Insulin-like growth factor characteristics of an acidic non-suppressible insulin-like activity

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The biological activities of an acidic form of non-suppressible insulin-like activity (ILA pI4.8) have been studied. ILA pI4.8 was isolated from Cohn fraction IV-1 of human serum by pH 5.5 ion-exchange chromatography on SP-Sephadex. Carrier-bound ILA was eluted at pH 9.7 and then sequentially gel chromatographed in 1% formic acid on Sephadex G-75 and Bio-Gel P-30. The low- M_r (7000) active material was subjected to flat bed isoelectric focusing. Overall recovery was 87 munit of insulin equivalents/100 g of Cohn fraction IV-1, with a specific activity in the range 4–10 munit/mg of protein, representing a purity of 1–6%. This material has been tested in a variety of insulin-like growth factor (IGF)/somatomedin assay systems. It stimulated, in a dose-related manner, [^{14}C]glucose conversion into lipid by isolated rat adipocytes, $^{35}\text{SO}_4^{2-}$ incorporation into weanling rat costal cartilage and [^3H]thymidine incorporation into DNA of cultured human fibroblasts. Like IGF-I and -II, ILA pI 4.8 was able to inhibit degradation of ^{125}I -insulin by crude homogenates of rat liver. In addition, the biological activity of ILA pI 4.8 was completely suppressible by a recently described inhibitor of IGF-I and IGF-II. ILA pI 4.8 was able to compete, in a parallel manner, with ^{125}I -IGF-I and ^{125}I -IGF-II and, at higher doses, with ^{125}I -insulin in a placental radioreceptor assay. No cross-reactivity was seen in a radioimmunoassay for IGF-I and -II C-peptides, but at higher concentrations parallel displacement was observed in a somatomedin C/IGF-I radioimmunoassay using two different antisera. These data indicate that ILA pI 4.8 does possess many of the biological activities previously reported for the IGFs. Since ILA pI4.8 does occur naturally in serum, it would appear reasonable to tentatively include it as one of the IGF/somatomedin family.

The insulin-like growth factors (IGF) or somatomedins are a family of growth hormone-dependent serum growth factors which circulate bound in a high- M_r complex. The long-held presumption that they regulate skeletal growth (Zapf *et al.*, 1978a; Phillips & Vasilopoulou-Sellin, 1980a, b; Daughaday, 1982) has now been substantiated in IGF-treated, hypophysectomized rats with highly purified preparations of IGF-I and -II (Schoenle *et al.*, 1982). In addition to such effects *in vivo* on growth, these factors have a variety of actions *in vitro*, including the stimulation of proteoglycan sulphation in cartilage tissue, acute insulin-like actions (non-suppressible by anti-insulin serum) in

adipose and muscle tissues (NSILA), and the stimulation of mitogenic and proliferative activity of a variety of cells in culture.

A number of biologically active forms of human IGF/somatomedin have been described, differing primarily in isoelectric point: IGF/somatomedin C (pI8.0–8.6) (Rinderknecht & Humbel, 1978a; Svoboda *et al.*, 1980; Klapper *et al.*, 1983), somatomedin A (pI ~ 7.4) (Hall & Fryklund, 1979) and IGF-II (pI6.5) (Rinderknecht & Humbel, 1978b). In addition, a fourth, biologically active, acidic form of NSILA (ILA pI4.8) has been reported (Cockerill *et al.*, 1980). This species has been largely ignored over the years, despite being recognized more than 10 years ago (e.g. Van Wyk *et al.*, 1971, 1974; Posner *et al.*, 1978). This may be due to its apparently low concentration in serum relative to the more abundant basic (IGF-I/

Abbreviations used: NSILA, non-suppressible insulin-like activity; ILA pI 4.8; an acidic form of NSILA; IGF, insulin-like growth factor.

somatomedin C) and neutral (somatomedin A, IGF-II) forms, and to the presence in human serum of a highly specific IGF inhibitor which is also acidic (pI ~4.4) (Herington & Kuffer, 1981).

Utilizing an IGF purification scheme first reported from this laboratory (Cockerill *et al.*, 1980), we have developed (Kuffer & Herington, 1984; the accompanying paper) a procedure which results in the isolation primarily of ILA pI4.8, at the expense of the other IGF/somatomedin peptides. We present here data to demonstrate that partially purified ILA pI4.8 possesses many of the characteristics and biological activities commonly associated with the members of the IGF/somatomedin family. The results suggest that ILA pI4.8 may well warrant inclusion within the family of IGF/somatomedins.

Materials and methods

Materials

Cohn fraction IV-I of human serum was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Chromatography gels were from Pharmacia, Uppsala, Sweden (Sephadex G-75, SP-Sephadex C-25, Sephadex IEF) or Bio-Rad Laboratories, Richmond, CA, U.S.A. (Bio-Gel P-30). Ampholines (pH 3.5–10) were obtained from LKB Produkter AB, Bromma, Sweden. Partially purified IGF-I and -II were gifts of Dr. R. E. Humbel, Zurich, Switzerland. All other reagents were of analytical grade.

Purification of acidic ILA

The procedure for purification of ILA pI4.8 is basically that described by Cockerill *et al.* (1980) as modified by Herington & Kuffer (1981). The semi-purified ILA pI4.8 had a specific activity of 4–10 munit of insulin equivalents/mg as assessed by the isolated rat adipocyte bioassay (Franklin *et al.*, 1976), representing between 1 and 6% purity when compared with the specific activities of highly purified IGF-I and -II [160 munit/mg and 364 munit/mg respectively (Zapf *et al.*, 1978b)].

Determination of bioactivities of acidic ILA pI4.8

ILA pI4.8 was tested for the following standard IGF bio-, receptor- or immuno-activities *in vitro*.

(a) Non-suppressible insulin-like activity was measured in the isolated rat adipocyte bioassay as previously described ([¹⁴C]glucose incorporation into [¹⁴C]lipid in the presence of anti-insulin serum) (Franklin *et al.*, 1976). Pig insulin (0.05–0.4 ng/ml) was used as a standard.

(b) Cartilage sulphation activity was measured in the fasted, weanling rat costal cartilage assay as previously described (Daughaday *et al.*, 1975).

Normal human serum (5 and 10%) was used as a standard.

(c) DNA synthetic activity was measured by the incorporation of [³H]thymidine into DNA in human skin fibroblasts by the method of Rechler *et al.* (1977). Fibroblasts were cultured in serum-free medium for 3 days in the presence or absence of varying doses of ILA pI4.8, and pulsed for 1 h with [³H]thymidine. These studies were carried out by Dr. Norbert Kraft.

(d) Inhibition of ¹²⁵I-insulin degradation was measured according to the method of Kahn *et al.* (1976) using crude homogenates of 36-day-old male rat liver as the source of insulin-degrading enzymes.

(e) Inhibition of activity by an IGF-specific inhibitor from normal human serum was measured by direct addition of inhibitor [isolated as previously described by Herington & Kuffer (1981)] to ILA pI4.8 in the isolated rat adipocyte and rat costal cartilage bioassays described above.

(f) Insulin and IGF receptor activity was measured in the human placental membrane or rat adipocyte systems (Marshall *et al.*, 1974; Zapf *et al.*, 1978a) as described for ¹²⁵I-insulin, ¹²⁵I-IGF-I and ¹²⁵I-IGF-II.

(g) IGF radioimmunoactivity was measured in C-peptide assays for IGF-I (Hintz *et al.*, 1980) and IGF-II (Hintz & Liu, 1982) (courtesy of Dr. Ray Hintz) and in a somatomedin C radioimmunoassay using a polyclonal (gift of National Pituitary Agency) and a monoclonal (TR-4) somatomedin C antibody (courtesy of Dr. Robert Baxter, Sydney, Australia) (Baxter *et al.*, 1982a, b).

Results

As a result of the isolation procedures used in this and earlier studies, several IGF-like chemico-physical characteristics of ILA pI4.8 have been determined. ILA pI4.8 has M_r ~7000, a pI of 4.8 and is stable to acid (pH 2.3) and basic (pH 9.7) conditions and to heating (56°C for 1 h or 100°C at pH 2.3 for 5 min). Like the IGFs, it is found in human serum and in Cohn fraction IV-1 bound in a high- M_r (100 000–150 000) acid-labile complex (Cockerill *et al.*, 1980; Herington & Kuffer, 1981; Kuffer & Herington, 1982, 1984).

The dose-dependent, stimulatory bioactivity of ILA pI4.8 in three standard IGF bioassays, i.e. the isolated rat adipocyte, rat costal cartilage and human fibroblast systems respectively, is given in Table 1. No purified IGF-I and/or -II was available for direct comparisons of relative potencies. However, the response to ILA pI4.8 in the adipocyte and cartilage bioassays was statistically linear and parallel with the response elicited by acceptable and traditional IGF/somatomedin

Table 1. Activity of ILA pI4.8 in three standard IGF bioassay systems

Each assay system is described in the Materials and methods section. Data are expressed as percentage stimulation of control activity, which for each bioassay was: 120 ± 3 c.p.m. of [^{14}C]glucose incorporated/mg of lipid in the isolated rat adipocyte bioassay, 6572 ± 275 c.p.m. of $^{35}\text{SO}_4^{2-}$ incorporated/mg dry wt. of cartilage in the weanling rat costal cartilage bioassay, and 100 ± 6 c.p.m. of [^3H]thymidine incorporated/well in the cultured human fibroblast bioassay. The doses of ILA pI4.8, insulin or the acid/ethanol extract of normal human serum (a crude mixture of IGFs) are given as μunit of insulin equivalents as determined in the standard ILA rat adipocyte bioassay. The dose of normal human serum used is given as a percentage of final assay volume. Data are given as means $\pm$ s.e.m. for $n = 3$ (a), 3–5 (b) or 6 (c) determinations respectively. $\dagger P < 0.02$, $\dagger\dagger P < 0.01$, $*P < 0.005$, $**P < 0.001$, by Student's unpaired t test.

Preparation	(a) Insulin-like activity		(b) Cartilage sulphation activity		(c) Thymidine incorporating activity	
	Dose (μunit)	Activity (% of control)	Dose (μunit or %)	Activity (% of control)	Dose (μunit or %)	Activity (% of control)
Control (buffer)	0	100 ± 2	0	100 ± 4	0	100 ± 6
ILA pI4.8	2.1	$185 \pm 2^{**}$	9.4	112 ± 6	8	$533 \pm 39^{**}$
	4.2	$279 \pm 8^{**}$	18.8	$142 \pm 8^{\dagger\dagger}$	16	$468 \pm 40^{**}$
	8.4	$340 \pm 12^{**}$				
	2.5	$182 \pm 12^*$			125×10^3	119 ± 11
Insulin	5.0	$296 \pm 2^{**}$				
	10.0	$366 \pm 6^{**}$				
	2.4	$170 \pm 5^{**}$				
Acid/ethanol extract	4.8	$217 \pm 3^{**}$				
	9.6	$264 \pm 8^{**}$				
Normal human serum			5	$136 \pm 12^{\dagger\dagger}$	12.5	$442 \pm 123^{\dagger}$
			10	$181 \pm 9^{\dagger\dagger}$		

standards for each assay, i.e. insulin and acid/ethanol extracts of normal human serum (adipocyte bioassay), and whole normal human serum (costal cartilage bioassay). Since all ILA pI4.8 preparations used in these studies contained no detectable activity in the neutral and basic pH regions (determined by isoelectric focusing and adipocyte bioassay) it is unlikely that any response seen was due to contamination with IGF-I and/or -II.

The IGFs have also been shown to inhibit degradation of ^{125}I -insulin by enzymes present in hepatic or renal tissue extracts (Burghen *et al.*, 1976; Kahn *et al.*, 1976). Fig. 1 demonstrates that, like insulin itself, ILA pI4.8 is equally able to inhibit the degradative effect of a crude rat liver homogenate. The effect is dose-dependent and, like a mixture of IGF-I and -II (NSILAs) (Kahn *et al.*, 1976), prevents degradation by interacting with the insulinase in a competitive manner (by Lineweaver-Burk analysis; results not shown). Although this action of the IGFs is not unique [a similar effect is exhibited by high- M_r non-suppressible insulin-like activity (NSILP) (Burghen *et al.*, 1976)] it is clearly consistent with the ILA pI4.8 being akin to the IGFs.

The recent description of a M_r 16000–18000 inhibitor with specificity for IGF-I and -II (Herington & Kuffer, 1981, 1982) has provided another probe for determining the IGF-like nature of ILA pI4.8. As shown in Fig. 2(a), the inhibitor

Fig. 1. Effect of ILA pI4.8 (□) and insulin (▨) on ^{125}I -insulin degradation by a crude insulinase preparation from rat liver homogenates

Degradation was assessed by the radioactivity precipitated by trichloroacetic acid following incubation of ^{125}I -insulin with or without (control) insulinase. The data are expressed as the percentage degradation compared with control (i.e. no insulinase) (□). The histograms are the means of duplicate determinations (●).

Fig. 2. Effect of a specific IGF inhibitor on ILA p14.8 activities

(a) Effect of a specific IGF inhibitor on rat adipocyte insulin-like activity of ILA p14.8, purified IGF-I and IGF-II, and a crude IGF preparation (i.e. an acid/ethanol extract of normal human serum). The effect of the inhibitor (final concn. $250\mu\text{g/ml}$) was measured after mixing with the appropriate stimulator and then assaying the mixture at the same doses as those used for stimulator alone, i.e. at three doses between 2.1 and $9.6\mu\text{unit}$ of insulin equivalents. The effect of the inhibitor (hatched) is expressed as a percent of the bioassay potency of the stimulator alone ($\square$, control, 100%). The vertical bars represent the 95% confidence limits of the calculated bioassay potencies. (b) Effect of a specific IGF inhibitor ($150\mu\text{g/ml}$) on fasting rat costal cartilage sulphation activity of ILA p14.8 ($25\mu\text{g/ml}$) and normal human serum (5% and 10% v/v). The results are expressed (means $\pm$ s.e.m.) as a percentage of basal sulphate incorporation. The number of replicate cartilage segments for each sample is shown in parentheses.

interferes with the insulin-like activity of purified preparations of IGF-I and -II, of a crude IGF mixture (an acid/ethanol extract of human serum) as well as of a partially purified preparation of ILA pI4.8. The apparent difference in the degree of IGF inhibition by the inhibitor is due to the dose-dependent nature of the inhibition (Herington & Kuffer, 1981) and simply reflects differences in the amount of the various IGF preparations used with a constant amount of inhibitor. The inhibitor also prevents the action both of normal human serum and of ILA pI4.8 in a cartilage sulphation assay (Fig. 2b).

A further criterion for classification as an IGF depends on the cross-reactivity in a variety of competitive binding assays. The IGFs are known to have their own receptors on human placental or rat adipocyte membranes and in addition to compete with insulin receptors which are also present. As shown in Fig. 3, ILA pI4.8 is able to displace ^{125}I -insulin, ^{125}I -IGF-II and ^{125}I -IGF-I from their respective placental or adipocyte receptors. For the insulin receptor, the displacement curve for ILA pI4.8 is parallel with that for both unlabelled insulin and a crude IGF preparation derived from an acid/ethanol extract of Cohn fraction IV-I of human serum. The equivalence of the potency of ILA pI4.8 with that of insulin for displacement of ^{125}I -insulin simply reflects the expression of ILA pI4.8 activity in munit of insulin equivalents as determined by the isolated rat

adipocyte bioassay. For the IGF-I and -II receptor assays, the ILA pI4.8 displacement curve was also parallel with that for a partially purified mixture of IGF-I (15–20%) and IGF-II (75%) obtained from Cohn fraction IV-I by the method of Svoboda *et al.* (1980), or to an acid/ethanol extract of Cohn fraction IV-I. Insufficient pure IGF-I or -II was available for a direct comparison of ILA pI4.8 with an IGF-I or -II displacement curve. Direct binding of ^{125}I -ILA pI4.8 to human placental or rat adipocyte IGF receptors was low (specific binding 2.8% and 2.7% respectively) and was perhaps due both to an apparent instability of the iodinated form and to the relative impurity of the mixture of acidic proteins obtained from isoelectric focusing.

Cross-reactivities of ILA pI4.8 have also been assessed in a variety of radioimmunoassays for IGFs. No cross-reactivity was observed with a low specific activity (0.4 munit/mg) preparation of ILA pI4.8 in the radioimmunoassay for IGF-I C peptide or IGF-II C-peptide (Fig. 4a) (assays by courtesy of Dr. Raymond Hintz). On the other hand, with a similar preparation a parallel displacement curve was obtained (albeit at high doses) with two polyclonal anti-(somatomedin C) sera [Tr4 (Dr. R. C. Baxter), results not shown, and NPA-somatomedin C (obtained through NIADDK), Fig. 4b] and a monoclonal anti-(somatomedin C) antibody (3D1/2/1; Dr. R. C. Baxter) (Fig. 4c). The potency of ILA pI4.8 in

Fig. 3. Effect of ILA pI4.8 on the binding of (a) ^{125}I -insulin to human placental membranes, (b) ^{125}I -IGF-II to human placental membranes and (c) ^{125}I -IGF-I to isolated rat adipocytes

Unlabelled preparations used for competition with labelled ligands were: ILA pI4.8; an acid/ethanol extract of Cohn fraction IV-I of human serum; a partially purified mixture of IGF-I (15–20%) and IGF-II (75%); and insulin. Binding in each case is expressed as $B/B_0 \times 100$. B_0 for the ^{125}I -labelled ligands was: insulin, 23.4%/200 μg of placental membrane protein; IGF-II, 5.4%/200 μg of placental membrane protein; IGF-I, 7.5%/10⁶ adipocytes.

Fig. 4. Cross-reactivity of ILA pI4.8 (0.4 munit of insulin equivalents/mg) and partially purified somatomedin C or IGF-II with antisera

The antisera used were (a) to IGF-II C-peptide, (b) a polyclonal National Pituitary Agency (NPA) anti-(somatomedin C) serum, (c) a monoclonal anti-(somatomedin C) antibody 3D1/2/1 raised by Dr. R. C. Baxter. These radioimmunoassays were kindly performed by Drs. R. Hintz and R. C. Baxter.

these radioimmunoassays was approx. 1/1000 that of IGF-I/somatomedin C preparations themselves and corresponds with the degree of purity of ILA pI4.8 based on insulin-like specific activities (0.4 munit/mg; cf. pure IGF-I, 160 munit/mg) (Zapf *et al.*, 1978b).

In other studies (Cornell, 1982) it has also been clearly shown that material that focuses at pI4.8 cross-reacts to some degree with the IGF-I antibody reported by Zapf *et al.* (1981), but more importantly cross-reacts quite strongly in the competitive binding protein assay described by Zapf *et al.* (1977) which utilizes a partially purified preparation of IGF-binding protein.

Discussion

The presence of acidic IGF or somatomedin activity in human serum has been recognized and reported several times in the past (Van Wyk *et al.*, 1971, 1974; Posner *et al.*, 1978; Cockerill *et al.*, 1980). It has remained largely unstudied because of its variable detection and/or its apparent low concentration in serum extracts. A major explanation for its apparently low concentration emerged, however, with the demonstration of a specific IGF inhibitor in serum which has an isoelectric point of approx. 4.4 (Herington & Kuffer, 1981, 1982). Its co-purification through many of the steps of conventional IGF purification procedures, in particular isoelectric focusing, ensures that detection of ILA pI4.8 will be markedly hindered. Effective separation of the inhibitor from the active IGF regions on Bio-Gel P-30, however, has allowed an assessment of the true distribution pattern of the various IGFs in serum. Under these

circumstances ILA pI4.8 may represent up to 21% of total NSILA activity in serum (Kuffer & Herington, 1982, 1984). A further reason for variable detection of ILA pI4.8 by many groups may well be the increasing use of assay systems (e.g. radioimmunoassays, radioreceptor assays) which detect only specific IGFs or somatomedins. The use of the broad-spectrum rat adipocyte bioassay for many of our studies has circumvented this problem.

Complete purification of ILA pI4.8 has proved difficult in our hands. The isolated material (4–10 munit/mg) is still only approx. 1–6% pure (based on a specific activity of 160–364 munit/mg for IGF-I and -II; Zapf *et al.*, 1978b). Whilst isoelectric focusing has proved extremely useful in purification of IGF-I (pI ~8.4) (Svoboda *et al.*, 1980) and IGF-II (pI ~6.5) (Cornell *et al.*, 1984), its efficacy is less pronounced for ILA pI4.8 since many extraneous serum proteins also focus in this pH region. Despite these problems of purification, the data presented demonstrate that ILA pI4.8, which has an M_r ~7000 and is carrier-bound in a high- M_r acid-labile complex in serum or Cohn fraction IV-I, possesses non-suppressible insulin-like activity, cartilage sulphation activity, and thymidine-incorporating activity into DNA. In addition, it competitively inhibited ^{125}I -insulin degradation by rat liver homogenates, it was inhibited by a specific IGF-inhibitor, it displaced ^{125}I -insulin, ^{125}I -IGF-II and ^{125}I -IGF-I in a parallel manner in radioreceptor systems, and it cross-reacted with polyclonal antisera or a monoclonal antibody to somatomedin C/IGF-I but not to antibodies to IGF-I or -II C-peptides. We have not yet been able to test for cross-reactivity in an

entire IGF-II immunoassay. Finally, it cross-reacted with IGF-I in the IGF-carrier protein binding assay (Cornell, 1982).

Thus, there are obvious similarities between ILA pI4.8 and the well-recognized IGFs I and II. The demonstrated effects of ILA pI 4.8 are most unlikely to be related to contamination with IGF-I or IGF-II, neither of which can be demonstrated by isoelectric focusing in the tested preparations. Similarly, the effects are not due to contamination with insulin (pI ~5.7; Righetti & Caravaggio, 1976), since firstly, no insulin was detectable in ILA pI4.8 by radioimmunoassay (results not shown), secondly, the insulin-like actions of ILA pI4.8 were determined in the presence of excess anti-insulin serum (Franklin *et al.*, 1976) and no alteration (stimulation) of this action was seen on omission of the anti-insulin serum, and thirdly, ILA pI4.8 was active in the cartilage sulphation bioassay, an effect not readily observed with insulin (Daughaday *et al.*, 1975). We have not yet been able to assess one of the major criteria required for classification of 'growth factors' as IGFs or somatomedins, i.e. its growth hormone dependence. Until a specific assay, which does not detect the other IGFs, is established for ILA pI4.8, we will be unable to answer this important question.

The questions now arise as to what is ILA pI4.8, what is its relationship to the other IGFs, if any, and what might be its physiological role and significance? The first two of these questions have been addressed in the accompanying paper (Kuffer & Herington, 1984), where we present evidence that ILA pI 4.8 may be a proteolytic conversion product of IGF-I and/or -II. A potential physiological significance for ILA pI4.8 is supported by several observations. First, it is detectable in appreciable amounts [21% of total IGF insulin-like activity (Kuffer & Herington, 1982, 1984)] in normal human serum which has been treated as simply as current methodology allows, i.e. acid gel filtration followed by isoelectric focusing. This suggests that it may well constitute a significant component of total IGF activity of native serum, although one still cannot unequivocally demonstrate that. Second, it is unlikely to be simply a degradation product, since it retains most IGF biological activities, it retains approximately equal potency with IGF-I in the IGF-I radioimmunoassay when the degree of purity is taken into account, and it exists in serum as a high-*M_r* carrier-complex, an unlikely observation for a degradation product.

The data presented in the present paper indicate that significant amounts of acidic IGF-like activity are detectable in serum fraction Cohn IV-I. The extent of detection is dependent on the removal of

the co-purifying acidic IGF inhibitor (Herington & Kuffer, 1981) and/or the nature of the assay system used. ILA pI4.8 possesses many of the biological activities and characteristics normally attributed to IGF-I and -II. Whilst the growth hormone-dependence of this material is yet to be determined, there would appear to be sufficient evidence that ILA pI4.8 does represent a significant proportion of naturally occurring IGF-like activity and that it may therefore have a particular, but as yet undefined, physiological role.

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