to control for membrane integrity). The subcellular distribution of the fluorescent signal was determined using CLSM. In all cells where the plasma membrane had been permeabilized but where the nuclear envelope remained intact (fluorescently labeled dextran being specifically excluded from the nucleus), IGFBP-3 was localized to the cell nuclei (Fig. 1A).

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Nuclear Transport of IGFBP-3 Is an Energy-Dependent Process Mediated by the Importin β Subunit—The role of individual components of the nuclear transport pathway can be examined by their selective addition to the in vitro nuclear transport assay. We compared the nuclear uptake of IGFBP-3 with that of N1N2 NLS:β-gal, which is transported to the nucleus by the conventional NLS-mediated nuclear protein import pathway utilizing Ran and the importin α/β heterodimer (45, 46). Following nuclear transport, the subcellular localization of unlabeled IGFBP-3 was monitored using indirect immunocyto-
Nuclear Import of IGFBP-3 and IGFBP-5

**Fig. 1.** Nuclear import of IGFBP-3 is a specific and saturable process. Following permeabilization of the plasma membrane, CHO cells were incubated in transport solution containing cytosol, an ATP-regenerating system and fluorescently labeled IGFBP-3 without (A, nil) or with a 10-fold (B) or 20-fold (C) excess of unlabeled IGFBP-3. The images were collected using CLSM and are representative of three independent experiments. Scale bar, 50 μm. Quantitation was carried out using NIH Image, and the results were expressed as the ratio of nuclear to cytoplasmic fluorescence (Fn/cy) ± S.E. (D).

chemistry, the control protein was directly fluorescently labeled, and both were detected using CLSM.

As was observed for fluorescently labeled IGFBP-3 (Fig. 1A), all cells with an intact nuclear envelope contained nuclear IGFBP-3 (Fig. 2A). When specific IGFBP-3 antiserum was replaced with nonimmune rabbit serum in similarly treated cells, only light background labeling was detected, indicating that the signal was specific to IGFBP-3 (data not shown). As previously shown, the N1N2 NLS directed nuclear accumulation of β-galactosidase (Fig. 2A) (46). In the absence of an ATP-regenerating system, both IGFBP-3 and N1N2 NLS:β-gal were localized to the cytoplasm, being generally excluded from the nucleus (Fig. 2B). A requirement for ATP in NLS-dependent nuclear import has been described for a number of proteins (26–28, 49).

When cytosolic proteins (in the form of RRL) were omitted from the transport solution, the pattern of nuclear accumulation of IGFBP-3 (Fig. 2C) was indistinguishable from that seen in its presence (Fig. 2A). Therefore, in contrast to N1N2 NLS:β-gal, which demonstrated cytosol-dependent nuclear transport (Fig. 2C), nuclear import of IGFBP-3 was independent of exogenously added cytosol. Previous studies have shown that although importin α is released following treatment of cells with digitonin, sufficient importin β may remain to sustain basal nuclear import (12, 47, 48). Therefore, the independence of nuclear uptake of IGFBP-3 on cytosolic factors suggests that importin α is not required for nuclear import, whereas the possibility remains that importin β may act alone as the transport receptor for IGFBP-3. NLS-dependent nuclear import, where the transported protein binds directly to importin β independently of importin α, has been documented for a number of proteins (23–25, 49).

Nuclear import of IGFBP-3 was examined following neutralization of importin β from the assay by the addition of an anti-importin β antibody in the absence of RRL. Results showed a significant reduction in the level of nuclear import of both IGFBP-3 and N1N2 NLS:β-gal (Fig. 2D). Because no importin α was added to the system in this experiment, the results suggest that importin α is unlikely to have a role in IGFBP-3 nuclear import. In a similar experiment where RRL was preincubated with the anti-importin β antibody prior to addition to the assay, nuclear import of IGFBP-3 was also reduced (data not shown). The role of GTP hydrolysis in nuclear transport of IGFBP-3 was examined following preincubation of the cytosol with the nonhydrolyzable GTP analogue GTPγS (E). Again nuclear accumulation of both IGFBP-3 and N1N2 NLS:β-gal was reduced in the presence GTPγS (Fig. 2E), with the pattern of IGFBP-3 labeling similar to that observed for cells treated with the anti-importin β antibody (Fig. 2D). These results suggest that nuclear accumulation of IGFBP-3 is an active process with a requirement for both importin β and GTP hydrolysis in its nuclear transport and appears to be independent of importin α. In addition, because some nuclear uptake of IGFBP-3 remains (Fig. 2D), other uptake/accumulation mechanisms may be operating in addition to that utilizing importin β. Although the cytosol-independent nature of IGFBP-3 nuclear import implies that a role for Ran is unlikely, other GTP-binding proteins may be involved in nuclear protein import, and the action of these proteins may constitute the basis of the inhibition of IGFBP-3 nuclear import by GTPγS.

**Fig. 2.** Nuclear import of IGFBP-3 is an energy-dependent process mediated by the importin-β subunit. Digitonin-permeabilized CHO cells were incubated with IGFBP-3 and N1N2 NLS:β-gal (a control for the conventional importin α/β-mediated nuclear import pathway directed by a bipartite NLS) and visualized by CLSM. In vitro nuclear transport (see “Experimental Procedures”) was carried out in the presence of cytosol and an ATP-regenerating system (A). The effect on nuclear transport of omitting the ATP-regenerating system (B) or cytosol (C) was examined. Transport studies were also carried out in the absence of an anti-importin-β antibody without the addition of cytosol (D) and following preincubation of cytosol with the nonhydrolyzable GTP analogue GTPγS (E). Images are representative of at least three independent experiments. Scale bar, 50 μm.
Alternatively, Ran may not have been fully depleted from the transport assay following permeabilization of the cells.

**Nuclear Accumulation of IGFBP-3 Is Prevented When the Putative NLS Is Mutated or Lost by Proteolytic Cleavage—**We have previously shown that the mutant, IGFBP-3[228KGRKR -> MDGEA], obtained by exchanging part of the putative NLS of IGFBP-3 for the corresponding sequences in IGFBP-1, is not transported to the nucleus of intact cells (35). However, we have also shown that this IGFBP-3 mutant is unable to bind to the cell surface (38), leading to the speculation that transport to the nucleus may be blocked at the level of the plasma membrane rather than at the level of entry into the nucleus. To address this issue, we compared nuclear uptake of wild-type and mutant IGFBP-3 (in cells were the plasma membrane had been permeabilized) using the fully reconstituted in vitro nuclear transport assay. In contrast to wild-type IGFBP-3 (Fig. 3A), the mutant, IGFBP-3[228KGRKR -> MDGEA], was not localized to the nucleus (Fig. 3B), suggesting that residues 228–232 within the basic region of IGFBP-3 are indeed required for nuclear accumulation as well as plasma membrane binding.

During an early round of purification of wild-type IGFBP-3, a 30-kDa proteolytic fragment was generated. The purified fragment was subject to N-terminal sequencing and shown to be an N-terminal fragment of IGFBP-3. It therefore lacks the basic region present in the C-terminal domain of the protein. When nuclear uptake was examined, this truncated form of IGFBP-3 did not accumulate in the nucleus (Fig. 3C). Therefore, with respect to nuclear transport, the proteolytic fragment behaved in a similar fashion to the mutant form of IGFBP-3, supporting the observation that sequences within the C-terminal domain are required for active nuclear transport.

**IGFBP-3 Binds to Insoluble Nuclear Components—**In the presence of a permeabilized nuclear envelope, soluble proteins are able to pass freely between the cytoplasm and nucleus. Under these circumstances, nuclear accumulation occurs only if the protein binds to insoluble nuclear components (42). To investigate whether IGFBP-3 was capable of such interactions, we used the in vitro transport assay on cells where the nuclear envelope had been permeabilized with the detergent CHAPS. To demonstrate that CHAPS was effectively permeabilizing the nuclear envelope, experiments were carried in the presence of FITC-dextran (molecular mass, ~77 kDa). Under these conditions, the dextran distributes evenly between the nucleus and cytoplasm. In the presence of a fully reconstituted assay and the absence of a barrier to nuclear entry, nuclear accumulation of wild-type IGFBP-3 was observed (Fig. 4A). The same field of cells visualizing the FITC-dextran signal (Fig. 4B) showed that accumulation of IGFBP-3 only occurred in those cells with a perforated nuclear envelope. In contrast, N1N2 NLS;β-gal did not accumulate in the nucleus, instead equilibrating between the nuclear and cytoplasmic compartments (data not shown).

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cells, respectively, with nuclear greater than cytoplasmic fluorescence (Table II). As expected, a double mutation of the IGFBP-3 NLS, which involved the first and third basic clusters prevented nuclear transport of the fusion protein (Table II). Mutation of both the first and second basic clusters resulted in a further decrease in nuclear transport (40.3%) of the fusion protein compared with the central mutant alone (62.2%). Together these results suggest that the sequences 228KGRKR(BP-3) and 214RGRKR(BP-5) are essential for nuclear import of the binding proteins but that the other basic residues within the NLS probably contribute to the overall efficiency of nuclear accumulation.

IGFBP-3 and IGFBP-5 Are Recognized by the Importin α/β Heterodimer through the Importin β Subunit—The α and β importin subunits constitute the high affinity NLS receptor used by many proteins to effect their nuclear import (12). The ability of full-length IGFBPs to be recognized by importin subunits was examined using dot blot (Fig. 6A) and Western ligand binding analysis (Fig. 6B) to examine interactions of native and denatured binding proteins, respectively. Wild-type IGFBP-3 showed strong binding by the importin complex (Fig. 6, A, lane 6, and B, top panel). This binding was of a similar intensity to that obtained for the T-ag NLS-β-galactosidase fusion protein (positive control) (Fig. 6A, lane 2). In contrast, the NLS mutant, IGFBP-3[228KGRKR→MDGEA], displayed weaker binding by the importin α/β heterodimer NLS (Fig. 6, A, lane 5, and B, top panel) compared with wild-type IGFBP-3. However, this binding appeared to be stronger than that obtained for β-galactosidase, which does not contain an NLS (negative control) (Fig. 6A, lane 1). Of the other binding proteins tested, IGFBP-5 was also recognized by the importin heterodimer (Fig. 6, B, top panel). IGFBP-1 and IGFBP-2, which are not translocated to the nucleus in intact cells (35), showed no detectable binding by the importin complex (data not shown).

Because IGFBP-3 has been shown to act as a carrier for IGF-I nuclear transport (34), we investigated whether the binary complex would bind more strongly to the importin subunits. However, when equimolar amounts of IGFBP-3 and IGF-I were equilibrated and subjected to dot blotting, there was no discernible difference in binding of either wild-type (Fig. 6A, lane 4) or mutant IGFBP-3 (Fig. 6A, lane 3) to the importin complex compared with these proteins in the absence of IGF-I (Fig. 6A, lanes 5 and 6). In addition, when IGF-I was added to the hybridization mix containing importin α/β, there was no change in the binding of importin to wild-type or mutant IGFBP-3 analyzed by Western ligand binding (data not shown).

Because nuclear transport of IGFBP-3 appears to be mediated by importin β (independently of importin α), we examined whether the observed importin α/β heterodimer binding was mediated by the importin α (Fig. 6B, middle panel) or β (Fig. 6B, bottom panel) subunit. The Western ligand binding assay clearly showed that IGFBP-3 and IGFBP-5 were both recognized by importin β (Fig. 6B, bottom panel). In contrast, neither binding protein was recognized to any great extent by importin α (Fig. 6B, middle panel), nor did mutant IGFBP-3 exhibit significant binding to either importin subunit (Fig. 6B, middle and bottom panels). Quantitation of binding indicated that, in the case of IGFBP-3, importin β binding accounted for almost 40% of the binding of the importin heterodimer (Fig. 6C). Although importin α binding to IGFBP-3 represented only a small proportion of importin α/β binding, it was still an appreciable amount compared with importin β binding only. However, in the case of IGFBP-5, importin β binding accounted for essentially all of the binding of the importin heterodimer (Fig. 6C). The mutant form of IGFBP-3 displayed less than 50%
DISCUSSION

This study identifies an 18-amino acid region of IGFBP-3 and IGFBP-5 that is necessary and sufficient for nuclear transport and accumulation. Our results suggest that nuclear import of IGFBP-3, and probably by analogy IGFBP-5, is an energy-dependent process mediated by importin β and following nuclear entry, IGFBP-3 can actively accumulate through binding to detergent-insoluble nuclear components. Mutation of the basic regions within the C-terminal domain of IGFBP-3 and IGFBP-5 attenuates nuclear import and/or accumulation and, as shown for IGFBP-3, reduces importin binding.

We have studied nuclear transport of IGFBP-3 using an in vitro nuclear transport assay that allows the role of individual components of the transport system to be examined. As a control, we examined the nuclear uptake of β-galactosidase fused to the N1N2 bipartite NLS (46). In the presence of cytosolic factors and an ATP-regeneration system, full-length wild-type IGFBP-3 was capable of nuclear uptake in all cells where the plasma membrane had been permeabilized, but the nuclear envelope remained intact. Previous studies in intact cells have shown that nuclear transport of IGFBP-3 is detected only in a low percentage of cells in the monolayer (33–36). Therefore, these data suggest that the plasma membrane is an important regulator of nuclear uptake of IGFBP-3 and consequently also of its subsequent function in the nucleus.

Nuclear protein import directed by bipartite NLSs conventionally requires cytosolic factors such as importin α/β, Ran, and nuclear transport factor 2 (44). However, we show here that nuclear transport of IGFBP-3 can occur efficiently in the absence of added soluble transport factors. Analogous observations have been reported for nuclear transport conferred by the HIV-I Tat NLS (26) and the heterogeneous nuclear ribonucleoprotein (hnRNP) K sequence KNS (28). The Wnt signal transduction pathway component β-catenin (27), which appears to be able to bind directly to nucleoporins, similarly appears not to require soluble factors for nuclear import; interestingly, nuclear transport of β-catenin appears to occur through a Ran-independent pathway (51). However, unlike IGFBP-3, the targeting signals of Tat and hnRNP K represent novel nuclear targeting signals not resembling the T-ag or bipartite NLSs and, furthermore, do not appear to be recognized by importin α/β. Previous studies have found that sufficient importin β, but not importin α, may remain associated with the nuclear pore complex to support a basal level of nuclear import subsequent to digitonin permeabilization (12, 47, 48). Therefore, nuclear import in the absence of added cytosol suggests that the adapter, importin α, is not required for nuclear transport but does not rule out the possibility that importin β alone is mediating uptake. Our findings that inclusion of an antibody specific to importin β, both in the presence and absence of added cytosol, inhibited nuclear import of IGFBP-3, suggests that importin β, but not importin α, is required to sustain basal nuclear import. Similarly, addition of the nonhydrolyzable GTP analogue, GTPγS, to the transport assay significantly reduced nuclear import of IGFBP-3. Together these results suggest that both importin β alone, and GTP hydrolysis are required for efficient transport. Importin β appears to be the sole nuclear targeting signal receptor used by parathyroid hormone-related protein (24), T-cell protein tyrosine phosphatase (25), and the yeast transcription factor, GAL4 (23). Finally, we cannot exclude the possibility that the cytosolic-independent nature of IGFBP-3 nuclear import is effected by other factors that, like importin β, may not be completely solubilized during digitonin permeabilization.

Nuclear import mediated by conventional NLSs such as those found in T-ag and retinoblastoma (42), as well as by novel binding to the importin α/β heterodimer and the individual importin subunits, compared with wild-type IGFBP-3. It was concluded that importin β, but not importin α, was able to recognize IGFBP-3 and IGFBP-5 and that there appeared to be a quantifiable difference in importin subunit binding to these IGFBPs.

FIG. 6. IGFBP-3 and IGFBP-5 are recognized by the importin αβ heterodimer through the importin β subunit. IGFBPs and controls were applied directly to membrane (A) or separated on 10% SDS-polyacrylamide gel electrophoresis prior to membrane transfer (B) and hybridization with a complex of importin αβ (A and B, top panels) or individual importin subunits (B, middle and bottom panels). The amount of protein added is indicated as pmol (A) or μg (B), and the positions of molecular mass markers are indicated. Control proteins were β-galactosidase (negative control) (A, lane 1) and the T-ag NLS fused to β-galactosidase (positive control) (A, lane 2). The binding proteins analyzed were wild-type IGFBP-3 (A, lane 6, and B, lanes 1 and 2), the IGFBP-3 NLS mutant, GKRKR → MDGEA (A, lane 5, and B, lanes 3 and 4) and wild-type IGFBP-5 (B, lanes 5 and 6). An equimolar amount of IGF-I was preincubated with either wild-type (A, lane 4) or the mutant form of IGFBP-3 (A, lane 3) prior to membrane application. Quantitation of the Western ligand blot was carried out using a Fujifilm FLA-3000 Imager and the Image Gauge software (C), where the mean signal intensities ± S.E. (n = 4) for importin α and importin β are expressed as percentages of those for binding by importin αβ.
NLSs of HIV-I Tat (26) and the hnRNP K (28) and hnRNP A1 M9 sequences (52), has been shown to be ATP-dependent; cytosolic factor-independent nuclear import of β-catenin has also been shown to require ATP (27). In the case of HIV-I Tat, ATP hydrolysis is believed to effect its release from cytoplasmic retention factors and enhance binding to nuclear components. Because nuclear import of IGFBP-3 was not observed in the absence of an ATP-regenerating system, IGFBP-3 may also require ATP for cytoplasmic release and/or enhanced nuclear binding. Alternatively, as is the case for many proteins transported to the nucleus, ATP may be involved in other, unknown actions that augment nuclear uptake.

In the presence of a permeabilized nuclear envelope, proteins are free to diffuse between the cytoplasmic and nuclear compartments and are only able to accumulate in the nucleus through binding to insoluble nuclear components such as chromatin and lamin (42). Under conditions where the nuclear envelope was permeabilized, IGFBP-3, but not the mutant, IGFBP-3[228KGRKR→MDGEA], was capable of nuclear accumulation. These results support the proposal that, upon entry into the nucleus, IGFBP-3 accumulates through nuclear binding and suggest that residues 228–232 are required for these nuclear interactions. The ability of IGFBP-3 to bind to structures within the nucleus supports the hypothesis that it may have a role in the nucleus, possibly in direct regulation of gene transcription (33–36). This has been suggested for granzyme A and B (53, 54) and parathyroid hormone-related protein (24), which can also accumulate in the nucleus even in the absence of an intact nuclear envelope.

Fusion of EGFP to isolated motifs has been used to study changes in subcellular distribution directed by these sequences (50). Using this approach, we investigated the role of the basic motifs of IGFBP-3 and IGFBP-5 in their nuclear transport. As the fusion proteins are expressed in living CHO cells, the proteins, factors and metabolic pathways present and active in living cells are able to exert their effects on nuclear transport, thus representing an in vivo nuclear transport assay. EGFP is small enough to enter the nucleus by passive diffusion, and as expected, the fluorescent signal derived from recombinant EGFP expressed in CHO cells was evenly distributed between the nucleus and cytoplasm. Fusion of the basic region within the C-terminal domain of IGFBP-3 and IGFBP-5 to EGFP caused nuclear accumulation of the fusion protein in greater than 90% of transfected cells, indicating that the basic regions are sufficient for nuclear import. However, these results do not distinguish between active nuclear transport and nuclear binding. Thus, there may be passive diffusion into the nucleus followed by accumulation resulting from interaction between these basic sequences and nuclear binding sites, as well as interaction of the basic residues with importins, leading to active nuclear import.

Mutation of the three basic clusters within the putative NLS of IGFBP-3 and IGFBP-5 caused different degrees of attenuation of nuclear transport of the EGFP fusion proteins. As was observed for the mutant form of full-length IGFBP-3, IGFBP-3[228KGRKR→MDGEA], the same mutation of both the IGFBP-3 and IGFBP-5 NLS when fused to EGFP, abolished nuclear uptake of EGFP. Mutation of both amino acids within the N-terminal basic cluster had a significant effect on nuclear transport of the EGFP fusion proteins, suggesting that the basic region may represent a classical bipartite NLS similar to that described for other proteins (9, 21). However, mutation of the central basic cluster reduced nuclear transport of the fusion proteins by approximately 60%, implying that these basic clusters also influence nuclear accumulation and that the NLS is more complex than a classical bipartite NLS. As discussed above, the ability of the fusion protein to diffuse freely into the nucleus means that a distinction cannot be drawn between enhancement of nuclear import and accumulation because of nuclear binding. Therefore, mutations affecting nuclear transport may relate to either or both effects. However, in vitro studies on the mutant, IGFBP-3[228KGRKR→MDGEA], suggest these sequences are involved in both effects. In this assay the basic sequences derived from IGFBP-5 behaved identically to those derived from IGFBP-3, suggesting that IGFBP-5, which is small enough to diffusion through the nuclear pore complex (molecular mass, 30 kDa), accumulates in the nucleus by a similar mechanism.

We found that the importin β subunit recognized both wild-type IGFBP-3 and IGFBP-5 but recognized only to a limited extent the mutant form of IGFBP-3. This suggests that transport of these binding proteins occurs by a signal-mediated pathway, consistent with the observation that importin β is required to effect in vitro nuclear transport of IGFBP-3. Interestingly, all the importin αβ binding to IGFBP-5 could be accounted for by importin β binding, whereas for IGFBP-3 importin β binding appeared significantly lower compared with its binding to the heterodimer. This was not compensated for by an appropriate increase in importin α binding. The explanation for this is unclear but may relate to differing affinities or accessibility of the binding proteins for the importin subunits. Alternatively, importin α may be binding to importin β in this assay and increasing the strength of its interactions with IGFBP-3. However, an essential role for importin α in IGFBP-3 nuclear transport is not supported by our findings that nuclear import occurs in the absence of exogenous cytosol.

An interesting question raised by this study is why IGFBP-5, and to a lesser extent IGFBP-3, possess functional NLSs when, because of their size, they have the potential to diffuse from the cytoplasm into the nucleus. There are several reasons why smaller proteins possess NLS-dependent mechanisms for nuclear import. As has been described for interleukin-5 (55), a functional NLS enables the cotransport of larger non-NLS containing proteins to the nucleus. In analogous fashion, IGFBP-3 or IGFBP-5 may possess functional NLSs to facilitate entry when part of a high molecular mass complex. Thus, they may require an NLS-dependent mechanism when cotransporting IGFs or other signaling molecules to the nucleus. Interestingly, a preliminary report suggests that IGFBP-3 interacts specifically with the retinoic acid X receptor-α (56). IGFBP-3 may thereby modulate the activity of nuclear transcription factors or have a specific signal transduction role in the nucleus. It may also regulate gene expression directly by binding to chromatin as has been reported for basic fibroblast growth factor (57) and the growth hormone receptor (58). Apart from the selective use of an NLS-dependent mechanism for the transport of high molecular mass complexes, the kinetics of nuclear import of IGFBP-3 and IGFBP-5 may be enhanced by their ability to interact effectively with importin. A major focus of future work in this laboratory is to distinguish between these possibilities. Understanding of the mechanisms of nuclear import of IGFBP-3 and IGFBP-5 should greatly assist in defining their nuclear functions.

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