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Functionally distinct insulin receptors generated by tissue-specific alternative splicing

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Cloning of the insulin receptor cDNA has earlier revealed the existence of two alternative forms of the receptor differing by the presence or absence of 12 amino acids near the C-terminus of the receptor α-subunit. This insert has been shown by others to be encoded by a discrete exon, and alternative splicing of this exon leads to tissue-specific expression of two receptor isoforms. We have studied the functional significance of the receptor isoforms and have confirmed that they are generated by alternative splicing. When cDNAs encoding the two forms of the insulin receptors are expressed in Rat 1 cells, the receptor lacking the insert (HIR-A) has a significantly higher affinity for insulin than the receptor with the insert (HIR-B). This difference in affinity is maintained when insulin binding activity is assayed in solution using detergent solubilized, partially purified receptors. These data, combined with the tissue specificity of HIR-A and HIR-B expression, suggest that alternative splicing may result in the modulation of insulin metabolism or responsiveness by different tissues.

Key words: alternative splicing/insulin binding/insulin receptor

Introduction

The biological effects of insulin are mediated by a membrane spanning cell surface receptor that has been shown to consist of disulfide-linked α- and β-subunits that are cleaved products of a common precursor. Binding of insulin to the receptor extracellular domain results in intracellular activation of a tyrosine-specific kinase activity and the generation of signals that determine the cellular response. Comparison of two insulin receptor primary structures revealed a 12 amino acid long sequence difference in the C-terminal region of the subunit (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). Since only a single insulin receptor gene has been identified, this suggested a post-transcriptional mechanism for the generation of receptor variants, which were termed HIR-A and HIR-B (Yarden and Ullrich, 1988). The 12 amino acid insert is encoded by a discrete exon (Seino and Bell, 1989) and the alternative splicing that generates the HIR-A and HIR-B forms of the receptor occurs in a tissue-specific fashion (Moller *et al.*, 1989). In this paper we confirm that the 12 amino acids of HIR-B are encoded by a small exon of the human insulin receptor gene and that this exon is alternatively spliced into insulin receptor mRNA in a tissue-specific manner. More importantly, when expressed in Rat 1 fibroblasts, human HIR-A and HIR-B receptors exhibit distinct binding characteristics for insulin. Our findings suggest the possibility of genetic regulation of cell type-specific responsiveness to stimulation by the same hormone using a single receptor gene.

Results

To investigate whether the nucleotide sequence encoding the HIR-B specific 12 amino acids [residues 717–728; Ullrich *et al.* (1985)] originated from the single insulin receptor gene, we used oligonucleotide probes to isolate from a human genomic library, recombinant CH30 clones which hybridized under stringent conditions. Hybridization analysis of *Eco*RI fragments of clone CH30-IR2 with either the 12 amino acid-specific HIR-B probe, an HIR-A control probe, or probes derived from sequences flanking the 12 amino acid insertion site (Figure 1A) demonstrated the presence of the insertion sequence as well as known flanking insulin receptor sequences within the same genomic fragment. Sequence analysis further confirmed this conclusion and showed that the HIR-B 12 amino acid sequence was encoded by a 36 nucleotide exon flanked by splice site consensus sequences and another HIR exon downstream containing the α/β subunit cleavage site and amino-terminal β-subunit (Figure 1B). This finding confirmed alternative splicing as the mechanism for the generation of insulin receptor variants HIR-A and HIR-B.

To investigate the biological significance of this observation, we first analyzed a panel of RNAs from human adult and newborn rhesus monkey for the presence of insulin receptor variant mRNAs. Using the polymerase chain reaction (PCR) technique and oligonucleotide primers from locations flanking the 12 amino acid sequence insertion site, we detected tissue characteristic expression of HIR-A and HIR-B mRNA variants (Figures 2A and B). The experiment yielded specific DNA fragments comigrating with HIR-A and HIR-B cDNA controls. Consistently, additional bands were detected with different primer combinations in various human and monkey tissues. Only the fragments corresponding to HIR-A and HIR-B sequences and the largest of the additional bands (which was shown to be S1 sensitive) hybridized to HIR probes. Expression of HIR-A mRNA was identified in human adult peripheral nerve, skin, kidney, striated muscle and fibroblasts; HIR-B was predominant in adult liver. Exclusive expression of the HIR-A variant

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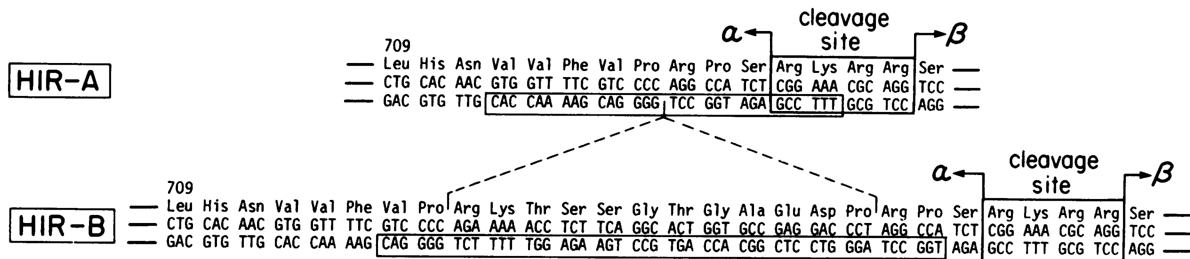


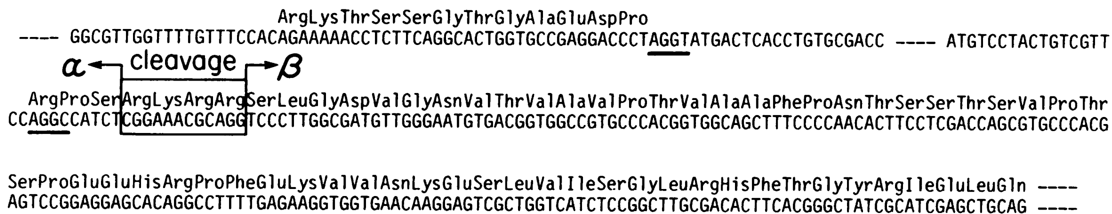
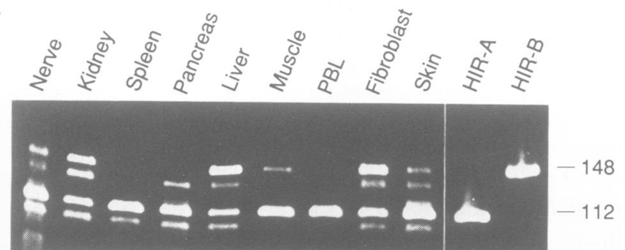


Fig. 1. Human insulin receptor variant sequence. **(A)** Comparison of HIR-A and HIR-B cDNA nucleotide and amino acid sequences. The 12 amino acid insertion in HIR-B is indicated by dotted lines. Two Pro-Arg sequences represent the borders of the insert. **(B)** Partial sequence of human insulin receptor chromosomal gene containing the exon coding for the 12 amino acid HIR-B insertion sequence. Splice consensus sequences are underlined and a downstream exon encoding the precursor cleavage site is shown.

mRNA was found in adult spleen and peripheral blood lymphocytes, and the newborn monkey brain.

Recently, Seino and Bell (1989) and Moller *et al.* (1989) have published data similar to the above, demonstrating tissue-specific expression of the two forms of the insulin receptor that are generated by alternative splicing. However, since only a functional difference between HIR-A and HIR-B variants would allow interpretation of these findings to be of general physiological significance, we constructed SV40 based plasmids for both receptor forms for expression studies in cultured cells. Transfection of Rat 1 fibroblasts with cDNA expression plasmids for either form of the normal human insulin receptor allowed the selection of several cell lines expressing high numbers of insulin receptors. Identity of the HIR-A or HIR-B clones was verified by hybridization analysis using specific DNA probes. Cellular DNA was digested with *Eco*RI, fractionated and blotted onto nitrocellulose. These filters were probed using either the 4.2 kb *Eco*RI fragment of the HIR-A cDNA (Ullrich *et al.*, 1985) or an end-labeled oligonucleotide corresponding to the sequence of the expression plasmid; this analysis demonstrated a 4.8 kb fragment of DNA in both cell lines which hybridized with the large insulin receptor cDNA probe. However, only the fragment from the HIR-B cells hybridized with the oligonucleotide representing the insertion sequence (not shown). PCR amplification of genomic DNA from these lines using primers flanking the insertion site also confirmed the presence in HIR-B or absence from HIR-A of the insert (not shown).

When extracts of cells labeled with [^{35}S]methionine were immunoprecipitated using a monoclonal antibody specific for the human insulin receptor, both HIR-A and HIR-B cells displayed labeled polypeptides corresponding to the 190 kd insulin receptor precursor and the processed 135 kd and 95 kd α- and β-subunits (Figure 3A). The α-subunits of the HIR-A and HIR-B receptors could not be differentiated by this electrophoretic technique. Analysis of insulin-induced autophosphorylation activity *in vitro* (Figure 3B) showed that Rat 1 transfectants expressed functional human insulin receptors and confirmed previous reports in the same and



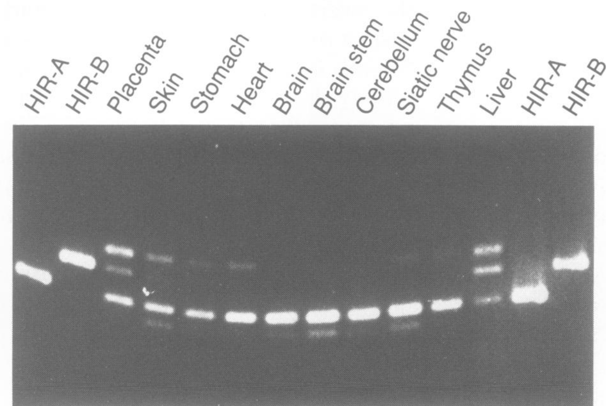


Fig. 2. PCR amplifications of HIR-A and HIR-B mRNA sequences in various human and monkey tissues. HIR-A and HIR-B insulin receptor fragments were separated by electrophoresis in 4% polyacrylamide gels (A) or 2% agarose gels (B). **(A)** Human adult tissues. PCR primers represented nucleotides 2136–2257 and 2327–2348. **(B)** Newborn Rhesus monkey. PCR primers: nucleotides 2163–2184 and 2500–2521. Amplified DNA fragments of HIR-A and HIR-B cDNAs were 358 and 394 bp in length, respectively (B) or 112 and 148 bp in length (A). Additional bands may represent single-stranded DNA or an additional, as yet unidentified, variant.

other cell systems that both forms of the receptor possess an active tyrosine kinase (Ellis *et al.*, 1986; Chou *et al.*, 1987; Ebina *et al.*, 1987; McClain *et al.*, 1987).

The location of the 12 amino acid insertion sequence in

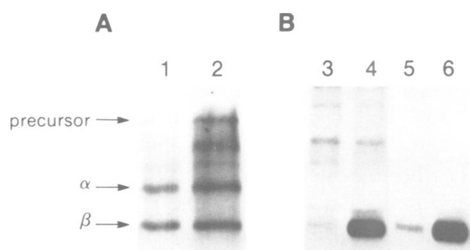


Fig. 3. Characterization of HIR-A and HIR-B expressed in Rat 1 cells. (**A**) Immunoprecipitation of [^{35}S-]methionine labeled receptors. The positions of unprocessed precursor and α- and β-subunits are indicated for HIR-A (lane 1) and HIR-B (lane 2). (**B**) *In vitro* autophosphorylation of HIR-A (lanes 3 and 4) and HIR-B (lanes 5 and 6) in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 300 nM insulin. **Panel A:** $\sim 10^6$ cells in a 60 mm dish were grown in the presence of 0.25 mCi [^{35}S]methionine for 12 h. Insulin receptors were purified from solubilized extracts of the cells using wheat germ agglutinin-agarose followed by immunoprecipitation by a monoclonal antibody specific for human insulin receptors. The other bands visible were non-specifically precipitated by non-immune serum. **Panel B**: To study receptor autophosphorylation, 200 fmol of lectin affinity purified receptors from cells transfected with HIR-A (lanes 3 and 4) or HIR-B (lanes 5 and 6) cDNAs were exposed to insulin (lanes 4 and 6) or buffer only (lanes 3 and 5). The receptors were then exposed to 50 μM [γ-^{32}P]ATP and 5 mM $MnCl_2$ for 1 h at 0°C. Human insulin receptors were then immunoprecipitated as in panel A.

the C-terminal region of the extracellular α-subunit suggested potential consequences for the ligand binding properties of the receptors. We therefore performed detailed binding studies on HIR-A and HIR-B receptor expressing rat fibroblasts (Figure 4). Binding of a tracer (4 pM) concentration of labeled insulin in the presence of increasing amounts of unlabeled insulin is shown. Binding studies were performed on five independently isolated HIR-A clones and six HIR-B clones. The level of expression of insulin receptors varied from 2×10^5 to 2×10^6 receptors per cell among both sets of clones. Binding studies (total $n = 18$, each done in triplicate) were performed using different numbers of cells to maintain bound/free ratios of insulin between 0.04 and 0.08. The competition curves were normalized and averaged for all HIR-A clones and all HIR-B clones. Untransfected Rat 1 cells bind < 1% as much insulin as do the transfectants, so the observed binding is mainly attributable to human (transfected) receptors and not endogenous rat receptors. It can be seen (inset) that on average HIR-A receptors exhibit half-maximal inhibition of binding (ED_{50}) at 0.24 nM free insulin, while for the HIR-B receptors the corresponding half-maximal value is significantly higher, 0.44 nM ($p <$ 0.01). While there is a range of ED_{50} values seen in the different clones, possibly reflecting clonal variation in receptor processing or density, there is no overlap of HIR-A and HIR-B clones. These ED_{50} values are both within the range reported for 'normal' insulin receptors, including the HIR-A receptor variant in CHO cells (Chou *et al.*, 1987) or rat fibroblasts (McClain *et al.*, 1987) and the HIR-B receptors in CHO (Ebina *et al.*, 1987; Ellis *et al.*, 1986) or 3T3 (Whittaker *et al.*, 1987) cells. The consistent differ-

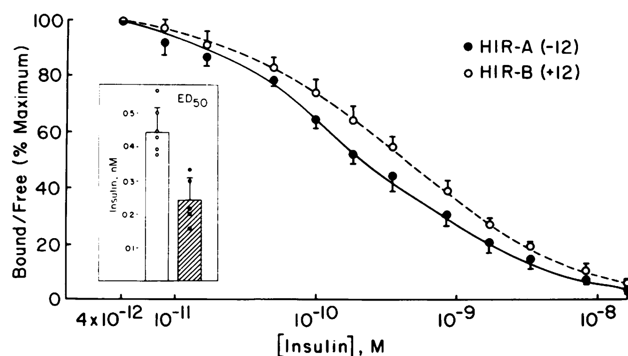


Fig. 4. Insulin binding to transfected cell lines. Cells were exposed to 4 pM [^{125}I]insulin and various concentrations of unlabeled insulin. Cell number was adjusted to keep the bound/free ratio of tracer insulin at ~6%. Competition of tracer [^{125}I]insulin binding by unlabeled insulin is shown, plotted as the percentage of maximal tracer binding as a function of free insulin concentration (●, HIR-A; ○, HIR-B). Results are the means ± SD of 18 experiments, each done in triplicate on five clones of HIR-A and six clones of HIR-B cells. **Inset:** Values for half maximal competition of insulin binding (ED_{50}) for HIR-B and HIR-A. Values for individual clones are shown (HIR-B, open circles; HIR-A, closed circles) as well as the average (± SD) for all HIR-B versus HIR-A clones. The averages differ significantly by *t*-test ($p <$ 0.01).

Table I. Insulin binding and receptor number in individual HIR-A and HIR-B clones

Receptor	ED_{50}(nM)*	Receptors/cell
HIR-A	0.16	1.5×10^6
	0.20	2.6×10^5
	0.22	3.3×10^5
	0.30	5.3×10^5
	0.33	3.1×10^5
HIR-B	0.37	2.0×10^6
	0.39	3.8×10^5
	0.43	4.0×10^5
	0.45	2.1×10^5
	0.50	4.9×10^5
	0.56	1.2×10^6

*The concentration of insulin required to inhibit binding of tracer [^{125}I]insulin half-maximally.

ences observed between HIR-A and HIR-B across all of these clones argues strongly that the differences in affinity are intrinsic to the receptors themselves and not a function of the host cell. Scatchard plots (Monson and Rodbard, 1980) of the binding data reveal that both receptors exhibit curvilinear binding isotherms, with the affinity difference manifest across the full range of insulin concentrations used (not shown). The differences in ED_{50} values do not correlate with the expression levels of the receptors in individual clones (Table I).

To confirm the ligand binding properties of HIR-A and HIR-B as well as to demonstrate that the differences in binding properties are intrinsic to the receptor itself, insulin binding was also measured in solution. Receptors were solubilized in 1% Triton X-100, partially purified by lectin chromatography, and adjusted in concentration so as to result in 10–20% binding of added insulin (17 pM). Competition curves of labeled tracer insulin by cold insulin are shown in Figure 5. These studies represent the average of seven experiments, each performed in quintuplicate, using HIR-A

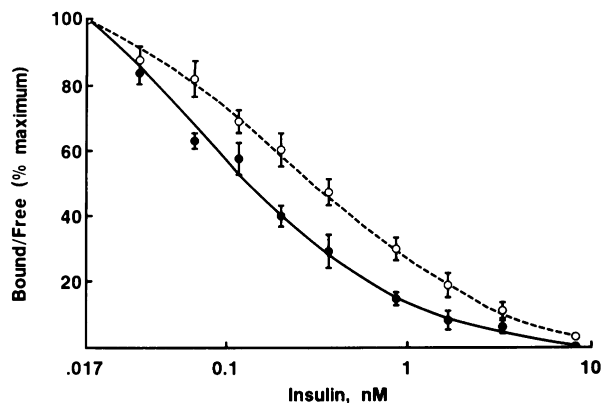


Fig. 5. Insulin binding to solubilized insulin receptors. Binding to 17 pM [^{125}I]insulin and increasing concentrations of unlabeled insulin was performed as described after an incubation of 24 h at 4°C in a volume of 100 μl. Receptor number was adjusted to yield a bound/free ratio of 10–20% of tracer insulin. Curves are the means of eight (HIR-A, ●) or seven (HIR-B, ○) independent experiments each performed in quintuplicate. C.p.m. bound at 300 nM insulin were subtracted to yield specific binding.

receptors from three clones of cells and HIR-B receptors from two clones. It should be noted that receptors from the highest affinity HIR-B clones and lowest affinity HIR-A clones were included. Thus, the HIR-A and HIR-B cells that were closest in affinities for insulin (Figure 4) were used in order to diminish the possibility of detecting differences that would be due to clonal variations in expression rather than to intrinsic receptor differences. Despite this attempt to minimize the chance of detecting differences between HIR-A and HIR-B, differences in ligand binding were still apparent. Both receptors have higher affinities for ligand in solution compared with the affinity as measured in whole cells, but soluble HIR-A receptors maintain a higher affinity for insulin than do soluble HIR-B receptors. The ~2-fold difference in ED_{50} for competition of tracer binding is similar to the fold difference noted using whole cells. The difference between HIR-A and HIR-B receptors depicted is statistically significant ($P = 0.016$ by Student's t-test).

Discussion

We have demonstrated that two isoforms of the insulin receptor are generated by alternative splicing of an exon encoding 12 amino acids, and that this splicing event gives rise to receptors that differ in an important functional characteristic, namely affinity for insulin. The generation of the receptors by alternative splicing has been previously demonstrated (Seino and Bell, 1989) and has been shown to occur with tissue specificity (Moller *et al.*, 1989). In addition to tissue specificity, possible developmental regulation of the splicing event has been inferred from the expression of HIR-B preferentially in liver but not hepatoma tissue (Seino and Bell, 1989).

The physiological significance of the regulation of the splicing event is unknown, but we report here that a fundamental property of the receptor, ligand binding, is affected by splicing. It had been previously known that both HIR-A and HIR-B possess the general properties associated with 'normal' insulin receptors, i.e. high affinity ligand binding, tyrosine-specific protein kinase activity, and the ability to mediate several biological responses to insulin (Ellis *et al.*, 1986; Chou *et al.*, 1987; Ebina *et al.*, 1987; McClain *et al.*, 1987; Whittaker *et al.*, 1987). Thus, only in a direct side-by-side comparison of HIR-A and HIR-B expressed in a common host cell could the quantitative differences in binding affinity emerge. The fact that insulin binding is affected by the presence or absence of a stretch of amino acids in the particular region of the α-subunit is not surprising. Although insulin binding is thought to occur in primarily the N-terminal end of the α-subunit (Yip *et al.*, 1988) it is known that mutations in the processing site for α/β subunit cleavage affect the receptor's affinity for insulin (Sasaoka *et al.*, 1989).

The precise physiological significance of a 2-fold difference in binding affinity is unknown, but there are several interesting potential consequences of the current findings. (i) With respect to tissue expression, the liver appears to contain a predominance of the HIR-B receptor type (Figure 2 and Moller *et al.*, 1985) and this receptor species binds insulin with lower affinity than the HIR-A receptor. It is important to note that *in vivo* the liver is exposed to portal insulin concentrations which are typically 2- to 3-fold higher than peripheral concentrations. Thus, changes in portal insulin concentration over the physiological range would lead to commensurate changes in binding to the HIR-B receptor subtype, since its affinity is lower, whereas the HIR-A receptor population would be relatively saturated at all portal insulin concentrations. Such a mechanism would allow the liver to respond appropriately to physiological changes in pancreatic insulin secretion in terms of insulin's hepatic bioeffects and hepatic clearance of insulin. (ii) Under chronic hyperinsulinemic conditions (which exist in obesity, and in some patients with non-insulin dependent diabetes mellitus and other conditions), relatively more of the high affinity HIR-A receptors would be occupied, leading to preferential down regulation of this receptor type. Thus, the relative expression of HIR-B receptors might be increased in down regulated tissues. If further studies show differences in biological signaling or other functional properties of HIR-B receptors, then this might contribute to the insulin resistance observed in these conditions. However, studies performed to investigate this point have shown no differences in the endocytotic itineraries of HIR-A and HIR-B receptors. When corrected for receptor occupancy, the rates of endocytosis, recycling, insulin degradation and down regulation are equivalent for the two receptor types (D.A.McClain, manuscript in preparation). (iii) The alternative splicing mechanisms that give rise to the receptor variants may be subject to metabolic regulation or altered in pathophysiological states, leading to modulation of tissue expression of the receptor variants, resulting in potential changes in insulin action. (iv) As demonstrated in this study for the human insulin receptor, alternative splicing of primary transcripts may represent a general mechanism for the generation of multiple, functionally distinct cell surface receptors from a single gene. This would not only dramatically expand the repertoire of intercellular communication in multicellular organisms, but suggest new possibilities for the molecular basis of pathological disorders.

Materials and methods

Cloning and PCR amplification of HIR-A and HIR-B sequences

Standard screening techniques (Maniatis *et al.*, 1982) were used to isolate CH30-IR2 from a partial *Sau*3A genomic library with a 26 nucleotide long

DNA probe coding for the 12 amino acid HIR-B insertion. Primer directed sequencing was used to obtain exon sequences shown.

For PCR amplifications, 1 μg of poly$(A)^+$ RNA was used to generate first strand cDNA using a cDNA synthesis kit (Invitrogen), following the manufacturer's instructions. Fifty pmol of synthetic oligonucleotide homologous to nucleotides 2858–2879 of the human insulin receptor sequence (Ullrich *et al.*, 1985) was used to prime specifically the cDNA. Without further purification, 40% of the cDNA reaction mix was subjected to 40 rounds of PCR amplification. Insulin receptor sequences were amplified in a 50 μl reaction volume containing 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl pH 8.8, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 170 μg/ml bovine serum albumin, 10 mM β-mercaptoethanol, 1 mM each of dNTP, 50 pmol of each oligonucleotide primer and 2 U of *Taq* polymerase (New England Biolabs). Samples were overlayed with mineral oil and placed in an automated temperature cycling device (Perkin-Elmer Cetus). After an initial denaturation for 10 min at 95°C, cycles were 1 min at 55°C, 2 min at 72°C and 1 min at 95°C except for the last cycle, where the extension time was 10 min. 5–10 μl of the PCR reactions were subjected to electrophoresis in either 2% agarose gels of 4% polyacrylamide gels.

Cell transfection and culture

Expression plasmids (pCVSVEHBVE400, European publication number 117050) were prepared containing the cDNAs for the two alternative forms of the normal human insulin receptor, HIR-A and HIR-B, and transfected into Rat 1 cells as described (McClain *et al.*, 1987). Methotrexate-resistant cell lines expressing high levels of human insulin receptors were screened by binding of radiolabeled insulin in the presence and absence of unlabeled insulin. Cells were subcloned by limiting dilution to select stable lines exhibiting high levels of insulin binding. The cells were grown in DME/F12 (50:50) medium supplemented with 10% fetal bovine serum (GIBCO).

Insulin binding and biochemical characterization of receptors

Techniques for analysis of transfected cells and their receptors have been previously described in detail (McClain *et al.*, 1987). Briefly, studies of insulin binding to cells were performed using cells passaged 2 days previously into 35 mm culture dishes. Cell density was adjusted to maintain a bound/free ratio of tracer insulin of 4–8%. Cells were exposed to 4 pM of A4 monoiodinated $[^{125}I]$insulin and different concentrations of unlabeled insulin for 3 h at 12°C in 1 ml of Krebs–Ringer's phosphate pH 7.6, 1% BSA. Cells were rinsed three times in cold saline (pH 7.6), solubilized in SDS, and counted. The c.p.m. bound at 300 nM cold insulin were subtracted from all values to give specific binding.

Receptors from transfected cell lines were partially purified from 1% Triton X-100 extracts of cells by means of chromatography on wheat germ agglutinin-agarose (Vector Labs, Burlingame, CA). Receptors were quantitated by $[^{125}I]$insulin binding followed by precipitation in 14% polyethylene glycol 8000 (Sigma) as described (McClain *et al.*, 1987). Autophosphorylation was measured on receptors exposed to 300 mM insulin for 1 h at 0°C. Receptors were then exposed to 50 μM ATP, 2 μCi of $[\gamma^{32}P]$ATP ($\sim$5000 Ci/mmol, Amersham) and 5 mM $MnCl_2$ for 1 h at 0°C. The reaction was stopped as described (McClain *et al.*, 1987) and the human receptors precipitated using a monoclonal antibody (83–14) kindly provided by Dr Kenneth Siddle (Cambridge University).

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