Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor

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Andress, Dennis L. Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E744–E750, 1998.—The finding that insulin-like growth factor (IGF)-binding protein-5 (IGFBP-5) binding to mouse osteoblasts was capable of being downregulated by IGFBP-5 suggested that the 420-kDa membrane protein, which interacted with IGFBP-5, may be a signaling receptor (Andress, D. L. J. Biol. Chem. 270: 28289–28296, 1995). In the current study, a carboxy-terminal IGFBP-5 peptide, IGFBP-5-(201–218), which was found to competitively inhibit [32P]IGFBP-5 binding and to specifically bind to osteoblast monolayers, was used to affinity-purify the 420-kDa membrane protein. Co-incubation of the affinity-purified membrane protein with [32P]ATP resulted in autophosphorylation at serine residues. Serine phosphorylation of the 420-kDa protein was enhanced by intact IGFBP-5, IGFBP-5-(1—169), and IGFBP-5-(201–218). When the IGFBP-5 receptor was incubated with dephosphorylated casein in the presence of [32P]ATP, casein became phosphorylated on serine residues. These data indicate that IGFBP-5 stimulates the phosphorylation of the IGFBP-5 receptor and suggest that serine/threonine kinase activation may be important in mediating some of the IGF-independent effects of IGFBP-5.

oestrogen

EXPERIMENTAL PROCEDURES

Materials. Recombinant forms of IGFBP-5, intact IGFBP-5 and IGFBP-5-(1—169), were expressed in baculovirus and purified as described previously (4). IGFBP-5 peptides IGFBP-5-(81—95), IGFBP-5-(94—106), IGFBP-5-(109—121), IGFBP-5-(123—136), IGFBP-5-(138—152), IGFBP-5-(167—185), IGFBP-5-(183—200), IGFBP-5-(201—218), and IGFBP-5-(235—252) were synthesized and purified by reversed-phase HPLC (Fred Hutchinson Cancer Center, Seattle, WA). Na125I was purchased from Amersham, and [32P]ATP was obtained from ICN Radiochemicals. Ig125I-IGFBP-5, Ig125I-IGFBP-5-(1—169), and Ig125I-IGFBP-5-(201—218) were prepared using chloramine T as described (3); specific activities ranged from 100 to 150 µCi/µg. Transforming growth factor-β1 (TGF-β1) was from R and D Systems (Minneapolis, MN), and IGFBP-6 was from Chiron (Emeryville, CA). Collagenase was purchased from Worthington. L-Lysine, casein, and ninhydrin were purchased from Sigma Chemical, and Affigel was obtained from Bio-Rad.

Cell culture. Neonatal mouse osteoblasts were released from calvaria with collagenase, as previously described (3), and grown for 1 wk in DMEM and 10% FCS. Confluent cells were then released by trypsin and plated onto 48-well plates (Costar) containing 10% FCS. IGFBP-5 binding to osteoblasts. Confluent monolayers of osteoblasts in 48-well plates were incubated in serum-free medium for 24 h. The cells were washed with PBS and then incubated in assay buffer (20 mM HEPES, 0.1 mg/ml BSA, pH 7.0) for 2 h at 4°C with intact Ig125I-IGFBP-5, Ig125I-IGFBP-5-(1—169), or Ig125I-IGFBP-5-(201—218) in the absence or presence of varying concentrations of unlabeled intact IGFBP-5, IGFBP-5-(1—169), and IGFBP-5-(201—218). At the end of the incubation period, the buffer was removed, and the cells were rinsed with PBS and solubilized with 1 N NaOH. Radioactivity of the cell lysates was quantified and specific binding was determined.

Preparation of extracellular matrix. Confluent osteoblasts were removed with trypsin-EDTA, plated onto 48-well plates, and grown to confluence over 24 h. The cells were washed with PBS and then incubated in assay buffer (20 mM HEPES, 0.1 mg/ml BSA, pH 7.0) for 2 h at 4°C with intact Ig125I-IGFBP-5, Ig125I-IGFBP-5-(1—169), or Ig125I-IGFBP-5-(201—218) in the absence or presence of varying concentrations of unlabeled intact IGFBP-5, IGFBP-5-(1—169), and IGFBP-5-(201—218). At the end of the incubation period, the buffer was removed, and the cells were rinsed with PBS and solubilized with 1 N NaOH. Radioactivity of the cell lysates was quantified and specific binding was determined.

Preparation of extracellular matrix. Confluent osteoblasts were removed with trypsin-EDTA, plated onto 48-well plates, and grown to confluence over 24 h. The cells were then rinsed with ice-cold PBS, and the cell membranes were extracted in 1% Triton X-100-PBS for 10 min on ice, followed by removal of nuclei and cytoplasm with 25 mM ammonium acetate, pH 9.0, for 10 min (2). The remaining extracellular matrix (ECM) was rinsed with PBS and immediately used for binding studies.
IGFBP-5-(201—218) affinity purification of the 420-kDa membrane protein. Membranes from mouse osteoblasts were prepared as previously described (2). Briefly, confluent primary cultures of osteoblasts were detached with 1 mM EDTA and pelleted; the cell pellet was resuspended in 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM iodoacetic acid. After sonication, the cell lysates were centrifuged at 12,000 g for 30 min, and the supernatant was centrifuged at 40,000 g for 1 h. The pellet was then suspended in 50 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM PMSF, and 2 mM magnesium sulfate overnight at 4°C and were recovered in 50 mM HEPES, pH 7.4, 1% Triton X-100, 0.15 M NaCl, 1 mM PMSF, and 2 mM magnesium sulfate and was centrifuged at 40,000 g for 30 min, and the supernatant after centrifugation at 12,000 g. The membrane preparation was applied to an IGFBP-5-(201—218) affinity column (10 mg of IGFBP-5-(201—218) bound to Affigel-10) overnight and equilibrated in 50 mM HEPES, pH 7.4, 1% Triton X-100. The column was washed with 50 ml of equilibration buffer, followed by 50 ml of 50 mM HEPES, pH 7.4, 0.05% Triton, 0.25 M NaCl, and 1 mM PMSF. Bound protein was eluted with 8 ml of 50 mM HEPES, 0.05% Triton X-100, 1.5 M NaCl, and 1 mM PMSF and concentrated with a Centricon-30 filtration device. The eluted protein was separated on a 5% SDS-polyacrylamide gel under reducing conditions and stained with silver. A similar membrane affinity purification was also attempted using an IGFBP-5-(183—200) affinity column.

Fig. 1. Competitive binding of intact 125I-IGFBP-5 and 125I-IGFBP-5-(1—169) to neonatal mouse osteoblasts. Confluent monolayers maintained in serum-free medium were exposed for 2 h at 4°C to intact 125I-IGFBP-5 without and with synthetic peptides that correspond to specific regions of IGFBP-5. Cells were then washed and solubilized, and cell-associated radioactivity was quantitated. Data are means ± SE of 6 wells from a representative experiment.

Table 1. Competitive binding of IGFBP-5-related peptides with intact 125I-IGFBP-5 in mouse osteoblasts

<table>
<thead>
<tr>
<th>Peptides, 30 µg/ml</th>
<th>Intact 125I-IGFBP-5 Bound, % of maximum</th>
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<tbody>
<tr>
<td>IGFBP-5-(81—95)</td>
<td>95 ± 0.3</td>
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<tr>
<td>IGFBP-5-(94—106)</td>
<td>89 ± 0.2</td>
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<tr>
<td>IGFBP-5-(109—121)</td>
<td>89 ± 0.5</td>
</tr>
<tr>
<td>IGFBP-5-(123—136)</td>
<td>95 ± 0.1</td>
</tr>
<tr>
<td>IGFBP-5-(138—152)</td>
<td>79 ± 0.3</td>
</tr>
<tr>
<td>IGFBP-5-(167—185)</td>
<td>97 ± 0.2</td>
</tr>
<tr>
<td>IGFBP-5-(183—200)</td>
<td>100 ± 0.1</td>
</tr>
<tr>
<td>IGFBP-5-(201—218)</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>IGFBP-5-(235—252)</td>
<td>98 ± 0.4</td>
</tr>
</tbody>
</table>

Confluent neonatal mouse osteoblast monolayers maintained in serum-free medium were exposed for 4°C to intact 125I-IGFBP-5 without and with synthetic peptides that correspond to specific regions of IGFBP-5. Cells were then washed and solubilized, and cell-associated radioactivity was quantitated. Data are means ± SE of 6 wells from a representative experiment.
isobutyric acid-0.5 M ammonium dihydroxide (5:3). The cellulose acetate was then dried overnight, and the second dimension was run with isopropanol-hydrochloric acid-water (7:1.5:1.5) as the solvent. The cellulose sheets were air dried, sprayed with ninhydrin to identify the phosphoamino acid standards, and then dried at 110°C for 30 min before identification of the 32P-labeled amino acids with the phosphoimager.

**RESULTS**

Competition binding studies using different IGFBP-5 peptides were performed to identify potential sites within IGFBP-5 that may be responsible for mediating its attachment to the osteoblast surface. In comparing unlabeled IGFBP-5-(1—169) and intact IGFBP-5 for their ability to compete for intact 125I-IGFBP-5 binding (Fig. 1A), we found that IGFBP-5-(1—169) was ineffective as a competitor. However, when 125I-IGFBP-5-(1—169) binding was examined, both unlabeled intact IGFBP-5 and IGFBP-5-(1—169) competed for binding, although IGFBP-5-(1—169) was a more effective competitor (Fig. 1B). It was unclear from these studies whether conformational differences due to truncation of IGFBP-5 can fully explain the differences in competitive binding. Thus, to further investigate whether amino acids within other portions of IGFBP-5 may be important in mediating its binding to osteoblasts, synthetic peptides from the variable region and from the carboxy terminus were used to compete for intact 125I-IGFBP-5 binding. As shown in Table 1, of the peptides tested, IGFBP-5-(201—218) was the major inhibitor of intact 125I-IGFBP-5 binding. Whereas most of these peptides are relatively neutral, the exceptions are IGFBP-5-(235—252), which is acidic, and IGFBP-5-(138—152) and IGFBP-5-(201—218), which are basic. Thus, because IGFBP-5-(138—152) did not inhibit intact 125I-IGFBP-5 binding substantially, the effect of IGFBP-5-(201—218) may not be solely attributable to its basic charge.

To more thoroughly evaluate the role of IGFBP-5-(201—218), competition binding studies were performed to evaluate the relative effectiveness of this peptide to function as a competitor for intact 125I-IGFBP-5 and 125I-IGFBP-5-(1—169) binding in mouse osteoblasts. Confluent monolayers maintained in serum-free medium were exposed to intact 125I-IGFBP-5-(201—218) without or with increasing concentrations of unlabeled IGFBP-5-(201—218). Inset, total specific binding to cell monolayers and to osteoblast-derived extracellular matrix (ECM). Cell-associated radioactivity was determined as described in Fig. 1.
blasts and osteoblast-derived ECM with $^{125}$I-IGFBP-5-(201–218) to determine its specificity for binding. As shown in Fig. 3 (inset), almost all of the $^{125}$I-IGFBP-5-(201–218) specifically bound to the monolayers compared with its binding to osteoblast-derived ECM. Competition binding studies with unlabeled IGFBP-5-(201–218) confirmed its specificity for cell binding.

Because these studies indicated that the 201–218 region within the carboxy terminus of IGFBP-5 is one of the sites that interacts with the osteoblast surface, we prepared an IGFBP-5-(201–218) affinity column to determine whether it would capture the 420-kDa osteoblast membrane protein previously recognized as a binding site for intact IGFBP-5 and for IGFBP-5-(1–169) (2). Osteoblast membrane preparations were applied to the affinity column, and after a wash with buffer containing 0.25 M NaCl, protein was eluted with 1.5 M NaCl, concentrated, and separated on a 4–15% SDS-PAGE. Shown in Fig. 4 is the silver stain of the 420-kDa protein that was eluted from the affinity column. Its size is identical to the osteoblast membrane protein we previously recovered using an intact IGFBP-5 affinity column (2). We did not detect the 420-kDa protein after applying similar amounts of membrane protein to the IGFBP-5-(183–200) affinity column (data not shown).

Our previous results with this membrane protein suggested that it not only functions to bind IGFBP-5 but may also act as a receptor for this ligand, because $^{125}$I-IGFBP-5 binding was rapidly downregulated by prior exposure to unlabeled IGFBP-5 and internalization of both intact $^{125}$I-IGFBP-5 and $^{125}$I-IGFBP-5-(1–169) could be demonstrated (2). To evaluate further the possibility that the 420-kDa membrane protein behaved as a receptor, we asked whether it was capable of becoming phosphorylated in response to IGFBP-5. Affinity-purified preparations of the 420-kDa membrane protein were incubated without and with intact IGFBP-5, IGFBP-5-(1–169), and IGFBP-5-(201–218). As shown in Fig. 5, intact IGFBP-5 (lane 1) and IGFBP-5-(1–169) (lane 2) caused an increase in $^{32}$P-ATP labeling of the 420-kDa membrane protein. IGFBP-5-(201–218) also stimulated phosphorylation of the membrane protein (Fig. 5B) at concentrations of 2 µg/ml (lane 2) and 20 µg/ml (lane 3). The stimulatory effect was specific, because phosphorylation was not stimulated with 20 µg/ml IGFBP-5-(183–200) or with 10 nM IGF-I, IGFBP-6, or TGF-$\beta$ (data not shown).

Constitutive autophosphorylation was present in lane 3 (Fig. 5A) and in lane 1 (Fig. 5B).

To determine the type of phosphorylation induced by IGFBP-5, phosphoamino acid analysis of the IGFBP-5 receptor was performed after stimulation with IGFBP-5-(201–218). As shown in Fig. 6, two-dimensional thin-layer chromatography of tryptic digests of the
32P-labeled IGFBP-5 receptor revealed that the 32P label comigrated with serine residues in the control (unstimulated) receptor preparation and that serine phosphorylation was enhanced on exposure to 20 µg/ml IGFBP-5-(201—218). Because some serine/threonine kinases can be stimulated by lysine (20), we used lysine to determine whether it could also stimulate phosphorylation and found that it stimulated the 32P labeling of serine and threonine residues of the IGFBP-5 receptor.

To demonstrate that the IGFBP-5 receptor could function as a protein kinase, casein was tested as a possible substrate for phosphorylation. Dephosphorylated casein became phosphorylated only in the presence of the IGFBP-5 receptor (Fig. 7A), and tryptic digestion of 32P-labeled casein revealed that serine residues were predominantly phosphorylated (Fig. 7B).

**DISCUSSION**

Our previous results demonstrating the binding and internalization of intact IGFBP-5 and IGFBP-5-(1—169) in neonatal mouse osteoblasts suggested that these ligands were interacting with a putative receptor (2). The 420-kDa osteoblast membrane protein, which was partially purified using an intact IGFBP-5 affinity column in those studies, is now also shown to be retrievable from an IGFBP-5-(201—218) affinity column. Because intact IGFBP-5, IGFBP-5-(1—169), and IGFBP-5-(201—218) all stimulate the phosphorylation of this membrane protein, and it in turn is capable of phosphorylating casein, these data indicate that intact as well as truncated versions of IGFBP-5 bind to a cell surface receptor that then stimulates serine/threonine kinase activity. This is the first indication that an IGFBP can directly stimulate protein phosphorylation and adds to the data showing that selected members of this family of proteins have effects on cells that are not mediated by the IGFs.

The finding that different regions of IGFBP-5 are capable of stimulating IGFBP-5 receptor activity suggests that there may be more than one site on IGFBP-5 that interacts with the receptor. Because not all of the regions within IGFBP-5 were tested for binding activity, we can only speculate that amino acids within the 1–81 and 153–169 regions may mediate the binding of IGFBP-5-(1—169). Similarly, we cannot exclude the possibility that amino acids within positions 170–200 and 219–234 may also be involved with binding the carboxy terminus of IGFBP-5 to its receptor. The
finding that IGFBP-5-(201—218) was a weak competitor of IGFBP-5-(1—169) binding suggests that basic residues within IGFBP-5-(1—169) may be important binding sites. However, it is possible that the differences in the competition binding studies may be due to the use of relatively small peptides, which would lack the potentially important secondary or tertiary structure needed for optimal binding. Despite these limitations, more than one region of IGFBP-5 is likely to be important for binding and possible stimulation of receptor activity. Thus it will be important to identify all potential receptor binding sites of IGFBP-5, because cell-specific proteolysis (19) likely plays a major role in the regulation of IGFBP-5 function and receptor activation.

The identity of the IGFBP-5 receptor is unknown. A review of the literature has revealed the existence of only one other large molecular weight membrane protein that contains serine/threonine kinase activity, the 400-kDa type V TGF-β receptor (14). This receptor has structural similarities to the type II TGF-β receptor (11), the activin receptor (12), and daf-1 gene products (7). Although the IGFBP-5 receptor and the type V TGF-β receptor are functionally similar in being able to phosphorylate casein on serine residues, their ligand binding characteristics appear to be different. Because TGF-β was not able to stimulate IGFBP-5 receptor phosphorylation (data not shown). It is still possible that structural similarities exist between the IGFBP-5 receptor and this class of membrane receptors within their membrane-spanning or cytoplasmic domains. It is also possible that the IGFBP-5 receptor may be structurally related to the IGFBP-3-specific membrane-associated proteins, which were recently shown to mediate IGF-independent growth inhibition of epithelial-like carcinoma cells (16). However, those membrane receptor proteins (20–50 kDa) are smaller than the IGFBP-5 receptor, and they have not yet been shown to internalize on ligand binding or to possess phosphorylation capability, which would classify them as signaling receptors.

The functional role of enhanced serine/threonine kinase activity in response to IGFBP-5 remains speculative at this time. In osteoblast-like cells, IGFBP-5-(1—169) (4) and intact IGFBP-5 (13) have both been shown to stimulate [3H]thymidine incorporation into DNA by IGF-independent mechanisms. Moreover, IGFBP-5 stimulates GH receptor synthesis in cultured rat osteoblasts (18), and IGFBP-5-(201—218) induces mesangial cell cytoskeletal reorganization and migration independent of IGF-1 stimulation (1). These seemingly diverse physiological functions most likely relate to cell-specific differences in IGFBP-5-induced stimulation and presumptive signaling. However, because of the lack of a unifying pattern of cell responsiveness (proliferative vs. differentiated function), it is unclear whether all of these IGFBP-5-inducible events are secondary to IGFBP-5 receptor signaling or are mediated by nonsignaling pathways.

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