Structure–Function Relationships in the Heparin-binding C-terminal region of Insulin-like Growth Factor Binding Protein-3


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SUMMARY. IGFBP-3 contains a carboxyterminal basic region which, when present as an isolated 18 amino acid peptide (P3), binds heparin, associates with cultured endothelial cells and stimulates glucose uptake. The P3 molecule has now been modified relative to charge, amino acid sequence and size to determine structure–function relationships relative to four properties of P3: affinity for heparin; inhibition of IGFBP-3 binding; stimulation of glucose uptake; and displacement of bFGF from the extracellular matrix of endothelial cells. Results indicate: (1) the presence or absence of heparin binding was concordant with the presence/absence of the other three properties; (2) the number of basic amino acids was an important, if not limiting, factor for each property; (3) the order of potency of the basic amino acids was arginine ≈ lysine > histidine; (4) the unrelated, basic protein, protamine, mimics all properties of P3; and (5) the putative consensus heparin-binding sequence of P3 was not essential for any of the P3 activities.

KEY WORDS: IGFBP-3, endothelial cells, heparin, bFGF, basic amino acids.

INTRODUCTION

Six IGF-binding proteins (IGFBPs) have been cloned and characterized. While their role was initially relegated to that of carrier proteins for the IGFs, it has become increasingly apparent that the IGFBPs are intimately involved in IGF function. The effects of IGFI and IGF-II, as well as their tissue distribution, are influenced by the IGFBPs present in the circulation and at the tissue level. In addition to serving as circulating carrier proteins, IGFBPs can act as inhibitors of IGF function and, in some cases, as enhancers of IGF actions. There is also evidence of direct effects of several of the IGFBPs, independent of inhibition or potentiation of IGF action. Because the vascular endothelium is bathed by IGFBPs, is a site of IGFBP production in vitro and in vivo, and likely plays a role in the movement of IGF from the circulation into underlying tissues, the interactions of the IGFBPs and their binding proteins with the vascular endothelium are of particular interest.

We have previously demonstrated that cultured vascular endothelial cells produce mRNA for five of the six IGFBPs, and secrete substantial quantities of IGFBP-2, -3, -4. Furthermore, IGFBP-3 and IGFBP-5 bind to endothelial cell monolayers and have specific affinity for the glycosaminoglycan, heparin. Other laboratories have demonstrated that heparin can alter properties of selected IGFBPs. Heparin interfered with the formation of the IGFI/IGFBP-3 complex, inhibited the formation of the IGFI/IGFBP-5 complex, and increased the rate of dissociation of IGF from the complex. A recent report, using mutants of IGFBP-5, indicated that basic amino acids (aa 201–218 region) contributed to heparin-binding affinity. The characterization of the heparin-binding regions of proteins, such as acidic and basic fibroblast growth factor, has indicated that heparin-binding sequences of proteins are highly basic regions enriched in arginine and lysine. Many, but not all, heparin-binding proteins contain a 'heparin-binding consensus' or Cardin-Weintraub sequence, XBBXXB or XBBBXXBX, where B is a basic amino acid. Of the six IGFBPs, IGFBP-3, IGFBP-5, and IGFBP-6 contain a Cardin-Weintraub sequence in the C-terminal third of the molecule. We previously reported studies with a synthetic 18 amino acid basic peptide (P3), corresponding to this C-terminal region of IGFBP-3.
and containing the Cardin-Weintraub sequence, YKKKQCRP. This peptide, P3, as well as synthetic peptides corresponding to the homologous regions of IGFBP-5 (P5) and IGFBP-6 (P6), inhibited the binding of IGFBP-3 and IGFBP-5 to endothelial cell surfaces and to the extracellular matrix (ECM) secreted by the cells. When 125I-labeled, P3, P5, and P6 also directly bound to endothelial monolayers, with predominant association with the extracellular matrix rather than with the cell surface, as observed with intact IGFBP-3 or IGFBP-5. The binding of the synthetic peptide (P3, P5 or P6) to the endothelial monolayer was inhibited by the homologous peptide, and by the highly basic protein protamine, but not by the intact binding protein. Additionally, we found that these peptides, at micromolar levels, stimulated glucose uptake by microvessel-derived endothelial cells, another property not shared by the intact binding proteins.

In the present study, the P3 molecule has been modified by charge, amino acid sequence, and size to further characterize the structural requirements of P3 for binding to the endothelial cell monolayer, binding to heparin, bioactivity, displacement of basic FGF from the extracellular matrix and localization within the endothelial monolayer.

MATERIALS AND METHODS

Materials

Recombinant, non-glycosylated human IGFBP-3 was a generous gift from Celsirx Pharmaceuticals (Santa Clara, CA). 14C-2-deoxyglucose (DOG) was obtained from Amersham (Chicago, IL). 3H-heparin and 14C-histamine were obtained from Dupont NEN (Boston, MA), protamine (Grade III, Clupeine) and heparin from Sigma Chemical Co. (St. Louis, MO). Basic FGF was purchased from R & D Systems (Minneapolis, MN). anti-bFGF type I from UBI (Lake Placid, NY)

Methods

Endothelial cell culture

Endothelial cells from microvessels (bovine periacute fat) and large vessels (bovine pulmonary artery) were prepared and characterized as pure endothelial cell cultures by methods previously described.

Peptide synthesis and purification

Peptides were synthesized using the t-bag technique which compartmentalizes the p-MBHA (p-methylbenzhydrylamine) resin in polypropylene bags as previously described. Confirmation of peptide identity for all the peptides except P3M was performed by fast atom bombardment (FAB) mass spectrometry by the high resolution mass spectrometry facility of the Department of Chemistry at the University of Iowa. A ZAB HF VG analytical mass spectrometer was used to confirm the complete deprotection and purity of the peptides in a thiohydrazide matrix. Analysis of this mass spectral data also gave a partial sequence for each peptide, and the sequence was analyzed by the gas chromatography mass spectrometry facility of the College of Medicine at the University of Iowa. A Nermag R10–10C Mass Spectrometer with Analytica Electrospray Interface, model 102047, was infused with sample at 53 ng/μl, 1 μl/min. Analysis was carried out in both neutral (50:50, methanol:water) and acidic (47:47:6, methanol:water:acetic acid) conditions with an EL range of 100–300. The observed peaks confirm the complete deprotection and purity of the sample.

2-deoxyglucose uptake

Glucose uptake was measured, using confluent cultures of microvessel endothelial cells (0.4×10⁶ cells/5 cm² well), utilizing the non-metabolizable glucose analog 14C-2-deoxyglucose according to modifications of the procedure of Ishibashi et al. All peptides were tested at 4 μM based on the previously published dose–response curve for P3.

IGFBP and synthetic peptide binding to endothelial cell monolayer

Pulmonary artery endothelial cells were grown to 0.2–0.3×10⁶ cells/5 cm² well, and microvessel endothelial cells to 0.4×10⁶ cells/5 cm² well, in M199 containing fungizone and gentamycin and 20% (pulmonary artery cells) or 10% (microvessel cells) fetal bovine serum (FBS, HyClone, Logan, UT). Endothelial cells were washed twice with 0.5 ml serum-free M199 containing 0.25% BSA, pH 7.8. 125I-IGFBP-3 or 125I-peptides, along with 4 μM competing unlabeled peptides, were incubated with endothelial monolayers in 0.3 ml serum-free M199 for 2 h at 22°C. After incubation, the medium was removed, monolayers washed twice with 0.4 ml cold phosphate buffered saline (PBS) and the monolayer (cells and underlying matrix) was removed with 0.3 ml 0.1 N NaOH and 2 subsequent washes with water. The media (plus washes), and the monolayer (plus washes) were counted in a gamma counter (Beckman Instruments, Palo Alto, CA). The amount bound to the monolayer was expressed as a percent of the total 125I added to the well. Recovery of the 125I was greater than 90%.
**ECM/cell surface binding**

For partition of binding between cells and extracellular matrix, $^{125}$I-P3, $^{125}$I-P3$_{34}$, or $^{125}$I-IGFBP-3 was incubated with endothelial monolayers as described above. After incubation, the supernatant was removed and the monolayer washed twice with PBS to remove non-associated counts. The cells were removed by a 3 min treatment of the monolayer with 0.05% TritonX-100, 0.02 M NH$_4$OH in PBS$^{22,40}$ followed by one wash with PBS (cell-associated binding). The ECM was subsequently removed with 0.1 N NaOH and the wells washed twice (ECM binding). When previously prepared ECM was incubated with labeled peptide or protein and subjected to this treatment, the Triton mixture stripped 10% of the peptide from the ECM and 28% of the IGFBP-3. Specific binding was calculated by subtraction of binding in the presence of 4 $\mu$M unlabeled P3 from total binding.

**FGF studies**

Endothelial monolayers or ECM were incubated for 90 min in serum-free M199 at room temperature with $\sim$30000 c.p.m. $^{125}$I-bFGF, the supernatant was removed and the monolayer/ECM was washed twice with PBS to remove non-associated $^{125}$I-bFGF. Fresh serum-free M199 was added (0.3 ml) along with 4 $\mu$M P3 and other test substances. The control received media only. After an additional 30 min at room temperature, the supernatant, along with two PBS washes, was counted (dissociated $^{125}$I-bFGF) and the monolayer/ECM treated with NaOH to remove the remaining associated $^{125}$I-bFGF. Per cent dissociation was calculated for each sample and dissociation in the presence of test substances compared with dissociation in the controls.

Anti-bFGF (10 $\mu$g/ml) was added to the standard 14C-2DOG uptake assay at the beginning of the incubation period to inhibit the effect of 2 ng/ml bFGF and potentially of 4 $\mu$M P3.

**Analysis of heparin binding by peptides**

Solid-phase heparin binding studies with synthetic peptides were performed similarly to those studies described for bFGF-derived peptides$^{29}$ using nitrocellulose filter circles and 0.05 M Hepes, 0.15 M NaCl, 20 $\mu$L/Tween 80, pH 7.5 (HNTT) as buffer. Filters were incubated overnight at 4°C with 0.7 $\mu$L of $^{3}$H-heparin in 0.3 ml HNT buffer containing 4% fatty acid-free BSA; 300 $\mu$g/ml unlabeled heparin was included to measure non-specific binding. A filter blank (no peptide spotted) was included in each set of assays. After incubation, the filters were washed 4 times with 1 ml HNT buffer and counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA) in 5 ml BioSafe II (RPI, Mount Prospect, IL). Specific binding was calculated by subtraction of non-specific from total binding.

**Statistics**

Data were analyzed by analysis of variance utilizing multiple range testing.$^{41}$ Dunnett's procedure was used to compare the effect of test peptides with the effect of the control or the control (P3) peptide and Student- Newman-Keuls test was used for comparisons among specific related test peptides. A square root transformation was performed on the heparin binding data in order to equalize the variances.

**RESULTS**

**Homologous 18 amino acid regions of IGFBP-1, 3, 4, 5, 6**

Peptides P1, P3, P4, P5, and P6 are 18 amino acid peptides corresponding to homologous regions of IGFBP-1 (aa 183–200), IGFBP-3 (aa 215–232), IGFBP-4 (aa 185–202), IGFBP-5 (aa 201–218) and IGFBP-6 (aa 165–182), respectively (Table 1). Studies were performed to assess: (1) the ability of the peptide to compete for $^{125}$I-IGFBP-3 and $^{125}$I-P3 binding to pulmonary artery endothelial cell monolayers; (2) the binding of heparin to the peptides; (3) the effect of the peptides on 2-deoxyglucose uptake by microvessel endothelial cells; and (4) displacement of $^{125}$I-bFGF from the extracellular matrix (ECM) of the endothelial monolayer. P3, P5 and P6 all inhibited binding of $^{125}$I-IGFBP-3 and $^{125}$I-P3, bound heparin, stimulated glucose uptake and released bFGF from ECM. In contrast, P1 and P4 manifested none of these properties. For heparin binding, 0.4 nmoles of P3, P5, and P6, specifically bound approximately 30 000 c.p.m. (4% of the added $^{3}$H-heparin), while 0.8 nmoles bound 60 000 c.p.m. P1 and P4 did not bind $^{3}$H-heparin (<100 c.p.m.). Non-specific binding, measured in the presence of 300 $\mu$g/ml heparin, was negligible (<500 c.p.m.) for all peptides. The filter blank was approximately 200 c.p.m. P3 stimulated glucose uptake (154±5% of control, n=15) as did P5 (141±8%, n=5) and P6 (135±4%, n=4) while P1 and P4 had no bioactivity.

**Effect of amino acid sequence, peptide size and basic amino acids**

P3, P5 and P6 differ from P1 and P4 in two obvious ways: the presence of a Cardin-Weintraub, heparin-binding consensus sequence and an increased number of basic amino acids with P3, P5 and P6 having 10, 10 and 8 basic amino acids, versus 3 basic amino acids in P1 and 5 in P4. To determine the importance of the consensus heparin-binding sequence, the amino acids of P3 were rearranged. The peptide P3F (RGKFKYQKCRPKSRGKR) was synthesized and had the same amino acid composition as P3 but with the sequence reordered such that P3F had no
Table 1. Peptides from homologous regions of IGFBPs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Binding*</th>
<th>Percent of control</th>
<th>Release†</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>KKGFKYKKQQRPSKGRKR</td>
<td>13 ± 2** 14 ± 2**</td>
<td>109 ± 13 154 ± 36</td>
<td>249 ± 17**</td>
</tr>
<tr>
<td>P4</td>
<td>RNGNYHPRQCEHSMGDGEA</td>
<td>122 ± 6 99 ± 14</td>
<td>0 ± 0** 102 ± 0</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>P5</td>
<td>RKGKFKYKQCRPSKGRKR</td>
<td>20 ± 3** 11 ± 3**</td>
<td>104 ± 34 141 ± 80</td>
<td>200 ± 14**</td>
</tr>
<tr>
<td>P6</td>
<td>HRGYFKYKQRCCRSSQGRQR</td>
<td>14 ± 3** 7 ± 1**</td>
<td>125 ± 34 135 ± 4</td>
<td>210 ± 28**</td>
</tr>
</tbody>
</table>

*Binding (% of control±SEM) of 125I-IGFBP-3 (P3) to endothelial monolayers in the presence of 4 μM unlabeled peptide. 100% control is binding in the absence of peptide, for both 125I-IGFBP-3 and 125I-P3, the control was ~25% of added 125I counts bound. **P<0.01 relative to control.

†Heparin binding measured as % of control (P5)±SEM, *P<0.01 as compared with P3. Specific heparin binding by 4 moles P3 was 4% of added 125I-heparin, approximately 30000 c.p.m.

Table 2. Comparison of modified peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Binding*</th>
<th>Percent of control</th>
<th>Release†</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>KKGFKYKKQQRPSKGRKR</td>
<td>13 ± 2** 14 ± 2**</td>
<td>109 ± 13 154 ± 36</td>
<td>249 ± 17**</td>
</tr>
<tr>
<td>P3a</td>
<td>YKKGFKYKQQRPSKGRKR</td>
<td>91 ± 3 109 ± 21</td>
<td>25 ± 14 102 ± 5</td>
<td>102</td>
</tr>
<tr>
<td>P3f</td>
<td>KGFKYKKQCRPSKGRKR</td>
<td>16 ± 3** 8 ± 1**</td>
<td>107 ± 24 148 ± 7</td>
<td>245 ± 78**</td>
</tr>
<tr>
<td>P3a4</td>
<td>FLNVLSPRQVHPNCDYK</td>
<td>12 ± 0** 22 ± 5**</td>
<td>142 ± 27 142 ± 14*</td>
<td>205 ± 7**</td>
</tr>
<tr>
<td>P5</td>
<td>KAEAKKQDNKRHKLQFSKE</td>
<td>66 ± 14** 83 ± 35</td>
<td>0 ± 0** 169 ± 1</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>P6</td>
<td>HRYFKYKQRCCRSSQQRQR</td>
<td>14 ± 3** 7 ± 1**</td>
<td>125 ± 34 135 ± 4</td>
<td>210 ± 28**</td>
</tr>
</tbody>
</table>

*Binding (% of control±SEM) of 125I-IGFBP-3 (P3). 125I-P3 (P4) to endothelial monolayers in the presence of 4 μM unlabeled peptide. 100% control is binding in the absence of peptide, for both 125I-IGFBP-3 and 125I-P3, the control was ~25% of added 125I counts bound. **P<0.01 relative to control.

†Heparin binding measured as % of control (P3)±SEM, *P<0.01 as compared with P3. Specific heparin binding by 4 moles P3 was 4% of added 125I-heparin, approximately 30000 c.p.m.

Cardin–Weintraub sequence. When compared to P3, P3F was as potent in all four properties previously demonstrated for P3 (Table 2) suggesting that the Cardin–Weintraub sequence, per se, is not essential for any of the properties studied, including the affinity of P3 for heparin.

Peptide size appeared important at two levels. First, the 8 amino acid peptide containing just the Cardin–Weintraub sequence (Table 2, P3a) was without effect. Peptides corresponding to several regions of P3 but having fewer than 13 amino acids, also were ineffective, regardless of the number of basic amino acids that the peptide contained (data not shown). Second, the site of localization within the endothelial monolayer was influenced by the size of the proteins/peptides. Three proteins/peptides, IGFBP-3, P3 and P3a4, a 34 amino acid portion of IGFBP-3 which is secreted and which was characterized, were labeled with 125I, incubated with microvascu-larized endothelial cell monolayers and the distribution of each peptide between the endothelial cell surface and the extracellular matrix determined. The ratio of specific cell binding (total binding minus non-specific binding) to specific ECM binding was binding was 91:9 for IGFBP-3, 26:74 for P3a4 and 9:91 for P3a. The inhibition of 125I-IGFBP-3 and 125I-P3, bound 125I-heparin, stimulated glucose uptake and increased 125I-bFGF displacement (Table 2).

The importance of basic amino acids was initially suggested by the difference in the number of basic amino acids in the 'active' P3, P5 and P6 versus the 'inactive' P1 and P4. The relevance of basic amino acids was further investigated in several ways. First, P3 was modified by substitution, for one basic amino acid (K→A at position 7 [P3C], R→A at position 11 [P3D]) or for two basic amino acids (K and P3, were labeled, incubated with microvascu-larized endothelial cell monolayers and the distribution of each peptide between the endothelial cell surface and the extracellular matrix was determined. The ratio of specific cell binding (total binding minus non-specific binding) to specific ECM binding was binding was 91:9 for IGFBP-3, 26:74 for P3a4 and 9:91 for P3a. The inhibition of 125I-IGFBP-3 and 125I-P3, bound 125I-heparin, stimulated glucose uptake and increased 125I-bFGF displacement (Table 2).

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Table 3. Effects of amino acid substitutions

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Binding$^a$</th>
<th>Percent of control</th>
<th>Glucose$^b$ Uptake</th>
<th>Release$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>K K G F Y K K K Q C R P S K G R K R</td>
<td>13 ± 2**</td>
<td>100 ± 13</td>
<td>154 ± 7</td>
<td>249 ± 17**</td>
</tr>
<tr>
<td>PSC</td>
<td>K K G F Y K K K Q C R P S K G R K</td>
<td>22 ± 3**</td>
<td>17 ± 4</td>
<td>75 ± 15</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>P3D</td>
<td>K K G F Y K K K Q C A P S K G R K</td>
<td>21 ± 3**</td>
<td>9 ± 4</td>
<td>72 ± 21</td>
<td>146 ± 4</td>
</tr>
<tr>
<td>P3CD</td>
<td>K K G F Y K K K Q C A P S K G R K</td>
<td>45 ± 3**</td>
<td>26 ± 8</td>
<td>23 ± 4</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>P3K</td>
<td>K K G F Y K K K Q C K P S K G R K</td>
<td>16 ± 5**</td>
<td>28 ± 11</td>
<td>82 ± 23</td>
<td>146 ± 16</td>
</tr>
<tr>
<td>P3R</td>
<td>R R G F Y R R R Q C R P S R G R R</td>
<td>13 ± 4**</td>
<td>9 ± 6</td>
<td>90 ± 17</td>
<td>220 ± 14**</td>
</tr>
<tr>
<td>P3H</td>
<td>H H G F Y H H H Q C P S H G H H</td>
<td>79 ± 30</td>
<td>34 ± 7</td>
<td>0</td>
<td>112</td>
</tr>
<tr>
<td>Proamine (≈ 4 μM)</td>
<td>12</td>
<td>25</td>
<td>175 ± 35</td>
<td>130 ± 6</td>
<td>245</td>
</tr>
</tbody>
</table>

$^a$Binding (% of control ± SEM) of [125I]-IGFBP-3 (BP3), [125I]-P3 (*P3) to endothelial cell monolayers in the presence of 4 μM unlabeled peptide. 100% control is binding in the absence of peptide, for both [125I]-IGFBP-3 and [125I]-P3, the control was 25% of added [125I] counts bound. **P < 0.01 relative to control.

$^b$Heparin binding measured as % of control (P3 ± SEM). **P < 0.01 as compared to P3. Specific heparin binding by α-mes P3 was 45% of added [3H]-heparin, approximately 30,000 c.p.m.

$^c$[125I]-bFGF dissociated from endothelial monolayers after 30 min in the presence of 4 μM peptide, measured as % of control (untreated). Data shown as SEM where available. **P < 0.01 as compared to control. Control bFGF dissociation was 15% of the bound [125I]-bFGF in 30 min. ND, not done.

R → A at positions 7 and 11 respectively (P3CD).

Some results could not be explained on the basis of number or type of basic amino acids. Foremost among these were findings with the peptide PA5, an 18 amino acid peptide corresponding to amino acids 128–145 of IGFBP-5. PA5 is a highly basic peptide with 8 basic amino acids, i.e. an identical number of basic amino acids as the active P6 (Table 2). PA5 inhibited [125I]-IGFBP-3 binding slightly (binding was 66% of control), had no effect on [125I]-P3 binding, did not bind heparin, stimulate glucose uptake nor displace bFGF (Table 2).

Release of bFGF from monolayers/extracellular matrix

Several peptides, including P3, caused release of [125I]-bFGF from endothelial monolayers and ECM that were preincubated with [125I]-bFGF. Since bFGF can directly stimulate glucose uptake in microvessel endothelial cells, it is synthesized by endothelial cells and is present/stored in the ECM of endothelial cell monolayers, the possibility was raised that the bioactivity of P3 was mediated through its release of bFGF from the ECM. To test this hypothesis, microvessel endothelial cells were incubated with bFGF (2 ng/ml), bFGF plus neutralizing antibody to bFGF, P3 (4 μM) alone or P3 plus bFGF antibody (Table 4). bFGF stimulated glucose uptake to 164% of control, whereas, in the presence of neutralizing antibodies, bFGF increased glucose uptake to only 107% (P < 0.01 relative to bFGF treatment, not significantly different from control). In contrast, P3 stimulated glucose uptake to 160% of control in the presence of absence of neutralizing

$^d$Additionally, PA5 did not inhibit [125I]-P3 binding (data not shown).
Table 4. Effect of neutralizing bFGF antibody on P3 activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose uptake</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100 ± 18</td>
<td></td>
</tr>
<tr>
<td>IGF-I 50 ng/ml</td>
<td>215 ± 12**</td>
<td></td>
</tr>
<tr>
<td>bFGF 2 ng/ml</td>
<td>164 ± 12**</td>
<td></td>
</tr>
<tr>
<td>bFGF plus 10 μg/ml antibody</td>
<td>197 ± 12</td>
<td></td>
</tr>
<tr>
<td>P3 4 μM</td>
<td>160 ± 15*</td>
<td></td>
</tr>
<tr>
<td>P3 plus 10 μg/ml antibody</td>
<td>160 ± 30**</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis of a single experiment performed in triplicate. **P < 0.01 compared with control. Data shown ± SEM.

bFGF antibody. Thus, it is unlikely that the stimulation of glucose uptake by P3 was mediated by bFGF.

DISCUSSION

IGFBP-3 contains a highly basic region in the C-terminal third of the molecule which appears to be responsible for adherence of the binding protein to endothelial cells and to the extracellular matrix secreted by these cells. A synthetic 18 amino acid peptide corresponding to this region of IGFBP-3 (P3), as well as homologous peptides from IGFBP-5 (P5) and IGFBP-6 (P6), inhibited IGF-I-IGFBP-3 and IGF-I-P3 binding to endothelial cell surfaces and extracellular matrices. P3, P5 and P6 also bound directly to the endothelial cell extracellular matrix, bound heparin, released [3H]-bFGF from the endothelial extracellular matrix and were bioactive, stimulating glucose uptake by microvessel endothelial cells. In comparison, IGFBP-3, a known heparin-binding protein, released bFGF from endothelial ECM, but did not compete for P3 binding nor stimulate glucose uptake.

To begin to define the structural specificities accounting for these properties of P3, the 18 amino acid peptide was modified relative to charge, size and amino acid sequence. Of these changes, the number of positively charged amino acids was the most relevant. P3 has 10 basic amino acids while P5 and P6, representing 18 amino acid peptides from the homologous C-terminal region of IGFBP-5 and IGFBP-6, mimic all P3 properties and, like P3, are enriched with basic amino acids, having 10 and 8 basic amino acids, respectively. Deletion of 2 (P3CD) basic amino acids from P3 resulted in significant loss of heparin binding, bioactivity, ability to compete for IGFBP-3/P3 binding and release of bFGF from ECM. Of the basic amino acids, arginine was equal to lysine with both being much more potent than the third basic amino acid, histidine. The importance of charge was further suggested by the finding that protamine, a highly basic protein structurally unrelated to P3, but containing large numbers of arginine, was as potent as P3.

Size of the peptide (when >13 amino acids) and specific amino acid sequence were less important than charge relevant to the four properties studied. Size did, however, appear relevant in determining the region of localization within the endothelial cell monolayer. In microvessel endothelial cell cultures, the 18 amino acid P3 bound to the extracellular matrix, IGFBP-3 prominently associated with the cell surface and the 34 amino acid P3A associated with both the extracellular matrix and the endothelial cell surface. The sequence of amino acids in P3 was least important. In fact, the consensus heparin binding sequence of P3, YKKKQCRP, was not critical for heparin binding nor for any of the other four properties studied. This implies that the mechanism for monolayer/ECM interactions of IGFBP-3 and its derived peptides may not be similar to that of other, better characterized, heparin-binding proteins such as bFGF where the presence of a Cardin–Weintraub sequence is essential for heparin binding. Furthermore, IGFBP-3 and P3 differ from bFGF and other heparin-binding proteins in their requirement for initial interaction with ECM proteoglycans in mediating their binding to cultured cells. Previous studies by Booth et al. and a recent report by Andress found that heparinase/heparitinase treatment of cell monolayers or growth in the presence of sodium chlorate to remove or prevent formation of heparin/heparan sulfate proteoglycans, respectively, did not decrease binding of IGFBP-3, P3 or IGFBP-5 to the monolayer or extracellular matrix of endothelial cells and osteoblasts. This suggested that in contrast to bFGF, IGFBP-3, P3 and IGFBP-5 did not require binding to heparin/heparan sulfate proteoglycans for association to cultured cells.

The finding that P3, as well as IGFBP-3, P3A and protamine, could dissociate bFGF from the endothelial extracellular matrix has several potential implications. Basic FGF is produced by endothelial cells, localizes in the ECM and affects many functions of endothelial and vascular smooth muscle cells, including differentiation and cell growth. To exert these effects, bFGF must form a complex between a lower affinity ECM heparan sulfate proteoglycan receptor and the cell surface receptor for bFGF. Although release of ECM bFGF was not the mechanism for P3 bioactivity, it remains to be determined whether basic proteins or basic peptides exert effects on vascular endothelium or smooth muscle, in vivo, by causing dissociation of bFGF from the basement membrane. For IGFBP-3, it is possible that the binding protein has limited access to the basement membrane or ECM of the intact vascular endothelium because of IGFBP-3 affinity for the endothelial cell surface. However, with damage to the endothelium, such as in atherosclerosis or at sites of neovascularization as observed in diabetic retinopathy, IGFBP-3 would have more direct exposure to the ECM.
We believe that dissection of the structure-function relationships within the C-terminal region of IGFBP-3 will be important in understanding the mechanisms of IGFBP-3 interaction with the vascular endothelium. Furthermore, recent studies in our laboratory indicate that thrombin, plasmin and pregnancy serum, which can be present, in vivo, at the endothelial cell surface in enriched concentrations, break down IGFBP-3 into fragments, some of which contain the P3 region of IGFBP-3 and possess several P3 properties, including stimulation of glucose uptake by endothelial cells. The properties of these P3-containing fragments and their relevance to IGF/endothelial cell function are presently under study.

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References


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