Distinct β -subunits are present in hybrid insulin-like-growth-factor-1 receptors in the central nervous system

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Previous work suggests the existence of different isoforms of the insulin-like-growth-factor-1 (IGF-1) receptor in various tissues. In the present study we provide support for the concept that heterogeneous IGF-1 receptors exist in the brain and that part of the heterogeneity is derived from IGF-1 receptor hybrids formed from different β -subunits. IGF-1 receptors were extracted from adult-rat forebrain synaptosomes and partially purified by wheat-germ agglutinin (WGA) chromatography. Hormone-binding studies in this preparation demonstrate the presence of receptors for IGF-1 and insulin. An antibody, a-RIR, specific for the rat insulin receptor was used to remove insulin receptors from the WGA extract. Studies with the immunodepleted material demonstrated two proteins of 92 and 99 kDa that are phosphorylated on tyrosine during incubation with low concentrations of IGF-1. Both proteins bound with high affinity and specificity to IGF-1 immobilized on agarose, and each underwent phosphorylation when the agarose beads were incubated with [γ - 32 P]ATP and $MnCl_2$. Two-dimensional

phosphopeptide maps after exhaustive trypsin treatment of the two proteins showed significant differences in their structure as well as differences from the phosphopeptide map for the β -subunit of the insulin receptor. The relationship of the two proteins to the IGF-1 receptor was further probed by an antibody (a-HF) raised against a specific sequence in the β -subunit of the human IGF-1 receptor, and a polyclonal antibody raised against the liver insulin receptor (L1) which cross-reacts with the IGF-1 receptor. Both antibodies immunoprecipitated the two phosphorylated proteins. However, reduction of the receptors to form receptor dimers or monomers showed that a-HF precipitated only the 99 kDa protein, whereas L1 precipitated primarily the 92 kDa protein. In conclusion, the brain IGF-1 receptor apparently has two structurally different β -subunits, one of 92 kDa and a second of 99 kDa. Interestingly, at least a portion of the IGF-1 receptor population has both isoforms in the same receptor.

INTRODUCTION

The insulin-like-growth-factor-1 (IGF-1) receptor mediates the growth-promoting actions and metabolic effects of IGF-1, an insulin-like molecule synthesized by many tissues during fetal and adult life (Rechler and Nissley, 1985). The amino acid sequence for the human IGF-1 receptor has been reported (Ullrich et al., 1986) and a partial sequence published for the IGF-1 receptor of rat (Werner et al., 1989). Comparison of the IGF-1 receptor sequence with the sequence of the insulin receptor demonstrated marked similarities that explained the similarities in the actions of IGF-1 and insulin and the ability of insulin and IGF-1 to cross-react with the opposite receptor at high ligand concentrations (Rechler and Nissley, 1985). Both receptors are members of the tyrosine kinase family, and both have an $\alpha_2\beta_2$ configuration (Rechler and Nissley, 1985). The extracellular α -subunits confer the hormone-binding activity, whereas the intracellular β -subunits provide the signalling activity derived from the tyrosine kinase activity (Rechler and Nissley, 1985).

A single gene on chromosome 15 is at present recognized as coding for the human IGF-1 receptor (Ullrich et al., 1986). There is, however, a significant amount of evidence for the existence of more than one IGF-1 receptor species (Jacobs et al., 1983; Jonas and Harrison, 1985; Ota et al., 1988; Alexandrides and Smith, 1989; Garofalo and Rosen, 1989; Hainaut et al., 1991). Thus two differently sized β -subunits have been found by several laboratories (Kull et al., 1983; Jacobs et al., 1983; Morgan et al., 1986; Ota et al., 1988; Alexandrides and Smith, 1989; Hainaut et al., 1991), and immunological differences using antibodies

against the insulin receptor have been shown (Jonas and Harrison, 1985; Alexandrides and Smith, 1989; Garofalo and Rosen, 1989; Hainaut et al., 1991). Changes during development in the IGF-1-receptor binding affinities for insulin and IGF-1 have been reported (Sara et al., 1983; Morgan and Roth, 1986). Using different approaches to evaluate the receptors for insulin and IGF-1 in fetal-rat muscle, Alexandrides and Smith (1989) reported an IGF-1 receptor distinct from the adult IGF-1 receptor.

Analysis of the IGF-1 receptor is further complicated by the formation of a hybrid receptor from the complexing of an α - β dimer of the insulin receptor with an α - β dimer of the IGF-1 receptor (Soos and Siddle, 1989; Moxham et al., 1989; Treadway et al., 1989). Evidently, cells that express both receptor types can potentially form the hybrid receptor, in addition to 'authentic' receptors for insulin and IGF-1 (Soos and Siddle, 1989; Moxham et al., 1989; Treadway et al., 1989).

In the present study, we have examined the structure of the IGF-1 receptor from adult rat brain and compared it with the structure of the insulin receptor. As initially reported by Garofalo and Rosen (1989) in studies of fetal-rat brain, we find two different β -subunits for the IGF-1 receptor from synaptosome membranes. By using sequence-specific antibodies against the rat insulin-receptor β -subunit and the human IGF-1-receptor β -subunit, it was possible to show that two distinct β -subunits contribute to the structure of the brain IGF-1 receptor and that neither subunit is the β -subunit of the structure of the insulin receptor. Although the results do not exclude differential splicing of IGF-1-receptor mRNA, the presence of both subunits in one

receptor molecule makes this possibility less likely, since a neuron would have to produce two quite differently spliced products. The findings most simply support coding by a different gene for the small β -subunit of the brain IGF-1 receptor, a situation that would predict a different α -subunit as well.

EXPERIMENTAL

Materials

Wheat-germ agglutinin (WGA)-agarose was purchased from Vector (Burlingame, CA, U.S.A.). Crystalline insulin was a gift from Eli Lilly (Indianapolis, IN, U.S.A.) and human recombinant IGF-1 was provided by KabiVitrum (Stockholm, Sweden). SDS, ATP, phenylmethanesulphonyl fluoride, *N*-acetylglucosamine, tosyl-lysylchloromethane ('TLCK')-treated trypsin, phosphotyrosine, phosphoserine, phosphothreonine and BSA were supplied by Sigma. Bio-Rad supplied Affigel-10 and all reagents for SDS/PAGE. The peptides used for immunizing animals were synthesized by Dr. Neil Bramson, University of Rochester School of Medicine and Dentistry. Immunization of rabbits and chicken was conducted by East Acres, Southbridge, MA, U.S.A. Calbiochem supplied keyhole-limpet haemocyanin and Pansorbin. Poly(ethylene glycol) was bought from VWR (S. Plainfield, NJ, U.S.A.). Pierce Chemical Co. provided protein A-agarose. Cellulose acetate plates were purchased from Analtech, Newark, DE, U.S.A. *N*-glycanase was bought from Genzyme Corp. 125 I-insulin was prepared by the chloramine-T method as previously described (Krupp and Livingston, 1980). 125 I-IGF-1 and [γ - 32 P]ATP were purchased from Amersham. Affinity-purified rabbit anti-chicken antibody was purchased from Sigma.

Antibodies

L-1 antibody, which was prepared against the insulin receptor purified from rat liver, has been previously described (Arsenis and Livingston, 1986a). a-RIR was prepared in rabbits against the peptide sequence corresponding to amino acid residues 1313–1324 of the rat insulin-receptor sequence (Goldstein and Dudley, 1990). A cysteine was added to the C-terminus of this peptide for coupling to keyhole-limpet haemocyanin by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as described by Herrera et al. (1985). Affinity-purified antibody was prepared by passage of immunized-rabbit serum over a peptide-Affigel column. After extensive washing of the column, the antibody was eluted in 0.25 ml fractions with 0.1 M glycine, pH 3.0. Fractions containing protein were immediately neutralized by addition of 0.1 ml of 1 M Tris, pH 7.4, containing 2 mg/ml BSA and 0.02 % $\text{Na}_2\text{S}_2\text{O}_3$. Ability to immunoprecipitate insulin receptors by the fractions was tested by using WGA extracts of brain synaptosomes that had been allowed to bind 125 I-insulin (50 pmol) overnight at 4 °C in the absence and presence of excess native insulin (Arsenis and Livingston, 1986b). A similar test was performed with 125 I-IGF-1. Fractions that contained significant amounts of insulin-binding activity were pooled and stored frozen in batches. In no case did the antibody fractions immunoprecipitate IGF-1-binding activity, although, as previously reported, the amount of IGF-1 receptors exceeds the amount of insulin receptors in this extract (Unger et al., 1989). The efficiency of a-RIR and L1 in precipitating the insulin receptor was compared in rat liver, a preparation that contains essentially no IGF-1 receptors. Both antibodies immunoprecipitated approx. 80 % of the insulin binding immunoprecipitated by poly(ethylene glycol).

a-HF was prepared from egg yolks after immunization of hens with the peptide based on the amino acid sequence 1284–1295

from the human IGF-1-receptor β -subunit (Ullrich et al., 1986). The peptide was coupled to keyhole-limpet haemocyanin through a cysteine residue added to the C-terminus as described by Herrera et al. (1985). Eggs were collected from chickens that tested positive for the presence of immunoprecipitating activity of IGF-1 receptor by using an assay similar to that described above for the insulin receptor and a-RIR, except that a rabbit anti-IGY antibody was used in the precipitation of IGY by Pansorbin. IGY was partially purified by poly(ethylene glycol) precipitation (Stuart et al., 1988; Gassman et al., 1990) and affinity-purified as described above for a-RIR, by using an Affigel column to which the IGF-1 receptor peptide was coupled.

Preparation of brain synaptosomes and WGA purification of receptors

Synaptosomes were prepared by the Percoll gradient method as described by Wang et al. (1988), from the forebrains of rats. Layer p3 was collected and the synaptosomes were pelleted after dilution in Krebs–Ringer buffer (Wang et al., 1988) by centrifugation at 10 000 *g* for 10 min. The membrane pellet (7 mg of protein) was solubilized by addition of a 1 % Triton X-100 solution containing 50 mM Tris, pH 7.4, 0.1 M NaCl and 1 mM phenylmethanesulphonyl fluoride. Non-soluble material in the Triton X-100 extract of synaptosomes (15 mg of protein) was removed by centrifugation and the supernatant was applied to a 2 ml WGA-agarose column. The column was washed with 30 ml of 50 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 0.05 % Triton X-100. Glycoproteins were eluted by addition of 0.3 M *N*-acetylglucosamine as described by Arsenis and Livingston (1986b). The protein content of the fractions was determined by the Bio-Rad method.

Autophosphorylation of the receptors for IGF-1 and insulin

WGA extract (approx. 5 μ g of protein) was incubated at 4 °C in 50 μ l of 50 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 10 mM MnCl_2 , 1 mM orthovanadate, 0.05 % Triton X-100, 0.1 mg/ml BSA and the indicated concentration of insulin or IGF-1. The phosphorylation reaction was initiated by addition of 4 μ M [γ - 32 P]ATP (5 μ Ci/assay), and the incubation was conducted at 21 °C for 20 min. The reaction was terminated by addition of Laemmli (1970) sample buffer, whereupon the sample was subjected directly to SDS/PAGE, or by addition of 150 μ l of 50 mM Tris buffer, pH 7.4, containing 1 mM orthovanadate, 0.05 % Triton X-100, 10 mM *p*-nitrophenyl phosphate, 50 mM NaF, 5 mM EDTA and 20 mM ATP when the receptors were to be immunoprecipitated.

Immunoprecipitation of the receptors and SDS/PAGE

The L-1 antibody was used at a 1/100 dilution in a 16 h incubation with samples at 4 °C. The immunoreaction product was collected with Pansorbin, and the immunoprecipitate was washed twice with 50 mM Tris, pH 7.4, containing 0.1 % Triton X-100 and 0.15 M NaCl, and once with the buffer in the absence of NaCl. Affinity-purified a-RIR was used at a concentration of 1 μ g/ml to immunoprecipitate the autophosphorylated receptors under the same conditions used with L-1. Affinity-purified a-HF (freshly prepared) was used at 2 μ g/ml concentration. The immunocomplex was precipitated with Pansorbin previously coated with rabbit anti-chicken IGY antibody.

For reduction of receptors to α - β dimers before immunoprecipitation, samples were quenched in the usual buffer, except that the pH was 8.5. They were then incubated with 1 mM

dithiothreitol (DTT) for 30 min at 21 °C, and 3 mM *N*-ethylmaleimide was added (Moxham et al., 1989). For reduction to α and β monomers before immunoprecipitation, the receptor preparation was incubated in 10 or 100 mM DTT for 30 min at 37 °C, followed by extensive dialysis to remove DTT before addition of the antibody.

The immunoprecipitates were boiled for 3 min in Laemmli (1970) sample buffer in the presence of 1% β -mercaptoethanol unless otherwise indicated. The extracts of the immunoprecipitates were applied to 5–12% gradient polyacrylamide gel for SDS/PAGE (Lerea and Livingston, 1983). The gel was fixed, stained with Coomassie Blue and dried for autoradiography. High-molecular-mass standard proteins (Bio-Rad) were used to determine the molecular masses of the phosphorylated proteins.

SDS/PAGE was also conducted on samples quenched in sample loading buffer and not immunoprecipitated. In these studies the gels were washed extensively in 10% trichloroacetic acid to remove free radioactive phosphate before gel drying and autoradiography.

Analysis of phosphoamino acid content and phosphopeptides

The phosphorylated proteins resolved by SDS/PAGE and autoradiography were cut from the dried gel, and rehydrated with 10% methanol followed by three changes of 50 mM NH_4HCO_3 . Each gel fragment was placed in 1 ml of 50 mM NH_4HCO_3 containing 200 μg of TLCK-treated trypsin that had been purified by reverse-phase h.p.l.c. After 8 h at 37 °C, another 200 μg of trypsin was added and allowed to incubate at 37 °C for 16 h. Approx. 85% of the radioactivity was removed from the gel piece by these procedures. The soluble radioactive material was dried in a Speed Vac apparatus, resuspended in water and redried. To determine the phosphoamino acid content, the pellet was suspended in 6 M HCl and hydrolysed by heating at 110 °C for 1 h under nitrogen (Jacobs et al., 1983). The acid hydrolysate was dissolved in 0.1 M formic acid and passed through Dowex 50 X 8 to remove unhydrolysed material. The flow-through material was dried, resuspended in water, dried again, and placed in electrophoresis buffer for spotting on cellulose t.l.c. plates (MN 300). The plates were subjected to electrophoresis in acetic acid/pyridine/water (10:1:89, by vol) at 1000 V. Phosphoamino acid standards were located by spraying the t.l.c. plate with a 0.5% solution of ninhydrin in acetone and heating at 80 °C for 10 min. Radioactivity was located by autoradiography.

The phosphopeptides were examined in a two-dimensional map after the trypsin treatment outlined above. The dried radioactivity from the gel fragment was solubilized in water and applied to MN300 cellulose thin-layer plates as described previously (Garofalo and Rosen, 1989). Electrophoresis for 35 min at 1000 V in formic acid/acetic acid/water (3:3:16, by vol.) was conducted, followed by drying of the plate. For chromatography in the second dimension, the plate was placed in butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.).

Binding of receptors to IGF-1-agarose

A 2 ml packed volume of Affigel-10 was rapidly washed in 0.1 M Mops buffer, pH 7.5, and mixed with 2 mg of IGF-1 contained in 5 ml of Mops buffer. After rocking overnight at 4 °C, 10 ml of 1 M ethanolamine, pH 7.5, was added and mixed for 1 h at 21 °C. The matrix was washed extensively with 4 M urea, followed by four washes with 50 mM Tris, pH 7.5, containing 0.1% Triton X-100. Then 50 μl of matrix was added to 100 μl of WGA extract, mixed carefully and allowed to incubate for 8 h at 4 °C in the absence or presence of the indicated concentration of

insulin or IGF-1. The IGF-1-agarose was washed three times with 50 mM Tris, pH 7.4, containing 0.15 M NaCl and 0.1% Triton X-100. The matrix was then suspended in this buffer, and 10 mM MnCl_2 and 4 μM [^{32}P]ATP were added. The incubation was contained for 20 min at 21 °C. The autophosphorylation reaction on the matrix was determined by addition of the termination buffer described above for the autophosphorylation/immunoprecipitation studies. The matrix was washed twice in 50 mM Tris, pH 7.4, containing 0.05% Triton X-100. Laemmli (1970) sample buffer was added, and the sample was boiled for 3 min. The matrix was pelleted by centrifugation and the supernatant subjected to SDS/PAGE.

RESULTS

Insulin stimulates phosphorylation of a 92 kDa protein and IGF-1 stimulates phosphorylation of a 92 and a 99 kDa protein in WGA extracts of synaptosomes

Figure 1 shows that insulin and IGF-1 treatment of the WGA extract from synaptosomes induces the phosphorylation of two proteins of 92 and 99 kDa that could be detected in the absence of immunoprecipitation. Although both proteins underwent phosphorylation with both hormones, insulin generated a larger amount of phosphorylation in the 92 kDa protein than in the 99 kDa. Furthermore, significant 99 kDa-protein phosphorylation was produced only with a high concentration of insulin. By contrast, IGF-1 stimulation generated a large amount of phosphorylated 92 kDa and 99 kDa material even at a relatively low IGF-1 concentration.

Figure 1 Effects of insulin and IGF-1 on the phosphorylation of 92 kDa and 99 kDa proteins

The WGA extract was incubated with 0, 3 or 100 nM hormone as indicated. Phosphorylation was carried out with 4 μM [^{32}P]ATP for 20 min at 21 °C. The incubation was terminated with Laemmli (1970) loading buffer and subjected to SDS/PAGE. The arrows indicate proteins migrating at 92 and 99 kDa.

Figure 2 a-RIR immunoprecipitation of phosphoproteins after insulin and IGF-1 treatment

Phosphorylation of receptor extract was carried out as in Figure 1 and followed by immunoprecipitation with a-RIR as described in the Experimental section. The immunoprecipitates were subjected to SDS/PAGE and autoradiography. Insulin stimulated phosphorylation of a 92 kDa protein.

a-RIR does not immunoprecipitate the 92 or 99 kDa IGF-1-stimulated protein

a-RIR, which is specific for the rat insulin receptor, precipitates only a 92 kDa protein after insulin stimulation (Figure 2). This antibody does not precipitate any proteins that have undergone phosphorylation during IGF-1 stimulation, including the 92 kDa protein shown in Figure 1. The same results were obtained when the receptors were reduced to α - β dimers by 1 mM DTT (Figure 3). Therefore, the 92 kDa protein that undergoes phosphorylation during insulin treatment is not the same 92 kDa protein that is phosphorylated during IGF-1 stimulation. Furthermore, a-RIR did not immunoprecipitate the 99 kDa protein, even though at high concentrations of insulin this protein is phosphorylated. Thus the 99 kDa protein probably results from the cross-over stimulation of the IGF-1 receptor that occurs with high concentrations of insulin. One other interesting result from these studies is that a high concentration of IGF-1 does not produce significant phosphorylation of the 92 kDa subunit of the insulin receptor. Thus IGF-1 does not cross-react with the central-nervous-system insulin receptor to any significant extent, or the interaction is not productive for the stimulation of β -subunit autophosphorylation.

Immunodepletion with a-RIR removes insulin receptors from the synaptosome WGA extract

To examine the 99 kDa and 92 kDa phosphoproteins found after

Figure 3 Phosphoproteins immunoprecipitated by a-RIR after receptor reduction with DTT

The WGA extract was incubated with 0, 3 or 100 nM hormone and phosphorylation was carried out as in Figure 1. After the samples had been quenched, they were treated with 1 mM DTT for 30 min at 21 °C, followed by 3 mM *N*-ethylmaleimide. Immunoprecipitation was then carried out with a-RIR. A single 92 kDa protein was phosphorylated in response to insulin.

IGF-1 stimulation, we removed the insulin receptor from the WGA extract by immunodepletion with a-RIR (results not shown). Treatment of the extract with a-RIR completely removed all insulin-stimulated 92 kDa phosphoprotein, since a second round of a-RIR treatment failed to precipitate any 92 kDa material.

The 92 and 99 kDa proteins phosphorylated in response to IGF-1 are associated with a high binding affinity for IGF-1

WGA extract that had been immunodepleted of insulin receptors was incubated with IGF-1-agarose beads in the absence or presence of the indicated concentrations of IGF-1 or insulin. Various concentrations of the two-hormones were used to compete with the immobilized IGF-1 in order to determine the hormone-specificity and binding affinity of the receptors in the WGA extract. Autophosphorylation studies were then carried out on the receptors bound to the beads, and the resulting phosphoproteins were resolved on SDS/PAGE (results not shown). In the absence of free IGF-1 or insulin, two phosphoproteins, of 99 and 92 kDa, were detected. The inclusion of relatively low concentrations of IGF-1 in the extract prevented both proteins from binding to the IGF-1 beads. In contrast, 100 nM insulin was required to prevent the two proteins from associating with the beads. The results of these studies support the contention that the 99 and 92 kDa proteins are β -subunits of the IGF-1 receptor.

Figure 4 Immunoprecipitation of phosphoproteins by antibody L-1 after treatment with insulin, IGF-1 and DTT

WGA extract immunodepleted of insulin receptor was incubated with 0, 3 or 100 nM insulin (INS) or IGF-1 as indicated. The extract was subjected to phosphorylation as described in Figure 1. After the reaction was terminated with the quench buffer (see the Experimental section), the samples were divided and incubated in the presence (+) or absence (–) of 1 mM DTT for 30 min. Antibody L-1 was added and allowed to incubate with the samples for 16 h at 4 °C; then Pansorbin was added and the immunoprecipitate was washed and boiled in Laemmli (1970) buffer, and the extract was subjected to SDS/PAGE. Arrows indicate proteins migrating at 92 and 99 kDa.

L-1 antibody directly immunoprecipitates the 92 kDa, but not the 99 kDa, IGF-1 receptor β -subunit

In order to probe further the IGF-1 receptor β -subunits, the insulin-receptor-depleted WGA extract was phosphorylated after insulin and IGF-1 stimulation and was subjected to immunoprecipitation with L-1, an anti-insulin-receptor antibody that will cross-react with the IGF-1 receptor (Arsenis and Livingston, 1986a). After insulin stimulation of the WGA extract (Figure 4), L-1, like a-RIR, immunoprecipitated a single 92 kDa phosphoprotein. With stimulation by 3 or 100 nM IGF-1, L-1 immunoprecipitated the 92 and 99 kDa proteins in equal amounts, again suggesting that the two proteins are β -subunits of the IGF-1 receptor. However, when immunoprecipitation was carried out after addition of 1 mM DTT to form α - β dimers (Moxham et al., 1989), L-1 immunoprecipitated primarily the 92 kDa protein (Figure 4). The results found in the absence and presence of DTT suggest that some fraction of the 99 kDa and 92 kDa subunits is located in the same tetrameric receptor. The results also indicate that the 92 kDa subunit is immunologically more similar to the insulin-receptor β -subunit than is the 99 kDa subunit.

a-HF antibody specifically immunoprecipitates the 99 kDa IGF-1-responsive protein

a-HF, an antibody generated against a specific amino acid sequence of the β -subunit of the human IGF-1 receptor, was used to probe further the 92 and 99 kDa proteins that become

Figure 5 Phosphoproteins immunoprecipitated by a-HF and L1 before and after treatment with DTT

The WGA extract was subjected to the standard phosphorylation conditions after incubation in the presence or absence of 0, 3 or 100 nM IGF-1. For (a), duplicate samples were phosphorylated in the absence or presence of 100 nM IGF-1, then were treated with 100 mM DTT followed by 100 mM *N*-ethylmaleimide, dialysed, and a-HF (a) was added to the samples. For immunoprecipitation with L1, the same protocol was followed, except that the samples were treated with 10 mM DTT (b). The immunoprecipitates were subjected to SDS/PAGE and autoradiography.

Figure 6 Phosphoamino acid analysis of brain and liver insulin-receptor β -subunit

WGA extracts were prepared from synaptosomes and rat liver. The extracts from synaptosomes were subjected to the phosphorylation reaction after incubations with 100 nM insulin; the rat liver WGA extract underwent phosphorylation in the presence of 100 nM insulin. Phosphorylated insulin receptor was immunoprecipitated by α -RIR, the β -subunit was resolved on SDS/PAGE, and the radiolabelled band was subjected to trypsin treatment as described in the Experimental section (a). Phosphoamino acid analysis was conducted on the trypsin-treated material as described in the Experimental section. For (b), WGA extract from synaptosomes was immunodepleted of insulin receptor with α -RIR. The depleted extract was subjected to phosphorylation under standard conditions in the presence of 100 nM IGF-1. The subunits were resolved by SDS/PAGE and their phosphoamino acid content was determined as described above. Abbreviations: PS, phosphoserine; PY, phosphotyrosine.

phosphorylated during IGF-1 stimulation. The results shown in Figure 5(a) demonstrate that both subunits are immunoprecipitated by this antibody when the extract is treated with IGF-1. Immunoprecipitation of both subunits by α -HF was completely blocked by addition of 1 μ M IGF-1 receptor peptide based on amino acid sequence 1284–1295 (results not shown). Immunoprecipitation with α -HF was also carried out after treatment of the autophosphorylation samples with 100 mM DTT to reduce the IGF-1 receptor completely to α and β monomers. α -HF immunoprecipitated both the 92 and the 99 kDa subunits under non-reducing conditions. After reduction, only the 99 kDa subunit was present in the α -HF precipitate (Figure 5a). When samples were treated with 100 mM DTT before immunoprecipitation with L1, the immunoprecipitation was rather poor, but the immunoprecipitate contained almost exclusively the 92 kDa subunit (results not shown). Figure 5(b) shows the results that were obtained with L1 after sample reduction with 10 mM DTT. There is very little 99 kDa subunit in the post-reduction immunoprecipitate, and the ratio of 92 kDa subunit is clearly greater in the post-reduction immunoprecipitate. The small amount of 99 kDa subunit present may have been due to incomplete reduction of the receptor, or to weak cross-reactivity of L1 for the 99 kDa subunit. Thus these results further support the existence of IGF-1 receptors that have 92 and a 99 kDa β -subunits in the same molecule. These findings also eliminate the possibility that the results with L-1 stemmed from its recognition of an epitope in the α -subunit of the IGF-1 receptor, rather than recognition of a 92 kDa β -subunit epitope.

Phosphoamino acid analysis reveals serine as well as tyrosine phosphorylation of the insulin and IGF-1 receptor β -subunits in response to ligand

Phosphoamino acid analysis illustrated in Figure 6(a) demonstrates that most of the phosphorylation occurs on tyrosine

Figure 7 Phosphopeptide analysis of the insulin-receptor β -subunit and the 92 kDa and 99 kDa IGF-1-receptor β -subunits

The phosphorylated β -subunits were resolved by SDS/PAGE, cut from the dried gel and subjected to trypsin treatment as described for phosphoamino acid analysis. After trypsin treatment, the dried radioactivity was solubilized in water and applied to cellulose thin-layer plates. Electrophoresis was carried out for 35 min at 1000 V in formic acid/acetic acid/water (3:3:16, by vol). After drying, the plate was placed in butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.) for chromatography. Radioactive peptides were detected by autoradiography (left panel). The right panel is a schematic of the left panel, with each peptide numbered for ease of comparison. Key: O, origin; INS, insulin.

residues in the insulin-receptor β -subunit of brain. The same is found for the 92 and 99 kDa subunits of the IGF-1 receptor (Figure 6b). One unusual feature of this system, however, is the presence of significant amounts of phosphoserine in the receptor subunits after hormone stimulation of brain WGA extract. This is in contrast with the results with insulin receptor from a WGA extract of rat liver (Figure 6a), which was processed in parallel with the brain extracts. As shown for the receptor from liver, there is almost no phosphoamino acid other than phospho-

Phosphopeptide maps of the β -subunits of the insulin and IGF-1 receptors

Two-dimensional phosphopeptide maps for the insulin-receptor and IGF-1-receptor β -subunits are shown in Figure 7. These maps for each subunit were reproducibly different from one another in each of several experiments. There is some overlap in phosphopeptide fragments; for example, peptide 1 of the insulin-receptor β -subunit corresponds to peptides 37 and 40 of the 92 and 99 kDa IGF-1-receptor β -subunit respectively. Also, the maps of the insulin-receptor β -subunit and the 92 kDa IGF-1-receptor β -subunit have clear similarities, such as peptides 5 and 25 and peptides 10 and 31. Similarly peptide 36 from the IGF-1-receptor 92 kDa subunit corresponds to peptide 50 in the 99 kDa β -subunit. Unique peptides are present in the insulin-receptor β -subunit (e.g. 6, 9 and 11); the 92 kDa IGF-1-receptor β -subunit also has unique peptides (e.g. 23, 32 and 35); and in the 99 kDa subunit unique peptides (e.g. 42, 45, 47 and 48) are present.

There are more phosphopeptides than expected in the map for the insulin-receptor β -subunit, given the limited number of tyrosine-phosphorylation sites and their relationship to the predicted sites of trypsin action. The cause of the additional fragments is likely to be serine phosphorylation of the subunit, which would add to the number of phosphopeptides. The sequence of the β -subunit of the rat IGF-1 receptor has not been reported. However, the serine phosphorylation found in the two β -subunits by phosphoamino acid analysis would also increase the number of phosphopeptides generated by the action of trypsin. The phosphorylation of a particular β -subunit could vary, depending on whether it is present in a classical or hybrid receptor. Thus differences in the peptide maps of the two 92 kDa subunits form suggestive, but not conclusive, evidence that they are structurally different proteins.

DISCUSSION

In receptor extracts of rat synaptosomes, as in extracts from a number of tissues and cell lines, IGF-1 stimulates the phosphorylation of two proteins of different molecular masses that have characteristics of the β -subunit of the IGF-1 receptor (Kull et al., 1983; Jonas and Harrison, 1985; Morgan et al., 1986; Ota et al., 1988; Garofalo and Rosen, 1989; Alexandrides and Smith, 1989). The differences in size of the subunits on SDS/PAGE found in these studies have been ascribed to differences in glycosylation or to differences in primary amino acid sequence (Jonas and Harrison, 1985; Morgan et al., 1986; Ota et al., 1988; Alexandrides and Smith, 1989; Garofalo and Rosen, 1989), i.e. two different isoforms of the β -subunit. It was not possible to exclude clearly the participation of the insulin-receptor β -subunit in contributing to the findings, because of the lack of a reagent specific for the rat IGF-1 receptor. In the present study we have developed epitope-specific antibodies against the rat insulin receptor and IGF-1 receptor to evaluate whether two isoforms with different primary sequences exist in the IGF-1 receptor of rat brain.

Removal of the insulin receptor with the highly specific antibody a-RIR allowed the examination of only the IGF-1 receptor. With this immunodepleted preparation of receptors, it was possible to show the presence of two proteins of 92 kDa and 99 kDa that undergo phosphorylation on tyrosine residues with stimulation by relatively low concentrations of IGF-1 and that do not respond to insulin. Furthermore, several lines of evidence indicate that the two proteins are both IGF-1 β -subunits. First, both undergo phosphorylation in roughly equal amounts. Secondly, both proteins were bound with high affinity and specificity

to immobilized IGF-1 and underwent phosphorylation on this matrix. And thirdly, an epitope-specific antibody for the human IGF-1 receptor and an antibody that cross-reacts with the rat IGF-1 receptor precipitated both proteins.

The studies also demonstrate the differences in the amino acid sequence of the two proteins. Phosphopeptide maps of the two proteins are clearly different, and each is different from the map for the β -subunit of the insulin receptor. Although some of these differences may be due to serine phosphorylation or to altered phosphorylation that occurs in hybrid receptors, the peptide maps support the existence of sequence differences in the subunits. Other evidence includes immunological differences between the proteins when the antibodies a-HF and L-1 are used to probe their structure after treatment with DTT. Overall, we conclude that the two proteins are β -subunits of the IGF-1 receptor and that differences exist in their primary structure.

Differences in the degree of glycosylation between the two subunits have not been ruled out by these studies. When the subunits are treated with N-glycanase, the difference in molecular mass is obscured, causing the subunits to migrate on SDS/PAGE as a broad band in the range of 87 kDa (results not shown). However, a difference only in glycosylation cannot explain the findings. If this were the case, the antibody a-HF, which was generated against non-glycosylated peptide sequence in the IGF-1-receptor β -subunit, should immunoprecipitate both β -subunits after DTT treatment, which it does not.

The other important finding is the presence of the two different β -subunits in the same molecule of the IGF-1 receptor. Although a-HF recognized only the monomeric 99 kDa subunit and L-1 antibody recognized only the 92 kDa subunit, both subunits were precipitated when the tetrameric receptor was probed with either antibody. It is not clear what proportion of the receptors have mixed β -subunits, but the amount is significant. In fact, since each subunit is present in roughly the same amount in the receptor preparation, based indirectly on the degree of ligand-mediated phosphorylation (see Figure 1), and a-HF and L-1 each precipitate roughly equal amounts of the non-reduced 92 kDa and 99 kDa species, we can speculate that there are more hybrid receptors than receptors with only one type of β -subunit isoform. However, the ability of a high concentration of insulin to stimulate autophosphorylation of the 92 kDa isoform, but not the 99 kDa species, suggests that an IGF-1 receptor with only the small subunit is present in the nervous system.

In human placenta and in several cell lines IGF-1 receptor heterogeneity is due to the presence of hybrid IGF-1 receptors formed by the combination of an insulin-receptor β -subunit and an IGF-1-receptor β -subunit (Moxham et al., 1989; Soos and Siddle, 1989; Garofalo and Barenton, 1992). Also, the assembly of such hybrid receptors can be demonstrated *in vivo* (Treadway et al., 1989). Somewhat surprisingly, no evidence of such receptor hybrids in adult-rat synaptosomes was found. a-RIR did not immunoprecipitate the 99 kDa protein under any circumstance and did not immunoprecipitate any proteins that underwent autophosphorylation in response to IGF-1, even after reduction of the receptors to form dimers. It remains possible that hybrids form between the 92 kDa IGF-1-receptor β -subunits and the insulin-receptor β -subunit. This entity, however, would have to be sensitive only to insulin, which is not true of hybrid receptors in other systems (Moxham et al., 1989). Thus, in adult-rat synaptosomes no evidence was found of hybrid receptors formed from insulin-receptor β -subunits and the β -subunits of the IGF-1 receptor.

Interestingly, in KB cells, which contain insulin-receptor-IGF-1-receptor hybrids, there also exists more than one species of IGF-1 receptor α -subunits (Garofalo and Barenton, 1992).

Distinct β -subunits associated with the different α -subunits would be expected, but cannot be deduced from these data. The homodimeric and heterodimeric IGF-1 receptor species from the KB cells also differ in their sensitivities to IGF-1 and IGF-II (Garofalo and Barenton, 1992).

Fetal brain has been reported to contain two different β -subunits in IGF-1 receptors. Garofalo and Rosen (1989) found a population of IGF-1 receptors in fetal-rat brain membranes that contain two immunologically distinct subunits, of 92 and 97 kDa, results in agreement with our findings. However, since all of the antibodies used were raised against the insulin receptor, the possibility could not be excluded that the IGF-1 receptor hybrids were formed by insulin- and IGF-1-receptor β -subunits.

Fetal-rat muscle is another tissue shown to have two distinct IGF-1-receptor β -subunits (Alexandrides and Smith, 1989). In addition to the usual 95 kDa insulin-receptor and 95 kDa IGF-1-receptor β -subunits found in adult muscle, fetal muscle also contained a 105 kDa subunit, which underwent phosphorylation in response to low concentrations of either IGF-1 or insulin. That study did not investigate whether or not hybrid receptor molecules composed of these different subunits existed. In summary, our data from adult-rat synaptosomes and the results from fetal-rat muscle and brain studies all support the presence of at least two distinct β -subunits for the IGF-1 receptor.

Whether the different β -subunits come from alternative splicing of a single gene transcript for the IGF-1 receptor or from different genes is not clear. Alternative splicing of the insulin-receptor transcript is well documented (Moller et al., 1989; Goldstein and Dudley, 1990), but no evidence has been collected of hybrid insulin receptors composed of the two resulting isoforms.

Alternative splicing of the IGF-1 receptor transcript has also been reported (Yee et al., 1989). As those authors point out, however, the alternatively spliced transcript identified cannot fully explain the molecular-mass difference in the two IGF-1-receptor β -subunits observed by us and others, since it involves a substitution of Arg for Thr-Gly. The proteins encoded by the two transcripts might undergo different post-translational modifications, resulting in a somewhat different molecular mass (Yee et al., 1989). However, our immunological data militate strongly against the possibility that the two β -subunits are the result of alternative splicing. In particular, it is unlikely that the polyclonal antibody L1 would react strongly with one splice variant and poorly if at all with the other. Also, it is improbable that the peptide against which a-HF was generated is coded for by the spliced-out sequence.

The other possibility is of a second, as yet uncloned, gene that codes for one of the two isoforms, i.e. that codes for the 92 kDa species, since an antibody specific for an epitope on the human IGF-1 receptor precipitates the 99 kDa form and not the 92 kDa subunit. Interestingly, there is a report of a gene with a sequence intermediate between the sequences for the insulin and IGF-1 receptors, predicted to encode the insulin-receptor-related receptor (IRR) (Shier and Watt, 1989). The IRR transcript has recently been identified in a number of human tissues (Zhang and Roth, 1992). The ligand for the IRR has not yet been identified, but is not a known member of the insulin family (Zhang and Roth, 1992). Thus studies in various tissues and cell lines continue to add to our knowledge of the diversity of subunit types and assembly in the insulin-receptor family (Alexandrides and Smith, 1989; Moxham et al., 1989; Treadway et al., 1989; Soos and

Siddle, 1989; Garofalo and Barenton, 1992; Zhang and Roth, 1992).

Given that the β -subunit is proteolytically processed from a proreceptor structure that contains the α -subunit of the receptor, it is possible that the hybrid receptors have different α -subunits as well if a separate gene contributes the small β -subunit. This possibility has not been examined, since epitope-specific antibodies to rat α -subunit have not been generated as yet. Although the source of the two different β -subunits is not clear, the idea that they arise from two different gene products is attractive. This system could markedly enrich the diversity of IGF-1 receptors and thus expand the cellular systems under control of IGF-1. Differential regulation of one or both genes could tailor the IGF-1 response of the cell to meet different challenges during neuronal differentiation.

We acknowledge the technical assistance of George Vamvakias and Matthew Krym, and the secretarial assistance of Helena M. Vatter and Karen M. Drake. This work was supported by an ADA Feasibility Grant, National Institutes of Health Grants DK 40394 and DK 44135 and a Wilms Cancer Research Fellowship (A.M.M.).

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