Proteolytic conversion of insulin-like growth factors to an acidic form(s)

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The relative amounts of the various forms of bioassayable insulin-like growth factors (IGF) isolated from human serum or serum fraction Cohn IV-1 depend on the purification procedure. With acid gel filtration or acid/ethanol extraction as the initial step, IGF-II (pI ~6.5) was the most abundant (40–70%) followed by somatomedin A (pI ~7.4; 15–23%), an acidic form of insulin-like activity (ILA pI4.8) (13–21%) and IGF-I (pI ~8.5; 5–27%). If, however, pH 5.5 ion-exchange chromatography on SP-Sephadex was used prior to acid gel filtration, the acidic pI4.8 form was the major (>90%) species recovered and was accompanied by a quantitative loss of the other IGF species. This suggested a possible conversion of IGF-I, somatomedin A and/or IGF-II to the acidic ILA pI4.8 form(s) during the SP-Sephadex procedure. Further experiments indicated that differences in the yields of ILA pI4.8 were not due simply to differences in the initial pH conditions of the various methods (i.e. acid versus neutral), although exposure to pH 9.7 (a pH experienced during elution of IGF activity from the SP-Sephadex) did appear to play a role. The involvement of the carrier protein in the conversion process was tested by subjecting carrier-free IGF-I and IGF-II to the SP-Sephadex procedure. No conversion of the free forms to ILA pI4.8 occurred. To examine the possible role of proteinase in the conversion of IGFs to ILA pI4.8, SP-Sephadex chromatography was performed in the presence of a broad spectrum proteinase inhibitor. The IGF distribution pattern obtained closely resembled the 'normal' pattern seen with acid gel filtration, indicating that proteinase inactivation had prevented conversion to ILA pI4.8. These data suggest that proteolytic conversion of IGF-I, somatomedin A and IGF-II to more acidic ILA pI4.8 form(s) (i) occurs during SP-Sephadex chromatography, (ii) is not prevented simply by prior acid exposure, and (iii) takes place only when IGF-I and -II are in their high- M_r carrier-bound forms. Since IGF-I and IGF-II, although homologous, have unique amino acid sequences, the conversion of both IGFs implies that at least two acidic ILA forms exist. Nevertheless, because ILA pI4.8 retains the full spectrum of IGF bioactivities *in vitro*, and significant quantities are present in normal human serum (21%), it would suggest that proteolytic conversion of IGF-I, somatomedin A and IGF-II to ILA pI4.8 *in vivo* may be a physiologically significant event.

In the accompanying paper (Herington & Kuffer, 1984) we have reported on the IGF/somatomedin-like characteristics and biological activities of an acidic form of serum NSILA. This species (ILA pI4.8) has been largely ignored over the years, perhaps due to the presence of a co-purifying specific IGF inhibitor (Herington & Kuffer, 1981) which has a similar isoelectric point

(pI ~4.4). Thus, the possibility that ILA pI4.8 may represent a physiologically significant and relevant entity deserves further study.

Cockerill *et al.* (1980) first reported a procedure which resulted in a high yield of the ILA pI4.8 species, with concomitant loss of IGF-I (i.e. somatomedin C; Klapper *et al.*, 1983) and IGF-II. This procedure involved an initial pH 5.5 ion-exchange chromatography step (on SP-Sephadex). These studies raised the question of whether the relative yields of the various IGF/somatomedin species were dependent on the initial steps of the purifica-

Abbreviations used: IGF, insulin-like growth factor; NSILA, non-suppressible insulin-like activity; ILA pI4.8, an acidic form of NSILA.

tion protocols used by different laboratories. Furthermore, it was important to determine the reason for the apparent increase in acidic material and concomitant loss of neutral and basic species in the SP-Sephadex procedure. We have addressed both issues in the present study and the data indicate that the initial purification step is important for the subsequent isolation of various IGFs and that the ILA pI4.8 species may be a proteolytic cleavage product of IGF-I, somatomedin A and IGF-II. The presence of ILA pI4.8 in serum and its retention of biological activity (Herington & Kuffer, 1984) suggest that it may be a biologically important IGF form.

Materials and methods

Materials

Cohn fraction IV-1 of human serum was obtained as a lyophilized powder from the Commonwealth Serum Laboratories, Melbourne, Australia. SP-Sephadex C-25, Sephadex G-75 and Sephadex IEF were obtained from Pharmacia,

Uppsala, Sweden. Bio-Gel P-30 was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Ampholines (pH3.5–10.0) were from LKB Produkter AB, Bromma, Sweden. Trasylol (100000k.i.u./10ml) was obtained from Bayer Australia, Botany, Australia. All other chemicals and reagents were of analytical grade. Spectrapor 3 dialysis tubing (M_r cut-off 3500) was obtained from Spectrum Industries, Los Angeles, CA, U.S.A.

Isolation procedures

Initial extraction. The isolation of IGF species was carried out primarily by two methods (Fig. 1) which differ only in the initial step of the procedure.

Method A involved dissolution of Cohn fraction IV-1 in acid (1% formic acid, pH2.3) prior to acid pH gel filtration on Sephadex G-75.

Method B involved a slightly acidic pH5.5 extraction (0.05M-ammonium acetate buffer) of Cohn fraction IV-1 prior to pH5.5 ion-exchange chromatography on SP-Sephadex C-25 as de-

Fig. 1. Flow chart for the two primary methods (A and B) used for isolation of the various forms of IGF from human serum Cohn fraction IV-1

The details are given in the Materials and methods section. The steps marked with an asterisk (*) represent those that were specifically investigated for a possible role in IGF conversion (see Table 2).

scribed by Cockerill *et al.* (1980) and modified into a batch procedure by Herington & Kuffer (1981). Urea was omitted from the previously reported extraction buffer.

To allow a wider comparison of the presence and relative yields of various IGF species, two other commonly used initial purification steps [i.e. acid/ethanol extraction (Franklin *et al.*, 1976) of Cohn fraction IV-1 (method C) and acidification of human serum to pH 2.3 with formic acid (Cornell, 1982) (method D)] were also substituted into the overall scheme shown in Fig. 1.

Acid gel filtration. The acidified Cohn fraction IV-1 or the active product from the SP-Sephadex procedure (resuspended in 1% formic acid) was gel chromatographed on a column (4 cm $\times$ 145 cm) of Sephadex G-75 in 1% formic acid. The low- M_r bioactive fraction (K_{av} , 0.4–0.8) was pooled, dialysed and lyophilized. To remove the co-purifying M_r 17000 IGF inhibitor (Herington & Kuffer, 1981) this fraction was rechromatographed on a column (2.3 cm $\times$ 48 cm) of Bio-Gel P-30 in 1% formic acid. The IGF-active region, K_{av} , 0.5–0.9, was pooled, dialysed and lyophilized.

Isoelectric focusing. The Bio-Gel P-30 active fraction was subjected to flat-bed isoelectric focusing over the pH range 3.5–10.0 in Sephadex IEF (16 h; 5 W; 1500 V; 4°C). In general 5–10 munit of insulin-like activity were applied to each run. Upon completion, the isoelectric focusing plate (11.1 cm $\times$ 24.3 cm $\times$ 0.4 cm) was divided into 23 1 cm wide segments, the Sephadex IEF from each region was placed into plastic centrifuge tubes with 1.6 ml of deionized water, centrifuged and the pH of the supernatant determined at 4°C. The corresponding supernatants and pellets were remixed and then mostly paired to provide 14 samples across the pH range 4–9.3 and placed in plastic minicolumns. The isoelectric focusing fractions were eluted with 1% formic acid and portions taken for dialysis prior to assay. After assay (a representative profile is shown in Fig. 2) the activity within the appropriate pH ranges (4.8 ± 0.5 ; 6.2 ± 0.5 ; 7.4 ± 0.4 ; 8.5 ± 0.5) was summed.

IGF bioassay

IGF activity was measured as NSILA in the isolated rat adipocyte bioassay (conversion of [14 C]glucose to [14 C]lipid in the presence of excess anti-insulin serum) (Franklin *et al.*, 1976). All isoelectric focusing fractions from a single run, together with an aliquot of 'starting material', were assessed together in the same assay. The inter- and intra-assay coefficients of variation were 22% and 10% respectively. All samples were assayed at three dose levels and potencies and estimates of parallelism with the insulin standard were calculated by computer by the method described by Borth

Fig. 2. Representative isoelectric focusing pattern of recovered IGF activity across the pH range 4–9.3 (●—●) following isolation by acidification of Cohn fraction IV-1 (method A).

Each bar represents a fraction assayed for IGF-like activity which is expressed as a percentage of activity recovered.

ted by computer by the method described by Borth (1960).

Studies on the possible cause of IGF conversion

The possible role of pH differences in the initial isolation steps of methods A and B was studied by the following manipulations: (a) extraction of Cohn fraction IV-1 at pH 5.5 (as in the SP-Sephadex procedure, method B) prior to acid gel filtration as in method A; (b) extraction of Cohn fraction IV-1 at pH 5.5 followed by adjustment to pH 9.7 (2 days) (as in method B) prior to acid gel filtration; (c) replacement of the pH 9.7 elution step in method B by a pH 7.4/1.0 M-NaCl elution step; (d) extraction of Cohn fraction IV-1 in pH 5.5 buffer (as in method B) followed by acidification to pH < 2.8 for 16 h. The extract was then readjusted to pH 5.5, dialysed against pH 5.5 buffer until the conductivity returned to normal and then subjected to the SP-Sephadex procedure (method B).

The possible role of proteolytic activity in the conversion of IGF was tested by inclusion in all buffers of the SP-Sephadex procedure (method B), of a broad spectrum proteinase inhibitor, Trasylol (1000 k.i.u./ml).

The relevance of the molecular nature of IGF (i.e. free or carrier-bound) for the conversion process was studied by subjecting carrier-free IGF-I and IGF-II (previously isolated by isoelectric focusing using method A) to the SP-Sephadex procedure (method B), either with or without prior mixing with a pH 5.5 extract of Cohn fraction IV-1 (as a source of added carrier protein and/or other factors responsible for conversion).

Results

Table 1 shows, for each extraction procedure used, the distribution pattern and relative yields

Table 1. *Distribution patterns and overall yields of recovered IGF activity within the pH regions 4.8 ± 0.5 , 6.2 ± 0.5 , 7.4 ± 0.4 and 8.5 ± 0.5 following isolation by acidification of Cohn fraction IV-1 (method A) (a), pH 5.5 SP-Sephadex ion exchange chromatography (method B) (b), acid/ethanol extraction of Cohn fraction IV-1 (c), and acidification of whole human serum (d)*

IGF activity has been expressed as munit of insulin equivalents recovered/100g of Cohn fraction IV-1 starting material (a, b and c) or as munit/litre of human serum (d). Values are means $\pm$ S.E.M. for $n = 3$.

Initial purification step	Bioassayable IGF-like activity (munit of insulin equivalents/100g of starting material*)				Overall yield
	pI4.8 ± 0.5	pI6.2 ± 0.5	pI7.4 ± 0.4	pI8.5 ± 0.5	
(a) Acidified Cohn fraction IV-1 (method A)	13.6 ± 2.8	38.6 ± 8.6	22.8 ± 7.1	15.5 ± 2.0	96.8 ± 14.0
(b) SP-Sephadex chromatography of Cohn fraction IV-1 (method B)	75.7 ± 22.8	4.3 ± 2.1	2.1 ± 1.4	0	87.5 ± 27.0
(c) Acid/ethanol extraction of Cohn fraction IV-1 (method C)	8.6 ± 4.3	27.1 ± 4.5	5.0 ± 1.4	2.1 ± 1.4	39.9 ± 7.6
(d) Acidified serum* (method D)	5.7 $\pm 1.1^*$	13.75 $\pm 5.3^*$	4.6 $\pm 2.5^*$	2.8 $\pm 1.1^*$	32.9 $\pm 7.7^*$

* munit/litre for serum.

(expressed as munit of insulin equivalents/100mg of Cohn fraction IV-1 starting material) [or/litre of normal human serum (method D)] of the four major regions of IGF activity recovered after isoelectric focusing (i.e. pH 4.8 ± 0.5 , 6.2 ± 0.5 , 7.4 ± 0.4 and 8.5 ± 0.5 , representing ILA pI4.8, IGF-II, somatomedin A and IGF-I/somatomedin C respectively). The distribution pattern is very similar for methods A, C and D with all four species present. Of particular interest is the relatively high proportion (13–21%) of pI4.8 material obtained by each of these standard methods. The greater abundance (3–5-fold) of IGF-II over IGF-I in serum (method D) is consistent with direct measurement by radio-immunoassay of IGF levels in acid-stripped sera (Zapf *et al.*, 1981).

The striking feature of this comparison is the isolation of only ILA pI4.8 by the SP-Sephadex procedure (method B). Since comparable overall yields of activity were obtained by methods A and B (96 ± 14 and 87 ± 27 munit/100mg of Cohn fraction IV-1 respectively), it is apparent that in the SP-Sephadex procedure a marked increase in ILA pI4.8 has occurred with a concomitant and quantitative loss of the other three species. Because the overall yields of IGF activity are the same it is highly unlikely that such loss is simply due to degradation of IGF-I, IGF-II and somatomedin A. Rather, it would suggest that a conversion of these species to another active product(s), i.e. ILA pI4.8, has occurred. The following studies were designed to test the nature and possible cause of such a conversion.

Effect of differences in pH conditions between methods A and B

A major difference in the initial steps of methods A and B is the pH (2.3 or 5.5 respectively) used for

initial extraction of Cohn fraction IV-1. Thus a series of cross-over experiments were performed.

Firstly, as shown in Table 2 (line 3), extraction of Cohn fraction IV-1 at pH 5.5 (as in method B) prior to acidification and acid gel filtration (as in method A), gave the distribution pattern expected for method A (Table 2, line 1). Thus, it appears that simple exposure to an initial pH of ~ 5.5 does not play a role in the conversion of IGF to ILA pI4.8.

A second major pH difference between methods A and B exists at the pH 9.7 elution step of the SP-Sephadex procedure. The involvement of this step was tested by extraction of Cohn fraction IV-1 at pH 5.5 followed by adjustment to, and maintenance at, pH 9.7 for up to 2 days (a pH and duration experienced in the batch procedure of method B), prior to acidification and acid gel filtration (as in method A). Table 2, line 4 indicates that the basic pattern of method A was retained although some loss of IGF-I (pI8.5) occurred. This was mirrored by a small (although not significant) increase in the yield of ILA pI4.8.

To test further the involvement of the pH 9.7 elution step of method B in IGF conversion, this particular step was replaced by a pH 7.4/1 M-NaCl elution step. This resulted in a distribution pattern resembling the acid gel filtration method (A) rather than the SP-Sephadex method (B) (Table 2, line 5), suggesting therefore that the pH 9.7 elution step of method B does play some role in IGF conversion.

Finally, to test the effect of simple acid exposure on the SP-Sephadex conversion process, Cohn fraction IV-1 was extracted at pH 5.5 (as in method B), acidified to pH ~ 2.3 and then reneutralized to pH 5.5 prior to being subjected to the normal SP-Sephadex procedure (method B). A distribution pattern very similar to that for method B was

Table 2. Distribution patterns of IGF activity recovered by isoelectric focusing following various manipulations (lines 3–9) of the standard purification protocols (methods A and B, lines 1 and 2)

These manipulations are described fully in the Materials and methods section. The IGF activity within the pH regions 4.8 ± 0.5 , 6.2 ± 0.5 , 7.4 ± 0.4 and 8.5 ± 0.5 has been expressed as the percentage of total IGF-like activity recovered across the entire isoelectric focusing range (pH 4–9.3). Data are given as mean $\pm$ S.E.M. ($n = 3$) or as individual values (lines 5, 8 and 9) where $n < 3$. The percentage data given for methods A and B (lines 1 and 2) are derived from the data expressed as munits/100g shown in Table 1.

Manipulation of purification steps	Bioassayable IGF-like activity (% of activity recovered)			
	pI4.8 $\pm$ 0.5	pI6.2 $\pm$ 0.5	pI7.4 $\pm$ 0.4	pI8.5 $\pm$ 0.5
1 Acidified Cohn fraction IV-1 (method A)	15.0 $\pm$ 3.1	42.6 $\pm$ 9.5	25.2 $\pm$ 7.8	17.1 $\pm$ 2.2
2 SP-Sephadex chromatography of Cohn fraction IV-1 (method B)	83.0 $\pm$ 23.7	5.2 $\pm$ 3.0	3.0 $\pm$ 2.3	0
3 pH5.5 extraction $\rightarrow$ acid G-75	14.9 $\pm$ 2.0	62.0 $\pm$ 13.4	6.9 $\pm$ 2.0	16.1 $\pm$ 4.7
4 pH5.5 extraction $\rightarrow$ pH9.7 $\rightarrow$ acid G-75	21.0 $\pm$ 8.4	68.7 $\pm$ 33.9	3.9 $\pm$ 3.9	6.5 $\pm$ 1.3
5 pH5.5 extraction $\rightarrow$ SP-Sephadex + pH7.4/1M-NaCl elution	10.7, 20.5	81.0, 63.2	5.4, 6.0	6.7, 10.2
6 pH5.5 extraction + acidification/reneutralization $\rightarrow$ SP-Sephadex	70.6 $\pm$ 10.2	26.9 $\pm$ 7.7	2.5 $\pm$ 0.8	0
7 pH5.5 extraction + proteinase inhibitor $\rightarrow$ SP-Sephadex	25.3 $\pm$ 5.8	51.3 $\pm$ 4.7	9.3 $\pm$ 1.9	7.2 $\pm$ 1.6
8 Free IGF-I and -II $\rightarrow$ SP-Sephadex	7	83	1	18
9 Free IGF-I and -II + Cohn fraction IV-1 extract $\rightarrow$ SP-Sephadex	23, 25	56, 68	1, 9	2, 4

obtained, i.e. isolation of ILA pI4.8 as the major product (Table 2, line 6). Thus conversion is not prevented simply by initial exposure to acid.

Effect of a proteinase inhibitor on IGF conversion

To test whether the action of a proteinase during IGF isolation led to conversion of IGF to ILA pI4.8, a broad spectrum proteinase inhibitor, Trasylol, was included in all extraction and elution buffers of the SP-Sephadex procedure (method B). As shown in Table 2 (line 7), the distribution pattern expected for method B was drastically altered and was now equivalent to that seen with the other methods (A, C and D), clearly indicating the involvement of a proteinase in the production of ILA pI4.8.

Influence of the carrier-bound state on IGF conversion

Another major difference between methods A and B, apart from the pH differences *per se*, is the state of the IGF during initial processing, i.e. carrier-bound or free. The initial acid gel filtration of Cohn fraction IV-1 (method A) ensures that all IGFs are in their carrier-free state during all stages of purification. On the other hand, in method B the IGFs remain in their carrier-bound state during the whole of the initial SP-Sephadex elution (Cockerill *et al.*, 1980; Herington & Kuffer, 1981). The influence, therefore, of the carrier proteins on IGF conversion was tested by processing, via the normal SP-Sephadex method (B), a mixture of carrier-free IGF-I and IGF-II (previously isolated by isoelectric focusing following method A). As shown in Table 2 (line 8), essentially only IGF-I and IGF-II were recovered, indicating that in the absence of carrier protein little or no conversion to

ILA pI4.8 occurred. This was further assessed by processing on SP-Sephadex the same preparation of free IGF-I and IGF-II following mixture with a normal pH5.5 extract of Cohn fraction IV-1. This extract would contain carrier-bound IGF, unsaturated carrier protein, and other factors (e.g. proteinases) which may be involved in IGF conversion. As shown in Table 2 (line 9), the majority of IGF recovered was as free IGF-II, but some loss of IGF-I in particular occurred. ILA pI4.8 was present in significant amounts ($\sim 25\%$), with the yield (in munits of ILA, not shown) being slightly greater than would be normally expected from processing that amount of Cohn fraction IV-1 alone through method B. This increased yield of ILA pI4.8 probably represents the normal SP-Sephadex conversion product plus some pI4.8 material converted from IGF-I and -II. The conversion of some 'free' IGF may be due to a limited recombination with the unsaturated IGF binding protein present in the added Cohn IV-1 extract. This result adds support to the view that only carrier-bound IGF is convertible to ILA pI4.8 and that failure of carrier-free IGF to convert is not due simply to prior removal of other high- M_r factors (e.g. proteinases) in other procedures (e.g. A).

Discussion

Of the various IGF species reported, the ILA pI4.8 form has not been widely studied although its existence has often been noted (Van Wyk *et al.*, 1971, 1974; Posner *et al.*, 1978; Cockerill *et al.*, 1980; Herington & Kuffer, 1982). The identification in serum of a specific IGF inhibitor (pI ~ 4.4)

(Herington & Kuffer, 1981), which co-purifies with IGF through most conventional purification procedures, is the most likely explanation for the failure of ILA pI4.8 to gain wider recognition. With the development of an ion-exchange SP-Sephadex procedure for the isolation of IGFs (Cockerill *et al.*, 1980; Herington & Kuffer, 1982), we were surprised to find that ILA pI4.8 was the major product isolated with little, if any, of the more widely recognized species IGF-I, -II and somatomedin A. We have since shown (Herington & Kuffer, 1984) that ILA pI4.8 possesses many of the biological activities of the other IGFs.

In an attempt to determine whether ILA pI4.8 might be an artifact of the isolation procedure, and/or whether in the absence of IGF inhibitor it represented a significant entity in normal serum, a comparison was made of the distribution patterns and relative yields of the IGFs obtained by a variety of reported isolation procedures. Indeed, even by the simplest procedure utilized (acid gel filtration of serum followed by isoelectric focusing; method D), ILA pI4.8 represented up to 21% of IGF activity present, a similar proportion to IGF-I. Its presumed presence in normal human serum, therefore, together with its retention of biological activities, suggests that it may be a physiologically significant species.

The association of an increased ILA pI4.8 yield with a concomitant and quantitative loss of the other IGF species during SP-Sephadex chromatography (method B) suggested some kind of conversion process. Since the overall IGF yields from methods A and B were equivalent (96 ± 14 and 87 ± 27 munit/100mg of Cohn fraction IV-1 respectively) it is unlikely that ILA pI4.8 is simply part of an IGF degradation process. Furthermore, the ability of a proteinase inhibitor to maintain the presence of IGF-I, -II and somatomedin A throughout the SP-Sephadex procedure rules out the possibility that the ILA pI4.8 is in fact a precursor molecule that is converted into the IGFs. Rather, the data suggest that the proteolytic event involves conversion of IGF-I, -II and somatomedin A to an ILA pI4.8 form(s), that it occurs on SP-Sephadex only when the IGFs are in the carrier-bound state, that it may be favoured by basic (pH9.7) conditions, and that it is not prevented simply by prior acid exposure. The data also suggest that IGF-I and somatomedin A may be more susceptible to conversion than is IGF-II.

The inability to convert carrier-free IGF-I and -II on SP-Sephadex also indicates that the conversion is not an artifact of the SP procedure *per se*. A similar conclusion can be drawn from two other reports. First, Svoboda *et al.* (1980) used SP-Sephadex to isolate the pI8.5 species, IGF-I/somatomedin C. Their initial extraction of Cohn

fraction IV-1 and application to SP-Sephadex was carried out at pH2.3, a condition in which none of the IGFs are in their carrier-bound form. Although they did not ascertain the presence or absence of ILA pI4.8 in these experiments, using this same protocol and a general IGF bioassay we observed only a small proportion ($\sim 5\%$) of ILA pI4.8 material (results not shown). Secondly, Cornell (1982) has demonstrated that acid gel filtration followed by application of the carrier-free forms of IGF to pH5.5 SP-Sephadex chromatography resulted in detection only of IGF-I and -II by specific radioimmunoassays or competitive binding protein assay. If, however, the chromatography order was reversed (SP-Sephadex then acid gel filtration, equivalent to our method B) then only ILA pI4.8 was observed, as detected by binding protein assay or bioassay. Cornell's (1982) study also indicated that there was some ILA pI4.8 cross-reactivity with the IGF-I radioimmunoassay. These observations raise the possibility that the proteinase responsible for conversion may in fact be one of the IGF carrier proteins, a subunit of the carrier proteins or otherwise associated with the carrier proteins. Whilst this question remains unresolved such a situation would be analogous to that for nerve growth factor and epidermal growth factor, both of which have serine proteinase as components of their high- M_r native complexes (Shooter *et al.*, 1981).

Finally, the mechanism and site of the proposed proteolytic cleavage is of some interest, given that a large drop in isoelectric point results from a cleavage which has only a marginal effect on M_r (Cockerill *et al.*, 1980). Simple deamidation is an unlikely explanation since (1) a major loss of amide groups would be required to lower the pI into the region (4.8) of the pK of the free carboxyl groups of the amino acid residues, (2) it is unlikely to be affected by basic pH alone, (3) it is unlikely to be affected by proteinase inhibitor, and (4) it is unlikely to show specificity for only carrier-bound forms of IGFs. An alternative hypothesis that would accommodate both requirements would be the cleavage of the extremely basic, but short, C-peptide sequences of IGF-I and -II. The C-peptide of IGF-I (twelve amino acids) contains two arginine residues, whereas that for IGF-II (eight amino acids) contains four (Rinderknecht & Humbel, 1978a,b). The absence of a C-peptide is tentatively supported by the observations (Herington & Kuffer, 1984) that no cross-reactivity of ILA pI4.8 occurs in radioimmunoassays for IGF-I and -II C-peptides but that some cross-reactivity was seen in radioimmunoassays for entire IGF-I with a variety of different antisera. Furthermore, if one examines the amino acid sequences in the IGF C-peptide regions there are several recognized cleav-

age points for proteinases, such as the kallikreins, the nerve growth factor and epidermal growth factor serine proteinases and endogenous insulinases. All of these possibilities require rigorous testing. A further consequence of the proteolytic conversion of both IGF-I and -II (and perhaps somatomedin A) is that due to their unique, although homologous, sequences there must be more than one ILA pI4.8 entity. This form has not yet been purified to homogeneity, thus one could assume that the acidic component does represent a mixture of at least two related molecules. However, since ILA pI4.8 does represent a significant proportion of the IGF able to be isolated from serum, and since it retains most biological activities of the other IGFs, the proteolytic conversion of IGFs to ILA pI4.8 may be a physiologically important event.

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