Use of Mutagenesis to Probe IGF-Binding Protein Structure/Function Relationships

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The IGF-binding proteins (IGFBPs) are multifunctional proteins that modulate IGF actions. To determine whether specific domains within these proteins account for specific functions, we and other laboratories have used *in vitro* mutagenesis. Prior experiments that used a variety of techniques had identified discrete regions within each protein that were proposed to account for specific functions. Alterations of these regions by substituting charged residues with neutral residues or hydrophobic residues with nonhydrophobic residues as well as domain swapping, *i.e.*, substituting a domain from one specific form of IGFBP for the homologous domain in another form, has resulted in the elucidation of the functions of many of these specific sequences. Because the areas of protein sequence that are altered involve a limited number of amino acids, they generally do not alter the conformation of the entire protein; therefore, these specific substitutions can often be correlated with the functional changes that occur after mutagenesis. Mutants have been particularly useful for performing functional analyses in which the purified mutant protein is added to a biological test system. In some cases it has been possible to overexpress the mutagenized protein and determine whether the constitutively synthesized, mutant form of IGFBP has altered functional activity. These results have revealed that discrete regions of IGFBP sequence can mediate important and specific functional properties of these proteins. (*Endocrine Reviews* **22: 800–817, 2001**)

I. Introduction

THE FUNCTIONAL ANALYSIS of IGF-binding proteins (IGFBPs) has been a topic of intense research interest for the past 15 yr. The IGFBPs were discovered as biological activities in the mid-1970s. Most of the early studies focused on purification of these proteins or the characterization of their ability to bind radiolabeled IGF-I or -II in solution. Assay methodologies were developed for separating bound and free radiolabeled IGF-I or -II, and these became the basis for monitoring their purification. Although some attempts were made to determine whether these proteins had inhibitory effects on IGF actions, many of these experiments were confounded by contamination of the binding protein extract itself with IGF-I or -II or by secretion of IGF-I or II by cells.

The first reports that suggested the existence of proteins that could bind to the IGFs were published in the late 1960s. At that time it was unclear whether these were higher molecular weight forms of the IGFs themselves or carrier proteins. Acidification of protein extracts that contained these higher molecular weight forms resulted in the appearance of lower molecular weight peptides that had IGF bioactivity, suggesting the possibility that the higher molecular weight proteins were carrier proteins (1, 2). In 1975 the first definitive report of an activity that would bind radiolabeled IGF-I was published (3). After this report, several groups attempted to purify these activities using IGF binding assays to monitor purification. These efforts focused on what later became termed "IGFBP-1," which was purified from human amniotic fluid (4–6), and what later became termed "IGFBP-3," which was purified from human serum (7). Attempts were also made to characterize and purify a binding protein activity from conditioned medium of a Buffalo rat liver cell line (8). This protein was eventually shown to be IGFBP-2. These attempts ultimately resulted in publication of limited amino-terminal amino acid sequence information for IGFBP-1 (4, 5, 9), IGFBP-2 (10), and IGFBP-3 (11). This sequence information was instrumental in developing cloning strategies that were used in the late 1980s to determine the complete primary structures of these proteins (12–16) by cDNA sequencing and for the subsequent sequencing and expression of all six forms of IGFBPs. This allowed sufficient quantities of purified, homogenous forms of these proteins to be widely available, which made functional studies possible for all interested laboratories. This greatly expanded the scope and number of publications in this area, and multiple studies of IGFBP functions were completed.

Abbreviations: ALS, Acid labile subunit; CHO, Chinese hamster ovary; ECM, extracellular matrix; IGFBP, IGF-binding protein; NMR, nuclear magnetic resonance; PAI-1, plasminogen activator inhibitor 1; PAPP-A, pregnancy-associated protein A; pSMC, porcine smooth muscle cells; SMC, smooth muscle cells.

The functions of all six binding proteins have been analyzed extensively. These studies, spanning the years of 1988–1996, in which structure/function relationships were the primary focus of several investigators, have been summarized in several reviews (17–19). Structural studies were undertaken that determined the relative affinities and IGF binding capacity of each form of IGFBP for IGF-I and IGF-II (20, 21). Proteolytic fragments of several forms of IGFBPs were isolated and then used in IGF binding assays to attempt to determine the critical regions in each protein that were required to bind to IGF-I and -II (22–24). Similarly, IGF-I and -II mutants were prepared and were used to identify the critical residues in IGF-I and -II that accounted for high-affinity binding (20, 21). Several functional studies were undertaken to determine the consequences of adding various forms of IGFBPs to biological test systems. The effects of IGFBPs on the ability of IGF-I or -II to stimulate DNA and protein synthesis or to inhibit protein breakdown were determined (25–30). Similarly, their effects on IGF-I-stimulated glucose transport were analyzed (31). Several studies were performed that analyzed the amount of IGF binding capacity that was present in serum and physiological fluids and the variables that controlled these changes (32, 33). Subsequently, RIAs for each form of binding protein were developed, and several studies correlated changes in binding capacity in physiological fluids with the changes in the absolute amount of each form of IGFBP (34–37). These reports were followed by studies that defined the variables that regulated the concentration of each form of IGFBP (38–42).

Studies were undertaken to correlate changes in IGFBP concentrations in extracellular fluids with changes in their functions (43–45). Intense interest was focused on the susceptibility of each of the binding proteins to proteolytic cleavage and characterization of different types of proteolytic activities in different physiological fluids (46–48). Further studies characterized the biological consequences of proteolytic cleavage (49–51). Other functional studies assessed the importance of binding to extracellular matrix (ECM) and to glycosaminoglycans, particularly heparin (52–56). The specific regions of the forms of IGFBPs that bound to glycosaminoglycans were identified. Studies also focused on the various types of posttranslational modifications that occurred, such as phosphorylation and glycosylation, and the functional consequences of these modifications (57–62). Cell surface association of IGFBPs and the effect of cell surface binding on IGF action were important functional parameters that were assessed (63–65). More recently, studies have focused on IGF-independent effects of IGFBPs and modulation of IGF receptor activity by cell surface association of binding proteins (66–70). Presently, there is a great deal of interest in identifying the cell surface proteins that interact with each form of IGFBP and the functional consequences of these interactions (64). Detailed summaries of these data are beyond the scope of this review. However, within each section, we will cite the most important findings that have been used as a rationale for preparing many of the IGFBP mutants, the functions of which are described below.

II. IGFBP Mutagenesis

The availability of cDNAs that encoded each of the IGFBPs made it possible to consider mutagenesis as an approach for analyzing structure/function relationships for each of these proteins. The major advantage mutagenesis offered over prior techniques was that very limited alteration of each protein could be made to diminish the probability that the structural alterations were affecting the functional properties of other domains within that specific form of IGFBP. This has allowed the effect of each discrete change to be correlated with changes in functional activity. By comparing the response of cells or tissues of the mutant form to that induced by the corresponding native form of IGFBP, one could deduce how important that particular residue or set of residues was for each specific function. Furthermore, the development of some of these mutants has made it possible to acquire reagents that have been useful for discovering or confirming new functions of IGFBPs. The first substitution mutant that was created determined the effect of altering the Arg/Gly/Asp sequence of IGFBP-1 (71) on its binding to the $\alpha5\beta1$ integrin and on its ability to stimulate cell migration (see below). Since that time, 30 papers have been published in which various mutant forms of IGFBPs have been created and used to assess structure/function activity relationships. These studies will be summarized in detail below.

A. IGF-binding site localization

To make rational predictions regarding the IGF-binding site, x-ray crystallography would have been the preferred method for predicting the critical regions of each protein that are necessary for binding. However, it has not been possible to obtain protein crystals to perform x-ray crystallography analysis. IGFBPs aggregate at relatively low concentrations, which has made crystal formation and subsequent analysis of crystals impossible. Recently, two-dimensional nuclear magnetic resonance spectroscopy using IGFBP fragments has been used successfully to identify potential binding sites (72). The importance of specific residues within these sites has been confirmed by mutagenesis (73, 74). Some success has also been achieved by using mutagenesis strategies that are based on altering highly conserved sequences (75). The recent determination of the disulfide bonding pattern in several forms of IGFBPs has aided with these analyses (76).

1. Identification of the IGF-binding site(s). The IGFBPs are modular proteins. Since the cysteine-rich regions are clustered in the amino- and carboxyl-terminal ends (Fig. 1), it has been speculated that the disulfide bonding pattern would predict that the two ends of the proteins folded in alignment with one another to create a high-affinity binding site. Some early empiric observations suggested that both the N- and C-terminal domains were important for binding. These included the initial observation that the amino-terminal fragment of IGFBP-3 was capable of binding IGF-I and -II, albeit with relatively low affinity (55, 67, 77, 78). Likewise, the C-terminal domain of IGFBP-2 was shown to have some binding activity (23). This suggested that important binding sites existed within both domains. Spencer and Chan (24) demonstrated that both the amino- and carboxyl-terminal

IGF Binding Protein Sequence Alignment

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                                  *    *      *
1  MSEVPVARVWLVLLLLTVQVGVTAG--------------APWQCAPCSAEKLALC--PPVS------------AS  22
2  MLPRVGCPALPLPPPPLLPPLPLLLLLGASGGGGARAEVLFRCPPCTPERLAACGPPPVAPPAAVAAVAGGARMP  38
3  MQRARPTLWAAALTLLVLLRGPPVARA-GASSGGLG----PVVRCEPCDARALAQCAPPP--------------AV  30
4  MLP--LCLVAA---LLLAA-GP------GPS---LG---DEAIHCPPCSEEKLARCR-PPVG--------------  21
5  M---------VLL--TAV--LLLLAAYAGPAQSLG----SFVHCEPCDEKALSMCPPSPLG--------------  24
6  MTPHR-----LLPPLLLLL-----ALLLAAS-PG-GALA----RCPGCGQGVQAGC---PGG--------------  19

   *         *  *  *  *        *        *       *
1  CSE--VTRS-A-GCGCCPMCALPLGAACGVATARCARGLSCRALPGEQQPLHALTRGQGACVQESDAS--------A  87
2  CAE-LV-REP--GCGCCSVCARLEGEACGVYTPRCGQGLRCYPHPGSELPLQALVMGEGTCEKRRDAEYGASPEQVA 111
3  CAE-LV-REP--GCGCCLTCALSEGQPCGIYTERCGSGLRCQPSPDEARPLQALLDGRGLCVNASAVSRLRAYLLPA 103
4  C-EELV-REP--GCGCCATCALGLGMPCGVYTPRCGSGLRCYPPRGVEKPLHTLMHGQGVCMEL--AEIEA-I-QES  91
5  C-E-LV-REP--GCGCCMTCALAEGQSCGVYTERCAQGLRCLPRQDEEKPLHALLHGRGVCLNEKSYREQVKIERDS  96
6  CVEEEDGGSPAEGCAEAEGCLRREGQCGVYTPNCAPGLQCHPPKDDEAPLQALLLGRGRCLPARAPAVAEENPKES  96

1  PHA---AEAG----SPESPESTEITEEELL--DN-FH-LMAPS---EEDHSILWDAIS-TYDGSKALHVTNIKKWK--  148
2  DNGDDH-SEGGLVENHVDSTMNMLGGGGSAGRKPLKSGMKELAVFREKVTEQHRQMGKGGKHHLGLEEP--KKLRPP  185
3  PPAPGNASESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIIIKKGHAKDSQRYKVDYESQSTDTQNFSSESKRE  178
4  LQPSDKDEGDHPNNSFSPCSAHDRRC----LQKHFAKIR------------DRSTS--GGK--MKVNGAPREDARPV  148
5  REHEEPTT-SEMAEE---TYSP---KIFRPKHTRISELKAEAVKKDRRKKLTQSKFVGGAENTAHPRIISAPEMRQE  176
6  KP--QAGTARPQDVNRRDQQRNPGTSTTP-SQPNSAGVQD-------------------------------------  134

           *                     *       *         * *
1  ---EPCRIELYRVVESLAKAQETSGEEIS-KFY---LPNCNKNGFYHSRQCETSMDGEAGLCWCVYPWNGKRIPG-S  217
2  PARTPCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRGECWCVNPNTGKLIQG-A  262
3  TEYGPCRREMEDTLNHLKFLNVLSPRGV-------HIPNCDKKGFYKKQCRPSKGRKRGFCWCVDKY-GQPLPGYT  249
4  P-QGSCQSELHRALERLAASQ--S-R-THEDLYIIPIPNCDRNGNFHPKQCHPALDGQRGKCWCVDRKTGVKLPG-G  219
5  SEQGPCRRHMEASLQELKA----SPRMVPRAVYL---PNCDRKGFYKRKQCKPSRGRKRGICWCVDKY-GMKLPG-M  234
6  TEMGPCRRHLDSVLQQLQTEVY---RGA-QTLY---VPNCDHRGFYRKRQCRSSQGQRRGPCWCVDRM-GKSLPG-S  202

        *
1  PEIRGDPNCQIYFNVQN------------------------------------------------------234
2  PTIRGDPECHLFYNEQQEARGVHTQRMQ------------------------------------------291
3  TKGKEDVHCYSMQSK--------------------------------------------------------264
4  LEPKGELDCHQLADSFRE-----------------------------------------------------237
5  EYVDGDFQCHTFDSSNVE-----------------------------------------------------252
6  PDGNGSSSCPTGSSG--------------------------------------------------------217
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FIG. 1. Amino acid sequence alignment of the complete amino acid sequences of all six human IGFBPs. The *stars* show the positions of the cysteines that are clustered in the amino- and carboxyl-terminal ends of each protein and are conserved in each of the proteins except IGFBP-6. The midregion of each protein does not contain cysteines. Residues that are conserved in at least four of the six proteins are shown in *bold*.

regions of IGFBP-3 had some binding activity, and Ho and Baxter (79) confirmed that the carboxyl-terminal fragment of IGFBP-2 had activity. Brinkman *et al.* (80) demonstrated that a significant deletion of the carboxyl-terminal region of IGFBP-1 resulted in a substantial reduction in its affinity for IGF-I.

Multiple investigators reported findings that suggested the presence of an important binding site in N-terminal domain fragments that terminated somewhere near the twelfth cysteine in each of the proteins (22, 24, 67, 77, 78, 81). Several residues in this region have side chains that could potentially interact with the appropriate side chains of residues in IGF-I or IGF-II that were known to be important for binding IGFBPs (20, 21). These investigators proposed that the N-terminal region contained the absolute basal binding subunit that was used by all six forms of IGFBPs (72, 81, 82). They further speculated that this region interacted with an important binding domain in the C terminus of each protein to establish the high-affinity binding site. Since empiric studies showed that the affinity of fragments containing this N-terminal domain-binding subunit was relatively low, a model was put forth that proposed that N-terminal region was absolutely required for binding and that the principal role of the C-terminal domain was to establish high affinity, but that it was not absolutely required for binding (72, 81, 82).

Based on these preliminary findings, two groups of investigators used the known information regarding the N-terminal binding domain of IGFBP-3 and prepared peptides that contained the putative binding domain. Hashimoto *et al.* (81) prepared a 41-residue peptide from positions Glu 52 to

Ala 92 of rat IGFBP-3. Using a solid-phase binding assay, they were able to demonstrate that this fragment had binding activity, although its relative affinity for IGF-I was only 0.008% of the native molecule. The affinity of a fragment containing residues 1–92 was decreased 25-fold. If the mid-portion of IGFBP-3 was included, the resulting fragment (1–186) had an affinity that was reduced 8-fold. Therefore, it was assumed that the basal binding subunit was contained in the sequence encompassing the Glu 52 to Ala 92, but that the flanking sequences in both the N-terminal and C-terminal regions adjacent to this sequence were important for folding. Disruption of the disulfide bonds, even within the short sequence between residues 52–92, resulted in complete loss of binding, suggesting that folding of this sequence into the correct conformation was critical.

This work was followed by an important paper by Kalus *et al.* (72), which analyzed this region of IGFBP-5 by two-dimensional nuclear magnetic resonance (NMR) spectroscopy. This region of IGFBP-5 is nearly identical in sequence to IGFBP-3. A fragment of IGFBP-5 containing residues 1–94 was prepared by limited proteolytic digestion of the whole protein, as well as a C-terminal fragment from residues 135–246 and a central fragment containing residues 95–134. An additional fragment, the sequence of which initiated at Ala 40 and ended at Glu 92, was contained in this proteolytic digest. BIAcore analysis was used to determine the binding affinities of each fragment for IGF-I and -II. Neither the C-terminal fragment (residues 135–246) nor the midregion fragment (residues 95–134) had detectable affinity. In contrast, the amino-terminal fragment (residues 1–94) and a

smaller fragment (containing residues 40–92) had affinities that were reduced 200-fold compared with full-length IGFBP-5, but binding was easily detectible. The association rate of IGF-I binding to the 40–92 fragment approximated that of the native protein, but its binding to this peptide was associated with a much more rapid dissociation rate. The affinity of the Ala 40-Glu 92 fragment was sufficient to inhibit IGF-II-stimulated phosphorylation of the IGF-I receptor. From these data, it was possible to construct a three-dimensional model of the region contained within the mini-IGFBP-5 sequence that is shown in Fig. 2. This structure formed a uniquely folded domain containing three antiparallel β-strands and two cysteine bridges that were responsible for its compact structure. The structure is further rigidified when IGF-I or -II binds to the peptide. Disulfide bonds are formed between cysteine 47 and cysteine 60 and cysteine 54 and cysteine 80. These pairings are also present in IGFBP-2, -3, and -6 (81, 83).

Using this model, it was predicted that Phe 48 of IGF-II would dock into the hydrophobic patch composed of Val 49, Leu 70, and Leu 73 within IGFBP-5. Glu 6 of IGF-II would interact with Lys 68, which is adjacent to the hydrophobic patch. When Kalus et al. (72) used NMR to analyze the largest interaction-induced spectral shifts that occurred in this peptide after IGF-II binding, it was noted that the residues Val 49, Tyr 50, Pro 62, and Lys 68 to Leu 74 within IGFBP-5
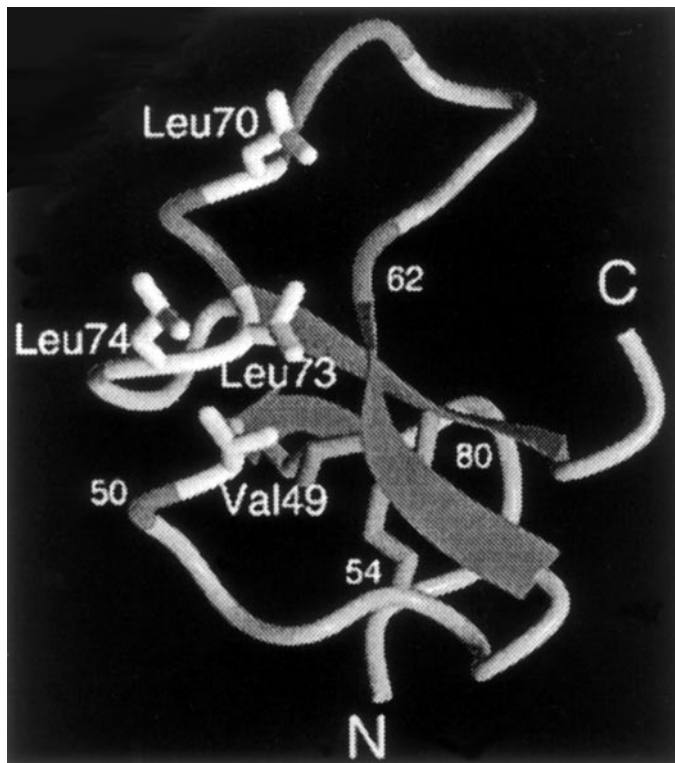

FIG. 2. Tertiary structure of the hydrophobic amino acid alignment of the IGF-binding pocket of the mini-IGFBP-5. The figure shows the position of the charged and hydrophobic amino acids that are believed to form the high-affinity binding pocket in the N-terminal region of IGFBP-5. These amino acids have been proposed to interact with amino acids containing specific side chains and charged groups within IGF-I and IGF-II. [Reprinted with permission from: W. Kalus et al.: EMBO J 17:6558–6572, 1998 (72).]

underwent the greatest changes, indicating that strong molecular interactions occurred at these residues within the binding cleft. Within the region Lys 68 to Leu 74 the strongest interactions were noted for Leu 70 and 73. When the 1–92 fragment was analyzed, the peaks were nearly identical to that of the 40–92 fragment, suggesting that the first 39 residues constitute an independent domain that has very little influence on the structure of the primary IGF-binding domain. Therefore, the investigators concluded that the primary binding site was comprised of Val 49, Tyr 50, Pro 62, and Lys 68 through Leu 74. Contrary to the C-terminal fragment data that had been generated for IGFBP-2 and -3, they could find no residual binding activity in the C-terminal fragment of IGFBP-5 that contained residues 134–246. The affinity constant of mini-IGFBP-5 fragment was in a range reported previously for the N-terminal fragment by Andress et al. (22) and for the N-terminal fragment of IGFBP-3 reported by Clemmons (84). Since there was no significant difference in affinity between the 1–92 fragment and the 40–92 fragment, the authors concluded that full affinity of the native protein is not due to these additional residues, but rather is due to complex formation with the C-terminal domain residues 135–246 and that this probably resulted in stabilization of the intact protein (see below).

The hydrophobic residues, Val 49, Leu 70, and Leu 74, expose their hydrophobic side chains in solution in a specific alignment that defines the hydrophobic patch on the surface of this IGFBP-5 fragment. In addition, Val 49, Leu 73, and Leu 74 of IGFBP-5 are conserved among all IGFBPs, suggesting that this module may be important for binding of other forms of IGFBPs to IGF-I or IGF-II. Site-directed mutagenesis studies of IGF-I and IGF-II have indicated that residues Glu 6, Phe 48, Arg 49, and Ser 50 are important for binding IGFBPs (21, 85–87). Phe 48 in IGF-II is located in a position where it could bind to Leu 73, Leu 74 of mini-IGFBP-5, and Glu 6 of IGF-II would interact well with Lys 68. Since the sequence between positions 62 and 69 is less conserved among other IGFBPs, it could be responsible for the variations in affinity for IGF-I and -II among the six binding proteins. This is supported by the observation that this region is conserved in IGFBP-3, which has an affinity for IGF-I and -II that is equal to IGFBP-5. Likewise, conservation of a basic residue at position 68, which occurs in IGFBP-3, -4, and -5 appears to be necessary for the highest affinity.

The important observations of Hobba and co-workers (88) support this model of IGFBP/IGF interaction. Specifically, in examining IGFBP-2 binding to IGF-I and -II, they noted iodination of Tyr 60 in bovine IGFBP-2 resulted in a 3-fold reduction in affinity for IGF-II. Furthermore, they were able to show that when IGF-II formed a complex with IGFBP-2, Tyr 60 was protected from iodination, suggesting that it was no longer surface exposed. Based on molecular modeling, they hypothesized that Val 59, Thr 61, Pro 62, and Arg 63 also contributed to IGF binding, partly because these residues are conserved in most forms of IGFBPs. To confirm this hypothesis, they used alanine scanning mutagenesis and mutated each residue between valine 59 and arginine 63. They determined that replacement of Tyr 60 with an alanine or phenylalanine reduced the affinity of IGFBP-2 for IGF-I by 4-fold and 8.4-fold, respectively, and for IGF-II by 3.5- and 4.0-fold,

respectively (89). In contrast, single mutations of the other residues did not significantly affect the affinity of IGFBP-2 for IGF-II. They concluded that Tyr 60 was either a key component of the binding site or was located in a strategic region of the binding site in such a way that binding site conformation was disrupted when this amino acid was substituted with a nonaromatic amino acid. This is consistent with the model of Kalus *et al.* (72), since Tyr 60 of IGFBP-2 is conserved in IGFBP-5 and corresponds to Tyr 50, which is a key component of the binding pocket. In keeping with the Kalus *et al.* (72) model, they noted using BIAcore analysis that IGF-II disassociated from Ala 60 IGFBP-2 6.4-fold more rapidly compared with native IGFBP-2.

A reccent study using mutagenesis has confirmed the importance of this amino-terminal binding site, and it supports the conclusions that the model proposed by Kalus *et al.* (72) accounts best for high-affinity IGF binding. Imai *et al.* (73) mutated five of the seven residues in the amino-terminal region of IGFBP-5 postulated by Kalus *et al.* to form the primary IGF-binding site. Four of these residues that were hydrophobic were changed to nonhydrophobic residues, and the charged Lys 68 was changed to a neutral residue. Unlike the studies of Kalus *et al.*, however, this study determined the effect of these substitutions on the affinity of the entire protein for IGF-I and IGF-II. These substitutions resulted in a substantial (>200-fold) reduction in affinity of IGFBP-5 for IGF-I as determined by solution binding assays. In addition, the substitutions resulted in marked attenuation of the ability of IGFBP-5 to inhibit IGF-I-stimulated receptor phosphorylation. The substitutions did not appear to markedly change the conformation of IGFBP-5, since it was fully susceptible to proteolytic cleavage. When the homologous substitutions were made in IGFBP-3, they also resulted in marked reduction of affinity of this protein for IGF-I. Specifically, when the same residues were altered in exactly the same way in IGFBP-3, its affinity for IGF-I was lowered approximately 400-fold, and the ability of IGFBP-3 to attenuate IGF-I-stimulated DNA synthesis was completely abolished. This *in vitro* mutagenesis study confirms the model of Kalus and colleagues, suggesting that these residues in the amino terminus of IGFBP-3 and -5 are required for the formation of a high-affinity IGF-I-binding pocket in the intact protein.

A third study supports the model of Kalus and associates and has extended that model by making substitutions in the C-terminal region of IGFBP-4 that were predicted to alter its folding pattern (82). These investigators attempted to model the entire IGF binding motif that is required to form the high-affinity binding site and to include alterations that would significantly change its tertiary structure. Multiple deletion mutants of IGFBP-4 were prepared. The results showed that the N-terminal sequence of IGFBP-4, Leu 72 through Ser 91, was essential for IGF binding. A mutant form of IGFBP-4 was made in which this 19-amino acid linear sequence had been deleted. In addition, fragments from His 121 to Glu 237 and Arg 142 to Glu 237 were prepared. Neither of these C-terminal fragments bound to IGF-I, but the loss of these regions resulted in a substantial reduction in IGF binding activity. Mini-deletion analysis of the region from Cys 205 to Val 214 revealed that this region was critical for op-

timum affinity, and this deletion resulted in a 6-fold reduction in its affinity. The authors concluded that the N-terminal sequence Leu 72 to Ser 91 contained the basal binding subunit and the region from Cys 205 to Val 214 was necessary for proper folding and attainment of high-affinity binding. When one examines Fig. 3, one can discern that Leu 72 in IGFBP-4 corresponds to Leu 73 in IGFBP-5 and that truncation at His 70 would result in loss of Leu 72. Although Kalus *et al.* (72) did not alter His 71 in IGFBP-5, it is located in a critical portion of the IGF-binding pocket, and a disruption of this residue might interfere with the conformation of this region. Leu 73 is a critical residue in the IGFBP-5-binding pocket, since multiple energy shifts were noted when IGF-II bound to this residue in IGFBP-5. Therefore, the model developed by Kalus *et al.* would predict that the equivalent substitution in IGFBP-4 (*e.g.*, Leu 72) would alter its affinity.

Bramani *et al.* (75) and Song *et al.* (90) have proposed that the C-terminal domain of IGFBP-5 is necessary for optimal IGF-I binding. They determined that two specific amino acids in the C-terminal region of IGFBP-5 were critical for maintenance of high-affinity binding. Specifically, these investigators used site-directed mutagenesis to mutate Gly 203 to Lys and Gln 209 to Ala. This caused an 8-fold reduction in affinity of IGFBP-5 for human IGF-I. Single substitutions resulted in 7- and 6-fold reductions, respectively. Because these residues are conserved in all six binding proteins, these investigators speculated that it would be possible to extrapolate this result to all six forms of IGFBPs. Since this region of IGFBP-5 has also been shown to be very important for ECM binding (see below), these investigators speculated that this may partially explain the finding that when IGFBP-5 binds to ECM, its affinity for IGF-I is reduced 8-fold. The investigators hypothesized that either this region contained a primary binding site or that these substitutions resulted in a major alteration in its alignment with the N-terminal region-binding site, thus disrupting the affinity of the whole protein. The latter explanation seems to be favored, based on the data of Kalus *et al.* showing that the C-terminal fragment has no intrinsic binding activity, thus suggesting that the role of this sequence is primarily to align correctly with the N-terminal sequence to form the high-affinity site. This work was followed by a paper (90) in which these investigators mutated residues that were adjacent to these sites (*e.g.*, R 201, K 202, K 206, and R 214). This resulted in a major attenuation in heparin binding, which had been shown previously (91), but resulted in no loss in affinity for IGF-I (90). Conversely, substitutions for Gly 203 and Gln 209 resulted in no change in the affinity of this form of IGFBP-5 for heparin. Since binding of IGFBP-5 to ECM or to heparin had been shown to

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IGFBP-1    48VATARCARGLSCRALPGEQQPLHALTR74
IGFBP-2    64VYTPRCGQGLRCYPHPGSELPLQALVM90
IGFBP-3    56IYTERCGSGLRCQPSPDEARPLQALLD82
IGFBP-4    48VYTPRCGSGLRCYPPRGVEKPLHTLMH74
IGFBP-5    49VYTERCAQGLRCLPRQDEEKPLHALLH75
IGFBP-6    49VYTPNCAPGLQCHPPKDDEAPLQALLL75
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Fig. 3. Sequence comparisons of the region within each form of IGFBP that contains the amino-terminal IGF-binding site. The residues that form the binding site have been determined for IGFBP-5 and are *underlined*.

lower the affinity of IGFBP-5 for IGF-I, this suggests that the heparin/ECM-binding domain and the IGF-binding domain overlap. The authors further proposed that the ability of heparin binding to alter the affinity of IGFBP-5 (56) for IGF-I is due, at least in part, to steric hindrance (90).

A study that further analyzed IGFBP-3 supports these conclusions. Firth *et al.* (92) demonstrated that substitutions for five amino acids in IGFBP-3 (K228–R232) with residues from the corresponding sequence in IGFBP-1 resulted in a substantial reduction (20-fold) in the affinity of IGFBP-3 for IGF-I as assessed by cross-linking. These residues are homologous to the R214–R218 sequence in IGFBP-5. They further showed that deletion of IGFBP-3 residues from 185–264 or from 89–184 markedly reduced IGF-I and -II binding. However, some affinity of the 1–89 fragment was retained, consistent with the results of Kalus *et al.* that the amino terminus contains a residual binding site.

In summary, the primary binding site in IGFBP-3 and 5 is comprised of seven residues that are located in the amino-terminal regions of each protein (Fig. 3). Comparisons of homologous residues in IGFBP-1, -2, -4, and -6 show that Val 49 of IGFBP-5 is conserved in all six forms of IGFBP and that Tyr 50 is conserved in all but IGFBP-1. Lys 68 of IGFBP-5 is conserved as a charged residue in IGFBP-3 and -4. Pro 69, Leu 70, and Leu 73 of IGFBP-5 are conserved in all six forms. A charged residue that corresponds to the His 71 position is conserved in IGFBP-1 and -4. Ala 72 is present in five of the six proteins (17). This remarkable degree of conservation of these residues in several of the forms of IGFBPs suggests that they are very important for IGF binding. However, definitive proof that the model of Kalus *et al.* is correct will require the creation of a combined mutant, where the C-terminal and N-terminal portions are both altered. More detailed analyses of the solution structure of the IGF-IGFBP complex by NMR would also help to determine which putative contact points are used.

The availability of these mutants has provided an important and useful set of reagents for assessing IGFBP functional activity. Recently, it has been determined that IGFBPs have several properties that are independent of their ability to alter IGF-I and IGF-II interactions with the IGF-I receptor. IGFBPs have been shown to attach to cell surfaces independently of IGF binding, to bind to heparin and proteoglycans, to bind ECM, to undergo nuclear localization, and, when cell surface associated, to lower the affinity of the IGF-I receptor for IGF-I. Likewise, fragments of IGFBPs have been shown to have inhibitory or stimulatory effects on IGF-I actions that are independent of IGF-I binding. Finally, IGFBPs have been shown to bind to several other proteins with relatively high affinity. Although the biological significance of most of these interactions is unknown, many occur independently of IGF binding. The availability of these mutants should make it possible to determine which of these activities can be easily detected in the absence of IGF-I binding. This will be of value in the analysis of complex cellular functions, such as induction of p53 or inhibition of cellular transformation, and in determining which structural features of the IGFBPs account for these activities. Since studies in which high concentrations of IGFBP-3 were administered to whole animals have not reported IGF-independent effects that are similar to those

reported in several *in vitro* studies, the ability to reproduce these *in vitro* findings in whole animals using these mutants will be critical for determining their physiological significance.

B. Proteolytic cleavage sites

Although the examples are limited, mutagenesis of proteolytic sites has been extremely successful in altering susceptibility of IGFBPs to proteolysis and for subsequently determining the physiological significance of proteolytic cleavage in well defined test systems. The existence of IGFBP proteases was first reported in 1990, when it was noted that pregnancy serum contained a protease that cleaved IGFBP-3 (46, 93, 94). Importantly, it was noted that the fragments that were generated from such cleavage had significantly reduced affinity for IGF-I or -II. Therefore, a major property of IGFBPs, that of forming a complex with the IGFs and preventing their access to receptors, was markedly altered by cleavage. Subsequently, proteolytic cleavage of IGFBP-1 through -6 has been described (55, 95–98). Generally, these proteolytic cleavages and their consequences have been studied using cells in tissue culture (98–103) although the results of some *in vivo* analyses have shown that proteases that cleave IGFBPs are present and are active in physiological fluids *in vivo* (104–110). In general, the proteolytic cleavage sites within individual forms of IGFBPs have been defined by purifying the fragments and then determining the N-terminal amino acid sequence of the fragment that is distal to the cleavage site. Other methods that have been used include ionization mass spectroscopy, and immunoblotting with antibodies that have very well defined, specific epitopes, although this method is less precise.

1. IGFBP-4 proteolysis. To determine the cleavage site of IGFBP-4, Chernausek *et al.* (78) purified a C-terminal fragment of rat IGFBP-4 that was generated after cleavage in the conditioned medium of rat neuronal cell line (B104 cells). These investigators had determined previously that the protease released by these cells was a serine protease (101). Therefore, it was reasonable to predict that dibasic residues might be the site of proteolytic cleavage. To determine this, the investigators purified the carboxyl-terminal fragment that occurred after cleavage and undertook amino acid sequencing to determine N-terminal sequence of the fragment. Sequence analysis revealed that Lys 120, His 121 was the probable cleavage site, since His 121 was the first amino acid in the C-terminal fragment that could be detected. Conover *et al.*, using a similar approach but a different cell line (*e.g.*, human fibroblast conditioned medium) showed that cleavage occurred at Lys 134 Met 135 (51). Both groups then undertook mutagenesis to prove their hypotheses. Chernausek *et al.* (78) mutated Lys 120 and His 121 to alanines and then expressed the mutagenic protein. This protein was purified to homogeneity and then tested for resistance to cleavage using the partially purified B104 cell protease. The protein was nearly completely resistant to proteolysis, although extending the incubation time resulted in cleavage at the site defined by Conover and co-workers (113) (Fig. 4). As further proof that this cleavage had physiological signif-
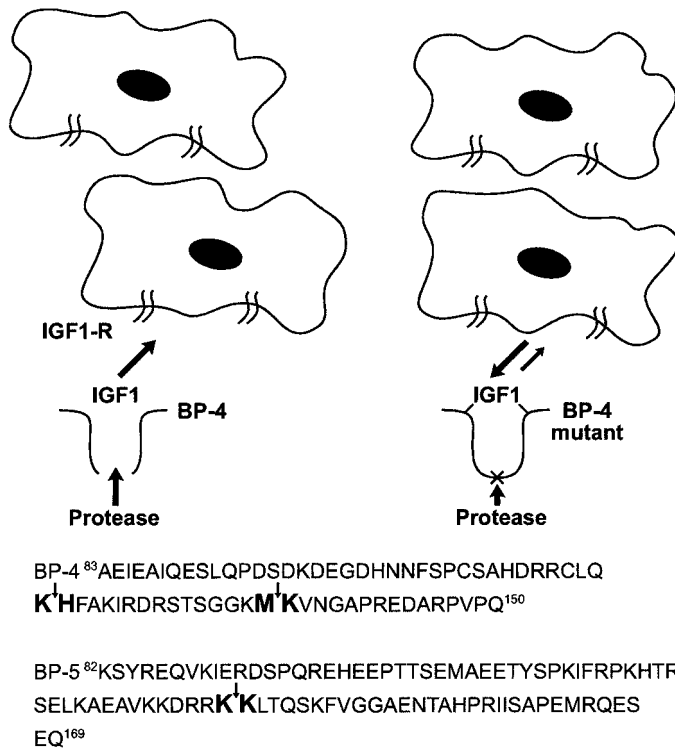
BP-4 [83]AEIEAIQESLQPDSDKDEGDHNNFSPCSAHDRRCLQ
Kʹ**H**FAKIRDRSTSGGK**M**ʹ**K**VNGAPREDARPVPQ[150]

BP-5 [82]KSYREQVKIERDSPQREHEEPTTSEMAEETYSPKIFRPKHTR
SELKAEAVKKDRR**K**ʹ**K**LTQSKFVGGAENTAHPRIISAPEMRQES
EQ[169]

FIG. 4. Proteolysis of IGFBP-4 and IGFBP-5. The *schematic diagram* illustrates the consequences of inhibiting IGFBP-4 proteolysis by mutagenesis. In normal physiological conditions IGFBP-4 is cleaved (*left panel*), and the bound IGF-I is released to receptor. Mutagenesis of the cleavage sites (*right panel*) causes less IGF-I to be released to receptors and inhibits IGF-I actions. The location of the two cleavage sites in IGFBP-4 that have been altered by mutagenesis are shown. The primary cleavage site in IGFBP-5 that has been altered by mutagenesis is shown for comparison.

icance, Chernausek *et al.* (78) added this protein to B104 cells and showed that it had greater capacity to inhibit the effects of IGF-I in these cells compared with the wild-type non-mutated form of IGFBP-4. Conover *et al.* (51) likewise demonstrated that a protease-resistant mutant prepared with alterations in Lys 134, Met 135 resulted in resistance to cleavage by human fibroblast conditioned medium. They also showed their mutant form of IGFBP-4 inhibited IGF-I-stimulated actions in both cultured fibroblasts and osteoblasts (98). Both groups were able to demonstrate that the alteration of the residues at these sites did not alter the affinity of IGFBP-4 for IGF-I, suggesting that mutagenesis did not severely alter the conformation of the protein and that these residues were not involved in IGF binding.

More recently, Qin *et al.* (111) have followed up this work, showing that protease-resistant forms of IGFBP-4 that have been prepared by mutagenesis are potent inhibitors of the effect of IGF-II on bone formation. Similarly, Rees *et al.* (112) showed that a K120N, H121N, human IGFBP-4 mutant was resistant to proteolysis and that it potently inhibited the smooth muscle cell replication response to IGF-I. Recently, the preparation of an IGFBP-4 mutant, in which all four residues that flanked the two cleavage sites were altered, has shown that this has greater protease resistance than either of the double mutants prepared previously. Most recently a

six-point IGFBP-4 mutant has been prepared. The same region of IGFBP-4 that contains the Lys 120, His 121 residues also contains an additional four basic residues. If all six basic residues are mutated to neutral residues, the protein is totally protease resistant. Currently, biological studies to characterize the usefulness of this protein as a reagent for inhibiting IGF action *in vivo* are being undertaken. Recently, pregnancy-associated protein A (PAPP-A), a metalloprotease that is present in human pregnancy serum, has been shown to cleave IGFBP-4 (113, 114). Qin *et al.* (115) have shown that PAPP-A does not cleave the M135 A K136A mutant IGFBP-4, suggesting that it is an important cleavage site for PAPP-A. An important property of IGFBP-4 proteolysis is that IGF-I or -II must bind to IGFBP-4 to detect cleavage. When Qin *et al.* (115) used a Lys 67 to His 74 deletion mutant of IGFBP-4 that did not bind IGF-II, the protease activity in human osteoblast medium had no ability to cleave IGFBP-4. In summary, it appears that both PAPP-A and an unknown serine protease cleave IGFBP-4 and that they use distinct cleavage sites. These studies have made it possible to create protease-resistant forms of IGFBP-4, and studies utilizing these mutant forms have allowed investigators to conclude that intact IGFBP-4 inhibits IGF actions in several biological test systems. Future studies using mutant forms of IGFBP-4 that are resistant to several proteases should allow more precise determination of the functions of this form of IGFBP *in vivo*.

2. IGFBP-5 proteolysis. A second form of IGFBP that has been studied extensively to determine the consequences of proteolysis is IGFBP-5. Initially, Imai and co-workers (50) identified the cleavage site of IGFBP-5 by isolating a C-terminal fragment that appeared after proteolytic cleavage by cultured human fibroblasts. Previous studies had shown that fibroblasts released a serine protease that degraded IGFBP-5 (116). After IGFBP-5 degradation, the C-terminal fragment was isolated and purified to homogeneity, and its amino-terminal sequence was determined. Sequencing revealed cleavage sites at Lys138–Lys139 and Ser143–Lys144 (Fig. 4). Cleavage at the latter site appeared to have occurred after the initial cleavage at Lys 138 and Lys 139 probably by an aminopeptidase that was activated. Based on these results, a mutant in which Lys 138 and Lys 139 were converted to alanines was prepared using site-directed mutagenesis. This mutant was then purified to homogeneity and tested for stability. After a 24-h incubation with the fibroblast cultures, no cleavage of this mutant could be detected. To further test the hypothesis, the protease was highly purified from fibroblast conditioned medium and retested against the mutant form. Even in the presence of purified protease, the K138N, 139N mutant was resistant to proteolytic cleavage. Other cell types, such as osteoblasts and porcine smooth muscle cells (pSMC) that release this protease, were used to show that this mutant form was completely resistant to cleavage. To determine the biological actions of this mutant, it was incubated with pSMC cultures. Increasing concentrations of the mutant were found to be far more potent than wild-type IGFBP-5 in inhibiting DNA synthesis in this cell type (50). Specifically, 200 ng/ml of the IGFBP-5 mutant caused a 50% reduction in the ^{3}H-thymidine incorporation response to 20 ng/ml of IGF-I. In contrast, between 5–10 μg/ml of native IGFBP-5

was required to achieve the same degree of inhibition. Analysis of the medium at the end of the experiment showed that most of the wild-type IGFBP-5 that had been added had been cleaved, whereas all of the mutant IGFBP-5 remained intact. To determine whether constitutive synthesis of this mutant could inhibit IGF-I actions, pSMC were transfected with the cDNA encoding the protease-resistant mutant. Subsequently, it was shown that the ability of IGF-I to stimulate protein synthesis was inhibited compared with cells that expressed similar amounts of the wild-type protein. As a control, des 1–3 IGF-I that does not bind to the mutant was tested. It was fully active in stimulating both protein and DNA synthesis, suggesting that expression of the mutant form of the protein did not have inhibitory activity that was independent of its capacity to inhibit IGF-I binding to the IGF-I receptor. To further ascertain whether this mutant could alter IGF-I signaling, the mutant was added with IGF-I, and the ability of IGF-I to stimulate the phosphorylation of PI3K was assessed. The mutant form was a potent inhibitor of PI3K induction by IGF-I. This suggested that the mutant was inhibiting IGF-I activation of its receptor, thus leading to inhibition of downstream signaling. This mutant has also been useful for determining whether an IGFBP proteolytic activity that is present in cell culture medium can be attributed to a known serine protease. For example, Zheng *et al.* (117) were able to use the K138N, K139N IGFBP-5 mutant to show that although thrombin cleaved IGFBP-5, it did not account for the IGFBP-5 protease activity that was secreted by fibroblasts. They showed that thrombin cleaved the IGFBP-5 mutant and that addition of the mutant IGFBP-5 to cultures that were also exposed to thrombin did not result in inhibition of IGF-I-stimulated fibroblast DNA synthesis.

In summary, these mutants have allowed us to assess the importance of IGFBP-5 cleavage in the extracellular fluids for altering IGF-I actions on cultured cells. In addition, they have been useful for determining the presence of proteases in physiological fluids that cleave IGFBP-5 at sites that have not been previously identified. Since fragments of IGFBP-5 have been shown to possess biological activity, the protease-resistant mutants offer a unique way to investigate the physiological significance of the accumulation of specific proteolytic fragments. Second, since IGFBP-5 has been shown to further potentiate and inhibit IGF actions, these mutants may be very useful in determining the role of proteolysis in regulating its bifunctional activities. Because they are very stable, they should be useful in future *in vivo* studies to further determine IGFBP-5 actions.

C. The use of mutagenesis to assess the physiological significance of binding to ECM

Biochemical analysis of the ECM derived from a variety of cell types has shown that IGFBP-5 is the most abundant form of IGFBP in ECM derived from connective tissue cells. Some studies have found that minimal concentrations of IGFBP-3 are present in ECM (54). This may be because the amino acids in IGFBP-5 that are major determinants of ECM binding are also conserved in IGFBP-3 (see below). However, in spite of this sequence similarity, there are major differences in the amounts of IGFBP-3 and IGFBP-5 that are contained in ECM.

This suggests that the region of IGFBP-5 that accounts for ECM binding is folded differently in IGFBP-3 such that there is less surface exposure of this sequence.

To determine the amino acids within IGFBP-5 that bound to ECM proteins, attention was focused on two highly basic regions that were present in the protein. One sequence was located between amino acids 131 and 141 and the other between amino acids 201 and 218. Small peptides were prepared that contained the sequences in each region, and their ability to inhibit IGFBP-5 binding to human fibroblast ECM was determined. Although the 131–141 region contained a higher percentage of basic residues, it accounted for very little ECM binding, and the principal ECM-binding site was located in the 201–218 region (54, 118) (Fig. 5). To further discern the importance of this region for ECM binding, mutagenic peptides were synthesized. Neutral substitutions for the basic residues at positions 211, 214, 217, and 218 in this peptide resulted in a major reduction in the ability of this mutant to compete with native IGFBP-5 for binding to ECM (119). A second peptide in which the charged residues at positions 202, 206, and 207 had been altered to neutral residues was also shown to have a major reduction in its ability to inhibit binding (119). Based on these data, mutant forms of intact IGFBP-5 that contained these combinations of substitutions were prepared. Table 1 shows the full range of substitutions that were tested. As shown in the table, by far the most potent inhibitors of ECM binding were the two combinations that were described above: substitutions for the basic amino acids at positions 211, 214, 217, and 218 with neutral residues or substitution for the combination of basic amino acids at positions 202, 206, and 207. Further detailed
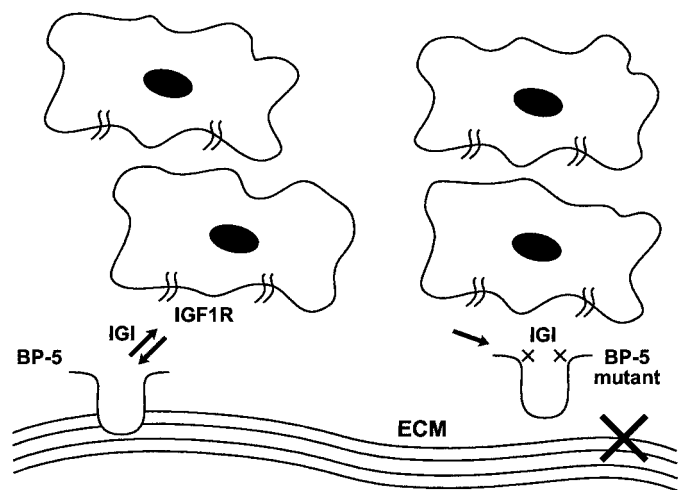
^{123}RISELKAEAV<u>KKDRRKKL</u>TQSKFVGGAENTAHPRIISAPEMRQESE
QGPCRRHMEASLQELKASPRMVPRAVTYLPNCD
<u>RKGFYKRKQCKPSRGRKR</u>$_{218}$

FIG. 5. ECM binding of IGFBP-5. Association of IGFBP-5 with ECM alters IGF-I actions. When IGFBP-5 binds to ECM, its affinity is lower, and bound IGF-I equilibrates more favorably with the IGF-I receptor. When the charged residues in the *underlined* sequence between amino acids 201 and 218 are altered, the IGFBP-5 mutant does not bind to ECM and it inhibits IGF-I binding to its receptor. A second sequence containing several charged residues between 133 and 139 is *underlined*. This region is important for binding to glycosaminoglycans and ALS.

TABLE 1. Relative affinities of IGFBP-5 mutants for ECM

Mutant	% of Maximum affinity
Nonmutated IGFBP-5	100
K211N	100
R201A/K202N	56
R201A/K202N/K206N/K208N	47
K217A/R218A	34
K207A/K211N	9.4
R214A	8.9
K202A/K206A/R207A	6.1
K211N/R214A/K217N/R218A	7.7

The affinity constants were determined using Scatchard analysis. The results are expressed as the affinity of each mutant divided by the affinity of the native IGFBP-5 × 100.

analysis revealed that a single mutation at R214 resulted in significant loss of binding, but no other single amino acid substitution caused a substantial reduction. Significant reductions were obtained with dual substitutions for 207 plus 211, 217 plus 218, or 201 plus 202. The effect of mutants with these substitutions was substantially less compared with the other three mutants discussed previously. This indicates that the most important residue is R214 and that R217, R218, K202, and R207 have some importance.

To determine the functional significance of this loss of ECM binding, two types of experiments were conducted. First, IGFBP-5 or its mutants were layered onto ECM. Cells were plated on top of this matrix, and then the ability of IGF-I to stimulate DNA synthesis or cell growth was determined. In all cases, the ability of IGF-I to stimulate cell growth correlated with the amount of IGFBP-5 that was present in the ECM (54, 118). Cultures that were exposed to either of the two mutants with the greatest reduction in matrix binding (*e.g.*, 211, 214, 217, 218; or 202, 206, 207 substitution mutants) had a marked reduction in their cell growth response to IGF-I (119). This ability of ECM-associated IGFBP-5 to enhance the cell growth response to IGF-I is believed to be due to the fact that when IGFBP-5 binds to ECM, there is an 8-fold reduction in its affinity for IGF-I (54). This allows much better equilibration between IGF-I bound to IGFBP-5 in the ECM and the IGF-I receptor. To further prove this hypothesis, cDNAs encoding these mutants were transfected into primary smooth muscle cell cultures. When the mutants were constitutively synthesized, these alterations of basic residues resulted in reduction in the amount of IGFBP-5 that was associated with the ECM (120). As was shown for mutant forms of IGFBP-5 that had been added exogenously, the ability of these cells to respond to IGF-I with an increase in ^{3}H-thymidine incorporation was proportionate to the amount of IGFBP-5 localized in the ECM (120). When cells expressing the mutants that bound poorly to ECM were compared with cells expressing wild-type IGFBP-5, there was a 7-fold difference in the amount of IGFBP-5 incorporated into ECM. Mutants with less disruption, such as the R217A, R218A IGFBP-5 mutant, showed intermediate levels of ECM association. When the ^{3}H-thymidine incorporation was assessed, the cells expressing the two mutant forms with the greatest decrease in ECM binding showed marked attenuation in their ^{3}H-thymidine incorporation responses to IGF-I. The levels of ^{3}H-thymidine incorporation were comparable to those seen with IGF-I

alone, whereas when wild-type IGFBP-5 was expressed, an approximately 2.5-fold greater increase in their ^{3}H-thymidine incorporation responses to 20 ng/ml of IGF-I was achieved. In summary, the region of amino acids between R201 to R218 in IGFBP-5 determines ECM binding. Constitutive expression of IGFBP-5 mutants that have neutral substitutions for basic amino acids in this region that are required for ECM binding is associated with a reduced response to IGF-I. This confirms the importance of ECM-associated IGFBP-5 for enhancing IGF-I action in mesenchymal cells. Since IGFBP-5 has been found to be localized in osteoblast and chondrocyte ECM (121, 122), this suggests that the findings obtained using SMC and fibroblasts may be relevant to these cell types.

Further molecular definition of the matrix-binding site, as deduced from the results of mutagenesis studies, has been possible. Specifically, when helical wheel analysis is conducted using the 201–218 region of IGFBP-5, an interesting pattern unfolds. As shown in Fig. 6, the residues that are the most critical for ECM binding are asymmetrically aligned in the wheel, suggesting that if this region of the protein does form an α-helix, it would result in alignment of four of these charged amino acids, and this region of high charge density could mediate a high-affinity electrostatic interaction with other ECM proteins. Interestingly, the residues that were altered by Song *et al.* that resulted in an 8-fold reduction in the affinity of IGFBP-5 for IGF-I are adjacent to this cluster of basic residues (90). This suggests that the ECM and IGF-I-binding sites in IGFBP-5 overlap, and that IGFBP-5 binding to ECM has an effect on its affinity for IGF-I that is similar to mutagenesis of residues 203 or 209. Therefore, IGFBP-5 binding to ECM would reduce the access of IGF-I to this site,
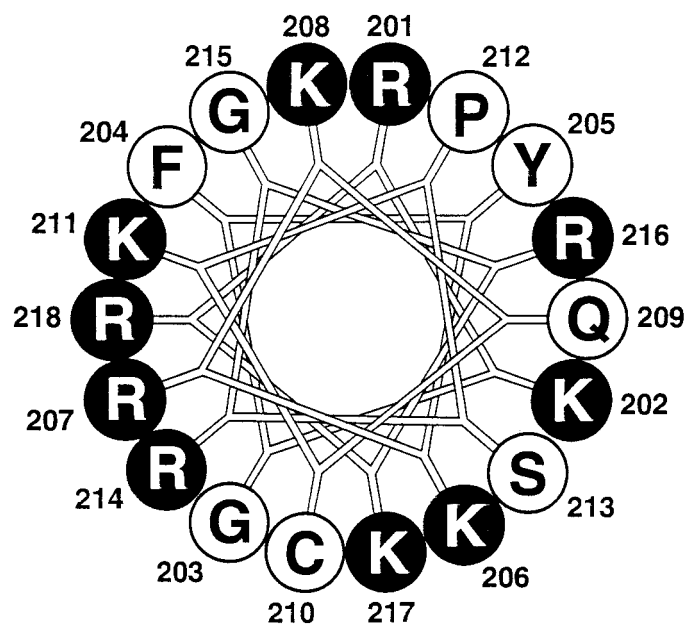

FIG. 6. Helical wheel alignment. The region between amino acids 201 and 218 of IGFBP-5 is shown. The amino acids that are charged are shown in *black*, and the residues that align asymmetrically (*e.g.*, 211–214) have been shown to be critical for ECM and glycosaminoglycan binding. If this region of IGFBP-5 forms an α-helix, these residues are likely to form the binding site.

thus resulting in an 8-fold reduction in its affinity for IGF-I. The explanation for why IGFBP-3, which contains charged residues at positions that are identical to those in IGFBP-5, does not bind as well to ECM remains undetermined. It is probable that this region is folded differently and that these residues are not surface exposed, thus limiting their ability to interact with ECM. It is also possible that the carbohydrate residues in IGFBP-3 function to diminish the interactions of the charged residues in this region with EMC components.

The only other IGFBP that has been shown to be significantly associated with ECM is IGFBP-2. Interestingly, this occurs only in the presence of high concentrations of IGF-I or -II (123). This suggests that IGFBP-2 must undergo a conformational change to unfold its structure in such a way that the ECM-binding site can be exposed. The location of the ECM-binding site in IGFBP-2 has not been determined.

The availability of these mutants has allowed definitive identification of the amino acids that mediate the binding of IGFBP-5 to ECM. More importantly, transfection of cDNAs encoding mutant forms of IGFBP-5 has allowed us to analyze the effects of constituitively synthesized mutants on IGF-I biological actions. Thus, constitutive expression of these mutants has permitted definitive evaluation of the importance of matrix association of IGFBP-5 in altering cellular responses to IGF-I that would not have been possible by simply adding IGFBP-5 exogenously to cells in culture.

D. Binding of IGFBPs to heparin and heparan sulfate-linked glycosaminoglycans

The use of IGFBP mutants has also been important in determining the specific amino acids that mediate binding to heparin and glycosaminoglycans. The importance of glycosaminoglycan binding for IGFBP function was initially delineated using IGFBP-3. Specifically, it was shown that glycosaminoglycans would result in the dissociation of the components of the ternary complex of IGFBP-3, IGF-I or II, and acid labile subunit (ALS) in serum (124, 125). Subsequently, it was shown that glycosaminoglycans would also interfere with binding of IGFBP-3 to cell surfaces and of IGFBP-5 to ECM (52, 53, 54). Furthermore, it was shown that heparin binding definitively reduced the affinity of IGFBP-5 for IGF-I by 8- to 10-fold; however, it had only a minimal effect on the affinity of IGFBP-3 (54, 56). The specific regions of IGFBP-3 and IGFBP-5 that accounted for heparin binding were determined using synthetic peptides and the same approach that had been used for determining the specific regions that accounted for ECM binding (91). It was shown that clusters of basic amino acids within IGFBP-5 between residues 131–141 and 201–218 contained basic amino acid sequences that mediated heparin binding (56). For IGFBP-3 it was determined that the region of sequence between amino acids 214 and 232 contained the predominant heparin-binding site. Since IGFBP-5 contained two regions that mediated heparin binding, the importance of each of these sequences for heparin binding was determined. These studies showed that the 201–218 sequence contained the predominant binding site. Based on these results, the significance of this glycosaminoglycan-binding domain in mediating IGFBP-3 and -5 binding to cell surface proteoglycans was determined.

Initially it was shown that a peptide containing the 214–232 region of IGFBP-3 could compete for binding with native IGFBP-3 to cell surface-associated proteoglycans (53, 126, 127). Subsequently, Andress showed that IGFBP-5 binds to a large cell surface protein of 420,000 kDa molecular mass and that heparin competitively inhibited this binding interaction (52). Further studies showed that a peptide containing amino acids 201–218 of IGFBP-5 competitively inhibited IGFBP-5 binding to this cell surface protein and to proteoglycans (52, 54, 118).

Further delineation of specific amino acids involved in glycosaminoglycan binding has been possible for IGFBP-5 and to some extent IGFBP-3 using mutagenesis. Using combinations of substitutions for charged amino acids in the 201–218 region of IGFBP-5, it was deduced that the heparin binding motif, K206 R207 K208 Q209 C210 K211(BBBXXB), contained the primary binding site (56, 90). Mutants with substitutions for several of the amino acids in this region showed these residues were required for heparin binding. R201 and K202 were also shown to be required for high-affinity binding (Table 2). Helical-wheel analysis of this region IGFBP-5 shown in Fig. 6 shows that of these residues, K211, R207, and R214, are aligned asymmetrically in a manner wherein they could easily form a tight binding pocket. Heparin binding to IGFBP-5 results in an 8- to 12-fold reduction in its affinity for IGF-I. When the effects of specific amino acid substitutions on this affinity shift were determined, it was noted that single substitution for K211 or a double substitution for K217, R218, resulted in no reduction in heparin binding but that these substitutions were associated with a marked reduction in the ability of heparin to alter the affinity of IGFBP-5 for IGF-I (91). As noted for ECM binding, the amino acids Q209 andG203 in IGFBP-5 that are important for formation of the high-affinity IGF-binding pocket are adjacent to these charged residues (Fig. 6). This suggests that heparin binding results in a conformational change within IGFBP-5 that reduces molecular interactions of IGF-I with residues in the high-affinity pocket, thus accounting for the 8-fold reduction in its affinity for IGF-I and that substitution for Gly 203 or Gln 209 results in similar change.

Identification of the residues that mediate the binding of IGFBP-3 to heparin has been analyzed much less extensively.

TABLE 2. Heparin binding activity of IGFBP-5 mutants

Forms of IGFBP-5	% of Maximal heparin binding
Native IGFBP-5	100
K134A/R136A	94
K134A/R136A/K211N	90
K211N	100
R207A/K211N	79
K217A/K202N	80
R201A/K202N	65
K202A/K206A/R207A	56
R201A/K202N/K206N/K208N	54
K211/R214A/R217A/R218A	42

Increasing concentrations of native IGFBP-5 or each mutant form were incubated with heparin sepharose beads, and the amount of IGFBP-5 that bound was quantified. Each result is expressed as the percentage of the total added IGFBP-5 mutant that was bound.

A mutant in which five residues in IGFBP-3 (*e.g.*, 228–232) that are equivalent to 214–218 in IGFBP-5 were substituted with five residues from IGFBP-1 (which does not bind to heparin) had a major reduction in binding to heparin but very little alteration in its affinity for IGF-I (92). Since these heparin-binding mutants of IGFBP-3 and IGFBP-5 have major reductions in their binding to glycosaminoglycans, it was predicted that their binding to ECM would also be reduced since ECM binding is partially mediated by proteoglycan binding.

In summary, these highly basic regions of IGFBP-3 and IGFBP-5 clearly account for the majority of glycosaminoglycan binding. Mutagenesis has been particularly helpful in determining the specific amino acids within these regions that mediate binding as well as those that mediate the reduction in the affinity of IGFBP-5 for IGF-I that occurs after glycosaminoglycan binding. Since definitive evaluation of the effect of glycosaminoglycan binding in altering IGFBP biological actions is difficult to analyze due to the complexities of glycosaminoglycan structure, these mutants should make it possible to better evaluate the consequences of changing glycosaminoglycan structure on IGFBP and IGF-I actions.

E. Cell surface association

Several studies have demonstrated that IGFBP-3 in particular, but also IGFBP-5 and in some cases IGFBP-1, can associate with the cell surface. Cell surface association of IGFBP-3 or IGFBP-5 has been shown to lead to both potentiation and inhibition of IGF actions (53, 68, 128, 129). Both proteins are believed to bind cell surface-associated proteoglycans (52, 53), and the existence of protein receptors has also been proposed (64, 130). The importance of glycosaminoglycan binding for IGFBP-2, -3, and -5 has been emphasized by the fact that heparin can easily compete for binding of these proteins to cell surface-associated binding sites (52, 53, 126, 130). The specific amino acids in IGFBP-3 and IGFBP-5 that bind to cell surface proteoglycans have not been definitively identified. However, because heparin was shown to be a potent inhibitor of cell surface binding, the heparin-binding domain of each protein has been evaluated. Synthetic peptides that contain this sequence and that bind to heparin also inhibit their cell surface binding, suggesting that these residues are an important component of the primary binding site (52, 52, 126, 127). A definitive study published by Firth and colleagues (92) in which they used the mutant form of IGFBP-3, wherein the residues from K228 through R232 had been substituted with residues from IGFBP-1, showed that this form of the protein associated much less well with cell surfaces, which suggested that these residues were an important component of the cell surface binding site. Clearly, future studies that determine other regions of IGFBP-3 and IGFBP-5 that are important for cell surface association are warranted.

F. ALS-binding mutants

Baxter and co-workers have demonstrated the importance of the ability of the IGFBP3/IGF-I complex in serum to associate with the third protein termed ALS (33). They have shown that the assembly of this ternary complex requires near-saturation of IGFBP-3 with IGF-I or IGF-II. The amount of IGFBP-3 that binds to ALS in the absence of IGF-I or IGF-II is significantly less (131). More recently, they have also shown that IGFBP-5 binds to ALS (132). To further probe the sites of interaction between ALS and these two forms of IGFBPs, these investigators have prepared IGFBP-3 and -5 mutants and used them to study the regions of the proteins that determine ternary complex formation. These investigators have used these mutants to study ternary complex assembly and its effect on controlling IGF half-life. Initially, Firth *et al.* showed that deletion of amino acids 189–264 resulted in total loss of IGFBP-3 binding to the ALS (92). A substitution mutant in which residues 228–232 in IGFBP-3 were substituted for the homologous residues from IGFBP-1 (a form of IGFBP that does not bind to ALS) resulted in a 90% reduction in the affinity of the mutant form of IGFBP-3 for ALS (94). These authors concluded that the region of sequence between amino acids 228 and 232 in IGFBP-3 was essential for normal ALS affinity. These studies were extended by Twigg *et al.* (133) who showed that the principal region of IGFBP-5 that accounted for ALS binding was also contained within its carboxyl-terminal region. They used domain swapping mutants in which the C-terminal domain of IGFBP-6 was substituted for the C-terminal domain of IGFBP-5. Similarly, they made a synthetic peptide to the region of IGFBP-5 that contained the residues that were analogous to the residues in IGFBP-3 that accounted for its ALS binding. They showed that this peptide could inhibit the formation of IGFBP-5/ALS/IGF-I ternary complexes. In further studies, Twigg *et al.* were able to show that an IGFBP-5 fragment that contained only residues 1–169 had some ALS binding activity, although it was greatly reduced compared with the intact protein (133). They further postulated that the region of basic amino acids in the central core of IGFBP-5 (*e.g.*, 132–140) might account for this binding and probably served as a second binding site. Hashimoto *et al.* (81) further confirmed that the C-terminal domain of IGFBP-3 was important for ALS binding. This was determined in two ways: first using a deletion mutant in which residues 186–265 were deleted and by substitution mutagenesis in which the carboxyl-terminal domain of IGFBP-2 beginning at position 186 was substituted for the homologous domain of IGFBP-3. Both the truncation mutant and the carboxyl-terminal substitution mutant showed marked reduction in ALS binding. Specifically, the IGFBP-2 substitution mutant showed a greater than 90% reduction in binding affinity for ALS in the presence of IGF-II, and the truncation mutant completely lost binding affinity, confirming that the C-terminal domain was required for ALS binding. In summary, the mutagenesis studies have shown that the heparin-binding domain of IGFBP-3 and, most importantly, residues 228–232 contain the major binding site for its association with the ALS. A secondary binding site in IGFBP-5 between residues 132 and 140 exists, although this binding site is less important.

G. Binding to other proteins

Recently, the IGFBPs have been shown to associate with several other proteins that are located in the ECM, on surface cell membranes, or in interstitial fluids. Mutagenesis studies have been used to determine the principal binding sites in IGFBP-3 for binding to plasminogen. Campbell and co-workers demonstrated that substitution for residues 228–233 in IGFBP-3 with residues from IGFBP-1 (*e.g.*, MDGEA) resulted in a 5-fold reduction in binding of IGFBP-3 to plasminogen, suggesting that this sequence within the heparin-binding domain was part of the binding site (134). This binding interaction is believed to be important for cleavage of IGFBP-3 by plasminogen, although its role in plasmin formation has not been determined.

Although IGFBP-5 binding to plasminogen has not been analyzed, Nam *et al.* showed that IGFBP-5 bound to plasminogen activator inhibitor (PAI-1). Using a series of IGFBP-5 mutants, they showed that amino acids K202 and K208 were the most important for IGFBP-5 binding to PAI-1 (135). These studies also demonstrated that other basic amino acids within the heparin-binding domain between residues 210 and 218 were not significantly important for binding. In contrast to those findings, several amino acids between K211 and R218, such as R214, are very important for binding to ECM. This suggests that PAI-1 is not one of the major determinants of IGFBP-5 binding to ECM. They also demonstrated that IGFBP-5 association with PAI-1 reduced its ability to inhibit the conversion of plasminogen to plasmin and that this effect was attenuated in the presence of the wild-type protein but not the PAI-1 binding-defective IGFBP-5 mutant.

These studies were extended by demonstrating that two other ECM proteins, osteopontin and thrombospondin, bound to IGFBP-5 within ECM (136). Unlike binding to PAI-1, IGFBP-5 substitution mutants that had reduced binding to thrombospondin or osteopontin also had decreased binding to ECM. The residues that were found to be the most important for osteopontin binding were R214, K217, and R218. To further confirm the importance of osteopontin as an ECM binding component, fibroblasts were transfected with a cDNA that contained substitutions for these residues in IGFBP-5 and binding to ECM was determined in cells expressing these mutant forms. These analyses showed that there was a substantial reduction in the amount of IGFBP-5 that was associated in the ECM. Thrombospondin binding to IGFBP-5 was reduced to the greatest extent when R201 and R214 were substituted with neutral residues, and mutant forms containing these substitutions had a significant reduction in ECM binding. The findings from these two studies suggest that although the ECM-binding domain in IGFBP-5 (R201–R218) contains residues that are important for it to bind to each of these three proteins, there is molecular specificity even within this short region of amino acids such that specific and distinct amino acids within this region mediate the interactions with each of these specific ECM components.

H. IGF-independent actions

IGFBPs have been shown to stimulate several biological effects that are independent of their ability to bind to the IGFs. After cell surface binding, IGFBP-3 alters IGF-I receptor affinity for ligands, usually as a negative regulator (137). In addition, proteolysis of IGFBP-3 at the cell surface has been shown to release fragments that have IGF-independent effects that can either enhance or inhibit cellular responsiveness to IGF-I (68, 70, 138, 139). There is also evidence to support the conclusion that these responses are mediated through altering access of IGF-I or -II to the receptor. It has been shown recently that IGFBP-3 or -5 can enter the cell and bind to several different targets in cytoplasm. Furthermore, it has been shown that both proteins can localize in the nucleus (140, 141) and bind to important growth-regulatory proteins, such as the RXR, an important protein for regulating cellular differentiation (142, 143). The biological significance of IGFBP-3 transport into the nucleus was recently investigated using mutagenesis (144). These studies demonstrated that nuclear localization required binding to importin B, a nuclear transport factor. Mutagenesis of residues 228–232 in IGFBP-3 or 214–218 in IGFBP-5 to noncharged residues reduced importin B binding and nuclear localization.

Mutagenesis has also been used to elucidate the importance of IGFBP-1 binding to a cell surface receptor (71). Since IGFBP-1 contains an arg-gly-asp (RGD) sequence, it was reasonable to propose that it might bind to a cell surface integrin (Fig. 1). Integrins are cell surface transmembrane proteins that bind to specific proteins in the ECM and transduce signals to cytoplasmic regulatory elements. Although integrins do not have intrinsic tyrosine kinase activity, they can influence the activity of a variety of kinases and phosphatases by localizing these enzymes in close approximation to their targets. Focal adhesion complexes have been shown to contain groups of proteins that bind to integrin cytoplasmic tails during focal adhesion complex formation. Protein kinases such as focal adhesion kinase can, upon stimulation of integrin clustering, signal downstream to various intracellular intermediary signaling proteins. While IGFBP-1 does not induce integrin clustering, it does bind to the $\alpha 5\beta 1$ integrin, and this binding has been shown to modulate focal adhesion kinase activity (145).

With the availability of mutants, it was shown that if the RGD in IGFBP-1 sequence was mutated to WGD, the protein would no longer bind to the $\alpha 5\beta 1$ integrin (71). More importantly, it would not signal because this mutant form of the protein could not stimulate cell migration. IGFBP-1 was shown to stimulate Chinese hamster ovary (CHO) cell migration 3-fold in a 24-h period in the absence of IGF-I. Since CHO cells do not respond to IGF-I with increased migration, it was concluded that IGFBP-1 was acting completely independently of IGF-I. The addition of the WGD mutant or transfection of the mutant cDNA into CHO cells resulted in no stimulation of migration, confirming that wild-type IGFBP-1 was acting through the $\alpha 5\beta 1$ integrin. In contrast, IGFBP-2, which also contains an RGD sequence, did not bind to the $\alpha 5\beta 1$ integrin or stimulate CHO cell migration. This is presumably due to a different folding pattern in which the RGD sequence is not surface exposed and therefore not able

to access this receptor site. This important property of IGFBP-1 has been shown to play a role in wound repair. Using an *in vivo* test system, Galiano *et al.* (146) demonstrated that the addition of wild-type IGFBP-1 with IGF-I to dermal ulcers in rabbits could stimulate a significant increase in reepithelialization. If the WGD IGFBP-1 mutant was used, there was no stimulation. This finding strongly suggests that IGFBP-1 stimulated cell migration *in vivo*.

Since many of the IGF-independent effects of IGFBPs are mediated by binding to cell surface proteins [*e.g.*, the IGF-I receptor (137)] or intracellular proteins [*e.g.*, the RAR (143)], mutagenesis should be very useful not only for determining the specific residues involved in these interactions but it should also prove to be a useful tool for probing the physiological significance of these interactions. Furthermore, since mutant forms that do not bind to IGFs can be used, their availability will make it possible to identify biological actions of IGFBPs that are truly IGF independent.

I. IGFBP phosphorylation

Three of the six forms of IGFBPs have been shown to undergo phosphorylation (17). IGFBP-1 and -3 have been shown to be phosphorylated on serine residues (57, 58, 60). The most significant functional consequence that has been demonstrated is that phosphorylation of IGFBP-1 results in an 8-fold increase in its affinity for IGF-I (57). The biological significance of this change became apparent when it was shown that nonphosphorylated forms of IGFBP-1 were associated with stimulation rather than inhibition of IGF-I actions (6, 25). Specifically, addition of nonphosphorylated IGFBP-1 to SMC or fibroblast cultures resulted in the ability of this protein to markedly potentiate the cell growth response to IGF-I. Conversely, phosphorylation of IGFBP-1 was shown to inhibit IGF-I actions (6). To further determine the significance of IGFBP-1 phosphorylation, the sites in IGFBP-1 that were phosphorylated were identified by radiosequencing. Jones *et al.* (59) labeled human IGFBP-1 produced by CHO cells with ^{32}P-orthophosphate. Radiosequencing showed that serines 101, 119, and 169 were radiolabeled (Fig. 7). Quantitative analysis showed that 60% of the labeling occurred at Ser 101, 34% at Ser 169, and the remainder at Ser 119. Therefore, the first two residues were the preferred sites of phosphorylation. Based on this result, a mutant was prepared in which the serines at positions 101 and 169 were converted to alanines, and this mutant was transfected into CHO cells. Biosynthetic labeling studies showed that there was a major reduction in the amount of phosphorylation after immunoprecipitation of the ^{32}P-labeled IGFBP-1 mutant (59). When this mutant was incubated with IGF-I, it enhanced IGF-I-stimulated DNA synthesis. Further use of these mutants was helpful in defining the enzymes that phosphorylate IGFBP-1. It was determined that casein kinase-2 and an isoform of casein kinase-1 were the principal enzymes that phosphorylated this protein in human hepatoma cells (147). To confirm that these kinases were active, *in vitro* assays using purified casein kinase-2 and the partially purified casein kinase-1-like enzyme and nonphosphorylated IGFBP-1 were performed. The mutants provided valuable control reagents to show that these residues
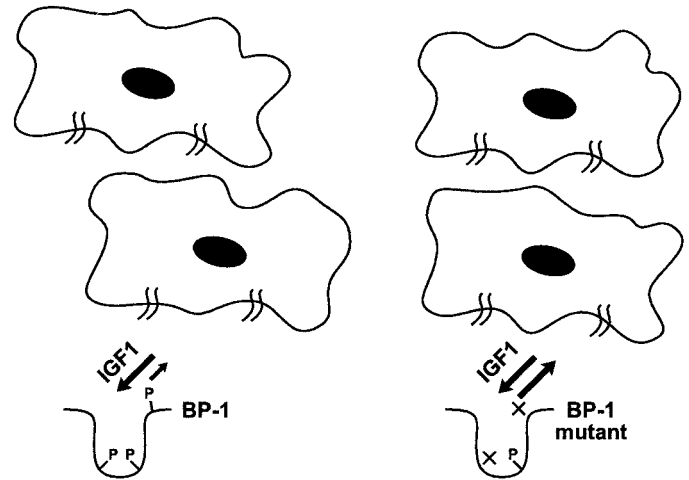
^{60}RALPGE**QQ**PLHALTRGQGACVQESDASAPHAAEAGSPE**S**PESTEI
TEEELLDNFHLMAP**S**EEDHSILWDAISTYDGSKALHVTNIKKWKEPC
RIELYRVVESLAKAQET**S**GEEIS^{174}

FIG. 7. Alteration in the distribution of IGF-I between its cell surface receptor and IGFBP-1 depends upon IGFBP-1 phosphorylation. When IGFBP-1 is phosphorylated (*left panel*), the equilibrium favors IGF-I binding to IGFBP-1 and not to its receptor. When the primary sites of phosphorylation have been altered by mutagenesis, the equilibrium shifts favoring binding to the receptor (*right panel*). The sites of serine phosphorylation are shown as *bold letters*. The two glutamines that are involved in transglutamination are also shown in *bold*.

were the primary phosphorylation sites and that the purified enzymes did not phosphorylate other sites *in vitro*.

Studies of IGFBP-3 phosphorylation have been equally revealing. Hoeck and Mukku (60) showed that IGFBP-3 was phosphorylated on serine residues. Coverley and Baxter (58) confirmed that serines 111 and 113 of IGFBP-3 were the sites of phosphorylation and showed that IGF-I could stimulate its phosphorylation. These investigators then further determined that casein kinase-2 was the principal enzyme that was responsible for phosphorylation of IGFBP-3, as had been shown for IGFBP-1 (148). Human fibroblasts were shown to also secrete phosphorylated forms of IGFBP-3. Recently, Coverley and Baxter (149) examined the effect of mutagenesis on phosphorylation of IGFBP-3 at these sites. When both serines were mutated to alanines, the amount of phospho-IGFBP-3 was reduced by 80%, suggesting that these are the primary phosphate-acceptor sites. To determine its biological activity, these investigators compared casein kinase-2-phosphorylated IGFBP-3 with native IGFBP-3 that had not been exposed to casein kinase-2. Unlike IGFBP-1, there was no difference in the affinity of phosphorylated and nonphosphorylated IGFBP-3 for IGF-I or -II. However, its ability to associate with the ALS was markedly altered, and phosphorylated IGFBP-3 showed less cell association (148). The phosphorylated form was shown to be more stable during exposure to proteases in cellular conditioned medium, and it was less susceptible to cleavage by plasmin over time. When biological activity was assessed, the casein kinase-treated IGFBP-3 had significantly reduced ability to augment

IGF-I-stimulated DNA synthesis in fibroblast cultures compared with native, nonphosphorylated IGFBP-3 (148).

J. Transglutamination

Several years ago it was reported that IGFBP-1 could polymerize in culture medium during exposure to fibroblasts if IGF-I was also present (150, 151). Polymerization was associated with the ability of IGFBP-1 to potentiate cell growth, *i.e.*, forms of IGFBP-1 that failed to polymerize did not result in enhancement of the cellular response to IGF-I. When the forms of IGFBP-1 that were present in medium from cell cultures in which enhancement of IGF-I action had been noted were analyzed, a significant fraction of the added IGFBP-1 had polymerized (150, 151). These polymers were shown to be covalently linked, *i.e.*, they did not completely disassociate even under denaturing conditions in SDS or with reduction (150). However, the molecular basis of this covalent interaction was not probed until recently. The enzyme tissue transglutaminase has been shown to catalyze the formation of covalently linked IGFBP-1 multimers *in vitro* (152). Mutagenesis was used to further analyze this process. Tissue transglutaminase has been shown to catalyze multimer formation by cross-linking proteins through adjacent glutamine residues. Direct exposure of IGFBP-1 to purified tissue transglutaminase resulted in covalently linked multimer formation. Phosphorylation of IGFBP-1 was shown to inhibit polymerization, and exposure to IGF-I was shown to enhance multimer formation. When the biochemical basis of the polymerization reaction was probed, it was shown that an IGFBP-1 mutant, in which glutamine 66 and 67 were converted to alanines, did not polymerize when incubated with tissue transglutaminase (Fig. 7). To determine whether multimer formation altered cellular responsiveness to IGF-I, SMC were exposed to Q66A, Q67A IGFBP-1, or native IGFBP-1 plus IGF-I, and their protein synthesis rates were compared. Although native IGFBP-1 inhibited IGF-I stimulation of protein synthesis, Q66A, Q67A IGFBP-1 had no effect. Since this transglutamination site is contained within the IGF-binding domain, it is possible that transglutamination is facilitated by the conformation changes that occur after IGF binding to IGFBP-1, thus allowing the enzyme better access to the Gln 66, Gln 67 site. These findings support the conclusion that polymerization is one mechanism by which cells autoregulate their response to exposure to IGFBP-1. The availability of this mutant should allow better definition of the biological consequences of this transglutaminase-catalyzed reaction.

III. Areas Not Yet Analyzed Using Mutagenesis

Several areas of IGFBP function that have been deemed to be important have not been analyzed by the mutagenesis approach. Specifically, although it has been shown in multiple publications that the heparin-binding domains of IGFBP-3 and -5 are important for cell surface localization, none of the heparin-binding mutants of IGFBP-5 have been analyzed in this regard. Similarly, the importance of each individual amino acid within the heparin motif in IGFBP-3 that mediate cell surface binding has not been determined. As a result, it has not been possible to quantify the functional significance of loss of cell surface binding in cells that constitutively express high concentrations of IGFBP-3 or -5 on the cell surface. Similarly, several protease activities have been characterized in terms of their ability to cleave IGFBPs, but the consequences of mutating the cleavage sites has been analyzed only for IGFBP-4 and -5. Since IGFBP-2 has been implicated in a major way in the role of IGF in modulating cancer cells, IGFBP-2 protease-resistant mutants could be extremely valuable for further delineating the role of this protein in tumorigenesis. Another area, which is newer and therefore has not had sufficient time for mutants to be prepared and analyzed, is the area of nuclear localization. The nuclear localization sequence within IGFBP-3 and -5 has been defined, but the biological significance of inhibition nuclear localization of these proteins on the cellular actions of IGFBPs has not been determined. Finally, by utilizing techniques such as the yeast two-hybrid system, many other proteins that bind IGFBPs have been identified; however, none of these has been analyzed by mutagenesis to determine the significance of these binding interactions on biological responses to IGFBPs in whole-cell systems.

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