Synthesis and Characterization of Insulin-like Growth Factor (IGF)-1 Photoprobe Selective for the IGF-binding Proteins (IGFBPs)

PHOTOAFFINITY LABELING OF THE IGF-BINDING DOMAIN ON IGFBP-2*

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Elevated insulin-like growth factor (IGF)-1 levels are prognostic for the development of prostate and breast cancers and exacerbate the complications of diabetes. In each case, perturbation of the balance between IGF-1/2, the IGF-1 receptor, and the IGF-binding proteins (IGFBPs) leads to elevated IGF-1 sensitivity. Blockade of IGF action in these diseases would be clinically significant. Unfortunately, effective IGF antagonists are currently unavailable. The IGFBPs exhibit high affinity and specificity for the IGFs and serve as natural IGF antagonists, limiting their mitogenic/anti-apoptotic effects. As an initial step in designing IGFBP-based agents that antagonize IGF action, we have begun to analyze the structure of the IGF-binding site on IGFBP-2. To this end, two IGF-1 photoprobe, N\(^{\text{Gly1}}\)-(4-azidobenzoyl)-IGF-1 (ab\(^{1}\)IGF-1) and N\(^{\text{Gly1}}\)-[2–6-(biotinamido)-2-(p-azido-benzamido)-hexanoamido]ethyl-1,3-dithiopropionoyl]-IGF-1 (bed\(^{1}\)IGF-1), selective for the IGFBPs were synthesized by derivatization of the α-amino group of Gly\(^{\text{N}}\), known to be part of the IGFBP-binding domain. Mass spectrometric analysis of the reduced, alkylated, and trypsin-digested ab\(^{1}\)IGF-1-recombinant human IGFBP-2 (rhIGFBP-2) complex indicated photocorporation near the carboxyl terminus of rhIGFBP-2, between residues 266 and 287. Mass spectrometric analysis of avidin-purified tryptic peptides of the bed\(^{1}\)IGF-1-rhIGFBP-2 complex revealed photocorporation within residues 212–227. Taken together, these data indicate that the IGFBP-binding domain on IGF-1 contacts the distal third of IGFBP-2, providing evidence that the IGF-1-binding domain is located within the C terminus of IGFBP-2.

Insulin-like growth factor (IGF)-1 and IGF-2 play central roles in a number of cellular processes, including growth, proliferation, differentiation, survival, transformation, and metastasis (1, 2). Enhanced activity of the IGFs has been implicated in diabetic complications and cancer. These effects are mediated by the IGF-1 receptor (IGF-1R), a member of the receptor tyrosine kinase family of cell-surface receptors. The IGF-2 receptor, which lacks signaling activity, plays a role in clearing IGF-2 from the cell surface (3, 4). The IGFs are regulated at the extracellular level by a family of six IGF-binding proteins (IGFBPs), designated IGFBP-1–6 (5–7). These six proteins exhibit higher affinities for the IGFs than the IGF-1R, while having negligible affinity for insulin.

Renewed interest in the function of the IGF system stems from the observations that IGF-1 and IGF-2, acting through the IGF-1R, increase the tumorigenic potential of breast and prostate cancer cells (8). Accordingly, increased serum IGF-1 levels have been shown to be prognostic for the development of prostate and breast cancers (9, 10). Alterations in IGFBP expression may also contribute to disease states. For example, IGFBP-3 is a target of the p53 tumor suppressor, and a common p53 mutation results in decreased IGFBP-3 secretion (11, 12), which is likely to cause an increased proliferative response to IGF-1. Also, reduced IGFBP-2 expression resulting from the hyperglycemia of diabetes was recently shown to enhance the sensitivity of renal mesangial cells to the growth and secretory effects of IGF-1, pushing the cells toward a glomerulosclerotic phenotype (13). Because IGF-1 can suppress apoptosis, cells lacking IGF-1 receptors, cells with compromised IGF-1R signaling pathways, or cells treated with the IGFBPs may selectively die by apoptosis (8). Taken together, these findings suggest that the IGFBPs serve a role as natural IGF antagonists.

IGF-1 and IGF-2 are homologous protein hormones of 70 and 67 amino acids in length, respectively (14). Based on studies of chemically modified and mutated IGF-1, a number of residues have been identified as being part of the IGF-1R contact site, in particular the aromatic residues at positions 23–25 (15). Cooke et al. (16) used NMR and restrained molecular dynamics to elicit the solution structure of IGF-1; this model clearly illustrates an IGFBP-interacting domain on the surface of IGF-1 and its lack of overlap with the receptor-docking site (see Fig. 1). Specifically, this site consists of the N-terminal tripeptide Gly-Pro-Glu (17) and residues 49–51 (18). These two regions come together to form an independent binding domain (see Fig. 1) (16). The analog des-1–3-IGF-1 binds to the IGF-1R with high affinity, but has dramatically reduced affinity for the IGFBPs, underscoring the importance of the N-terminal con-
tact site on IGF-1 for binding protein specificity (17). In addition, mutations of Glu3 or residues 49–51 result in a ligand with severely reduced binding activity (19). In good agreement with these findings, insulin lacks these residues common in the IGFBP-binding region and thus does not bind to the IGFBPs with high affinity.

The IGFBPs are globular proteins containing 18 spatially conserved cysteine residues participating in the formation of nine disulfide bonds. They range in size from 200 to 300 amino acids and, based on their high degree of homology, can be divided into three distinct domains, each constituting about one-third of the protein (20). The N- and C-terminal regions, designated domains 1 and 3, respectively, share the highest homology (20, 21), whereas the intervening region (domain 2) is highly variable (<30% homology) (6). Domains 1 and 3 contain 12 and 6 spatially conserved cysteine residues, respectively, except in the case of IGFBP-6, which is missing 2 cysteine residues in domain 1 (5); IGFBP-4 has 2 additional cysteine residues in domain 2. Because of the high homology of these proteins in domains 1 and 3 (37–70%) (6), the IGF-binding domain has been proposed to reside within one of these regions. Support for this notion is based on studies in which N- or C-terminal IGFBP fragments were found to retain high affinity binding activity for IGF-1 and/or IGF-2 (19). IGFBP-related protein-1 and -2, also known as IGFBP-7/Mac25 and IGFBP-8/connective tissue growth factor, respectively, have homologies in their N termini to IGFBP-1–6 (23). These proteins have lower affinities for the IGFs compared with the IGFBPs and have been reported to interact with insulin (24). The precise mechanism by which the IGFBPs inhibit IGF-1 and IGF-2 action is presently unknown. It is thought to involve high affinity binding of the IGFs by the IGFBPs, thereby limiting their access to the IGF-1R. A complete understanding of this inhibitory action will come with the solution of the three-dimensional structure of the IGFBPs. As of yet, the IGF-binding domain on the IGFBPs has not been defined.

To date, extensive use of molecular techniques has been applied to assess the binding domain on the IGFBPs. However, only sparse structural information has been obtained. Currently, a debate exists as to which domain(s) of the IGFBPs are most crucial for IGF binding. This is further confounded by the paucity of precise structural information about the IGFBPs as recently reviewed by Baxter (25). To more precisely identify the points of contact between IGF-1 and IGFBP-2, we chose to pursue a photoaffinity labeling approach, which has been used to identify sites of interaction between a number of interacting proteins. On this basis, we derivatized the N-terminus of IGF-1 (abG1IGF-1) and their successful application to photoaffinity labeling analyses, our results indicate that the C terminus of IGF-1 is essential for high affinity binding of IGF-1 to the IGFBPs and thus does not bind to the IGFBPs with high affinity.

IGFBP-binding region and thus does not bind to the IGFBPs with high affinity. This is further confounded by the photoreactive group is inserted within the region shown to be essential for high affinity binding of IGF-1 to the IGFBPs. A complete understanding of this affinity binding of the IGFs by the IGFBPs, thereby limiting their access to the IGF-1R. A complete understanding of this inhibitory action will come with the solution of the three-dimensional structure of the IGFBPs. As of yet, the IGF-binding domain on the IGFBPs has not been defined.

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and 50 mM HEPES (pH 7.4) with 0.05% sodium azide. Diallyl and lyophilized conditioned medium was dissolved in 40–45 ml of 50 mM HEPES (pH 7.4) containing 150 mM NaCl (Buffer A). Insoluble material was removed by centrifugation at 3000 × g for 20 min. 2 ml of IGF-1-agarose in Buffer A were then added, and the slurry was incubated overnight at 4 °C with gentle agitation. The column was subsequently washed with 100 ml of Buffer A, followed by 50 ml of 10% Buffer A. Proteins bound to the column were then eluted with 15 ml of 0.5 M acetic acid, and the column was washed with 20 ml of Buffer A plus 1 ml of 1 mM HEPES (pH 7.4), followed by 40 ml of Buffer A. The eluate was dried in vacuo using a SpeedVac concentrator. The dried eluate was stored at −20 °C until further purification as described above.

**IGFBP-2 Binding Assay**—Soluble IGFBP-2 binding assays were carried out using polyethylene glycol precipitation and centrifugation (31). 1 ng of rhIGFBP-2 was combined with various concentrations of IGFBP-1, abG1IGF-1, or bedG1IGF-1 ranging from 30 fm to 100 nm in binding assay buffer (100 mM HEPES (pH 7.4), 44 mM NaHCO3, 0.01% bovine serum albumin, 0.01% Triton X-100, and 0.02% NaN3), followed by the addition of 10 nCi of 125I-IGF-1 (Amersham Pharmacia Biotech). After a 4-h incubation at room temperature, 250 μl of 0.5% bovine γ-globulin were added, followed by 500 μl of 25% polyethylene glycol (average Mr of 8000, Sigma). The samples were incubated for 10 min at room temperature and centrifuged for 3 min at 15,000 × g. The pellets were washed with 1 ml of 6.25% polyethylene glycol, and bound radioactivity was quantified in a CompuGamma spectrometer (LKB-Wallac, Turku, Finland). Counts bound in the presence of 1 μM or 100 nM IGFBP-1 (nonspecific binding) were subtracted to obtain specific binding. IC50 values were calculated using the equation B = Bmax/(1 + [lg(IGFBP)]Bmax), where B is the concentration of bound ligand and Bmax is the maximal binding observed. The Microsoft Excel 97 Solver was used to minimize the sum of the squares of the differences from the mean IC50 values for each IGFBP-1 concentration by optimizing B restrained by the above equation. The calculated IC50 values were used to generate smooth curves.

**Photoactivity Labeling**—Equimolar quantities of abG1IGF-1 or bedG1IGF-1 and rhIGFBP-2 were allowed to attain equilibrium binding by co-incubation for 4 h at 25 °C in 100 mM HEPES (pH 7.4) containing 44 mM NaHCO3 and 0.01% Triton X-100. The sample was then placed in ice water and irradiated for 2 h with a prewarmed Fotodyne handheld, single-wavelength UV lamp (2 × 4-watt 300-nm bulbs) at a distance of 2 cm. The mixture was dried in vacuo, and the proteins were reduced and alkylated using tris(2-carboxyethyl)phosphine (32) and 4-vinylpyridine (33). abG1IGF-1 and bedG1IGF-1-photo labeled IGFBP-2-proteins were separated from unreacted IGFBP-2 by reversed-phase HPLC and trypsinized as described below.

**Tryptsinization of IGFBP-1, IGFBP-2, and Photoactivity-Labeled Complexes**—Trypsinization of reduced and alkylated proteins was performed according to Honegger and Humbel (34). Proteins (20 μM) were dissolved in 100 mM N-ethylmorpholine acetate (pH 8.5) to which was added sufficient trypsin (1 μg/μl) to achieve a 1:50 enzyme/substrate ratio. Following a 2-h incubation at 37 °C, trypsin was inactivated by the addition of an equal volume of 0.1% trifluoroacetic acid. The mixture was dried in vacuo or applied directly to a C18 reversed-phase HPLC column.

**Avidin Chromatography of bedG1IGF-1-IGFBP-2 Trypptic Peptides**—Tryptic peptides generated from the bedG1IGF-1-IGFBP-2 complex were applied to an UltraLink™ monomeric avidin column. Flow-through fractions were collected and pooled. The column was eluted with low pH buffer, and the eluate fractions were pooled. The flow-through and eluate fractions were dried and further analyzed by reversed-phase HPLC on a C18 column equilibrated in 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile. Eluted peaks were analyzed by MALDI-TOF-MS.

**Immunoblot—**Samples were dissolved in SDS sample buffer with or without dithiothreitol and resolved on a 10 or 12.5% SDS-polyacrylamide gel according to the procedure of Laemmli (35) using a Hoefer Scientific Instruments apparatus. The proteins were transferred to nitrocellulose and immunoblotted using a commercial antiserum against intact bovine IGFBP-2 (Upstate Biotechnology, Inc., Lake Placid, NY). Blots were developed with horseradish peroxidase-labeled secondary antibody (Chemicon International, Inc., Temecula, CA) and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). For sequential anti-IGF-I/anti-IGFBP-2 antibody analysis of the same blot, the membrane was stripped for 20 min at 60 °C in 1× Tris (pH 6.7) containing 10% SDS and 0.1 mM β-mercaptoethanol. The stripped membrane was washed twice with Tris-buffered saline containing Tween for...
carried out in the dark to avoid photoactivation of the probe, and recombinant human IGF-1 was also digested in parallel to serve as a control for subsequent HPLC and mass spectrometric analyses.

For each photoprobe, HPLC purification of the pepsin digestion products revealed that the retention times for the derivatized AE fragments were significantly increased compared with the underivatized AE fragment (data not shown). This was predicted based on the added hydrophobicity of the additional functional groups. Fig. 4A shows the MALDI-TOF-MS of abG1AE. This fragment had the correct mass (predicted average mass of 2419.7 Da; observed mass of 2419.8 Da) and exhibited loss of nitrogen as a result of photoactivation by the 337-nm laser, to yield a second peak at 2394.3 Da. The identities of the CG and D fragments, containing Lys65/Lys68 and Lys27, respectively, were also confirmed by MALDI-TOF-MS and were in good agreement with those of Forsberg et al. (28).

IGF-1 N-terminal Contact Sites on IGFBP-2
complex was first reduced and alkylated with tris(2-carboxyethyl)phosphine and 4-vinylpyridine prior to electrophoresis. As discussed above, reduction and alkylation of bedG1IGF-1-IGFBP-2 should yield IGFBP-2 biotinylated at the site of photoincorporation. As shown in lanes 4–6, two bands were again detected with reduction and alkylation of the samples. The upper band had the same electrophoretic mobility as uncross-linked, reduced, and alkylated IGFBP-2 and was detectable with anti-IGFBP-2 antibodies. However, roughly one-half of the content of this band was retained on the avidin-agarose column (lane 5) and reacted with NeutrAvidin™-peroxidase (lane 6), indicating that the band in lane 4 contains both uncleaved and biotinylated IGFBP-2. The lower band reacted with anti-IGFBP-2 antibodies, was retained by the avidin-agarose column (lane 5), and could be labeled with NeutrAvidin™-peroxidase (lane 6), indicating that it represents a biotinylated fragment of IGFBP-2. These results indicate that reduction of the bedG1IGF-1-IGFBP-2 complex allowed greater access of avidin-peroxidase to the biotin moiety on photolabeled IGFBP-2.

Identification of the abG1IGF-1 Photoincorporation Site—To identify the site of photoincorporation of abG1IGF-1, the abG1IGF-1-IGFBP-2 complex was isolated by HPLC following reduction and alkylation of the photolysis reaction mixture. Owing to the similar retention times of the free and cross-linked species, the two components overlapped significantly (Fig. 8A). Subsequent re-chromatography of each component provided sufficient resolution to attain a high level of purification of the abG1IGF-1-IGFBP-2 complex (Fig. 8B). Immunoblot analysis of the column fractions indicated that >40% photoincorporation was achieved.

Fractions containing purified abG1IGF-1-IGFBP-2 were pooled, dried, and trypsinized. It was anticipated that tryptic digestion of the abG1IGF-1-IGFBP-2 complex would yield a cross-linked peptide containing a tryptic fragment of IGFBP-2 (BP2T) and the N-terminal tryptic fragment of abG1IGF-1 (abIGF1T). Since abIGF1T alone (residues 1–21) is 2521 Da, this represents the minimum mass for the resulting cross-linked peptide (BP2T-abIGF1T). The tryptic digest was applied...
to a C18 column and eluted with a shallow gradient of acetonitrile to obtain optimal separation of the tryptic peptides (data not shown). MALDI-TOF-MS was then performed on each fraction to locate BP2T-abIGF1T. The MALDI mass spectrum of the identified fraction is shown in Fig. 9

A. Although not pure, we obtained a significant enrichment of BP2T-abIGF1T in this fraction with an observed mass of 5295.6 Da. These results suggest that abIGF1T (predicted mass of 2521 Da) was covalently incorporated into the C-terminal tryptic peptide of IGFBP-2 corresponding to residues 266–287 (BP2T predicted mass of 2772 Da), yielding a complex with a predicted mass of 5293 Da. To further validate this assignment, the fraction containing this complex was subdigested with Staphylococcus aureus V8 protease and analyzed by MALDI-TOF-MS. As illustrated in Fig. 9 (B and C), MALDI-TOF-MS of this fraction identified four distinct peptides consistent with the proposed structure. These data indicate that when attached to the a-amino group of Gly1, the azidobenzoyl moiety contacts IGFBP-2 in its distal C-terminal end within the tryptic peptide corresponding to residues 266–287 (see Fig. 11

B. Identification of the bedG1IGF-1 Photoincorporation Site—To determine the site of photoincorporation of bedG1IGF-1 into IGFBP-2, the photolyzed reaction mixture obtained as described under “Experimental Procedures” was then analyzed with tris(2-carboxyethyl)phosphine and 4-vinylpyridine, resulting in cleavage of IGF-1 from the complex and biotinylation of IGFBP-2 at the site of photoincorporation. The mixture was then desalted on a C4 column, dried in vacuo, and digested with trypsin. The tryptic peptides were loaded onto an UltraLink™ monomeric avidin column, which was washed with 6 column volumes of phosphate-buffered saline. To release retained biotinylated tryptic peptides, the column was eluted with regeneration buffer (0.2M glycine (pH 2.8)). The flow-through and eluate fractions were pooled separately and dried, and the peptides present in each were resolved by HPLC on a C18 column. As expected, the majority of peptides eluted in the flow-through fraction of the avidin column (Fig. 10A), resembling a representative combined tryptic digest of uncross-linked IGF-1 and IGFBP-2 (data
not shown). Two major peaks were observed in the low pH elution of the column. The first peak exhibited a mass of 2595.7 Da when analyzed by MALDI-TOF-MS (Fig. 10A). When corrected for the mass of the biotin and remaining cross-linker residues from the bed moiety (653.3 Da), this corresponded to a BP2T fragment of 1942.3 Da (Fig. 10A) (41), these two sites are likely to be this domain (Fig. 11B) (43). Although we cannot rule out the possibility that this side chain results in the labeling of regions of the IGFBPs outside the IGFBP-binding domain, including Glu3, which plays an essential role in maintaining high affinity binding to the IGFBPs (43). Alternatively, Forbes et al. (42) prepared monoclonal antibodies to residues 188–196 and 222–227 (bedG1IGF-1) (Fig. 11A). The labeling of these different sites can be attributed to the different side chain lengths of the two IGF-1 photoprobes. abG1IGF-1 contains an azidobenzoyl moiety and thus lacks an appreciable side chain between the 6-amino group of Gly1 and the aryl azide. In this case, the aryl azide resides near the outer edge of the IGF-1-binding domain, defining the site of contact for Gly1. The longer, more flexible side chain present on the aryl azide in bedG1IGF-1 has the potential to define a contact site within the vicinity of the other residues of the IGFBP-binding domain, including Glu3, which plays an essential role in maintaining high affinity binding to the IGFBPs (43).

Introduction of photoactivable aryl azide moieties within the IGFBP-binding domain on IGF-1 (residues 1–3 and 49–51) resulted in the selective photoaffinity labeling of two separate sites in the C terminus of IGFBP-2, within tryptic peptides 266–287 (abG1IGF-1) and 212–227 (bedG1IGF-1) (Fig. 11A). The labeling of these different sites can be attributed to the different side chain lengths of the two IGF-1 photoprobes. abG1IGF-1 contains an azidobenzoyl moiety and thus lacks an appreciable side chain between the ω-amino group of Gly1 and the aryl azide. In this case, the aryl azide resides near the outer edge of the IGF-1-binding domain, defining the site of contact for Gly1. The longer, more flexible side chain present on the aryl azide in bedG1IGF-1 has the potential to define a contact site within the vicinity of the other residues of the IGFBP-binding domain, including Glu3, which plays an essential role in maintaining high affinity binding to the IGFBPs (43).

Although we cannot rule out the possibility that this side chain results in the labeling of regions of the IGFBPs outside the IGFBP-binding domain, we believe that the likelihood of this occurring is minimal based on the previous identifications of C-terminal binding activity described above. Given the globular nature of the IGFBPs and the disulfide bonding pattern in this domain (Fig. 11B) (41), these two sites are likely to be closely apposed in three-dimensional space. Although the two photoprobes labeled sites within the C terminus of IGFBP-2 that are separated by ~40 amino acids in the primary sequence, these sites are likely to be much closer when the secondary structure of the protein is taken into consideration (Fig. 11B). Indeed, the two labeled sites cannot be farther apart than the total length of the two photoprobe spacer arms combined (~300 nm).

Based on chemical modifications, site-directed mutagenesis

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**DISCUSSION**

In this report, we present evidence for a C-terminal contact site on IGFBP-2 for IGF-1 based on direct photoaffinity labeling studies with two unique N-terminally modified photoaffinity derivatives of IGF-1. We interpret these findings as an indication of the presence of a high affinity binding site for IGF-1 within the C terminus of IGFBP-2. Consistent with this finding are a number of reports describing the C terminus of IGFBP-2 as the domain containing the high affinity IGF-binding site. Wang et al. (38) and Ho and Baxter (39) each identified C-terminal fragments of IGFBP-2 with high affinity IGF-binding activity. In preliminary studies, we have characterized a 15.8-kDa C-terminal fragment of rhIGFBP-2 from our transfected Chinese hamster ovary cell cultures that exhibits an affinity for IGF-1 identical to that of intact rhIGFBP-2. This fragment lacks N-terminal and mid-region epitopes based on tryptic peptide mapping studies. Additional support for the C terminus as the site of IGF binding stems from studies of Brinkman et al. (40), who deleted the last 20 amino acids from the C terminus of IGFBP-1 and thereby abolished IGF-1-binding activity. Similarly, Forbes et al. (41) generated a series of four sequential C-terminal truncation mutants of bovine IGFBP-2 and concluded that residues 222–236 are required for high affinity IGF interactions. Finally, Schuller et al. (42) prepared monoclonal antibodies to residues 188–196 and 222–227 of IGFBP-1 that blocked IGF-1 binding. These investigators concluded that the regions surrounding these epitopes were important for IGF binding.

**IGF-1 N-terminal Contact Sites on IGFBP-2**

The labeling of these different sites can be attributed to the different side chain lengths of the two IGF-1 photoprobes. abG1IGF-1 contains an azidobenzoyl moiety and thus lacks an appreciable side chain between the ω-amino group of Gly1 and the aryl azide. In this case, the aryl azide resides near the outer edge of the IGF-1-binding domain, defining the site of contact for Gly1. The longer, more flexible side chain present on the aryl azide in bedG1IGF-1 has the potential to define a contact site within the vicinity of the other residues of the IGFBP-binding domain, including Glu3, which plays an essential role in maintaining high affinity binding to the IGFBPs (43). Although we cannot rule out the possibility that this side chain results in the labeling of regions of the IGFBPs outside the IGFBP-binding domain, we believe that the likelihood of this occurring is minimal based on the previous identifications of C-terminal binding activity described above. Given the globular nature of the IGFBPs and the disulfide bonding pattern in this domain (Fig. 11B) (41), these two sites are likely to be closely apposed in three-dimensional space. Although the two photoprobes labeled sites within the C terminus of IGFBP-2 that are separated by ~40 amino acids in the primary sequence, these sites are likely to be much closer when the secondary structure of the protein is taken into consideration (Fig. 11B). Indeed, the two labeled sites cannot be farther apart than the total length of the two photoprobe spacer arms combined (~300 nm).

Based on chemical modifications, site-directed mutagenesis

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**FIG. 9.** Analysis of abG1IGF-1-IGFBP-2 photoaffinity-labeled complex. 

A, shown is a MALDI-TOF mass spectrum of a unique peak having a mass (5295.6 Da) that lacks correspondence to IGF-1 or IGFBP-2 tryptic peptides. B, the column fraction containing the 5295.6-Da fragment was dried and subdigested with *S. aureus* V8 protease. Shown is the MALDI-TOF mass spectrum of the digestion products. C, the peptides identified in the V8 digest in B are indicated.

**FIG. 10.** Analysis of bedG1IGF-1-IGFBP-2 photoaffinity-labeled complex. A, elution profile of the reduced, alkylated, and trypsin-digested photolysis reaction mixture following chromatography on a column of monomeric avidin. Shown are the HPLC column profiles of the column flow-through/washes and eluate. B, MALDI-TOF mass spectrum of the peaks identified by arrows in A. The first peak exhibited a mass of 2595.7 Da. After correcting the observed mass for the associated biotin and spacer, it was identified as tryptic peptide 212–227. The second biotinylated component (second arrow) was identified as having a mass of 1014.1 Da, which corresponds to a truncated form of the same tryptic peptide (residues 212–220) and suggests rupture of the peptide backbone of IGFBP-2 during photoincorporation. This finding explains the composition of the smaller band observed in Fig. 7B (loss of 7 kDa; residues 221–289). Z, pyridylethylated cysteine.
studies, and the identification of fragments with IGF-binding activity, the N terminus of the IGFBPs has also been described as the site of the IGF-binding domain. Iodination studies on bovine IGFBP-2 in isolation or as a complex bound to IGF-2 resulted in efficient iodination of Tyr residues at positions 71, 98, 213, 226, and 269; Tyr60 was protected from iodination by bound IGF-2 (44). Huhtala et al. (45) isolated a 21-kDa N-terminal fragment of IGFBP-1 that retained some IGF-1-binding activity. More recently, Kalus et al. (46) reported that N-terminal fragments of IGFBP-5 representing residues 1–104 and 40–92 exhibit weak IGF-binding activity. Based on NMR studies, they defined a hydrophobic patch comprising residues 49, 50, 62, and 68–75 that potentially represents the primary IGF-binding site on IGFBP-5. This hypothesis was subsequently tested by the construction of full-length IGFBP-5 and IGFBP-3 mutants in which combined substitutions at residues 68, 69, 70, 73, and 74 resulted in a 1000-fold reduction in binding affinity (47). Contrary to these mutagenesis results, Ständker et al. (48) reported that the C terminus of IGFBP-5 contains the IGF-binding domain responsible for binding selectivity for IGF-1 via its IGFBP-binding domain. The second low affinity site (N terminus) binds to IGF-1 via its IGF-1R-binding domain and thus plays a role in blocking IGF-1 binding to the IGF-1R. This model would likely require a conformational change to take place within the N terminus following IGF binding to the C terminus. Evidence to support this comes from NMR analyses of IGF/IGFBP complexes (50, 51). It is possible to speculate that such conformational changes may reflect the ability of the IGFBP, following initial binding to the N terminus (IGFBP-binding domain) of IGF-1, to interact with the IGF-1R domain of IGF-1. This could provide a sterically mechanism through which inhibition of IGF-1R activation is accomplished. This in turn explains low affinity insulin binding to the N terminus of IGFBP-related protein-1 (9). In addition, a conformational change explains why des-1–3-IGF-1 does not bind the IGFBPs well, even though it still contains an intact IGF-1R-binding domain. This scheme may serve to explain how N-terminal fragments and truncation mutants of the IGFBPs are able to bind to IGF-1. This would also support a sterically hinderance-based model of IGFBP inhibition of IGF-1 action at the IGF-1R. Following high affinity binding between the IGFBP-binding domain on IGF-1 and its contact site within the C terminus of IGFBP-2, the ensuing conformational rearrangement enables high affinity interaction of the N terminus of IGFBP-2 with the IGF-1R binding domain on IGF-1. Obviously, this is one of several potential models one might propose; confirmation of any such model will require a more definitive understanding of the three-dimensional structure of the IGFBPs. Future photolabel-
ing studies with abK²GIF-1 and bedK²GIF-1 may aid in addressing the issue of whether there are N-terminal contact sites on the IGFBPs for the IGF-1R-binding domain on IGF-1.

Based on their ability to block IGF actions, we have chosen to pursue the design of IGF antagonists based on the structure of the IGF-binding domain on the IGFBPs. This clearly requires more detailed information than is currently available concerning IGFBP structure. We have employed a photoaffinity labeling approach as a means of identifying the IGF-1 site of contact on IGFBP-2. To this end, a photoreactive derivative of IGF-1 was prepared, exploiting the reactivity of primary amines in the protein to covalently attach an aryl azide moiety within the IGFBP-binding domain. This approach has been used for many photoaffinity labeling studies, including the insulin (52) and IGF-1 (36) receptors. It has the drawback of incorporating this reagent in photolabeling a ligand-binding site.

In conclusion, this study represents the first report defining IGF-1 N-terminal Contact Sites on IGFBP-2.

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Synthesis and Characterization of Insulin-like Growth Factor (IGF)-1 Photoprobes Selective for the IGF-binding Proteins (IGFBPs): PHOTOAFFINITY LABELING OF THE IGF-BINDING DOMAIN ON IGFBP-2
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