Fragments of Bovine Insulin-like Growth Factors I and II Stimulate Proliferation of Rat L6 Myoblast Cells

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ABSTRACT: The active sites of bovine insulin-like growth factor (IGF) I and II fragments were studied. Overlapping fragments of IGF I (residues 1–25, 11–35, 21–45, 31–55, and 41–70) and of IGF II (residues 1–24, 10–34, 20–44, 30–54, and 40–67) were chemically synthesized. The activity of the fragments was measured by stimulating the proliferation of rat L6 myoblast cells. Two fragments of IGF I (residues 21–45 and 31–55) and two fragments of IGF II (residues 20–44 and 30–54) were active while the other fragments were inactive in stimulating cell proliferation. Although the activity of these fragments was observed only at a high concentration of 0.1 mM, the results imply that the active site is located around residues 31–45 for IGF I fragments and residues 30–44 for IGF II fragments. Consequently, an IGF I fragment (residues 26–50) having a five-residue extension to both the N- and C-terminal sites of residues 31–45 also stimulated the proliferation of L6 myoblast cells. Furthermore, the substitution of Ile-35 in two IGF II fragments (residues 21–45 and 31–55) by Ser inactivated these fragments. This suggests that Ile-35 is an essential residue for IGF II fragment activity. Ser-35, which was reported in the original sequencing of bovine IGF II, is incorrect in the sequence and furthermore has been consistently found to be an Ile-35 in our hands. The results were unexpected because there is little sequence homology between residues 31–45 of IGF I and residues 30–44 of IGF II; i.e., nonhomologous peptides showed the same biological activity. However, residues 31–45 of IGF I and residues 30–44 of IGF II show a similar hydrophobicity profile. This indicates that hydrophobicity may be an important property for activity in the fragments.

Once a protein is sequenced, it is highly desirable to characterize its biological function and identify the active site. Several approaches have been used to predict or identify the active site of a protein. If the active site of the homologous protein has been identified already, the corresponding region of the protein may be the active site. The application of this method is limited to proteins in which a homologous protein is well characterized already. Another method involves the chemical modification of amino acids in a protein in order to cause a substantial loss of activity. These amino acids must make significant contributions to the activity. This method would be used to identify the types of amino acids involved in the activity rather than identifying the individual functional residue in a protein. Recent advances in site-specific mutagenesis enable a residue to be substituted by other amino acids in order to identify the functional residues in the activity. However, the amino acid substitution of an essential residue at the active site is not the only factor that may inactivate the protein. Amino acid substitutions of certain residues outside of the active site may cause conformational changes, improper folding, destabilization towards temperature or enzymatic digestion, poor solubility causing aggregation, etc., and these changes may induce the inactivation of the protein. Furthermore, since each residue is substituted by another amino acid, which can be selected arbitrarily, the substituting residue itself may cause the activity loss of the protein.

If an active site of a protein is localized in a certain region of the protein, a fragment including this region may show activity. The advantage of this method is that there is no chemical modification or amino acid substitution which may affect the functions or properties of the protein. The disadvantage of this method is that the fragment may have a flexible conformation in solution, and therefore, the population containing the active conformation of the fragment may be small, which results in a low activity of the fragment compared to that of the entire protein. Or some fragments may show activity by accident. In order to search active fragment systematically, the fragments were chosen to be 24–25 residues in length and with 15 residues of overlap between consequent fragments, which could be long enough to include an active site.

This method has been applied to bovine IGFs[1] I and II and is described in this paper. IGFs I and II are polypeptide hormones that have structural and functional homologies with insulin. Insulin and both IGFs produce similar biological effects in most cells, including stimulation of hexose, amino acid uptake, and DNA synthesis (Zapf et al., 1981; Rechler et al., 1981). Rat L6 myoblasts represent a myogenic cell line that can form multinucleated myotubes concomitant with the cessation of DNA synthesis (Yaffe, 1968). The myoblast cells bind substantial amounts of IGFs I and II and can be stimulated to grow (Richman et al., 1980) and to differentiate by IGF (Ewton & Florini, 1981). It is thought that IGF I acts through type I receptors on rat L6 myoblast cells. However, IGF II can also bind to type I receptors and stimulate the proliferation of the cells (Ballard et al., 1986). Thus, we have measured the stimulation of the proliferation of rat L6 myoblast cells as a bioassay of IGF I and II fragments. Five fragments (residues 1–25, 11–35, 21–45, 31–55, and 41–70) were synthesized for IGF I and five fragments (1–24, 10–34,

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[1] Abbreviations: IGF, insulin-like growth factor; Fmoc, (9-fluorenylmethoxy)carbonyl; TFA, trifluoroacetic acid; DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; ABI, Applied Biosystems.

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20–44, 30–54, and 40–67) were synthesized for IGF II for activity measurements.

Experimental Procedures

Peptide Synthesis and Purification. The peptides were prepared by conventional solid-phase peptide synthesis using (9-fluorenylmethoxy)carbonyl (Fmoc) amino acids (Stewart & Young, 1984). Except for serine, threonine, and arginine, the amino acids used in the synthesis were Fmoc-L-amino acid pentafluorophenyl esters. These esters were purchased from either Advanced ChemTech or Milligen. A *tert*-butyl group was used to protect the side chains of Asp, Glu, and Tyr. A *tert*-butyloxycarbonyl group was used to protect the side chain of Lys. The side chain of Cys was protected by an acetamidomethyl group during the synthesis and also in the assay. Fmoc-L-serine oxobenzotriazine ester and Fmoc-L-threonine oxobenzotriazine ester were used for Ser and Thr, respectively, and were purchased from Milligen. Fmoc-L-arginine [(4-methoxy-2,3,6-trimethylphenyl)sulfonyl] was used for Arg and purchased from Advanced ChemTech and Milligen. The *p*-methylbenzylhydrylamine hydrochloride resin used for peptide synthesis was purchased from Fluka Chemical Corp. (Phenylacetamido)methyl *tert*-butyloxycarbonyl Ala or Glu(*O*-benzyl) resin was used to synthesize the C-terminal fragment of IGF I or II, respectively, and was purchased from Applied Biosystems. The *tert*-butyloxycarbonyl group on the resin was removed by treatment with 50% trifluoroacetic acid (TFA)/methylene chloride for 2 min followed by a second treatment for 30 min. The resin was washed with methylene chloride for 2 min three times and dried before being used in peptide synthesis. As in conventional Fmoc peptide synthesis, unreacted amino groups were capped by acetic anhydride after each coupling, and the synthesis was terminated at a point in which the peptide product retained the Fmoc group. The peptides on the resin were then washed twice with methylene chloride and dried under vacuum. The peptides were cleaved from the resin by using the conventional HF cleavage procedure at −5 to 0 °C for 1 h. A mixture of 10% anisole and 5% methyl sulfide was used as scavenger. The cleaved peptides were dissolved in 3 mL of TFA, filtered through a polypropylene filter (Econo-column, Bio-Rad), and then precipitated by dropping the peptide solution into ethyl ether. Since the truncated byproducts were acetylated, only the full-length product had the Fmoc group, and this Fmoc group was used as an identification marker to purify the product from the truncated byproducts.

Before the removal of the Fmoc group from the product, the peptides were dissolved in 4 mL of dimethyl sulfoxide and purified on a reverse-phase high-performance column (C4, 10 × 250 mm, SynChrom, Inc.) using a 5–70% acetonitrile gradient in water in the presence of 0.3% TFA. The elution profile was monitored by the absorbance at 300 nm using the Fmoc group as a marker. The purified peptides were lyophilized and dissolved in 4 mL of *N*,*N*-dimethylformamide. The Fmoc groups on the purified peptides were then removed by adding 2 mL of piperidine to the peptide solution and stirring for 60 min. The peptides were precipitated by dropping the peptide solution into 30 mL of ethyl ether, since dibenzogulvene and *N*-(fluorenylmethyl)piperidine, which are the products of Fmoc group cleaved by piperidine, *N*,*N*-dimethylformamide, and piperidine are soluble in ethyl ether. The peptides were further washed with ethyl ether three times. The purity of the deprotected peptides was assessed by use of an analytical reverse-phase high-performance column (C4, 4.6 × 250 mm, Vydac) with 5–65% acetonitrile gradient in water in the presence of 0.1% TFA. The elution profile was monitored by the absorbance of the peptide bonds at 210 nm. The removal of a very hydrophobic Fmoc group from a product shifts the retention time of the peptide by 3–8% in acetonitrile concentration. The complete disappearance of Fmoc-peptide was also ascertained in the chromatography. The peptides were characterized by amino acid analysis. For some peptides mass spectroscopy was also used to confirm the identification.

Some of the peptides (IGF I residues 4–45 and residues 16–45; IGF II residues 40–67) were made by the *t*-Boc method. All the reagents and protected Boc-amino acids were purchased from ABI. The synthesis was performed on a fully automated peptide synthesizer (ABI 430A). The amino acids were sequentially incorporated, as the preformed symmetric anhydride, into the solid support (PAM resin) by using the standard ABI coupling protocol (Clark-Lewis et al., 1986). No capping step was included in this procedure. Peptides were cleaved from the resin by HF in the presence of 5% anisole. The crude peptide was then purified by HPLC in a similar manner as described above.

Rat L6 Myoblast Bioassay. The biological activity of IGF I and IGF II fragments was assessed by measuring enhancement of myoblast proliferation in vitro. Rat L6 myogenic cells (Yaffe, 1968) have been shown to respond to IGF I (0.5–10 nM), IGF II (2–50 nM), and insulin (0.1–1 μM) by several groups (Kotts & Baile, 1985; Ballard et al., 1986; Ewton et al., 1987). A standardized bioassay using these cells for measuring proliferation-promoting factors in serum has been described (Kotts, et al., 1987). This assay was used with some modifications. Briefly, cells were plated onto 2-cm^2 wells (24-well plates, Corning) at 1000 cells/cm^2 in Dulbecco's minimum essential medium (DMEM, Dibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco). After 24 h, the cells were rinsed with 1 mL of DMEM, and then the test medium containing the IFG fragment (1–100 μM) in DMEM plus 2% FBS was applied (1 mL/well). Stock solutions of fragments were prepared in 10 mM HCl at a concentration of 10 mg/mL. Fresh test medium was applied again 24 h later. After an additional 48 h, cell number was estimated by measuring the content of DNA on each well (Kissane & Robins, 1958). DNA content was correlated with cell number by using a standard curve consisting of known numbers of L6 cells as counted on a Coulter counter (Model ZM, Coulter Electronics, Hileah, FL). Controls received DMEM containing 2% FBS and appropriate volumes of 10 mM HCl. Positive controls received various concentrations (1–50 nM) of recombinant human/bovine IGF I (Monsanto Co., lot no. S105, St. Louis, MO) in DMEM plus 2% FBS. All incubations were carried out at 37 °C, 10% CO_2, and 100% humidity. Results are presented as percent increase in cell number over controls (DMEM + 2% FBS) within each assay and defined as stimulation. Four individual bioassays were carried out to obtain the activity of each peptide, with intraassay variation averaging 5.1% (±1.2%). Interassay variation is listed in Table I for each peptide or protein.

Hydrophobicity Profile of Amino Acid Sequence. Although many hydrophobicity properties have been reported, most of them are classified into three types: (1) hydrophobicity of free amino acids at high ionic strength so that the charge contribution of the ionizable side-chain groups is suppressed, (2) hydrophobicity of free amino acids at low ionic strength so that the charge contribution of the ionizable side-chain groups is included, and (3) hydrophobicity of amino acid residues in proteins defined as the depth to which an amino acid residue is buried inside a protein from X-ray crystallographic data (Kidera et al., 1985). In this paper the third type of hydro-

Table I: Stimulation of Rat L6 Myoblast Cells Proliferation by IGF I, IGF II, and Their Fragments

protein or fragments	concn	stimulation of cell proliferation	activity
control[a]		100	
IGF I	0.5 nM	115 ± 4[b]	active
	1 nM	126 ± 5	
	2 nM	143 ± 6	
IGF I-(1–25)	0.1 mM	91 ± 7	inactive
IGF I-(11–35)	0.1 mM	97 ± 2	inactive
IGF I-(21–45)	0.1 mM	116 ± 2	active
IGF I-(31–55)	0.1 mM	125 ± 4	active
IGF I-(41–70)	0.1 mM	101 ± 5	inactive
IGF I-(4–45)	0.1 mM	129 ± 2	active
IGF I-(16–45)	0.1 mM	131 ± 6	active
IGF I-(26–50)	0.1 mM	124 ± 4	active
IGF II	1 nM	107 ± 2	active
	2 nM	116 ± 4	
	10 nM	137 ± 11	
	50 nM	156 ± 9	
IGF II-(1–24)	0.1 mM	99 ± 2	inactive
IGF II-(10–34)	0.1 mM	107 ± 3	inactive
IGF II-(20–44)	0.1 mM	131 ± 6	active
IGF II-(30–54)	0.1 mM	123 ± 3	active
IGF II-(40–67)	0.1 mM	49 ± 6	inhibitory
[Ser^{35}]IGF II-(21–45)	0.1 mM	90 ± 5	inactive
[Ser^{35}]IGF II-(31–55)	0.1 mM	97 ± 1	inactive

[a] A negative control was measured with DMEM plus 2% FBS at each assay. [b] Interassay variation (intraassay variation was averaged, 5.1 ± 1.2%, and is not shown in this table).

phobicity is used. The hydrophobicity of each residue varies drastically along the amino acid sequence in general. Thus, a line connecting the hydrophobicity of each residue along the sequence gives a very noisy curve, which is very difficult to interpret. The hydrophobicity profile of the sequence of IGF I is shown in Figure 1A. Various smoothing mathematical functions have been used to smooth the noisy curve of Figure 1A. An example of the hydrophobicity profile smoothed by cubic splines is shown in Figure 1B [for details of this function refer to ICSSCU in IMSL (1982)]. The major disadvantage of the smoothed hydrophobicity profile is that the profile is strongly dependent on the funtion used for the smoothing and the degree of the smoothing. Also, the hydrophobicity of the individual residue is averaged with that of its neighboring residues along the sequence, which affects the details of the hydrophobicity profile of the sequence. Most importantly, the degree of smoothing is determined by the user arbitrarily. In order to overcome the disadvantages of the conventional hydrophobicity profile, we have developed a novel way of displaying the hydrophobicity in which the total hydrophobicity of residues 1–i in a protein, i.e., $\sum_{j=1}^{i}\mathrm{HP}(j)$, is displayed at the ith residue, where HP(j) is the hydrophobicity of the jth residue. Thus, the hydrophobicity of the kth residue is displayed as the difference of the hydrophobicities displayed at the kth and the (k − 1)th residues, i.e.

$$\mathrm{HP}(k) = \sum_{j=1}^{k}\mathrm{HP}(j) - \sum_{j=1}^{k-1}\mathrm{HP}(j) \qquad (1)$$

The hydrophobicity of a certain region of a protein or a whole protein can be displayed clearly by using eq 1. The hydrophobicity of a fragment or whole protein from the kth residue to the mth residue, HP(k–m), is the difference between the hydrophobicities displayed at the kth and the mth residue, i.e.

$$\mathrm{HP}(k\text{–}m) = \sum_{j=1}^{k}\mathrm{HP}(j) - \sum_{j=1}^{m}\mathrm{HP}(j) = \sum_{j=k}^{m}\mathrm{HP}(j) \qquad (2)$$

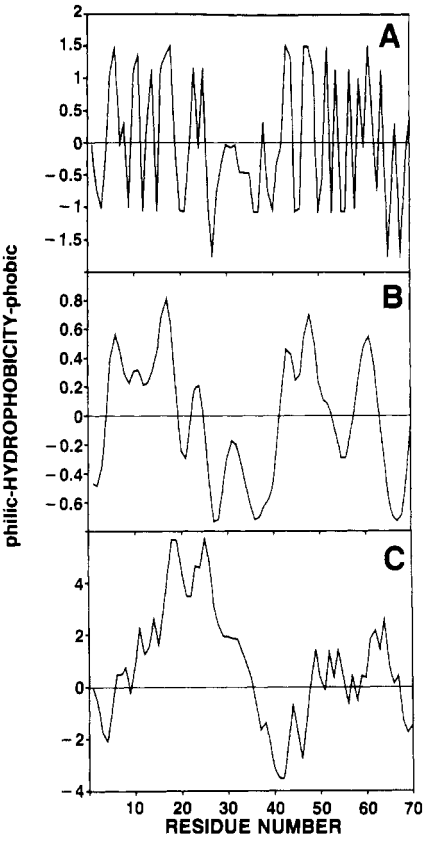


FIGURE 1: Hydrophobicity profile of IGF I. Panel A is a plot of the hydrophobicity of each residue at its residue number with its corresponding linear connection. The curve is too noisy to interpret the overall hydrophobicity profile of the sequence. The curve in panel A is smoothed in panel B by using cubic splines [ICSSCU in IMSL (1982)] with a smoothing factor of 0.8. The curve has less noise depending on the smoothing factor used and illustrates the hydrophobic/hydrophilic regions of the sequence. However, the hydrophobicity of the individual residue is no longer presented. The hydrophobicity of each residue is plotted in panel C. Here, the summed hydrophobicity of the first to the ith residues is plotted at the ith residue and the hydrophobicity of the (i + 1)th residue is added to this to plot the hydrophobicity at the (i + 1)th residue. Thus, the hydrophobic region of the protein shows a positive slope such as in the region of residues 5–18. The hydrophilic region of the protein shows a negative slope such as in the region of residues 27–42. Regions such as residues 50–60 mix the hydrophobic and hydrophilic residues with a net neutral hydrophobicity.

The advantage in this type of display is that the hydrophobicity profile of a protein is illustrated without losing the hydrophobicity information of each individual residue. Figure 1C shows the hydrophobicity profile of IGF I according to this method. The overall hydrophobicity profile of IGF I includes an N-terminal region (residues 5–18) that is hydrophobic with a net hydrophobicity of about +8. The middle of the molecule (residues 26–41) is very hydrophilic with a net hydrophobicity of about −9.5, and the C-terminal region (residues 48–67) is neutral with a net hydrophobicity of about 0. The same type of hydrophobicity profile is used in this paper for each corresponding peptide fragment.

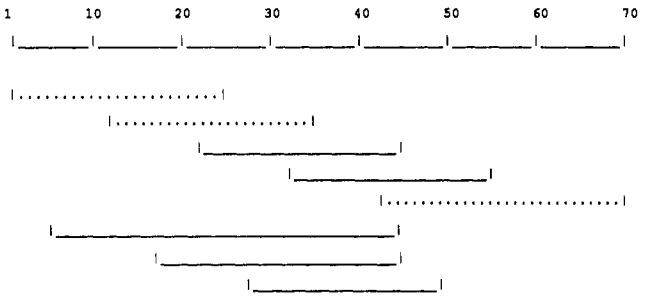
Results and Discussion

The bioassay responses to native recombinant bovine IGF I and IGF II are shown in Table I. Both proteins are potent stimulators of L6 myoblast proliferation; however, approximately 5 times the medium concentration of IGF II is required to achieve similar proliferative activities as those observed with IGF I. These relative differences in bioactivities have been reported by others (Ballard et al., 1986; Ewton et al., 1987).

Type I and II receptors have been demonstrated to occur on these myogenic cells (Beguinot et al., 1985; Read et al., 1986). The relative functions of these two receptor types in mediating biological response are currently under investigation. There is evidence that blocking the type II receptor does not interfere with IGF II stimulation of amino acid or glucose uptake or protein synthesis in L6 myoblasts (Kiess et al., 1987). These data suggest that at least these IGF II responses are probably mediated by the type I receptor in L6 cells as well as IGF I responses.

Activity of IGF I and II fragments was investigated in a concentration range of 1–100 μM. None of the fragments showed activity at 10 μM or lower. At 0.1 mM, two fragments (residues 21–45 and 31–55) of IGF I and two fragments (residues 20–44 and 30–54) of IGF II showed a 16–37% stimulation of the proliferation of rat L6 myoblast cells. Since a 1 nM concentration of IGF I or II shows activity, the activity of these fragments is extremely low compared to that of the native protein. Nonetheless, the stimulation by these active fragments as well as other active IGF I or II fragments in this paper was statistically significant, with a p value that was less than 0.01 and reproducible. Thus, the active sites of the fragments were suggested to be in the overlapping regions of these active fragments, i.e., residues 31–45 of IGF I and 30–44 of IGF II.

In order to confirm these results, a fragment (residues 26–50) of IGF I, which has extensions of five residues at both the N- and C-terminals of the common region (residues 31–45) of the active fragments, was synthesized. Indeed, this peptide (at 0.1 mM) showed a 24% stimulation of the proliferation rat L6 myoblast cells. Furthermore, the longer fragments (residues 16–45 and 4–45) which include this region and some of the B domain of IGF I did not show any higher activity compared to the shorter peptides. The results are summarized as



where the active fragments are shown with solid lines and the inactive fragments are shown with dashed lines. The common region of the active fragments is residues 31–45. Since the active fragments have comparable activity, the majority of the activity comes from the common residues 31–45, and residues 4–30 would not have any significant contribution to the stimulation of proliferation of rat L6 myoblast cells. A similar argument can be applied to identify residues 30–44 as the active site of the active IGF II fragments.

A sequence of bovine IGF II has been reported by Honegger and Humbel (1986). Ser-35 reported by these authors was found to be Ile-35 in IGF II isolated from bovine colostrum by Francis et al. (1988) later. Since the 35th residue is contained within the active fragments, we synthesized two fragments (residues 21–45 and 31–55) in which the 35th residue was replaced by Ser and analyzed their activity. Neither peptide showed any activity even at a high concentration of 0.1 mM (Table I). This suggests that Ile-35 is essential for fragment activity.[2]

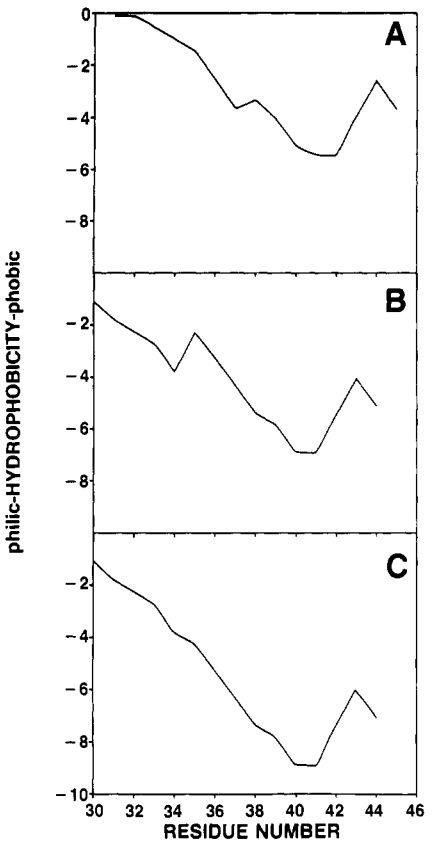


FIGURE 2: Hydrophobicity profile of IGF fragments. The IGF I fragment, residues 31–45 (panel A), the IGF II fragment, residues 30–44 (panel B), and the [Ser^{35}]IGF II fragment, residues 30–44 (panel C), are plotted by using the same method as in Figure 1C.

A sequence comparison of bovine IGF I and bovine IGF II is shown:

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        |------------ B domain -------||-C domain--||---- A domain -----

                  10        20        30        40        50        60
IGF I     GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRA-PQT-GIVDECCFRSCDLRRLEMY
            ::::: :::: :::::::::::::  :     :::       ::: ::::::::::  :: :
IGF II  AYRPSETLCGGELVDTLQFVCGDRGFYFSRP-----SSR-INRRSRGIVEECCFRSCDLALLETY
                  10        20        30              40        50

          -||-- D domain

                  70
IGF I     CAPLKPAKSA
          ::
IGF II    CATPAKSE
          60
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The symbol ":" in the comparison represents identical amino acids between the two proteins. Although the B- and A-domains of the IGFs are homologous, the C-domains of the IGFs show quite different sequences including insertion and deletion of amino acids. It is very surprising that the active regions of IGF I fragments (residues 31–45) and of IGF II fragments (residues 30–44) have weak sequence homology. However, since both stimulate the proliferation of L6 myoblast cells, there must be some common feature in this region. Figure 2A shows the hydrophobicity profile of residues 31–45 of IGF I, and Figure 2B shows the hydrophobicity profile of residues

[2] Three residues (Val-20, Ser- or Ile-35, and Glu-45) are different between (Ser-35)-IGF II-(21–45) and IGF II-(20–44). Val-20 is unlikely to be involved in the activity because IGF II-(30–54) missing Val-20 is also active. Thus, the lack of Val-20 in [Ser^{35}]IGF II-(21–45) should not cause the loss of the activity. Glu-45 is unlikely to influence the activity because both IGF II-(20–44) missing Glu-45 and IGF II-(30–54) containing Glu-45 are active. Thus, addition of Glu-45 in [Ser^{35}]IGF II-(21–45) should not cause the loss of the activity. Therefore, the loss of the activity of [Ser^{35}]IGF II-(21–45) should be due to the substitution of Ile-35 by Ser. Similar arguments can be applied to [Ser^{35}]IGF II-(31–55) and IGF II-(30–54).

30–44 of IGF II (see Experimental Procedures for details of the hydrophobicity profile). Although the sequences of IGF I and IGF II are quite different in this region as shown above, the hydrophobicity profiles of these sequences are homologous; i.e., most of the residues in this region are hydrophilic except for three residues, namely, Ala-38, Ile-43, and Val-44 of IGF I and Ile-35, Ile-42, and Val-43 of IGF II. Interestingly, the substitution of Ile-35 by Ser eliminates the hydrophobic hump as shown in Figure 2C and inactivates the peptide. These results suggest that the hydrophobicity/hydrophilicity of this region may be important for activity. Since Ile-35 of IGF II appears to be essential, the corresponding residue Ala-38 of IGF I would be important for its activity. The conserved residues around Ala-38 of IGF I or Ile-35 of IGF II are Ser-Ser-Arg (residues 34–36 of IGF I and residues 32–34 of IGF II) and Gly-Ile-Val (residues 42–44 of IGF I and 41–43 of IGF II). Some of those conserved residues between IGF I and IGF II may be involved in the fragment activity.

The active site of IGF has not been identified previously. Certain residues have been suggested to be involved with the binding to the type I receptor. For example, the IGF I–type I receptor complex protected Tyr-24, Tyr-31, and Tyr-60 of IGF I from iodination (Maly & Luthi, 1988). Although this suggests that these tyrosines are at or close to the binding site of IGF I, it does not mean that these tyrosines are involved in binding to the receptor. Tyr-24 of human IGF I was further suggested to be involved in binding to the type I receptor because the mutation of Tyr-24 to Leu or Ser affects receptor binding. However, the binding affected may be due to indirect effects rather than the loss of an essential Tyr from the 24th residue. An example of the indirect effect has been suggested for IGF I. It has been found that the deletion of the N-terminal three residues increases the IGF I activity approximately 7 times in the protein synthesis bioassay. This increase in activity was suggested to be due to an indirect effect between the three N-terminal residues and the binding of IGF I to nonreceptor carrier proteins (Francis et al., 1986; Szabo et al., 1988). The removal of the three N-terminal residues may decrease the binding to the carrier proteins, resulting in an increased concentration of the IGF I analogue, which binds to the type I receptor (Ballard et al., 1987). Tseng et al. (1987) and Chen et al. (1988) used hybrid molecules of insulin and IGF I to identify the active site of IGF I. The activities of the hybrid molecules as measured by [^{3}H]thymidine incorporation into chick embryo fibroblasts were in the order of IGF I (relative potency = 100) > the hybrid molecule between the A-domain of IGF I and the B-domain of insulin (16.6) > insulin (3.3) > the hybrid molecule between the A-domain of insulin and the B-domain of IGF I (0.25). Furthermore, Danho et al. (1981) reported that a human insulin A-chain disulfide has weak growth-promoting activity (0.2–0.3% of IGF I). These results indicate that the A-domain (residues 42–62) of IGF I is significantly involved in its growth-promoting activity.

In this paper we measured the activity of IGF fragments, and some of them containing the C-domain and the N-terminal portion of the A-domain showed activity at a high concentration of 0.1 mM compared to IGF activity at nanomolar concentrations. C-Terminal fragments of IGF containing A- and D-domains did not show activity. This indicates that the C-domain contributes to the activity of IGF fragments. Since the activity of the fragments is so weak, the active regions in IGF *fragments* may not be the active site of IGF *proteins*. Even so, it is quite interesting and surprising that two nonhomologous regions of IGF (residues 31–45 of IGF I and residues 30–44 of IGF II) showed the same biological activity to stimulate the proliferation of L6 myoblast cells.

Conclusions

Fragments of IGF I and IGF II (residues 4–45, 16–45, 21–45, 26–50, and 31–55 of IGF I and residues 20–44 and 30–54 of IGF II) stimulated the proliferation of rat L6 myoblast cells at a concentration of 0.1 mM. Even though the activities of these fragments relative to the corresponding entire proteins are very low, the results indicate that the active sites of IGF I and II *fragments* are located in the regions of residues 31–45 and 30–44, respectively. It is very interesting that these two regions have weak sequence homology yet showed the same biological activity. The hydrophobicity profiles of these regions in IGF I and IGF II were similar, suggesting that hydrophobicity may be a significant factor for the fragment activity.

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Ethoxyformylation of Tubulin with [^{3}H]Diethyl Pyrocarbonate: A Reexamination of the Mechanism of Assembly Inhibition†

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ABSTRACT: In this study we reexamined the basis for the profound inhibitory effects of low concentrations of diethyl pyrocarbonate (DEP) on tubulin's ability to assemble into microtubules [cf. Lee, Y. C., Houston, L. I., & Himes, R. H. (1976) *Biochem. Biophys. Res. Commun. 70*, 50–56]. Assembly inhibition at low DEP concentrations can be resolved into two components: a component reversible with hydroxylamine (attributed to monoethoxyformylation of histidyl residues) that contributes ~40% of the inhibition and a hydroxylamine-resistant component (attributed to ethoxyformylation of non-histidyl residues) that contributes ~60% of the inhibition. Comparisons between the extent of assembly inhibition associated with each component and the degree of residue modification argue for the involvement of a small number of highly reactive residues in the inhibition process. To identify these residues, tubulin was reacted with limiting concentrations of [^{3}H]DEP and subjected to tryptic digestion and HPLC analysis. Only one moderately reactive histidyl residue was detected. This residue (~2–3-fold more reactive than the bulk histidyl residues) eluted in an apparently large, hydrophobic fragment. We failed to detect any non-histidyl residues that were exceptionally reactive to [^{3}H]DEP. However, we did observe that the N-terminal methionyl residues in native protein were ethoxyformylated at rates comparable to that of the bulk histidyl residues. In denatured protein these methionyl residues were ethoxyformylated to a much larger extent (~3–4-fold) than the bulk histidyl residues. We suggest that the N-terminal methionyl residues in tubulin are partly buried or are in a salt-bridge interaction in native protein and that ethoxyformylation of these residues disrupts tubulin structure and interferes with microtubule assembly.

Microtubules are cylindical structures several hundred angstroms in diameter and typically several microns long that participate in a variety of diverse functions in eucaryotic cells including mitosis, morphogenesis, and vesicle transport (Soifer, 1986). A molecular understanding of these functions will involve in part detailed knowledge of the properties of the major constituent protein of the microtuble, tubulin, and its two homologous subunits, α- and β-tubulin. These subunits are each ~450 amino acid residues in length (Ponstingl et al., 1981; Krauhs et al., 1981), display ca. 40% sequence homology, and have different functional roles which at present are only poorly understood (Luduena, 1979; Cleveland, 1987).

We have had a longstanding interest in the α-subunit. This subunit contains a highly reactive lysyl residue, Lys 394, whose methylation renders tubulin assembly incompetent (Szasz et al., 1982, 1986). Chemical modifications of this reactive residue have been implicated in the pathophysiology of diabetes (Williams et al., 1982) and in alcohol-induced hepatic necrosis (Jennett et al., 1987, 1989), two disorders that result in impaired microtubule function. Lys 394 is thought to be in a positively charged cluster consisting of Arg 390, His 393, and Lys 394 which renders this residue reactive as a nucleophile (Szasz et al., 1986). The role of this cluster in assembly is not understood, although it has been suggested that the cluster interacts with highly negatively charged regions in the C-terminal domain of α-tubulin to stabilize a conformation essential for microtubule assembly (Blank et al., 1986).

Lee et al. (1976) reported that microtubule assembly is inhibited by low concentrations of DEP[1] and proposed that

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