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Modifications in the B10 and B26–30 regions of the B chain of human insulin alter affinity for the human IGF-I receptor more than for the insulin receptor

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Summary Inversion of the natural sequence of the B chain of human insulin (HI) from $Pro^{B28}Lys^{B29}$ to $Lys^{B28}Pro^{B29}$ generates an insulin analogue with reduced tendency to self-associate. Since this substitution increases the homology of insulin to insulin-like growth factor-I (IGF-I), we have examined the affinity of a series of insulin analogues with the general modified structure $Xaa^{B28}Pro^{B29}$ HI for binding to both human placental insulin and IGF-I receptors. The $Xaa^{B28}Pro^{B29}$ HI series is approximately equipotent to HI in binding to the insulin receptor with the exception of when Xaa = Phe, Trp, Leu, Ile, and Gly (40–60 % relative to HI). Substitution with basic residues in the B28 position increased the relative affinity to the IGF-I receptor approximately 1.5–2-fold ($Arg^{B28}Pro^{B29} > Orn^{B28}Pro^{B29} = Lys^{B28}Pro^{B29}$). Substitution with acidic residues reduced relative affinity for the IGF-I receptor approximately 2-fold ($Cya^{B28}Pro^{B29} = Glu^{B28}Pro^{B29} > Asp^{B28}Pro^{B29}$). Combination of Asp^{B10} substitution in conjunction with a modification in the B28–29 position (e.g. $Asp^{B10}Lys^{B28}Pro^{B29}$ HI) showed an additional 2-fold selective increase in affinity for the IGF-I receptor, suggesting that these two effects are additive. Addition of Arg residues at B31–32, on the backbone of either HI or Asp^{B10} HI, increased affinity for the IGF-I receptor 10 and 28 fold, respectively, compared to HI, confirming the significance of enhanced positive charge at the C-terminal end of the insulin B-chain in increasing selectivity for the IGF-I receptor. This relative increase in IGF-I receptor affinity correlated largely, but not completely, with enhanced growth promoting activity in human mammary epithelial cells. In the case of $Lys^{B28}Pro^{B29}$ HI, growth activity correlated with dissociation kinetics from the insulin receptor which were shown to be identical with those of human insulin. [Diabetologia (1997) 40: S54–S61]

Keywords Insulin, insulin analogues, insulin receptor, insulin-like growth factor-I receptor, proliferation, mammary epithelial cells.

Current commercial insulin formulations do not possess the most desirable onset of action since the duration of time from injection to peak activity can be up to 3 h [1]. This makes it difficult to match insulin administration with meals, and may increase the risk of postprandial hypoglycaemia [2]. One of the limitations in absorption of subcutaneously administered insulin is the rate of dissociation of hexamer to dimer (and possibly monomer) at the injection site [3]. Therefore, insulin analogues that self-associate more weakly than human insulin would be anticipated to act more rapidly [4]. This has been demonstrated clinically with insulin analogues such as $Lys^{B28}Pro^{B29}$ insulin [5, 6] and Asp^{B10} insulin [7].

Structure/function studies employing insulin analogues have suggested that amino acid residues in insulin essential for insulin receptor binding affinity include Gly^{A1}, Gln^{A5}, Tyr^{A19}, Asn^{A21}, Val^{B12}, Tyr^{B16}, and Phe^{B24}-Tyr^{B26} [8, 9]. Truncation of the insulin B chain sequentially from the C- terminal end does not significantly reduce insulin binding until Tyr^{B26} is removed, generating des-(B26–30) insulin (despentapeptide

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Abbreviations: HI, human insulin; DiArg HI, $Arg^{B31}Arg^{B32}$ human insulin; TriArg HI, $Arg^{A0}Arg^{B31}Arg^{B32}$ human insulin, IGF-I, insulin-like growth factor I; HMEC, normal human mammary epithelial cells

insulin, or DPI) with approximately 20–50% the activity of insulin [10, 11]. However, DPI-Phe^{B25} amide has full potency [12]. This would suggest that the B26–30 region is not particularly critical for insulin receptor recognition. However, it is clearly important in mediating the formation of insulin dimers [13, 14]. For this reason, structural modifications of insulin in this region would be anticipated to generate monomeric insulin analogues with minimal alteration in insulin receptor affinity. Insulin-like growth factor I (IGF-I) is a 70-amino acid polypeptide which induces proliferation and differentiation in a number of cell types [15]. IGF-I is a single-chain molecule with A and B domains that exhibit 45–50% homology with the A and B chains of insulin. Unlike insulin, however, the A and B regions of IGF-I are connected through a 12-residue C domain and the C-terminal end of the A domain contains an 8-residue D region extension. The IGF-I and insulin receptors also exhibit substantial structural homology to each another, and both ligands have weak, but measurable, affinity for the heterologous receptor [15].

Early studies with insulin/IGF-I mixed hybrid molecules (e.g. A^{IGF-I}-B^{Insulin}) suggested that the A domain of IGF-I possessed most of the selectivity for the IGF-I receptor [16]. However, more recent studies employing site-directed mutants of IGF-I have demonstrated that substitution of residues 42–56 in the IGF-I A domain with the homologous residues of insulin had no effect on IGF-I receptor affinity [17]. Several studies have suggested that the key regions of IGF-I responsible for inducing selectivity for the type I receptor are located in the C region and the C-terminal portion of the B domain [18, 19].

Homology of the C-terminal region of the B chain of insulin with the corresponding regions of IGF-I (and the closely related molecule IGF-II) is enhanced in Lys^{B28}Pro^{B29} insulin and Arg^{B28}Pro^{B29} insulin, respectively (Fig. 1). For this reason, we investigated the relative affinities of the entire Xaa^{B28}Pro^{B29} insulin series compared to human insulin for binding to the human placental insulin and IGF-I receptors to determine if these analogues possessed differential affinity for the two receptors.

Materials and methods

Materials. Insulin analogues were made by either: 1) recombinant DNA methods; 2) trypsin-catalysed semisynthesis in a mixed organic solvent system [20] using des-(B23–30) forms of porcine insulin and Asp^{B10} human insulin, and the corresponding synthetic octapeptide modified in the B28–29 positions [21]; or 3) chain combination [22] using recombinant human insulin A chain with a synthetic B chain. Arg^{B31}Arg^{B32} (DiArg) insulin and Asp^{B10}DiArg insulin were obtained by limited tryptic hydrolysis of recombinant human proinsulin and Asp^{B10} human proinsulin, respectively. tert-Butoxycarbonyl (Boc) amino acids were purchased from Applied Biosystems (Foster City, Calif., USA). All other chemicals were of analytical grade or higher. Natural sequence human IGF-I was of recombinant DNA origin (Lilly). Iodinations were performed by the lactoperoxidase procedure, and in the case of insulin or insulin analogues, the Tyr^{A14} monoiodinated species (specific activity approximately 360 μCi/μg) was employed [23]. Protein was determined by the BCA (bicinchoninic acid) method (Pierce Chemical, Rockford, Ill., USA) using bovine serum albumin (BSA) as standard. Placental membranes were obtained from full term human placenta by a modification of the procedure of Grupusso et al. [24]. RIA-grade BSA was obtained from Sigma (St. Louis, Mo., USA). HepG2 cells were obtained from the American Type Tissue Collection (Rockville, Md., USA).

Cell growth. Mitogenicity was assessed by measuring insulin analogue-stimulated growth of human mammary epithelial cells (HMEC) in culture. HMEC were obtained from Clonetics Corporation (San Diego, Calif., USA) at passage 7 and were expanded and frozen at passage 8. A fresh ampule was used for each experiment so that cells were not grown beyond passage 9. Cells were maintained in MCDB 170 medium containing bovine insulin (5 μg/ml), recombinant human epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml), bovine pituitary extract (50 μg/ml) and gentamycin/amphotericin B. For a growth experiment, cells were plated in 96-well trays at a

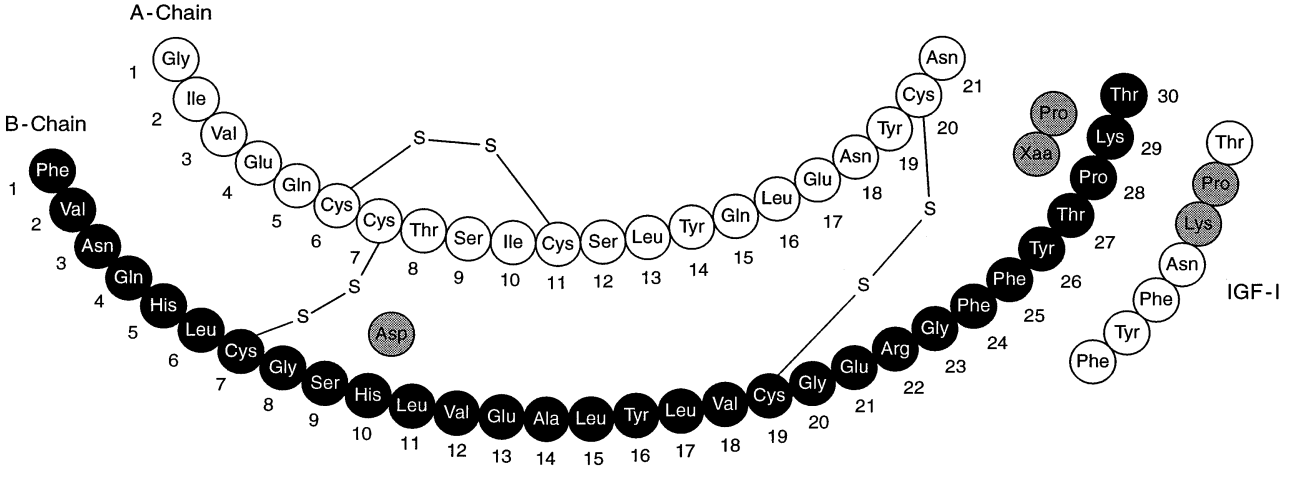

Fig. 1. Primary structure of human insulin, indicating position of B10 and B28–29 modifications. Also shown is the sequence of IGF-I homologous to the B25–30 portion of insulin

Table 1. EC_{50} values and relative potencies (insulin = 1.00) of insulin analogues for binding to the human placental insulin and IGF-I receptors

Insulin analogue	Insulin receptor		IGF-I receptor	
	EC_{50} (nmol/l)	Relative potency	EC_{50} (nmol/l)	Relative potency
Insulin	0.43 ± 0.02	1.0	326 ± 23	1.0
IGF-I	32.6 ± 2.4	0.016 ± 0.002	0.42 ± 0.02	697 ± 52
Ala	0.50 ± 0.07	0.78 ± 0.12	422 ± 14	0.51 ± 0.11
Gly	0.91 ± 0.01	0.44 ± 0.003	746 ± 112	0.29 ± 0.01
Ile	0.83 ± 0.12	0.61 ± 0.02	1179 ± 215	0.41 ± 0.06
Leu	0.81 ± 0.14	0.63 ± 0.02	1239 ± 177	0.38 ± 0.05
Met	0.53 ± 0.04	0.76 ± 0.13	362 ± 7	0.98 ± 0.21
nNle	0.62 ± 0.06	0.73 ± 0.07	1019 ± 130	0.38 ± 0.03
nVal	0.51 ± 0.02	0.97 ± 0.08	619 ± 28	0.57 ± 0.11
Pro	0.40 ± 0.04	0.97 ± 0.06	339 ± 19	0.63 ± 0.08
Val	0.52 ± 0.10	0.85 ± 0.05	833 ± 192	0.61 ± 0.18
Phe	1.11 ± 0.06	0.43 ± 0.06	1293 ± 281	0.33 ± 0.02
Trp	1.13 ± 0.04	0.47 ± 0.06	1693 ± 119	0.25 ± 0.02
Tyr	0.63 ± 0.08	0.87 ± 0.14	808 ± 292	0.56 ± 0.13
Asn	0.41 ± 0.02	1.12 ± 0.09	320 ± 15	1.10 ± 0.21
Gln	0.59 ± 0.09	0.81 ± 0.03	460 ± 85	0.92 ± 0.04
Ser	0.45 ± 0.02	1.0 ± 0.04	367 ± 107	1.13 ± 0.27
Thr	0.43 ± 0.07	1.09 ± 0.03	332 ± 59	1.15 ± 0.45
Asp	0.52 ± 0.03	0.78 ± 0.09	733 ± 99	0.52 ± 0.08
Cya	0.50 ± 0.03	0.88 ± 0.12	978 ± 113	0.36 ± 0.06
Glu	0.46 ± 0.05	0.87 ± 0.06	693 ± 50	0.38 ± 0.03
Arg	0.48 ± 0.04	0.91 ± 0.11	190 ± 23	1.89 ± 0.14
His	0.50 ± 0.10	0.94 ± 0.18	331 ± 56	1.19 ± 0.13
Lys	0.51 ± 0.04	0.84 ± 0.06	229 ± 33	1.57 ± 0.16
Orn	0.49 ± 0.05	0.88 ± 0.04	234 ± 39	1.59 ± 0.24
$Lys^{B28}Lys^{B29}$	0.46 ± 0.05	0.94 ± 0.18	74 ± 2	2.28 ± 0.29
$desPro^{B28}$	0.29 ± 0.03	1.33 ± 0.10	79.7	2.3
$desLys^{B29}$	0.36	1.08 ± 0.22	197 ± 16	1.02 ± 0.09
$des^{B28–30}$	0.44 ± 0.04	0.77 ± 0.15	304 ± 28	0.66 ± 0.06
Gly^{B29}	0.50 ± 0.03	0.78 ± 0.11	513 ± 68	0.40 ± 0.06
Ala^{B26}	0.39 ± 0.03	0.87 ± 0.06	271 ± 3.5	0.55 ± 0.03

Data are mean ± SEM
Single amino acids refer to the B28 position of $Xaa^{B28}Pro^{B29}$ HI. Relative potency was determined by comparing each analogue to an insulin control within each experiment, and then averaging the relative potency over the number of experiments performed. Therefore, a comparison of the average EC_{50} for any analogue with the average EC_{50} for insulin will not generate the same value

density of 12 500 cells/cm^2 in the above-medium modified as follows: 0.1 % BSA was added and 5 μg/ml bovine insulin was substituted by a graded dose of human insulin or analogue from 0 to 1000 nmol/l final concentration. Trays were incubated for 72 h and the cells were counted by Coulter counter (Coulter Electronics, Hialeah, Fla., USA) after trypsinization. Typically, the maximal growth response was between 3- and 4-fold stimulation over basal and did not differ between analogues. Response data were normalized to between 0 and 100 % response equal to 100 × (response at dose X – response at zero dose) divided by (response at maximal dose – response at zero dose). Dose-response data were fit by non-linear regression employing JMP (SAS Institute, Inc., Cary, N. C., USA).

[^{125}I]-Insulin and [^{125}I]-IGF-I binding assays. The placental assay employed incubating 30–40 μg of membrane protein with approximately 10 fmol of iodinated ligand in a final volume of 500 μl of 100 mmol/l HEPES, pH 7.8, 120 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 8 mmol/l glucose and 0.25 % BSA for 16–18 h at 4 °C. Membranes were collected on glass fiber filters pre-treated with 0.1 % polyethyleneimine by using a cell harvester (Skatron, Lier, Norway). Binding assays with mammary epithelial cells were performed under similar conditions using confluent cells on either P12 (IGF-I receptor) or P6 (insulin receptor) plates (Costar, Cambridge, Mass., USA). Monolayers were incubated in insulin-free medium for 24 h prior to initiating the binding assay. Cells were incubated in the above buffer containing iodinated IGF-I and cold competing ligands for 16–18 h at 4 °C. After washing the monolayers with cold assay buffer, cells were solubilized in 0.1 N NaOH and counted for ^{125}I. EC_{50} values were determined by fitting displacement data to a four-parameter model by non-linear regression. Dissociation constants for Tyr^{A14}-[^{125}I]-Insulin, – $Lys^{B28}Pro^{B29}$ HI, and -Asp^{B10} HI were obtained according to the method of Drejer et al. [25].

Results

Placental insulin and IGF-I receptor binding. Table 1 shows the EC_{50} values for binding of the $Xaa^{B28}Pro^{B29}$ series of insulin analogues to both the placental insulin and IGF-I receptors. In terms of insulin receptor affinity, these analogues differed little from native insulin, with the exception of when Xaa = Gly, Ile, Leu, Phe or Trp, where the affinity was reduced approximately 50 %. Selective removal of the B28 and B29

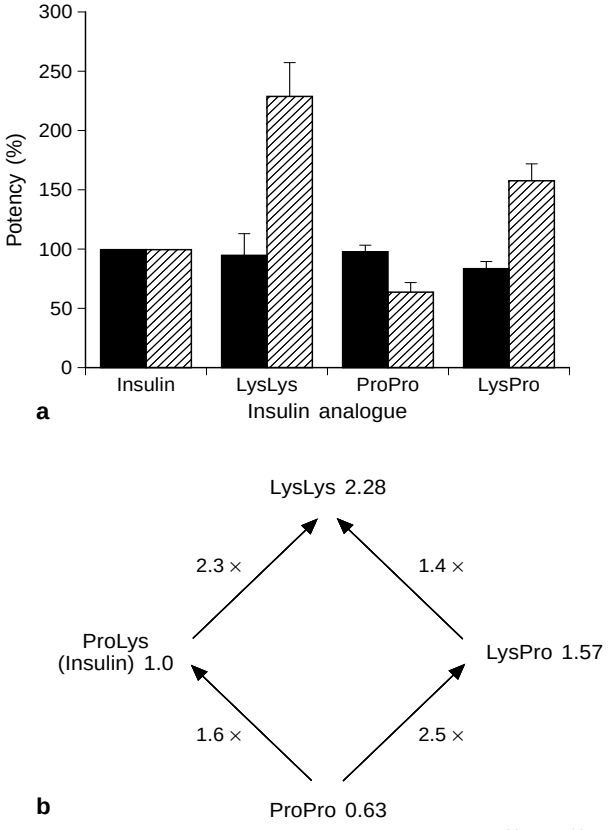

Fig. 2 a, b. Comparison of human insulin to $Lys^{B28}Pro^{B29}$ HI via the "intermediate" analogues $Lys^{B28}Lys^{B29}$ HI and Pro^{B28}-Pro^{B29} HI. Insulin receptor = ■, IGF-I receptor = ▨

residues, as well as the entire terminal tripeptide also had little effect. Interestingly, Gly^{B29} was only marginally less potent than native insulin compared to the 50% reduction in activity observed with the Gly^{B28} substitution.

Analysis of the same analogue series in the IGF-I receptor binding assay suggested that the IGF-I receptor was more sensitive to changes in the B28-B29 region than was the insulin receptor. IGF-I receptor affinity was reduced in the same series of B28-substituted aliphatic and aromatic analogues as observed with the insulin receptor (Xaa = Gly, Ile, Leu, Phe or Trp), but also with Xaa = Ala, Pro and Val, but, interestingly, not Met. The presence of charged residues in the B28 position of $Xaa^{B28}Pro^{B29}$ insulin was of particular interest, since basic residues (Xaa = Arg, Lys and Orn) appeared to slightly increase IGF-I receptor affinity, while acidic residues (Xaa = Asp, Cya and Glu) reduced binding affinity. The more basic Arg residue enhances IGF-I receptor affinity to a greater extent than does either of the less basic Lys or Orn residues. The acidic residue substitutions appear to follow a similar order, with the more acidic Cya showing the greater effect. Interestingly, the simple removal of Pro^{B28} resulted in a 2.3-fold enhancement in IGF-I receptor affinity, while the Gly^{B29} and Ala^{B26} substitutions reduced affinity by 50–60%. None of these analogues had significantly altered affinity for the insulin receptor.

The sequential effect of substitution in the B28–29 position is shown in Figure 2, where the affinity of native insulin for both the insulin and IGF-I receptors has been set to 100%. Replacing Pro^{B28} of insulin with Lys ($Lys^{B28}Lys^{B29}$ insulin) increases the relative IGF-I receptor affinity almost 2.5-fold, while additional substitution of Lys^{B29} with Pro ($Lys^{B28}Pro^{B29}$ insulin) reduces the affinity increase by approximately 35%. This is quantitatively similar to the reduction in IGF-I receptor affinity observed when comparing insulin to $Pro^{B28}Pro^{B29}$ insulin, which again reflects the effect of a Lys^{B29} to Pro^{B29} substitution. This suggests that the more important modulator of the altered IGF-I receptor affinity is the charged residue in the 28 position, not the B29 Pro.

To assess further the effect of altered positive charge in this region on IGF-I receptor affinity, $Arg^{B31}Arg^{B32}$ insulin (DiArg insulin) was examined. This molecule possesses two additional Arg residues at the C-terminal end of the insulin B chain, and, in fact, is a naturally occurring intermediate in the biosynthetic processing of proinsulin to insulin. Although previous studies have indicated that DiArg insulin is approximately equipotent with native insulin in terms of insulin receptor binding and stimulation of glucose uptake into adipocytes [26], we found in the present study that DiArg insulin is approximately 1.5-times more potent than insulin in the placental insulin receptor binding assay (Table 2). However, it was found to be 10.2 times more potent than insulin at displacing [^{125}I]-IGF-I from placental IGF-I receptors. Extending the $GlyA^1$ position of both insulin and DiArg HI with Arg at the A^0 position reduced the affinity for both the insulin and IGF-I receptors approximately 2–3-fold.

Substitution of Asp for His at B10 increased IGF-I receptor affinity to an extent similar to that reported previously for binding to the insulin receptor [27], and this effect was additive when added onto a background of $Xaa^{B28}Pro^{B29}$ insulin with Xaa = Asp, Lys or Val (Table 2). Superimposing the Asp^{B10} substitution on top of the $Arg^{B31}Arg^{B32}$ addition resulted in a 28-fold increase in IGF-I receptor affinity relative to insulin, again showing that substitutions in these two regions are additive (Table 2). This was further supported by the analogue series generated from sequential truncation of the C-terminal five amino acids on top of the Asp^{B10} substitution. Removal of Thr^{B30} (leaving a terminal Lys) increased affinity for the IGF-I receptor to a greater extent than it did for the insulin receptor, while removal of Lys^{B29} (leaving a terminal Pro) resulted in a 60% decrease in affinity for the IGF-I receptor compared to a 30% reduction for the insulin receptor. Interestingly, complete truncation to $Asp^{B10}DesB^{26–30}$ resulted in minimal alteration in insulin receptor affinity but an approximately 60% reduction in IGF-I receptor affinity.

Table 2. EC_{50} values and relative potencies of insulin analogues for binding to the human placental insulin and IGF-I receptors

Insulin analogue	Insulin receptor		IGF-I receptor	
	EC_{50} (nmol/l)	Relative potency	EC_{50} (nmol/l)	Relative potency
Asp^{B10}	0.29 ± 0.02	1.98 ± 0.17	86 ± 14	3.02 ± 0.38
$Asp^{B10}Lys^{B28}Pro^{B29}$	0.23 ± 0.02	2.32 ± 0.10	68 ± 4	4.53 ± 0.42
$Asp^{B10}Val^{B28}Pro^{B29}$	0.24 ± 0.01	1.78 ± 0.13	145 ± 16	1.18 ± 0.25
$Asp^{B10}Asp^{B28}Pro^{B29}$	0.17 ± 0.01	2.24 ± 0.15	169 ± 24	1.38 ± 0.08
$Asp^{B10}Glu^{B28}Pro^{B29}$	0.27	1.52	167	1.1
$Asp^{B10}Gln^{B28}Pro^{B29}$	0.24	1.71	79.8	2.3
Asp^{B10}	0.29 ± 0.02	1.98 ± 0.17	86 ± 14	3.02 ± 0.38
$Asp^{B10}DesB^{30}$	0.10	2.75 ± 0.15	40.7 ± 2.1	5.0 ± 0.86
$Asp^{B10}DesB^{29–30}$	0.15 ± 0.01	1.92 ± 0.11	107.5 ± 13.4	1.90 ± 0.18
$Asp^{B10}DesB^{28–30}$	0.17 ± 0.06	2.57 ± 0.59	79.8 ± 20.5	3.31 ± 0.90
$Asp^{B10}DesB^{27–30}$	0.10	2.75 ± 0.15	66.0 ± 0.71	3.13 ± 0.66
$Asp^{B10}DesB^{26–30}$	0.16	1.72 ± 0.09	160.5 ± 28.3	1.28 ± 0.05
Arg^{A0}	0.57 ± 0.05	0.48 ± 0.05	208 ± 8	0.22 ± 0.08
DiArg	0.31 ± 0.06	1.53 ± 0.21	12.5 ± 2.0	10.2 ± 1.8
TriArg	0.38 ± 0.06	0.93 ± 0.10	37 ± 14	2.90 ± 0.40
Asp^{B10}DiArg	0.14 ± 0.005	4.68 ± 0.37	5.3 ± 0.05	28.1 ± 1.1

Data are mean ± SEM
DiArg and TriArg HI are $Arg^{B31}Arg^{B32}$ HI and $Arg^{A0}Arg^{B31}Arg^{B32}$ HI, respectively

Table 3. Comparison of the HMEC cell growth and IGF-I receptor binding assays for select insulin analogues

Insulin analogue	Cell growth		IGF-I receptor	
	EC_{50}	Relative potency	EC_{50}	Relative potency
IGF-I	0.34	57	0.35 ± 0.23	1324 ± 980
Asp^{B10}DiArg	0.55 ± 0.01	37.7 ± 5.0	2.7 ± 0.13	88.9 ± 10.0
DiArg	1.9 ± 0.21	9.2 ± 0.85	11.6 ± 3.8	21.8 ± 3.9
Asp^{B10}	5.1 ± 0.60	3.4 ± 0.37	72.5 ± 7.7	3.83 ± 0.62
TriArg	9.6 ± 0.89	1.6 ± 0.57	ND	ND
$Asp^{B10}Des^{B28–30}$	10.3 ± 1.5	1.75 ± 0.22	ND	ND
Insulin	17.6 ± 1.2	1.0	264 ± 21	1.0
$Lys^{B28}Pro^{B29}$	18.6 ± 4.1	0.89 ± 0.10	183 ± 11.5	1.58 ± 0.10
Ala^{B26}	42.5 ± 0.03	0.45 ± 0.01	ND	ND

Data are mean ± SEM
ND, Not determined

Human mammary epithelial cells (HMEC) growth and IGF-I receptor binding. Several key insulin analogues from the above-mentioned series were examined in terms of their ability to stimulate growth of normal human mammary epithelial cells. These cells were chosen because they can readily be cultured under defined medium conditions and insulin (or IGF-I) is a required growth factor. Table 3 and Figure 3 summarize the EC_{50} values and relative potencies for these analogues. When all analogues were considered, relative potency in the mitogenicity assay correlated highly with placental IGF-I receptor binding activity ($p < 0.01$), but not insulin receptor affinity, suggesting that it is largely an IGF-I receptor-mediated event. Table 3 also shows binding data for these analogues to the HMEC IGF-I receptor. Characterization of the HMEC insulin and IGF-I receptors by LIGAND [28] indicated a very low level of insulin receptor compared to IGF-I receptor (2200 vs 44 000 receptors/cell, respectively). Since [^{125}I]-IGF-I competitive binding assays in HMECs with insulin, Asp^{B10} HI, DiArg HI, and Asp^{B10}DiArg HI confirmed the same relative potency observed in the placental binding assay and the HMEC mitogenesis assay, IGF-I receptors from both sources are characterized by the same pharmacology and therefore the enhanced IGF-I receptor affinity of this series does not appear to be mediated through atypical IGF-I receptors present on placenta only [29, 30]. As in placental preparations, HMEC IGF-I receptor affinity correlates highly significantly with potency in HMEC growth stimulation.

Interestingly, $Lys^{B28}Pro^{B29}$ HI was indistinguishable from insulin in the HMEC growth assay while it was more potent than insulin in both the placental and HMEC IGF-I receptor binding assays. Asp^{B10}DiArg HI and DiArg HI also appeared to show less of an enhancement in the growth assay potency relative to insulin than would have been predicted from their increased IGF-I receptor affinity. Degradation for the duration of the growth assay could explain this, at least for $Lys^{B28}Pro^{B29}$ HI. Media from cells treated with 10 nmol/l insulin, $Lys^{B28}Pro^{B29}$ HI, or Asp^{B10} HI were recovered after 72 h and insulin receptor-recognizable ligand was determined in a placental

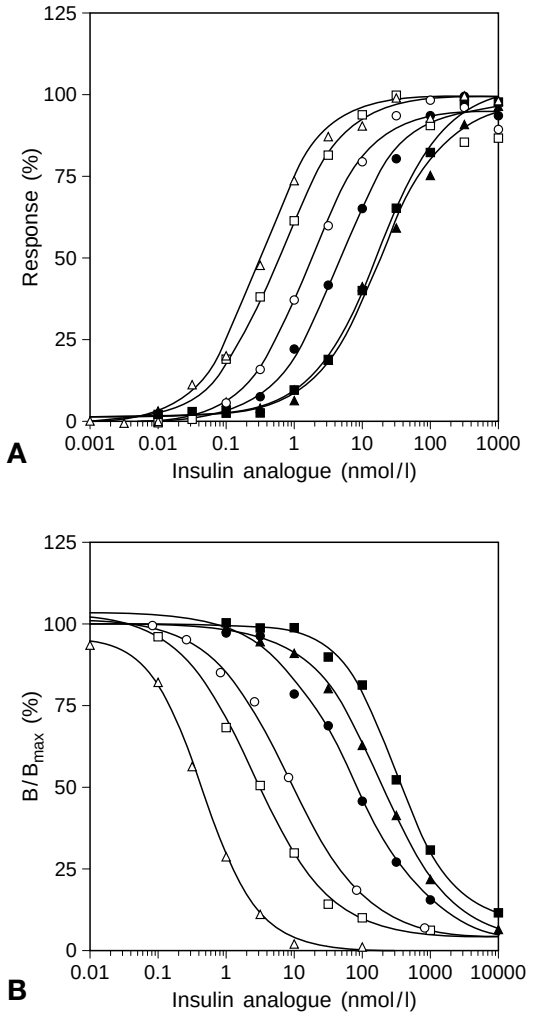

Fig. 3. A. Growth of normal human mammary epithelial cells (HMECs) stimulated by IGF-I (△), Asp^{B10}DiArg HI (□), DiArg HI (◆), Asp^{B10} HI (●), Lys^{B28}Pro^{B29} HI (▲) and human insulin (■). See Materials and methods for definition of % Response. **B.** [^{125}I]-IGF-I competitive binding curves in HMECs. Legend as in **A**

membrane receptor binding assay employing separate control standard curves for each analogue. The percent degradation was: insulin, 3.5 %; Lys^{B28}Pro^{B29} HI, 14 %; Asp^{B10} HI, 0 %. These data suggest that increased degradation of Lys^{B28}Pro^{B29} HI compared to insulin is not sufficient to be responsible for artifactually altering their relative potencies in this assay.

Alternatively, De Meyts et al. [31] and Hansen et al. [32] have suggested that mitogenicity of insulin analogues may be associated with residency time on the insulin receptor such that higher mitogenic activity would correlate with slower dissociation kinetics. Measurement of dissociation kinetics of ^{125}I-human insulin, – Lys^{B28}Pro^{B29} HI, and -Asp ^{B10} HI from HepG2 human hepatoma cells at 4°C according to the method of Drejer et al. [25] indicated that insulin and Lys^{B28}Pro^{B29} HI had identical off-rates (insulin $k_d = 2.96 \pm 0.19$ e^{-4} s^{-1}; Lys^{B28}Pro^{B29} HI $k_d = 2.98 \pm 0.25$ e^{-4} s^{-1}, $n = 3$), while Asp ^{B10} HI dissociated 1.9-fold slower ($k_d = 1.56 \pm 0.14$ e^{-4} s^{-1}).

Discussion

The results of this study indicate that despite the high sequence homology between insulin and IGF-I, their respective receptors recognize the C-terminal portion of the B chain and extensions into the C chain quite differently. The inversion of the B28 proline to the B29 position in the Xaa^{B28}Pro^{B29} insulin analogues series had little effect on insulin receptor selectivity, unless large aliphatic or aromatic residues were present at the B28 position. Binding to the IGF-I receptor, however, showed much greater sensitivity to structural changes in this region. This sensitivity was not only to steric bulk, but also to charge, since comparison of an Arg at B28 to a Glu at the same position resulted in a greater than 5-fold difference in placental IGF-I receptor affinity with no change in insulin receptor binding. The presence of additional basic residues in the form of Lys^{B28}Lys^{B29} HI or Arg^{B31}Arg^{B32} HI enhanced placental IGF-I receptor affinity 2.3 and 10-fold, respectively, suggesting that absolute location of the basic residues is important but not absolutely critical. However, substitution of Lys for Pro in the B29 position (starting from either Pro^{B28}ProB^{B29} HI or Lys^{B28}ProB^{B29} HI) increases IGF-I receptor affinity only 1.5-fold in both cases, while the corresponding substitution in the 28 position of either insulin or Pro^{B28}ProB^{B29} HI increases IGF-I receptor affinity approximately 2.5-fold. This would suggest that the IGF-I receptor can distinguish between a basic residue at positions B28 and B29. Asp^{B10}DiArg insulin is approximately 30 and 90 times more potent than insulin at binding to the placental and HMEC IGF-I receptor, respectively, showing the additivity of the effects at both the B10 and B31–32 sites. To our knowledge, no other insulin analogue has been reported to show that magnitude of affinity for the IGF-I receptor. Other superpotent insulin analogues, such as His^{A8} His^{B4} Glu^{B10} His^{B27} insulin [25] and Asp^{B10} Des $^{B26–30}$ Tyr^{B25} -NH_2 insulin [33] have been reported to show 7–10 times greater affinity than insulin for the insulin receptor, but no apparent selectivity for the IGF-I receptor.

It is interesting to note that an Arg-Arg sequence exists in the C domain of IGF-I. It has been reported that substitution of the Arg^{36}Arg^{37} residues of IGF-I by Ala reduces IGF-I receptor affinity 15-fold, confirming the importance of this portion of the C domain in mediating IGF-I receptor selectivity [34]. Interestingly, substitution of Lys^{65} and Lys^{68} in the D region with Ala also reduced IGF-I receptor affinity approximately 10-fold, suggesting that multiple basic residues (Lys^{27}, Arg^{36}, Arg^{37}, Lys^{65} and Lys^{68}) in IGF-I may mediate IGF-I receptor specificity. Using chimeric insulin/IGF-I receptors, Gustafson and

Rutter [35] demonstrated that 56 and 52 amino acid stretches in the cysteine-rich regions of the insulin and IGF-I receptors, respectively, contained major determinants for ligand binding specificity. Others have confirmed the importance of the cysteine-rich region of the IGF-I receptor in ligand specificity [36–38], but have suggested that the N-terminal 68 amino acid portion of the insulin receptor α-subunit contains the key residues for insulin selectivity [38]. It is interesting to note that the C-terminal 20 amino acids of the region of the IGF-I receptor described by Gustafson and Rutter contains one basic residue and five acidic residues, which might interact with the essential basic residues on IGF-I.

In general, analogue potency in stimulation of HMEC growth correlated with IGF-I receptor binding affinity. This is in contrast to previous reports which suggested that Asp^{B10} HI is considerably more potent in growth assays than would be predicted by its relative affinity to either the insulin or IGF-I receptor [32, 39, 40]. Whether this is due to differences between assays (measurement of [^{3}H] thymidine incorporation vs actual increase in cell number) or differences between cell types used is unclear. Although we cannot rule out involvement of the insulin receptor, the dramatic enhancement in mitogenic activity of Asp^{B10}DiArg HI compared to its relatively modest increase in insulin receptor affinity would suggest that the IGF-I receptor is involved in the mitogenic response for at least the more potent analogues.

$Lys^{B28}Pro^{B29}$ HI, which has slightly increased IGF-I receptor binding affinity relative to insulin in both the placental and HMEC binding assays, was indistinguishable from insulin in the HMEC cell growth assay. Since this does not appear to be entirely an artifact of decreased stability of $Lys^{B28}Pro^{B29}$ HI compared to insulin during the duration of the assay, several explanations are possible. First, the precision of the growth assay was insufficient to resolve small differences (1.5–2-fold) in relative potency. This seems unlikely, since the assay did resolve insulin and Ala^{B26} HI, whose activity in both the IGF-I receptor and growth assay was 50% that of insulin. Alternatively, as suggested by Hansen et al. [32], the mitogenic activity of insulin analogues may be inversely proportional to their dissociation rate from the insulin receptor, even in the presence of a large excess of IGF-I receptors. This is particularly striking when the analogue k_d is less than 25% that of insulin [32]. Since the dissociation rate constants for insulin and $Lys^{B28}Pro^{B29}$ HI are identical, this could explain their equivalence in the HMEC growth assay.

The potential increase in IGF-I activity in insulin analogues recently became an important issue when Asp^{B10} insulin was found to be associated with an increased incidence of breast tumours in rats during 12-month toxicity studies [41, 42] and was subsequently removed from clinical trials. Since several in vitro studies have shown that Asp^{B10} HI has enhanced growth promoting activity compared to insulin [32, 39, 40], it has been speculated that this is the mechanism behind the observed mammary tumours [41]. However, independent 12-month toxicity studies with $Gly^{A21}Arg^{B31}Arg^{B32}$ HI [43], which would be expected to have enhanced IGF-I receptor affinity as a result of the DiArg substitution, and with $Lys^{B28}Pro^{B29}$ HI [44] have not demonstrated an association with breast tumours. These results would suggest that in vitro mitogenic activity or IGF-I receptor binding affinity *per se* do not appear to correlate with increased tumour incidence in vivo.

In summary, we have demonstrated that the IGF-I receptor exhibits greater selectivity for charge differences in the C-terminal portion of the B chain of insulin than does the insulin receptor. This may be due to the importance of basic residues in positions Lys^{27}, Arg^{36}, Arg^{37}, Lys^{65} and Lys^{68} of IGF-I, some of which have been previously shown to be critical for IGF-I receptor affinity [34]. In general, analogues with substantially increased IGF-I receptor affinity are more potent in stimulating HMEC growth. However, with certain insulin analogues of only marginally enhanced IGF-I receptor affinity, such as $Lys^{B28}Pro^{B29}$ human insulin, no enhanced mitogenicity relative to human insulin is observed, suggesting that IGF-I receptor binding and mitogenicity do not completely correlate.

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