Insulin-like growth factors (IGFs) play a central role in the integration of proliferative and survival responses of most mammalian cell types. IGF-binding protein-3 (IGFBP-3) influences IGF action directly as a carrier of IGFs but also modulates these actions indirectly via independent mechanisms involving interactions with plasma, extracellular matrix and cell surface molecules, conditional proteolysis, cellular uptake, and nuclear transport. Here we demonstrate that a short C-terminal metal-binding domain (MBD) of IGFBP-3 mediates binding to metals. MBD epitopes, sequestered in the intact molecule, are unmasked by incubation in the presence of ferrous (but not ferric or zinc) ions. An isolated 14-mer MBD peptide triggered apoptotic effects in stressed HEK293 cells as effectively as IGFBP-3. The MBD, which encompasses a nuclear localization sequence and an adjacent putative cavelolin-binding sequence, mobilizes rapid cellular uptake and nuclear localization of unrelated proteins such as green fluorescent protein and streptavidin-horseradish peroxidase conjugate. Metal ions stimulate MBD-mediated cellular/nuclear uptake in vivo. Cross-linking studies showed a direct physical interaction of MBD with integrins αv and β1, cavelolin-1, and transferrin receptor. MBD-mediated protein mobilization and pro-apoptotic effects are inhibited by nystatin but not chlorpromazine, suggesting an involvement of cavelolin-mediated endocytosis. However, MBD effects are inhibited by antibodies to transferrin receptor or integrins. These results are discussed with particular reference to the cell target specificity of IGFBP-3 in disease processes such as cancer and atherosclerosis.

IGFBP-3's modulates the biological effects of IGFs directly by sequestering them and indirectly by independent mechanisms that may involve specific binding to a number of plasma, extracellular matrix and cell surface molecules, conditional proteolysis, rapid internalization of IGFBP-3 (or its fragments) into target cells, and translocation into the nucleus (reviewed in Ref. 1). In the nucleus, IGFBP-3 interacts with transcription factors such as retinoid X receptor-α (2).

IGFs trigger cellular effects via their cognate plasma membrane receptor tyrosine kinases. By binding IGFs, the IGF-binding proteins (IGFBPs) can modulate these effects directly. Other biochemical actions of IGFBPs at the target cell surface are not well understood. IGFBP-3 is known to bind a number of extracellular matrix components including collagen, fibronectin, and heparin (3–5), and the existence of a cell-surface receptor has been suggested (52). Cross-talk between IGF receptor tyrosine kinases and integrins has also been reported (6).

The role of iron in the biological actions of IGFs and IGFBPs is not well understood. In plasma, IGFBP-3 is known to participate in high affinity interactions with the iron-binding proteins lactotransferrin (7) and transferrin (8), but the functional significance of these interactions is not known. Unlike the documented binding interactions of IGFBP-3 with pre-kallikrein, plasminogen, fibrinogen, collagen, and fibronectin (3, 4, 9–11) (all of which are unaffected by IGF ligand occupancy), binding to lactotransferrin competes with IGF binding (7). Iron-saturated transferrin binds IGFBP-3 with twice the affinity of apotransferrin (8). Selective release of molecular iron from transferrin (15) is believed to be enhanced at low pH environments typically found in iron-rich tumors (16) and atherosclerotic lesions (17). Acidic environments promote a dramatic acceleration of lipid oxidation rates in the presence of iron (18).

Caveolae, specialized flask-shaped invaginations on the cell surface, are composed of cholesterol, sphingolipids, and structural proteins termed caveolins; functionally, these plasma membrane microdomains have been implicated in signal transduction (19). However, in most cells, caveolin-1 plays additional roles in membrane dynamics, including organization of non-caveolar detergent-insoluble lipid rafts and trafficking of cholesterol (20). Caveolin has profound effects on cellular oxidative metabolism (21, 22) via physical sequestering of endothelial nitric-oxide synthase (23, 24).

A role for caveolin in IGF signal transduction is suggested by the observation that IGF-I receptor colocalizes with caveolin 1 in the lipid raft-enriched fractions of plasma membranes. IGF-I induces caveolin 1 phosphorylation at tyrosine 14, which results in the translocation of caveolin 1 and in the formation of membrane patches on the cell surface. These actions are IGF-I-specific (as opposed to insulin-specific) (25). Insulin-induced protein tyrosine phosphorylation cascade and signaling molecules are also localized in a caveolin-enriched cell membrane domain (26) where caveolin binds to the insulin receptor and stimulates signaling (27). Lipid rafts may represent a spatial integration point for insulin/IGF signaling and membrane traffic (28).

Both IGF-I and insulin (but not IGF-II) enhance lipid oxidation (29), and caveolae are implicated in human disease states
characterized by altered membrane lipid structure. Chronic disease states such as rheumatoid arthritis, human immunodeficiency virus infection, multiple sclerosis, amyotrophic lateral sclerosis, atherosclerosis, diabetes, chronic obstructive pulmonary disease, and cancer (30–37) are characterized by profound changes to cellular iron and/or oxidative metabolism.

IGFBP-3 treatment has been observed to sensitize some types of tumor cell lines to secondary apoptotic stimuli such as exposure to radiation or selected chemical and biological agents (38), and these effects are postulated to be IGF-independent (39). The pleiotropic nature of the IGFBP-3 molecule makes it difficult to establish unequivocal “IGF independence” of observed biological effects in experiments using the intact IGFBP-3 molecule. In this work we use short synthetic peptides to identify domains within the IGFBP-3 molecule that are necessary and sufficient for cellular uptake, nuclear localization, and pro-apoptotic effects.

MATERIALS AND METHODS

Reagents—Laboratory chemicals were purchased from Sigma unless otherwise specified. Monoclonal antibodies purchased from BD Biosciences are as follows: anti-GFP JL-8, anti-fibronectin, anti-integrin-β3, and anti-transferrin receptor-1. Monoclonal antibodies obtained from Sigma are as follows: anti-integrin-αv, P2W7, anti-ı6 VLA60c, and anti-transferrin. Polyclonal antibody to human caveolin 1 was purchased from Upstate Biotechnology (Lake Placid, NY). Preparation of recombinant human (rh) IGFBP-3 in glycosylated (g-IGFBP-3) and non-glycosylated (ng-IGFBP-3) forms, rhIGF-FrhIGFBP-3 complex, and a polyclonal antibody to intact IGFBP-3 have been described (40, 41). For this work, unless otherwise specified, rhIGFBP-3 refers to non-glycosylated IGFBP-3. Custom synthesis of all peptides and preparation of anti-MBD5 polyclonal antibody were performed under contract at Genemod Synthesis, Inc. (South San Francisco, CA). Each peptide was purified by HPLC to >80% purity and analyzed by mass spectroscopy.

Cell Culture—Human embryonic kidney (HEK) 293 cells (ATCC) were used for all mammalian cell culture experiments in this work. 293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% calf serum (VWR Scientific) and penicillin/streptomycin. Cells were grown in LB broth (BD Biosciences) containing 50 μg/ml ampicillin broth overnight, diluted 1:100 in the same medium, grown to 0.4, and induced with 1 mM isopropyl-1-thio-D-galactopyranoside. Cells were harvested by centrifugation and disrupted using a sonicator. Cells were frozen and stored at -80 °C.

FIG. 1. Metal-binding domain of IGFBP-3. A, C-terminal sequence of IGFBP-3 molecule (residues 215–241 of the mature protein) including the conserved CD74-like motif. The core 12-mer cysteine loop is underlined, and the putative caveolin-interaction sequence is boxed. For iron independence studies, serum-free media containing 10% calf serum was used instead of Dulbecco’s modified Eagle’s RPMI culture medium was used instead of Dulbecco’s modified Eagle’s medium.

Metal Binding—Nitrolotrionic acid (NTA)-agarose was purchased either uncharged or pre-charged with manganese or zinc (Sigma). The His-Bind kit (Novagen, Madison, WI) was also used according to the kit protocol. For charging NTA, nickel chloride (VWR Scientific, West Chester, PA), ferrous and ferric chlorides (VWR Scientific, West Chester, PA), cobalt chloride, and magnesium sulfate solutions were used. Each was charged with 100 μg of protein. Each construct contains a hexahistidine tag to facilitate protein purification. Bacterial cultures of constructs exhibiting the expected sequence (GFP-31, -32, -34, -35, and -36) were grown in LB/ampicillin broth overnight, diluted 1:100 in the same medium, grown to OD600 = 0.4, and induced with 1 μM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation and disrupted using a Branson sonicator. Extracts were clarified by centrifugation, and GFP proteins were purified by immobilized metal-affinity chromatography using the His-Bind kit (Novagen, Madison, WI) followed by a desalting step on an Amersham Biosciences Hi-Trap desalting column using an Amersham Biosciences LKB LCC-500 Plus fast protein liquid chromatography apparatus. Proteins were stored in phosphate-buffered saline (PBS) at −80 °C.

ELISAs—Each extract was assayed in triplicate. 100 μl of cell extract per well was incubated in a 96-well plate for 1 h at room temperature. The plate was washed with 1× phosphate-buffered saline (PBS) plus 0.5% Tween (VWR Scientific) 3 times and blocked for 3 h with 3% bovine serum albumin. The primary antibody(a) at a 1:3500 dilution (200 μl) was added to each well and incubated at room temperature for 1 h and
then washed with 1× PBS plus Tween 3 times. Peroxidase-labeled secondary antibody (Amersham Biosciences) at a 1:5000 dilution (200 µl) was added to wells and incubated for 1 h at room temperature. After 1 h the plates were washed 3 times with 1× PBS and Tween. 100 µl of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added to each well and incubated for 45 min at room temperature in the dark. An absorbance reading was taken at 655 nm using a Bio-Rad plate reader.

**Apoptosis Assays**—Cell extracts were prepared 6 h after addition of peptides to cultured cells. Extracts were assayed in triplicate for caspase-3 using a commercial kit (Clontech, Palo Alto, CA). Cell extracts were assayed for Bax and Bcl-2 mRNA levels using Quantikine mRNA kits from R&D Systems (Minneapolis, MN). Alternatively, cell supernatants were assayed in triplicate for nuclear matrix protein at 2-h intervals using the Cell Death Detection Kit (Calbiochem).

**Nuclear Uptake**—Peptides and proteins were added directly to 85–90% confluent 293 cells in culture dishes and incubated at 37 °C for 20 min. For GFP experiments, GFP protein was directly added to the medium of 293 cells in culture (200 ng/ml). For MBD-mediated SA-HRP experiments, a complex was first formed in vitro by coincubating a molar excess of biotinylated MBD peptide with 3 µg of streptavidin-horseradish peroxidase conjugate (Sigma) for 30 min at 37 °C. This complex was purified by filtration (Centricon-30 filter) to remove free peptide, and the specific activity of the peroxidase complex was checked by enzymatic assay before experimental use. Complexes were directly added to the medium of 293 cells in culture (350 ng/ml complex final concentration, unless otherwise stated). After 20 min of incubation with cells, media were removed from the plates, and the cells were washed with 1× PBS plus 1% calf serum twice. Cell extracts were assayed for peroxidase activity (in SA-HRP experiments) or for GFP by ELISA (in GFP experiments). Assay results were read using a Bio-Rad plate reader at 655 nm.

**Cross-linking Assays**—Approximately 100,000 (per aliquot) HEK293 cells grown to 85–90% confluency and subsequently harvested from cell culture plates were cooled down for 1 h at 4 °C. 1 µg of biotinylated MBD peptide was added to each cell aliquot, and incubation was continued at 4 °C. At various times (0, 30, 60, 90, and 120 min) 25 mM bis(sulfosuccinimidyl) suberate cross-linking reagent (Pierce) was added to an aliquot of cells. After 20 min, the reaction was stopped with 100 mM Tris-HCl, pH 7.5. Cells were washed twice with Tris Buffer and centrifuged at 1500 rpm for 3 min to remove excess material. Cell

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Fig. 2. Unmasking of MBD epitopes by ferrous iron. A, ELISA measuring cross-reactivity of increasing amounts of each MBD peptide or IGFBP-3 against anti-IGFBP-3 or anti-MBD5 polyclonal antibodies. IGFBP-3 (500 ng) was statistically significant against all MBD peptides. B, ELISA measuring cross-reactivity of IGFBP-3 to anti-IGFBP-3 (left) or anti-MBD5 (right) polyclonal after incubation in the presence of Fe^{2+}, Fe^{3+}, or Zn^{2+} salts in vitro for the indicated times in the presence or absence of equimolar IGF-I. Open squares, IGFBP-3 alone, no addition; open circles, Fe^{2+}; closed circles, Fe^{3+} plus IGF-I; triangles, Fe^{2+}; diamonds, Zn^{2+}. Fe^{2+} sample was statistically significant against all other groups at 60 min.
Fig. 3. MBD-mediated apoptosis. a, induction of apoptosis in 293 cells. Three representative cell stress assays are shown: nutrient stress/caspase-3 (left panel), chemical stress/caspase-3 (middle panel), and chemical stress/nuclear matrix protein assay (right panel). In each case, "-" is unstressed cells, and "++" is stressed cells. The pro-apoptotic effects of exogenously added IGFBP-3 (50 ng/ml; stippled bars) or MBD2 (50
C-terminal MBD of IGFBP-3 Mediates IGF-independent Effects

RESULTS

Metal-binding Domain—A homology BLAST search of the amino acid sequence of IGFBP-3 revealed the presence of a conserved C-terminal domain (residues 215–241) homologous to a zinc-finger-like motif conserved in human CD74 and other vertebrate proteins of unrelated function. We asked if intact IGFBP-3 might also be able to bind metals. Purified samples of IGFBP-3 were passed through nickel-NTA columns charged with each of several metals known to bind NTA. The results (Fig. 1, A and B) showed that IGFBP-3 bound nickel, cobalt, zinc, iron, magnesium, and manganese but not calcium. Nickel-NTA binding (and ferrous-NTA binding, not shown) was inhibited by IGF-I but was unaffected by the glycosylation status of IGFBP-3. We next asked if peptides made to the above motif (putative metal-binding domain, MBD) within the IGFBP-3 sequence might also bind metal. As shown in Fig. 1C, a 12-residue cyclic peptide (residues 224–235) is sufficient to specify Ni-NTA binding.

Effect of Metals on IGFBP-3 in Vitro—We observed that a polyclonal antibody raised to the peptide MBD5 efficiently recognized MBD2, MBD5, and MBD9 but did not recognize intact rhIGFBP-3 (Fig. 2A, right panel). A second independent anti-MBD5 polyclonal antibody generated identical results (not shown). Conversely, anti-IGFBP-3 polyclonal antibody recognized IGFBP-3 but did not recognize any of the MBD peptides (Fig. 2A, left panel). One explanation of these results is that MBD epitopes are “buried” in the intact molecule.

We then considered the possibility that MBD epitopes might be “unmasked” by pre-treating IGFBP-3 with metal ions in vitro. Aliquots of IGFBP-3 or IGF-I-IGFBP-3 complex were pre-incubated for various times with each of several metal ions. As shown in Fig. 2B, MBD epitopes are rapidly unmasked by pre-incubation in 100 μM ferrous (but not ferric) or zinc chloride solution. Simultaneously, IGFBP-3 epitopes appear to be lost. We have not been able to distinguish whether this effect of ferrous metal on IGFBP-3 occurs via partial proteolysis or by the induction of a conformational change in IGFBP-3. Iron-mediated epitope unmasking is dramatically inhibited in the presence of IGF-I.

As shown in Fig. 7C, ~10–15% of the starting IGFBP-3 is converted to MBD mol equivalents (i.e. “epitope-unmasked” form) within 60 min in ferrous chloride buffer. The loss of IGFBP-3 immunoreactivity during that time (Fig. 2B, left panel) appears to be about 50%. This discrepancy can be explained by rapid proteolysis of unmasked IGFBP-3. IGFBP-3, even in highly purified form, is known to be highly sensitive to rapid proteolytic degradation unless “stabilized” by high salt or by binding to heparin or IGF-I. From its chromatographic behavior in a number of systems, some investigators have tentatively concluded that IGFBP-3 can exist in at least two dramatically different conformations, with respect to proteolytic sensitivity. We suspected that iron, by exposing MBD epitopes, might trigger degradation of IGFBP-3 (or vice versa). A series of complementary experiments using plasminogen/plasmin, kallikrein, and other reported IGFBP-3 proteases to try and establish the reverse ontology (proteolysis, therefore exposure of epitopes) failed, in our hands, to provide supportive evidence for that option.

MBD peptides trigger apoptosis in stressed cells. HEK293 cells stressed chemically (sublethal doses of paclitaxel) or nutritionally (2% serum) can be induced to undergo apoptosis when 50 ng/ml rhIGFBP-3 is added to the growth medium. As shown in Fig. 3a (a typical experiment is shown of several dozen performed, all yielding essentially identical results), MBD2 peptide triggers apoptosis as effectively as intact IGFBP-3 in these assays when used at the same concentrations. Even conservative amino acid substitutions in the MBD core sequence (such as in the homologous region of the closely related protein, IGFBP-5) completely abolish this pro-apoptotic effect (Fig. 3b). There is a sharp peptide length optimum for this effect (Fig. 3c) with the 14-mer, MBD2, consistently exhibiting the highest activity in this assay. Caspase-3 levels induced in cells treated with pro-apoptotic MBD peptides correlate exactly with the levels of Bax mRNA measured in the same cells (Fig. 3d). The pro-apoptotic effects of intact IGFBP-3 in this assay are inhibited by [Y60L]-IGF-I, an IGF-I mutant incapable of binding to cell membrane IGF receptors, but able to bind IGFBP-3 as avidly as wild type IGF-I (42). However, MBD-mediated apoptosis is not inhibited by [Y60L]-IGF-I (Fig. 3e). This suggests that the MBD of IGFBP-3 can mediate unequivocally IGF-independent effects on cultured cells.

MBD-mediated Cell Uptake and Nuclear Localization—Purified GFP proteins that had been engineered to contain short MBD sequences at their N termini were added directly to HEK293 cells in culture. GFP32 contains most of the MBD domain, including the sequences shown to be sufficient for metal binding and for mediating pro-apoptotic effects. As shown in Fig. 4A, the added GFP32 protein rapidly disappears from the culture medium and simultaneously appears in the nuclear compartment of HEK293 cells. Uptake is essentially complete within 20 min. When various MBD-GFP fusions were tested, the core 14-mer sequence present in MBD35 was found to be sufficient for cellular uptake, but the so-called “basic domain” peptide used by numerous investigators in past studies (53) was not able to mobilize GFP at all (compare GFP35 and GFP36). Thus, the integrity of the 12-mer cysteine loop appears to be important for this activity.

MBD-mediated cell uptake was further investigated using biotinylated peptides MBD9 and MBD21. Each peptide was pre-incubated with streptavidin-horseradish peroxidase (SA-HRP) conjugate and then added to cultured cells. MBD21 con-
tains a putative caveolin-binding sequence (FCWAVDKY), partially overlapping the conserved MBD motif. The presence of this sequence appears to enhance nuclear uptake of SA-HRP (compare MBD9 and MBD21 in Fig. 4C). Unlike the case of GFP uptake, there is substantial localization of SA-HRP in the cytoplasmic as well as the nuclear compartments. Perhaps molecular mass (SA-HRP complexes are nearly 5 times as large as GFP) affects the rate of intracellular compartmentalization. However, the overall rates of nuclear uptake are very similar for GFP32 and SA-HRP-MBD9 (about 0.2–0.4% of the initially added reagent can be found in the nuclei of HEK293 cells within 10 min of being added to the culture medium; data not shown).

Inhibition of MBD-mediated Effects—Pre-incubation of HEK293 cells with nystatin (an inhibitor of caveolae), LY294002 (inhibitor of phosphoinositol 3-kinase), antibodies to transferrin receptor, integrin \( \beta_1 \), and integrin \( \alpha_6 \), significantly inhibited MBD-mediated cell/nuclear uptake of both GFP32 and SA-HRP, as well as pro-apoptotic effects of MBD2 (Fig. 5). On the other hand, chlorpromazine (an inhibitor of clathrin-mediated endocytosis) had no effect on GFP uptake and only modest effects on SA-HRP uptake and apoptosis.

Cross-linking MBD Peptide to Cell Surface Markers—Chilled HEK293 cells were mixed with the biotinylated peptides MBD21 or MBD9 and incubated for various intervals at 4 °C. Chemical cross-linking was then performed for 20 min at the same temperature. MBD-linked proteins were detected by ELISA (Fig. 6). The results indicate a progressive, time-dependent association of MBD21 peptide with integrins, caveolin, and transferrin receptor. Except for integrin \( \alpha_6 \), physical interactions of MBD21 with cell surface markers were reduced when cells had been pre-treated with nystatin, and the levels of cross-linking of MBD9 peptide to various cell surface markers in this experiment were negligible except at early times (see Fig. 6, B and D).

Iron Requirements for Biological Effects—Nuclear uptake of MBD21-SA-HRP complex by HEK293 cells is dependent upon the concentration of ferrous iron in the growth medium (Fig. 7A), with an optimum observed at around 10 \( \mu \)M iron. However, iron is not required for the apoptotic effects of either IGFBP-3 or MBD2 (Fig. 7B). There appears to be a dose-dependent effect of iron upon the unmasking of MBD epitopes in the intact IGFBP-3 molecule in vitro (Fig. 7C).

DISCUSSION

In this work we used synthetic peptides modeled on sequences surrounding a cysteine loop in the C-terminal domain of IGFBP-3. Sequences in this region have been implicated previously (1) in binding to plasma and extracellular matrix components, transportin, and retinoid X receptor-\( \alpha \), a nuclear transcription factor. We were intrigued by the possibility that an IGFBP-3 domain exhibiting homology to a zinc finger-like motif conserved in human CD74 and other vertebrate proteins...
of unrelated function (IGFBP-3 residues 215–241) might be able to bind metals. We found that peptides containing a 12-amino acid cysteine loop within this CD-74-like domain were able to bind metal ligands in NTA-immobilized metal affinity chromatography. We refer to this domain of IGFBP-3 as the metal-binding domain (or MBD). Full-length IGFBP-3 was also observed to bind nickel, iron, zinc, and other metals, but the presence of IGF ligand inhibited binding to nickel- or ferrous iron-charged resin.

Surprisingly, anti-MBD sera did not recognize intact IGFBP-3 and anti-IGFBP-3 sera did not recognize MBD peptides. However, incubation of intact IGFBP-3 in 100 μM ferrous (but not ferric or zinc) salt solutions unmasked MBD epitopes in a time-dependent manner. One explanation for these observations is that the MBD domain is sequestered in the intact IGFBP-3 molecule, but a conformational change induced by ferrous ions unmaskd MBD epitopes. The simultaneous loss of ELISA reactivity to anti-IGFBP-3 sera during this process may reflect enhanced proteolytic degradation in the new conformation. Proteolytic degradation of IGFBP-3 is influenced by the presence of high salt, heparin, and other agents, which suggests a conformation-dependent susceptibility to proteolysis (9). The exquisite specificity of action exhibited by ferrous (versus ferric) ions on IGFBP-3 suggests a possible physiological role for IGFBP-3 in oxidative metabolism.

The potential relevance of metals, and specifically iron, in the biological actions of IGFBP-3 has not been noted previously. However, specific binding of IGFBP-3 to the iron-binding proteins transferrin and lactoferrin in serum has been shown (7, 8), and IGF is known to modulate the availability of transferrin receptors on cell surfaces (44). Binding of IGFBP-3 to iron-binding proteins, unlike its binding to numerous other known ligands, may be affected by IGF ligand occupancy of IGFBP-3, raising the formal possibility that iron occupancy and IGF ligand occupancy of IGFBP-3 could be mutually exclusive. In our experiments, IGF-I-IGFBP-3 complex bound poorly to a Ni-NTA column, whereas IGFBP-3 alone bound efficiently.

It is interesting to speculate that iron may directly mediate the formation of heterodimers between IGFBP-3 and various iron-binding proteins, and that this iron-protein-bound state is characteristic of so-called “IGF-independent” pathways of IGFBP-3 action. In this view, cellular uptake of IGFBP-3 might be dependent on iron-transport mechanisms. We have demonstrated in the present study that cellular actions of MBD peptides are entirely dependent upon the availability of cell surface transferrin receptors, an observation consistent with this view. However, our data do not suggest a requirement for iron in the IGF-independent biological actions of IGFBP-3, only a potential role in unmasking MBD epitopes. Iron is not required for the apoptotic effects of either IGFBP-3 or MBD2.

A number of peptides containing the core MBD region were tested for activity in HEK293 cell stress apoptotic assays. A 14-mer MBD peptide exhibited potent coapoptotic effects, whereas homologous peptides modeled on domains from closely related proteins such as IGFBP-5 did not. Even relatively conserved sequence substitutions in this 14-mer region completely abolished the apoptotic activity of these peptides. We show that the pro-apoptotic actions of MBD peptides are unequivocally IGF-independent, as they are unaffected by the presence of equimolar amounts of [Y60L]-IGF-I, a mutant IGF-I molecule that retains its ability to bind IGFBP-3 but not its cognate receptor IGF-1-R (42).

These data illustrate a formal distinction from prior descriptions of IGF-independent cellular actions of wild type IGFBP-3. The term IGF-independent has often been used to describe “IGF-receptor-independent” effects in cell systems where physical sequestration of IGFBP-3 by IGFs (formally, an “IGF-dependent” effect) would still be capable of influencing IGFBP-3 action. This significant point is addressed in our experiments through the use of the [Y60L]-IGF mutant, which inhibits the pro-apoptotic effects of IGFBP-3 but not of MBD2 peptide.

MBD peptides mobilized cell uptake, internalization, and nuclear translocation of GFP or SA-HRP. Rapid uptake into the nuclear fraction of HEK293 cells was observed in both cases, and this mobilization was MBD sequence-specific. In particular, the core 14-mer sequence straddling the cysteine loop was sufficient for mobilization; efficient mobilization of SA-HRP into cell nuclei additionally required the integrity of a caveolin-binding homology region (“caveolin box”) that partially overlaps the core MBD sequence. By using cross-linking studies, we have demonstrated a time-dependent physical association of
MBD sequences with transferrin receptor, caveolin, and with β and α integrins at the cell surface. A phosphatidylinositol 3-kinase inhibitor, LY294002, abolished uptake, as did pre-incubation of cells with antibodies to integrin β1, integrin αv, or transferrin receptor. However, whereas nuclear uptake was efficiently inhibited by nystatin, it was hardly affected by chlorpromazine, a classical inhibitor of clathrin-mediated endocytosis. The pro-apoptotic properties of the MBD peptides were also inhibited by nystatin and by antibodies to transferrin receptor, integrin β1, or integrin αv, but not by chlorpromazine.

The canonical “caveolin box” in the MBD region is defined by three appropriately spaced aromatic residues in the IGFBP-3 sequence. MBD21 mobilizes SA-HRP into the nucleus of HEK293 cells more efficiently than does MBD9, a similar peptide lacking one of the three aromatic residues of the caveolin box. However, GFP32 (which contains an N-terminal extension

FIG. 6. Cross-linking of MBD to cell surface proteins. Biotinylated MBD21 peptide was added to chilled 293 cells, and cross-linking with bis[sulfo]sulfoalkyl] succinimidy] carbonate was carried out for 20 min beginning at the indicated times post-addition. All the above manipulations were carried out at 4 °C. Cell extracts were prepared after cross-linking was terminated with Tris-HCl. Extracts were captured on streptavidin-coated 96-well plates and assayed for the indicated proteins by ELISA. The y axis of each panel shows ELISA units (arbitrary). a and b = p < 0.01 versus MBD21 + nystatin and MBD9 groups, respectively. A, β1 integrin; B, αv integrin; C, α6 integrin; D, caveolin 1; E, transferrin receptor.
FIG. 7. Effect of iron in growth media on MBD-mediated transport. A, HEK293 cells were grown in media containing the indicated concentrations of ferrous chloride, and nuclear uptake of MBD21-SA-HRP complex was measured. Fe^{2+} does not affect the complex formation between MBD21 and SA-HRP. B, iron independence of apoptotic effects. HEK293 cells were grown in iron-free and serum-free RPMI media overnight and pre-incubated with 0, 10, or 100 μM ferrous chloride for 30 min prior to addition of 25 ng/ml IGFBP-3 or 25 ng/ml MBD2 peptide. Caspase-3 was assayed in cell extracts as described under “Materials and Methods.” C, calibration of epitope unmasking effects. A 1 mg/ml solution of purified IGFBP-3 was incubated at room temperature for 60 min in the indicated buffers. The yield of MBD5 molar equivalents was measured by ELISA and expressed as a percentage of the starting material. A typical experiment is shown. Similar results were obtained when IGFBP-3 was incubated at concentrations ranging from 0.25 to 2 mg/ml. D, speculative model for bifurcation of IGFBP-3 effects in vivo. IGF ligand occupancy is the dominant event in the life cycle of the IGFBP-3 protein. When IGF ligand is present, IGFBP-3 exerts its well documented IGF-dependent effects. When IGF ligand is not present, a (irreversible?) metal-induced conformational change in IGFBP-3, possibly accompanied by proteolysis and/or interactions with iron-binding proteins such as transferrin, define an alternative IGF-independent pathway of action. In this proposed model, iron (and possibly other metals), although required to induce a conformational change, may or may not be required for subsequent biological actions of (modified) IGFBP-3. At the cell surface, multimolecular complexes containing IGFBP-3 (or its fragments), transferrin, α and β integrins, caveolin, and transferrin receptor may serve as spatial integration foci for diverse metabolic signals derived from IGF signal transduction, cytoskeletal-extracellular matrix interactions, as well as from iron and lipid trafficking. IGFBP-3 interacts with these complexes via its MBD and thus serves as a key participant in this type of signal integration. These complexes may further be associated with recycling endosomes that can transport IGFBP-3 (or its fragments) to a perinuclear location, from where the MBD can localize to the nucleus.
similar in sequence to MBD9) is readily taken up into cell nuclei. The relative efficiency of uptake of GFP32 compared with MBD9-SA-HRP is comparable (in both cases about 0.2–0.4% of the added protein enters the nucleus within 10 min of being added to the culture medium; data not shown), but it is difficult to make direct comparisons because the molecular mass of GFP is about 5-fold smaller than that of SA-HRP complex, and the methodology employed in the two assay systems differs in several other respects.

It is interesting to note that although MBD36 protein was not able to mobilize into cell nuclei in our assay, a similar fusion protein has been reported to be fully functional in a nuclear localization assay (53). In that work, the investigators transfected cells with a vector specifying an enhanced GFP fusion protein, which ensured that the protein was generated intracellularly. In our case, the purified proteins were added to intact cells. Our sequence requirements therefore encompass both a cellular uptake step and a nuclear localization step, only one of which is a requirement in the experimental system of the previous report (53).

Taken together, our data suggest the spatial integration of at least three target cell surface elements in initial interactions with the MBD (and, by extension, with IGFBP-3): cytoskeletal organization (integrins), iron transport (transferrin receptor), and membrane lipids (caveolin).

A potential involvement of integrins has been documented previously for IGF-I signaling (6), but not for the IGF-independent actions of IGFBP-3. It has been postulated that the regulation of integrin vesicular traffic facilitates cell migration by internalizing integrins at the rear of the cell and transporting them forward within vesicles for exocytosis at the leading edge to form new contacts with the extracellular matrix. The RAB family of GTPases control key targeting events in the endo/exocytic pathway; therefore, these GTPases may be involved in the regulation of cell-matrix contact assembly. In mouse 3T3 fibroblast cell lines, internalized αβ2 and αβ1 integrins were transported through RAB4-positive early endosomes and arrived at the RAB11-positive, perinuclear recycling compartment ~30 min after endocytosis. From the recycling compartment, integrins were recycled to the plasma membrane in a RAB11-dependent fashion (45). RAB5 and RAB11 are strictly segregated GTPases localized to sorting and recycling endosomes, respectively (46).

Although the transferrin receptor is considered to be a canonical clathrin-coated pit marker, RAB11-positive recycling endosomes have been shown to contain transferrin as well as caveolin. In epithelial Madin-Darby canine kidney cells, recycling endosomes are enriched in molecules known to regulate transferrin recycling but lack proteins involved in early endosome membrane dynamics, indicating that recycling endosomes are distinct from conventional early endosomes. In these cells, the apical endocytic pathway intersects the transferrin pathway. Strikingly, recycling endosomes are enriched in the raft lipids sphingomyelin and cholesterol as well as in the raft-associated proteins caveolin-1 and flotillin-1 (47). The direct involvement of α and β integrins, and a requirement for phosphatidylinositol 3-kinase, is also indicated in this work. In this regard it is interesting to note that localization of integrins to lipid rafts can modulate signal transduction in some cell types (48, 49) and that caveolae profoundly influence cytoskeletal organization (50).

Glycosphingolipids are internalized from the plasma membrane of human skin fibroblasts by clathrin-independent caveolar endocytosis and are rapidly delivered to early endosomes by a RAB5a-dependent mechanism, where they colocalize with transferrin. Later they are fractionated into two major pools, one that is transported via late endosomes to the Golgi apparatus in a process dependent on microtubules, phosphatidylinositol 3-kinase, Rab7, and Rab9 and the other that is returned to the plasma membrane via the recycling compartment (51).

It is clear from our data that the optimal sequence requirements for metal-binding apoptotic effects and cell internalization, although overlapping, are not identical. It is not clear to us whether cell uptake and nuclear localization of MBD peptide is an obligate step in generating an apoptotic response. However, our data do suggest at least a partial overlap in the cell surface markers involved in these processes.

A key unanswered question is whether the IGF-independent effects of IGFBP-3 are enhanced for certain cell types (or for altered physiological states) relative to IGF-dependent effects. In particular, it might be important to establish whether such discrimination, if it exists, could lead to the development of therapeutic molecules that can target cell environments characteristic of disease processes such as cancer or atherosclerosis. αβ2 integrin is a major integrin displayed by HEK293 cells in culture and serves as a coreceptor for adenovirus (43). αβ1 integrins, in general, are preferentially displayed by invasive tumors, as well as in inflammatory disease, retinopathy, and other disease states (12, 13); a cationic nanoparticle coupled to an integrin αβ2-targeting ligand can deliver genes selectively to angiogenic blood vessels in tumor-bearing mice (14). Markedly increased iron uptake via the transferrin pathway has been observed in tumors and atherosclerotic lesions (16, 17).

A role for metal ions (especially iron) in an early step required for the IGF-independent cellular actions of IGFBP-3 is suggested by our data. More experiments will be needed to further elucidate this novel aspect of IGFBP-3 metabolism, and whether metal occupancy is indeed a defining characteristic of bifurcation into an IGF-independent pathway.

REFERENCES

Insulin-like Growth Factor-independent Effects Mediated by a C-terminal Metal-binding Domain of Insulin-like Growth Factor Binding Protein-3
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