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Heterogeneity of Insulin Receptors in Rat Tissues as Detected with the Partial Agonist B29,B29′-Suberoyl-Insulin

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SUMMARY

Using the insulin receptor partial agonist B29,B29′-suberoyl-insulin, a covalently dimerized insulin derivative, we previously demonstrated a heterogeneity of signal transduction by insulin receptors in two cell systems. The present study was designed to characterize the heterogeneity of insulin receptors in different rat tissues with this agent. Binding of ^{125}I-insulin to insulin receptors and its inhibition by B29,B29′-suberoyl-insulin or by unlabeled insulin were assayed in plasma membranes from brain, spleen, adipocytes, and liver. IC_{50} values of B29,B29′-suberoyl-insulin were different in all tissues investigated (brain < spleen < adipocytes < liver). In contrast, IC_{50} values of insulin were identical, with the exception of spleen membranes (spleen < brain = adipocytes = liver). Furthermore, the IC_{50} ratios (B29 dimer/insulin) were significantly different, ranging from 0.7 (brain) to 12.8 (liver). Solubilization and partial purification of insulin receptors failed to abolish the marked difference between brain and liver (IC_{50} ratios of 1.8 and 7.1, respectively). The apparent molecular masses of the α subunits of insulin receptors, as labeled with a photoreactive insulin derivative, appeared identical in liver and spleen but were significantly lower in adipocytes and brain (liver = spleen > adipocytes > brain). The tissue-specific expression of the known insulin receptor isoforms generated by alternative splicing (insulin receptor types A and B), as assessed by polymerase chain reaction amplification with oligonucleotide primers flanking exon 11, was not correlated with the differences in the IC_{50} values and ratios for insulin and B29,B29′-suberoyl-insulin. Furthermore, IC_{50} values of both insulin and the B29 dimer were 3-fold lower in membranes from Rat1 cells overexpressing insulin receptor type A, compared with membranes with insulin receptor type B; the IC_{50} ratios were identical. No additional alternative splicing of insulin receptor mRNA was found by polymerase chain reaction amplification and digestion with *Hae*III and *Alu*I of seven overlapping domains of the receptor α subunit. These data suggest a heterogeneity of insulin receptors in rat tissues that is unrelated to alternative splicing of the insulin receptor gene.

The insulin receptor is a member of the tyrosine kinase family of receptors, which consist of an intracellular, highly conserved, tyrosine kinase domain and an extracellular, ligand-specific, binding domain (1). Insulin receptors exert pleiotropic responses, in that they produce rapid alterations of metabolic functions, e.g., glucose transport and glycogen synthesis, induce or suppress the expression of specific genes, e.g., c-*fos* or phosphoenolpyruvate carboxykinase, and stimulate DNA synthesis and cell growth (2, 3). It has been debated whether these pleiotropic responses are mediated by a unique insulin receptor or whether subtypes of the receptor exist. Indeed, several lines of evidence have suggested that insulin receptors are heterogeneous. Firstly, the apparent molecular mass of the α subunit was different in various tissues, with the most striking difference (10–15 kDa) being observed between brain and all other tissues (4–6). Secondly, the kinetic characteristics of the tyrosine kinase activity appeared to differ between tissues (7, 8). Finally, the cloning of insulin receptors revealed that two isoforms, IR-A and IR-B, are generated by alternative splicing of the receptor mRNA and are expressed in a remarkably tissue-specific manner (9–11).

The covalently dimerized insulin derivative B29,B29′-suberoyl-insulin (12) is a partial agonist of the insulin receptor (13, 14). Consequently, it inhibits the effects of insulin under certain conditions. Surprisingly, the derivative acted as a partial agonist in some systems, e.g., 3T3-L1 cells, but as a full agonist in others, e.g., rat fat cells (14). Thus, we reasoned that the dimerized insulin derivative might differentiate different receptor isoforms. The present study was designed, therefore, to corroborate this hypothesis by a comparison of the binding affinities of B29,B29′-suberoyl-insulin for insulin receptors in various tissues. The data indicated that the binding of the dimerized insulin derivative was highly heterogeneous in membranes from different rat tissues (liver, adipocytes, brain, and spleen). Therefore, we tested the possibility that the observed heterogeneity was due to the tissue-specific expression of the

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ABBREVIATIONS: IR-A, insulin receptor type A; IR-B, insulin receptor type B; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

two known receptor isoforms or another, as yet unknown, isoform generated by alternative splicing.

Materials and Methods

Preparation of B29,B29′-suberoyl-insulin. B29,B29′-suberoyl-insulin was synthesized as described previously (12). The purity of the covalent dimer was evaluated by high performance liquid chromatography, and no monomeric insulin was detected.

Cell culture. IM-9 lymphocytes (CCL 159; American Type Culture Collection) were cultured as described, in RPMI 1640 medium supplemented with 10% fetal calf serum. Rat1 fibroblasts expressing the insulin receptor isoforms IR-A and IR-B were obtained from Dr. A. Ullrich (Max-Plauck-Institut fur Biochemie, Martiusried, Germany) and were grown as described (10).

Preparation of membranes. Plasma membranes from rat adipocytes (15), human placenta (16), and differentiated 3T3-L1 cells (17) were prepared according to previously published procedures. Crude brain membranes were prepared from two to four rats by homogenization of total brains (devoid of cerebellum) in a 50-ml Potter Elvehjem homogenizer (catalogue no. 3431-E25; A. H. Thomas, Philadelphia, PA) (specific clearance, 0.15 mm) by 15 strokes at 1100 rpm, in 30–60 ml (15 ml/rat) of a Tris-buffered (20 mM, pH 7.4) sucrose (0.25 M) solution containing 1 mM EDTA. After centrifugation of the homogenate at 600 × *g* for 10 min, the supernatant was centrifuged for 40 min at 45,000 × *g*. The resulting pellet was resuspended in Tris buffer (20 mM, pH 7.4) and centrifuged again. The washing step was repeated, and the final pellet was resuspended in the same buffer to a protein concentration of approximately 10 mg/ml.

A crude membrane preparation from liver or spleen was obtained by homogenization of rat liver or spleen with a Potter Elvehjem homogenizer (see specifications above; 10 strokes at 1100 rpm) in Tris-buffered sucrose (3 ml/g tissue; 20 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 7.4). After centrifugation of the homogenate at 600 × *g* for 10 min, the supernatant was centrifuged for 25 min at 45,000 × *g*. The resulting pellet was layered onto a cushion of 38% sucrose and was centrifuged at 100,000 × *g* for 60 min. Membranes were collected from the interface, washed once with 20 mM Tris buffer (pH 7.4), and resuspended in the same buffer to a protein concentration of approximately 3 mg/ml.

Rat1 fibroblasts were homogenized and fractionated according to a modification of a protocol developed for 3T3-L1 adipocytes (17). Confluent cells were washed twice with TES homogenization buffer (20 mM Tris, 1 mM EDTA, 255 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride) that had been equilibrated at 4°. The cells were scraped off the culture dishes with a rubber policeman and were homogenized with 15 strokes (by hand) in a Potter Elvehjem homogenizer (see above) precooled on ice. The homogenate was centrifuged at 12,000 × *g* (JA20 rotor, 10,000 rpm) and 4° for 15 min. The resulting pellet was layered onto a 38% sucrose cushion and centrifuged for 60 min at 100,000 × *g* (SW27 rotor, 23,000 rpm). Plasma membranes were collected from the surface of the sucrose cushion.

Assay of insulin binding. Samples of plasma membranes (30–150 μg of protein/sample) were incubated at 4° overnight with monoiodinated ^{125}I-insulin (Amersham, Braunschweig, Germany), at a final concentration of 20–40 pM, and the desired concentrations of unlabeled insulin or B29,B29′-suberoyl-insulin. The membranes were separated by centrifugation (45 min, 15,000 × *g*) in a refrigerated microfuge. After removal of the supernatants, the tips of the tubes containing the pellets were cut off, and the bound radioactivity was determined by scintillation counting. The data were corrected for nonspecific binding, as determined with samples containing 10^{-6} M unlabeled insulin. An attempt to obtain K_d values by evaluation of the binding data with a computerized fit (LIGAND program) indicated that neither a two-site model nor a one-site model was applicable. Therefore, bound tracer insulin was plotted against the concentrations of unlabeled ligand, and IC_{50} values were deduced from the graphs. Means ± standard errors of IC_{50} values are given in Table 1. In addition, to facilitate the comparison of binding curves obtained for membranes from different tissues, the data for each membrane preparation were normalized to maximum binding and mean values were plotted against the concentrations of unlabeled insulin (see Figs. 1 and 2).

Insulin binding in preparations of partially purified receptors was assayed as described previously (16). Solubilized receptors were purified by absorption to wheat germ agglutinin-agarose and were eluted with buffer supplemented with 0.3 M *N*-acetylglucosamine (13). Binding was allowed to equilibrate overnight at 4°, and bound and free insulin were separated by precipitation with polyethylene glycol (16).

Tyrosine kinase activity of insulin receptors. Membranes (1 mg) from Rat1 fibroblasts overexpressing IR-A or IR-B were solubilized with Triton X-100 (1%), and insulin receptors were partially purified as described (13). Insulin-binding activity as assayed in aliquots of the preparations was 2-fold higher in preparations of IR-B than in preparations of IR-A. Aliquots of the purified receptor preparation were incubated with the desired concentrations of insulin or B29,B29′-suberoyl-insulin for 20 min at room temperature. The phosphorylation of the synthetic substrate poly-Glu/Tyr by the activated insulin receptors in a period of 10 min at room temperature was assayed as described previously (13).

PCR amplification of domains of the insulin receptor α subunit from cDNA. Total RNA was prepared from brain, spleen, liver, and adipose tissue by homogenization in guanidinium thiocyanate and centrifugation through a cushion of caesium chloride. First-strand cDNA synthesis was initiated with the antisense oligonucleotide primer 5′-AGGCCAGAGATGACAAGTGAC (complementary to the rat insulin receptor sequence starting at position 2438) (11). PCR amplification of fragments comprising the alternatively spliced exon 11 was started with the initial primer and the sense oligonucleotide 5′-TTCATTCAGGAAGACCTTCGA-3′ (corresponding to the insulin receptor sequence from position 2181). In addition, seven different overlapping domains covering the whole insulin receptor α subunit were amplified by PCR with specific oligonucleotide primers. The reactions were allowed to proceed for 30 cycles of 60 sec at 95°, 60 sec at 60°, and 30 sec at 72°. The reaction products were separated on nondenaturing polyacrylamide gels (5 or 8%) and detected by ethidium bromide fluorescence. In some experiments, the reaction products were digested with the restriction enzymes *Hae*III or *Alu*I before electrophoretic separation.

Photolabeling of insulin receptors. B29-(4-azidobenzoyl)-insulin (18) was prepared by a modified procedure.[1] It was radioiodinated and isolated via Sep-Pak adsorption as described previously (19) and was stored in solution at −20°. Samples of partially purified insulin receptor (13) containing approximately equal amounts of binding activity were incubated with the photoreactive insulin derivative (150,000 cpm/sample) at 4° overnight. The samples were photolyzed by six flashes (setting of 1000 W × sec) from the high pressure mercury lamp of a LIZZY photolysis flash (Raytest, Straubenhardt, Germany). Insulin receptors were precipitated with polyethylene glycol 6000 (13%, w/v, final concentration) in the presence of 0.1 mg/ml IgG as carrier protein and were separated by centrifugation in a refrigerated microfuge (15,000 rpm, 30 min). The resulting pellets were washed once with acetone and were separated by SDS-PAGE (7% gels). Gels were dried and autoradiographed for 2–14 days.

Results

Fig. 1 illustrates the inhibition of binding of ^{125}I-insulin by unlabeled insulin in membrane preparations from rat brain, liver, spleen, and adipocytes. IC_{50} values derived from these experiments are presented in Table 1. In membranes from brain, liver, and adipocytes, the affinities of insulin receptors for insulin were essentially identical. In contrast, IC_{50} values assayed in plasma membranes from spleen tissue were approx-

[1] J. Kleinjung, unpublished.

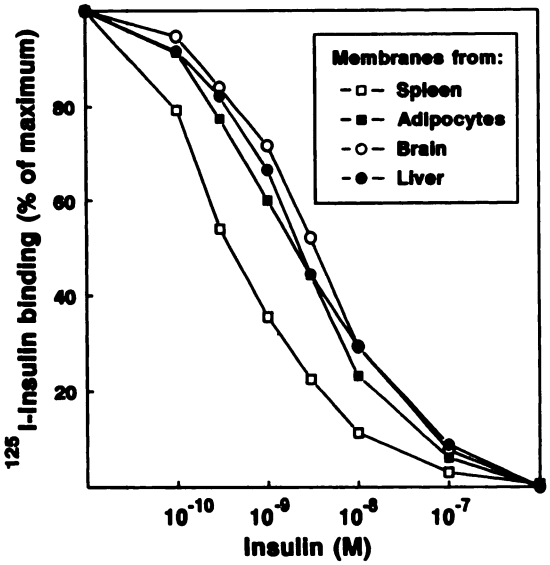

Fig. 1. Inhibition by unlabeled insulin of ^{125}I-insulin binding in membranes from brain, liver, adipocytes, and spleen. Membranes prepared from rat spleen, adipocytes, brain, and liver as described in Materials and Methods were incubated at 4° for 16 hr in the presence of ^{125}I-insulin and the indicated concentrations of unlabeled insulin. Bound and free fractions were separated by centrifugation, and the bound radioactivity was determined by scintillation counting. Nonspecific binding (determined in the presence of 10^{-6} M insulin) was subtracted, and the data were normalized to maximum binding. The data represent means of at least three separate experiments, each assayed in duplicate samples. Means ± standard errors of IC_{50} values determined from these experiments are summarized in Table 1.

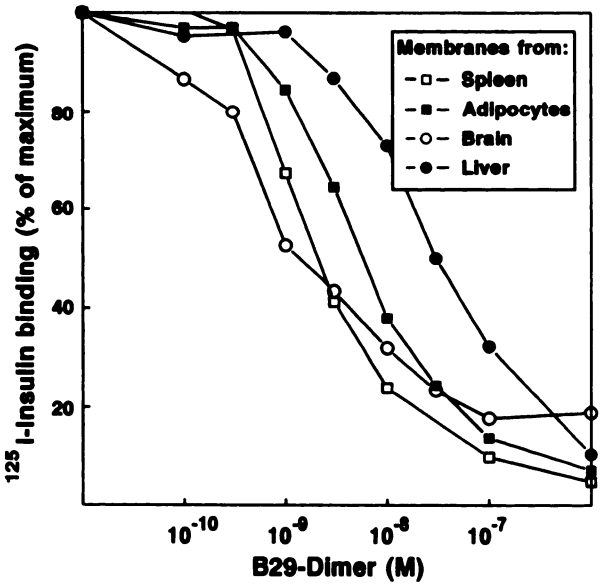

Fig. 2. Inhibition by B29,B29′-suberoyl-insulin of ^{125}I-insulin binding in membranes from brain, liver, adipocytes, and spleen. Membranes prepared from rat spleen, adipocytes, brain, and liver were incubated at 4° for 16 hr in the presence of ^{125}I-insulin and the indicated concentrations of unlabeled B29,B29′-suberoyl-insulin. Bound and free fractions were separated by centrifugation, and the bound radioactivity was determined by scintillation counting. Nonspecific binding (determined in the presence of 10^{-6} M insulin) was subtracted, and the data were normalized to maximum binding. The data represent means of at least three separate experiments, each assayed in duplicate samples. Means ± standard errors of IC_{50} values determined from these experiments are summarized in Table 1.

TABLE 1

Relative affinities of insulin and B29,B29′-suberoyl-insulin for insulin receptors in membranes from various tissues and cells

^{125}I-Insulin binding was assayed in the presence of increasing concentrations of unlabeled insulin or B29,B29′-suberoyl-insulin as described in Materials and Methods. IC_{50} values were derived from plots of the amount of bound radioligand versus the concentration of competing ligand, and means ± standard errors of the number of experiments given in parentheses are presented. For Rat1 cells, the difference between the two membrane preparations studied is given in parentheses.

Receptor preparation	IC_{50} of ^{125}I-insulin binding		Ratio of IC_{50} values
	Insulin	B29 dimer	
	nM		
Membranes			
Rat liver (4)	2.38 ± 0.18	30.5 ± 4.1	12.8
Rat spleen (4)	0.47 ± 0.06	2.13 ± 0.17	4.5
IM-9 lymphocytes (3)	0.51 ± 0.12	2.11 ± 0.61	4.1
Human placenta (3)	0.67 ± 0.01	4.60 ± 1.3	6.9
Rat adipocytes (3)	2.03 ± 0.23	6.10 ± 0.5	3.0
3T3-L1 adipocytes (4)	2.48 ± 0.51	4.58 ± 1.6	1.9
Rat1 cells expressing IR-A (2)	3.7 (0.3)	5.5 (1.6)	1.5
Rat1 cells expressing IR-B (2)	12.0 (2)	20.0 (3)	1.7
Rat brain (7)	2.60 ± 0.16	1.76 ± 0.21	0.7
Partially purified receptors			
Rat liver (3)	0.22 ± 0.05	1.56 ± 0.34	7.1
Rat brain (3)	0.22 ± 0.08	0.40 ± 0.14	1.8

imately 5-fold lower than those in the other membranes (Fig. 1; Table 1).

When the binding of ^{125}I-insulin was inhibited by B29,B29′-suberoyl-insulin, all four membrane preparations exhibited different binding affinities (Fig. 2). As judged from the IC_{50} values derived from these experiments (Table 1), the rank order of the affinities of insulin receptors for the dimerized insulin was liver < adipocyte < spleen < brain. It should be noted that the binding curve assayed in brain membranes extended over >3 orders of magnitude, suggesting that at least two distinguishable binding sites (or affinity states) are present in this tissue. Moreover, it should be noted that a small (membranes from liver, adipocytes, and spleen) or substantial (membranes from brain) portion of the labeled insulin could not be displaced by the dimer (Fig. 2).

The heterogeneity of insulin receptor binding in rat tissues became increasingly apparent when the ratios of IC_{50} values for insulin and the B29 dimer were calculated (Table 1). In addition, we assayed insulin binding in several other membrane preparation (from IM-9 lymphocytes, human placenta, 3T3-L1 adipocytes, and Rat1 cells overexpressing IR-A or IR-B). Table 1 ranks the different tissues according to the IC_{50} ratios. The largest difference was observed between receptors from rat brain and liver (IC_{50} ratio of 0.7 and 12.8, respectively), with the difference being due to the different affinities for the B29 dimer. Insulin receptors in membranes from spleen, supposedly consisting mainly of lymphocytes, were strikingly similar to those from IM-9 lymphocytes and human placenta in their high binding affinity for insulin. Insulin receptors from rat adipocytes differed from those in other tissues in their affinity for the B29 dimer (Table 1) but were similar to those from the murine preadipocyte cell line 3T3-L1 (IC_{50} ratios of 3.05 and 1.85, respectively).

It was conceivable that the observed heterogeneity of insulin receptor binding was due to factors intrinsic to the membrane preparation, e.g., the lipid environment or receptor-associated proteins, rather than to structural heterogeneity of the receptor itself. To test this possibility, we partially purified insulin

receptors from liver and brain by affinity absorption on wheat germ agglutinin-agarose and assayed insulin binding in these preparations (Table 1). Like the receptors in the particulate preparation, the partially purified receptors from liver and brain differed markedly with respect to the IC_{50} values of the B29 dimer and consequently also in the IC_{50} ratios of B-29 dimer/insulin (Table 1). It should be noted that the difference between the receptors from liver and brain, as judged from the IC_{50} ratios, appeared somewhat smaller in the purified preparation than in the membranes. Furthermore, the purification markedly increased the affinity of insulin receptors from both brain and liver.

Alternative splicing of exon 11 in the insulin receptor gene leads to the tissue-specific expression of two receptor isoforms, IR-A and IR-B, differing by 12 amino acids in the carboxyl-terminal regions of their α subunits (9–11). It is well recognized that rat liver expresses predominantly IR-B, whereas in brain and spleen only the IR-A isoform is detected (11). Therefore, we tested the possibility that the differences in the receptor affinities observed here reflected the different ratios of these known insulin receptor isoforms in the tissues under investigation. Binding of insulin and the B29 dimer was assayed in membranes from Rat1 cells overexpressing the two isoforms (Fig. 3). In addition, we examined the expression of the two known isoforms in the different tissues by PCR amplification of a domain comprising exon 11 (see Fig. 5). As anticipated (10), insulin receptors from Rat1 cells overexpressing IR-A exhibited an approximately 3-fold higher affinity for insulin, compared with those from cells overexpressing IR-B (Fig. 3, *upper*). A similar difference was observed with the B29 dimer (Fig. 3, *lower*). Consequently, the IC_{50} ratios of IR-A and IR-B were essentially identical (1.5 and 1.7, respectively; Table 1). It should be noted that the binding characteristics of IR-A and IR-B from Rat1 cells were markedly different from those in placenta, from which these receptors were originally cloned.

B29,B29′-suberoyl-insulin is a partial agonist of the insulin receptor kinase isolated from adipocytes or 3T3-L1 cells (13, 14). Similarly, as illustrated in Fig. 4, the dimerized insulin derivative is a partial agonist of both receptor isoforms, IR-A and IR-B, isolated from Rat1 cells. There was no apparent difference between the two receptor isoforms with respect to the effects of the dimerized derivative on the phosphorylation of the synthetic substrate poly-Glu/Tyr; its maximal effect at 10^{-6} M was 24% of that of insulin with IR-A and 18% with IR-B. Furthermore, there was no apparent difference between the two receptor isoforms in the concentration of insulin producing a half-maximal effect (approximately 10^{-8} M). The maximal effect of insulin was higher in the receptor preparation from cells overexpressing IR-B, reflecting an approximately 2-fold higher receptor concentration in the IR-B preparation.

To assess the expression of the known insulin receptor isoforms, cDNA from brain, spleen, liver, and adipose tissue was prepared and was amplified by PCR with two oligonucleotide primers flanking the alternatively spliced region of exon 11 (11). Fig. 5 illustrates the electrophoretic separation of the PCR products. As anticipated (11), only a single product, representing the IR-B isoform, was observed in rat liver. From cDNA

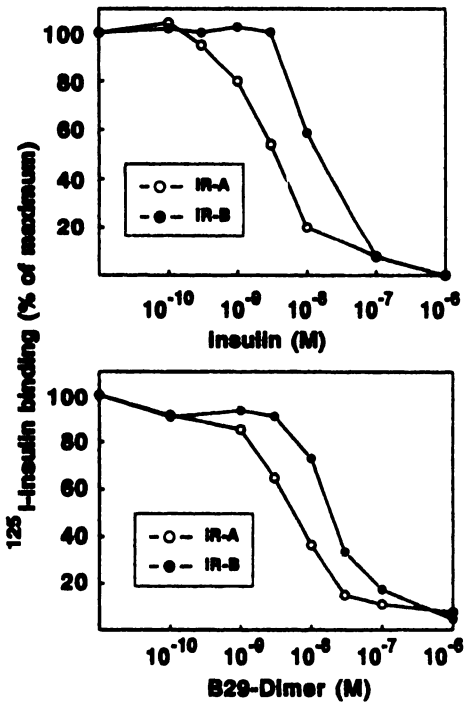

Fig. 3. Inhibition by insulin and B29,B29′-suberoyl-insulin of ^{125}I-insulin binding in membranes from cells overexpressing either IR-A or IR-B. Membranes prepared from Rat1 cells overexpressing IR-A or IR-B were incubated at 4° for 16 hr in the presence of ^{125}I-insulin and the indicated concentrations of unlabeled insulin (*upper*) or B29,B29′-suberoyl-insulin (*lower*). Bound and free fractions were separated by centrifugation, and the bound radioactivity was determined by scintillation counting. Non-specific binding (determined in the presence of 10^{-6} M insulin) was subtracted, and the data were normalized to maximum binding. Data represent means of two experiments.

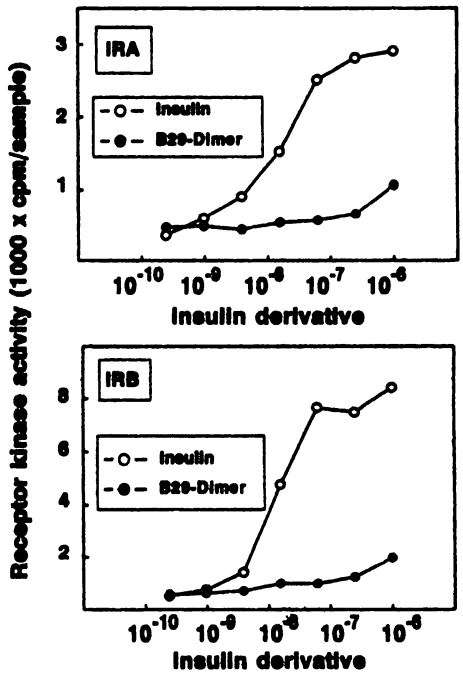

Fig. 4. Effects of insulin and B29,B29′-suberoyl-insulin on the insulin receptor tyrosine kinase activity isolated from Rat1 cells overexpressing IR-A or IR-B. Insulin receptors were partially purified from Rat1 cells overexpressing IR-A (*upper*) or IR-B (*lower*), as described in Materials and Methods. After incubation with the indicated concentrations of insulin or B29,B29′-suberoyl-insulin for 20 min, phosphorylation of the synthetic substrate poly-Glu/Tyr in a period of 10 min was assayed at room temperature as described. Insulin-binding activity was 2-fold higher in samples with IR-B than in those with IR-A. The data represent means of duplicate samples from a representative experiment.

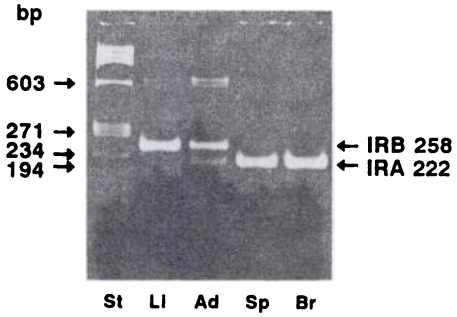

Fig. 5. Tissue-specific splicing of insulin receptor mRNA in liver, adipose tissue, spleen, and brain. Total RNA was prepared from liver (*Ll*), adipose tissue (*Ad*), spleen (*Sp*), and brain (*Br*) as described in Materials and Methods. After reverse transcription with an oligonucleotide primer corresponding to the insulin receptor gene, fragments comprising the region of the alternatively spliced exon 11 were amplified by PCR and were separated by nondenaturing PAGE (8%). IR-A generates a 222-base pair band and IR-B corresponds to the 258-base pair fragment. *St*, standards.

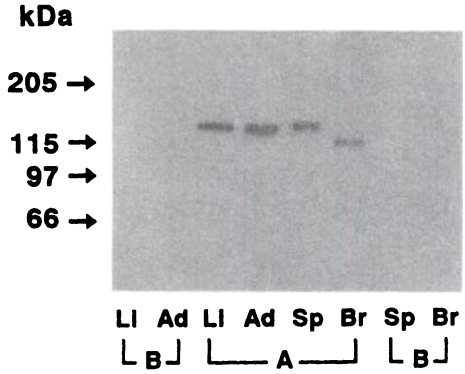

Fig. 6. Heterogeneity of photolabeled insulin receptors from liver, adipose tissue, spleen, and brain. Insulin receptors from liver (*Ll*), adipocytes (*Ad*), spleen (*Sp*), and brain (*Br*) were partially purified and photolabeled with radioiodinated B29-(4-azidobenzoyl)-insulin in the absence (*A*) or presence (*B*) of 1 μM unlabeled insulin, as described. The receptors were separated by SDS-PAGE (7%) under reducing conditions, and the gel was autoradiographed for 14 days.

isolated from rat brain or spleen, a single, faster migrating, product, representing the IR-A isoform, was amplified. In cDNA from adipose tissue, PCR products from both receptor isoforms were detected (Fig. 5). Because brain and spleen exhibited the same pattern of expression of the IR-B isoform, one would expect identical insulin-binding characteristics in these tissues. However, this was not the case (Figs. 1 and 2). Thus, the expression of the two receptor isoforms in the various rat tissues can explain only partially, if at all, the observed heterogeneity of insulin binding.

To examine the possibility that additional alternatively spliced mRNA species of the insulin receptor α subunit exist, seven overlapping domains covering the whole α subunit were amplified (data not shown). As judged from the mobility of these fragments in polyacrylamide gels and from their fragmentation pattern after digestion with *Hae*III and *Alu*I, the fragments generated from cDNA from brain, liver, adipose tissue, and spleen were identical except for the alternative splicing of exon 11 (data not shown). Thus, IR-A and IR-B appear to be the only insulin receptor isoforms, with heterogeneity of the receptor α subunit being generated by alternative splicing.

It was shown previously that α subunits of insulin receptors from brain, adipocytes, and liver differ in their electrophoretic mobility in reducing SDS gels (5, 6, 20). To compare the apparent molecular masses of the insulin receptor α subunits in our membrane preparations, we covalently labeled the receptors with a photoreactive insulin derivative (21). As illustrated in Fig. 6, the apparent molecular masses of the insulin receptor α subunits from liver and spleen appeared identical. In contrast, the apparent molecular masses of the receptors from adipocytes and brain were approximately 2 or 10 kDa lower, respectively (Fig. 6).

Discussion

The present data indicate that ligand binding to insulin receptors in rat tissues is highly heterogeneous. The partial receptor agonist B29,B29′-suberoyl-insulin revealed marked differences in the binding affinities of insulin receptors in all tissues investigated (brain, liver, spleen, and adipocytes). Most strikingly, there was a 15-fold difference in the IC_{50} values between liver and brain membranes, with the latter apparently containing two sites with considerably different binding affinities. Furthermore, insulin receptors in membranes from spleen tissue exhibited a 4–5-fold higher affinity for insulin than did those isolated from brain, liver, or adipocytes.

The previously demonstrated heterogeneity of the electrophoretic mobilities of insulin receptor α subunits has been assumed to largely reflect different glycosylation of the proteins (5, 6, 22). However, more recently it has been shown that the α subunit of insulin receptors from human brain and placenta can be distinguished with a monoclonal antibody (MC51) directed against the binding domain (23). Similarly, another monoclonal antibody against the binding domain ($10D_9$) reacted with human insulin receptors from adipose and muscle tissue but not with receptors from liver (20). Like the present findings with the B29 dimer, these data demonstrate a profound functional heterogeneity of the ligand binding site of insulin receptors.

The present data indicate that heterogeneous expression of the known insulin receptor isoforms IR-A and IR-B can only partially, if at all, account for the observed heterogeneity of ligand binding. The largest difference in the binding affinities of insulin receptors for the B29 dimer was observed between brain and liver. Accordingly, in brain exclusively IR-A was detected, whereas in liver only IR-B was found. However, all other observed differences cannot be explained by the differential expression of IR-A and IR-B. Most notably, brain and spleen express only IR-A but exhibit a large difference in insulin binding. Moreover, the IC_{50} ratios of insulin and B29 dimer binding were comparable in membranes from Rat1 cells overexpressing IR-A or IR-B (1.5 and 1.7, respectively). In contrast, the IC_{50} ratios were considerably different in rat tissues, ranging from 0.7 (brain) to 12.8 (liver). Thus, according to these criteria it appears reasonable to conclude that alternative splicing of exon 11 does not sufficiently explain the heterogeneity of antagonist binding to insulin receptors.

We have examined the possibility that the observed differences in the binding affinities of insulin receptors in tissues are related to additional, as yet unknown, alternative splicings of the insulin receptor mRNA. PCR amplification of seven overlapping domains of the α subunit and digestion of the PCR products with restriction enzymes indicated that in liver, brain, spleen, and adipose tissue only the known receptor isoforms IR-A and IR-B (9–11) are generated from the insulin receptor

gene. In a recent report the same conclusion was drawn on the basis of heteroduplex mapping carried out with mRNA from brain, liver, skeletal muscle, kidney, and spleen (24). Thus, it is concluded that the observed heterogeneity of insulin receptors in rat tissues is unrelated to alternative splicing of the insulin receptor gene.

The structural basis of the heterogeneity of insulin receptor binding in rat tissues remains unclear, but several mechanisms can be considered. Firstly, it cannot be excluded that other genes exist that encode additional insulin receptor isoforms. Recently, the gene of an 'insulin-related receptor' was cloned from genomic DNA (25) and was shown to be expressed in human kidney, heart, skeletal muscle, liver, and pancreas (26), as well as in thymus and stomach (27). However, the product of this gene does not appear to bind insulin (26). To date there is no evidence that additional insulin receptor genes exist. Secondly, the heterogeneity might be caused by tissue-specific post-translational modification of the receptors, e.g., heterogeneous glycosylation (5), by heterogeneity of disulfide bridges between or within the α subunits, or by formation of a variable amount of hybrids between insulin receptors and homologous receptors. The formation of hybrids between $\alpha\beta$ heterodimers of insulin receptors and insulin-like growth factor I receptors has been described previously (28). Thirdly, we do not fully discount the possibility that receptor-associated proteins that are co-purified with the receptors on wheat germ-agglutinin modify the affinity of the receptors.

Acknowledgments

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