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A model for the quaternary structure of human placental insulin receptor deduced from electron microscopy

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ABSTRACT **Electrophoretically pure and functionally intact human placental insulin receptor was studied by electron microscopy with negative-staining techniques. The quaternary structure of the detergent-solubilized receptor was determined. The receptor had the shape of a letter T ≈24 nm in height and 18 nm in width with a thickness of the stem and the crossbar of 3–4 nm. No consistent change in ultrastructure of the receptor could be detected after the addition of insulin alone or insulin and Mn^{2+}/Mg^{2+}/ATP. After partial reduction of the $\alpha_2\beta_2$ heterotetrameric receptor into $\alpha\beta$ heterodimers, the electron micrographs showed a clear reduction in average size of the molecule with disappearance of the T profiles characteristic of the $\alpha_2\beta_2$ heterotetramers. By incubation of the heterodimers in a phosphorylation medium containing insulin, a reassociation to molecules with molecular weights of the $\alpha_2\beta_2$ heterotetramer took place judged from SDS/PAGE. Electron microscopy showed that the molecule formed larger aggregates, and only a few solitary T-shaped copies were seen.**

The insulin receptor is an integral membrane protein composed of two α subunits (M_r 130,000) and two β subunits (M_r 95,000). In the functional heterotetrameric complex $\alpha_2\beta_2$, an α and a β subunit are held together by disulfide bonds, termed class II disulfides, and two $\alpha\beta$ heterodimers are joined by a second class of disulfide bonds, termed class I disulfides (for reviews, see refs. 1 and 2). The receptor is a tyrosine kinase stimulated by insulin and capable of autophosphorylation as well as exogenous substrate phosphorylation (3). Cloning of the insulin receptor gene and deduction of the amino acid sequence from the nucleotide sequence has provided valuable information about receptor structure and function (4, 5).

Previous attempts to visualize the isolated, heterotetrameric receptor (6) and a secreted form of the receptor ectodomain (7) by electron microscopy (EM) have not contributed to modeling structure of the intact receptor in any detail. In this study we have visualized the $\alpha_2\beta_2$ heterotetrameric insulin receptor in solubilized form by EM with the techniques of negative staining and determined its gross quaternary structure. Additionally, we have studied the insulin receptor after cleavage by partial reduction into $\alpha\beta$ heterodimers and after reassociation through a phosphorylation procedure (8, 9).

MATERIALS AND METHODS

Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin A, 1,10-phenanthroline, benzamidine hydrochloride, and disuccinimidyl suberate were all obtained from Sigma. Triton X-100 (Für elektronische Zahlung von Milchzellen) was from Merck. CNBr-activated Sepharose 4B was obtained from Pharmacia. Porcine insulin and human insulin monolabeled with ^{125}I on the A chain were obtained from Novo Nordisk, Bagsværd, Denmark. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. The molecular weight markers—cytochrome *c*, ovalbumin, bovine albumin, β-galactosidase, and cross-linked phosphorylase b—were from Sigma.

Wheat Germ Agglutinin–Sepharose. Wheat germ agglutinin was isolated as described by Vretblad (10) and coupled to CNBr-activated Sepharose 4B. The coupling was performed in 0.2 M sodium citrate buffer, pH 6.5, and the amount of wheat germ agglutinin bound to the gel was 3.3 mg/ml.

Insulin–Sepharose. Insulin was coupled to Sepharose 4B essentially as described by Fujita-Yamaguchi *et al.* (11). The amount of insulin bound to Sepharose 4B was 0.32 mg/ml.

SDS/PAGE. SDS/PAGE was performed in 0.1% SDS as described by Laemmli (12), and gels were silver-stained (13). Under nonreducing conditions the separating gel was 6% acrylamide, and under reducing conditions, the run was done in 13% acrylamide gel. Samples were boiled for 5 min in 1.5% SDS with or without 20 mM dithiothreitol. The series of marker proteins, cross-linked phosphorylase b with M_r 97,000, 194,000, 291,000, 388,000, 487,000, and 584,000, were used as reference to control uniformity of the nonreduced-receptor preparations.

Purification of Insulin Receptors. Methods for isolation of plasma membranes and purification of insulin receptors were, with minor modifications, as described by Fujita-Yamaguchi *et al.* (11). Normal human placentae, obtained within 1 hr of delivery, were homogenized at 4°C in 500 ml of 0.25 M sucrose containing 50 mM Tris·HCl, pH 7.4/leupeptin at 10 μg/ml/0.5 mM PMSF. The membranes were washed once and stored in homogenization buffer at a protein concentration of ≈30 mg/ml at −80°C until use. Membranes were solubilized in 2% Triton X-100 in 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/leupeptin at 10 μg/ml, 0.5 mM PMSF/aprotinin at 0.05 unit/ml, 1 μM pepstatin A/1 mM phenanthroline/25 mM benzamidine while being stirred at room temperature for 15 min. The detergent-to-protein weight ratio was 4:1. The supernatant obtained after centrifugation at 200,000 × *g* for 45 min was applied to a wheat germ agglutinin–Sepharose 4B column (45 ml) at a rate of 50 ml/hr. The column was washed overnight with 10 column volumes of 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/0.1% Triton X-100/leupeptin at 10 μg/ml/0.5 mM PMSF/0.02% sodium azide and eluted with the same buffer containing 0.3 M *N*-acetylglucosamine but without $MgCl_2$. Fractions (20 ml) containing insulin-binding activity were pooled, made 1 M in NaCl, and applied to an insulin–Sepharose column (25 ml) at a rate of 5 ml/hr. For EM we found it essential that all buffers used after this chromatographic step be purified by ultrafiltration through a PM 10 filter (Amicon) before addition of Triton X-100 to the appropriate concentration. The column was washed overnight with at least 10 column volumes of 50 mM Tris·HCl, pH 7.4/1 M NaCl/0.1% Triton X-100/leupeptin at 10 μg/ml/0.5 mM PMSF/0.02% sodium azide. The column was eluted with 50 mM sodium acetate, pH 5.0/1 M NaCl/0.05% Triton X-100/10% (vol/vol) glycerol/0.02%

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

sodium azide. Fractions of 5 ml were collected and immediately adjusted to pH 7.6 with 1 M Hepes, pH 8.0. All chromatographic procedures were done at 4°C.

Insulin-Binding Assay. Insulin receptors were incubated with 0.1 nM ^{125}I-labeled insulin in a final volume of 0.1 ml of 50 mM Tris·HCl, pH 7.4/0.1% Triton X-100/0.1% bovine serum albumin/0.1 mM PMSF for 16 hr at 4°C. Free ^{125}I-labeled insulin was separated from the bound hormone by adding 25 μl of 0.4% bovine γ-globulin and 125 μl of 20% (wt/vol) polyethyleneglycol, M_r 6000. After 10 min at 4°C the samples were centrifuged at 12,000 × *g* for 2 min in a Beckman Microfuge. The pellet, suspended in 100 μl of 0.5% Triton X-100, was counted in a γ counter. Nonspecific binding was determined in the presence of 4 μM unlabeled insulin. For Scatchard analysis, a concentration range of 0.4–32 nM unlabeled insulin was used.

Insulin Receptor Cross-linking and Autophosphorylation. Insulin receptors were incubated at 4°C for 16 hr with 0.4 nM ^{125}I-labeled insulin with or without 16 μM unlabeled insulin in 50 mM Tris·HCl, pH 7.4/0.1% bovine serum albumin/0.1% Triton X-100. The insulin was cross-linked to the receptor by adding disuccinimidyl suberate freshly dissolved in dimethyl sulfoxide. The final concentration of disuccinimidyl suberate was 20 mM, and the reaction was stopped after 15 min at 4°C by adding Tris, pH 8.0, to a final concentration of 100 mM. The samples were analyzed by SDS/PAGE followed by autoradiography on Kodak XAR-5 film.

Autophosphorylation was done by incubating freshly prepared receptor with and without 100 nM insulin in 50 mM Hepes, pH 7.6/10 mM $MgCl_2$/ 2 mM $MnCl_2$. After 1 hr at room temperature 4 μCi of [γ-^{32}P]ATP was added. The final ATP concentration was 5 μM. The reaction was stopped after 10 min by adding EDTA to a concentration of 30 mM. Samples were analyzed by SDS/PAGE followed by autoradiography on Kodak XAR-5 film with Cronex Lightning Plus intensifying screens.

Reduction of Insulin Receptors into αβ Heterodimers and Their Reassociation by Phosphorylation. Heterodimeric (αβ) insulin receptors were prepared essentially as described (8, 9). Freshly prepared insulin receptor (80 μg) in a 200-μl volume was adjusted to pH 8.5 with 1 M Tris and left at room temperature for 25 min. Dithiothreitol was added to 2 mM, and the reaction was stopped after 5 min by two consecutive centrifugations at 1000 × *g* for 5 min through a prespun column (3-ml syringe) packed with desalting gel (Bio-Gel B-6DG, Bio-Rad) equilibrated in 50 mM Hepes, pH 7.6/10% (vol/vol) glycerol/0.1% Triton X-100/0.02% sodium azide. The eluate showed >90% conversion of $\alpha_2\beta_2$ heterotetramers to αβ heterodimers, as analyzed by SDS/PAGE.

For reassociation of heterodimers into tetramers, according to Wilden *et al.* (9), insulin was added to 100 nM in 50 mM Hepes, pH 7.6/50 μM ATP/10 mM $MgCl_2$/2 mM $MnCl_2$/10% glycerol/0.1% Triton X-100, and the mixture was left to react at room temperature for 1 hr. The degree of reassociation was assessed by SDS/PAGE.

EM. Fractions from the insulin–Sepharose column with peak insulin-binding capacity were pooled and immediately concentrated by using Centricon-30 microconcentrators (Amicon). The salt concentration was lowered to ≈150 mM by adding 50 mM Hepes, pH 7.6/10% glycerol/0.02% sodium azide. Receptor concentration was 15 μg/ml measured as insulin-binding capacity. When the insulin receptor was studied under conditions of phosphorylation, insulin and Mn^{2+}/Mg^{2+}/ATP were added in the same concentrations as the ones used for reassociation of heterodimers into heterotetramers (100 nM insulin/100 mM $MgCl_2$/2 mM $MnCl_2$/ 50 μM ATP for 1 hr.

For EM the preparation was adsorbed to a very thin carbon-coated Formvar film, carried on EM grids and exposed to plasma glow before use. The preparation was negatively stained with 2% (wt/vol) sodium silicotungstate, pH 7.2, as given in ref. 14. The specimens were examined and photographed at ×60,000–70,000 primary magnification in a JEOL 100 CX electron microscope equipped with a high-resolution objective-lens pole piece [spherical aberration coefficient (C_s) ≈ 0.7 mm] and operated at 80 kV with focus setting around Scherzer focus. Images were recorded on Agfa Scientia 23D56 emulsion by using a low-dose exposure technique (35–50 $e^-/Å^2$ of specimen). Plates were developed in Kodak D-19 solution to an electron speed of 0.7 $\mu m^2/e^-$. Analysis of Thon diffractograms ensured that images could be interpreted to a resolution of 1 nm without contrast reversal (15). Magnification of the microscope was calibrated using a Fullam SiO_2 grating replica no. 6002.

In all experiments, we also examined the ultrafiltrated buffers before and after addition of Triton X-100. The buffer blanks were devoid of EM-detectable structures. After addition of Triton X-100, spherical micellar structures, 6–12 nm in diameter, were present in the negatively stained preparations, declining in numbers and size proportional to the degree of washing of the specimen with staining solution. Examination of several brands of Triton X-100 showed the selected Merck variety to be notably free of contaminating nonmicellar particles.

RESULTS

Insulin receptors were isolated from human placenta. The purity of the receptor preparation was ascertained by SDS/PAGE (Fig. 1*A*). After silver-staining, the nonreduced receptor preparation developed a heavy band corresponding to an apparent M_r of 350,000, whereas only faint traces of lower molecular weight material could be detected. One such faint band could be discerned at a position corresponding to an apparent M_r of 290,000. After exhaustive reduction of the preparation, two distinct bands developed at positions of M_r 130,000 and 95,000, corresponding to the receptor α and β subunits. Only very faint traces of other material could be discerned—in particular, no band corresponding to a partially degraded β subunit [β′ subunit, M_r 45,000, (11)], was observed.

Functional characterization of the receptor was obtained through the insulin-binding assay. Scatchard analysis showed curvilinear plots with K_d values of 0.5–1 nM. Cross-linking of ^{125}I-labeled insulin to the receptor followed by SDS/PAGE under nonreducing and under reducing conditions revealed autoradiographic spots corresponding to the intact receptor and to the α subunit, respectively. Further, the insulin receptor preparation showed insulin-activated autophosphorylation (data not shown). Thus, the preparation corresponded in biochemical and functional terms to the data reported for the insulin receptor in the literature.

Seven independent preparations of the receptor were examined by EM. Three of the preparations were further examined after addition of insulin or insulin in combination with Mn^{2+}/Mg^{2+}/ATP, and two preparations were examined after partial reduction and reassociation. Electron micrographs of negatively stained preparations of the heterotetrameric insulin receptor in detergent solution are shown in Fig. 1 *B–E* (*B* and *D* derive from preparations as they elute from the insulin-affinity column, posttreated only by ultrafiltration to raise receptor concentration and lower salt concentration; *C* and *E* derive from similar preparations subjected to insulin-stimulated phosphorylation). We have been unable to detect any consistent structural change after addition of either insulin or insulin combined with Mn^{2+}/Mg^{2+}/ATP. The following description, thus, applies to all three forms.

Assuming minimum M_r of 310,000 and partial specific volume of the insulin receptor of ≈0.75 cm^3/g, the molecular

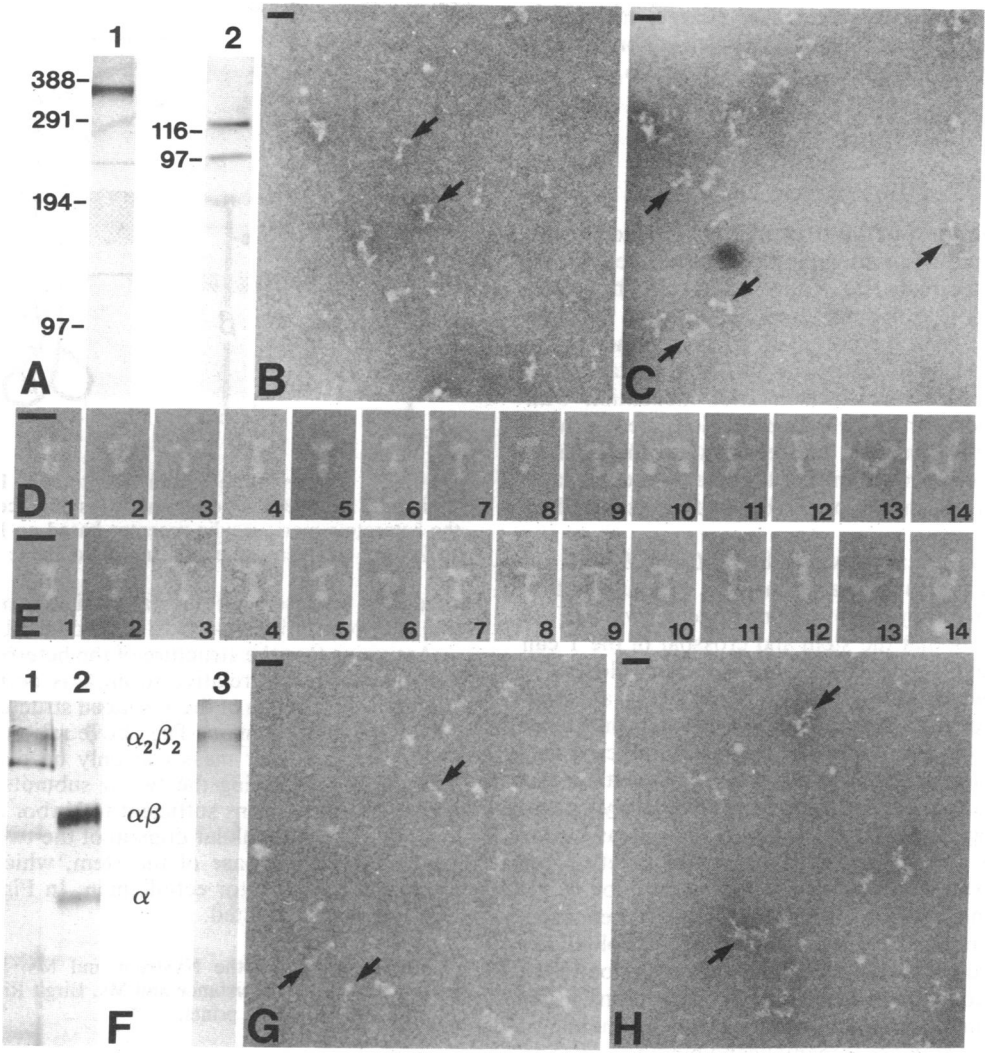

FIG. 1. SDS/polyacrylamide gels and electron micrographs of insulin receptors. (*A*) SDS/PAGE of insulin-receptor preparation in unreduced form (lane 1) and after reduction (lane 2). Positions of M_r standards are indicated. (*B*) Electron micrograph of a freshly prepared and negatively stained insulin-receptor preparation as shown in *A*. Arrows indicate characteristic T-shaped profiles. (*C*) Preparation analogous to *B* of a receptor preparation exposed to 100 nM insulin/10 mM $MgCl_2$/2 mM $MnCl_2$/50 μM ATP for 1 hr. (*D* and *E*) Selection of characteristic profiles seen in nonphosphorylated (*D*) and phosphorylated (*E*) insulin-receptor preparations. (*F*) SDS/PAGE of receptor preparation before (lane 1) and after (lane 2) partial reduction, and after reassociation induced by insulin-stimulated phosphorylation (lane 3). (*G*) Electron micrograph of a partially reduced preparation. Arrows indicate rod-shaped profiles probably representing $\alpha\beta$ heterodimers. (*H*) Electron micrograph of the preparation shown in *G* after phosphorylation with 100 nM insulin/10 mM $MgCl_2$/2 mM $MnCl_2$/50 μM ATP for 1 hr. Arrows indicate molecular aggregates. (Sodium silicotungstate negative staining; scale bars indicate 25 nm.)

volume of the receptor will have a minimum value of 390 nm^3. All our preparations contained some aggregated material with larger volumes in which structural details could not be resolved. Similarly, the preparations contained some structures much too small relative to the expected volume of the receptor. Most of the observed profiles were within the size range relevant for the $\alpha_2\beta_2$ form of the receptor and did conform with the structures shown in Fig. 1 *D* and *E*, with those of *4–10* being the most common and consistently present in all our preparations. This structure has the shape of the letter T $\approx$24 nm in height and 18 nm in width, the thickness of the stem and the crossbar each being 3–4 nm. Most of these profiles exhibited thickening at the base of the stem. Some less common profiles are depicted in *D* and *E 1–3* and *11–14*; those of *1* and *2* are characterized by a split stem, whereas those of *3* appear cleaved, except toward the base of the stem. The profiles of *11–12* could have derived from the T shape by bending the two ends of the crossbar upward to align with the stem axis; those of *13* and *14* appear as twins, joined at the stem base.

After partial reduction of the receptor preparations, SDS/PAGE clearly demonstrated cleavage of the receptor into fragments compatible with the $\alpha\beta$ heterodimeric species, as well as some smaller fragments probably representing some cleavage down to the α and β subunits (Fig. 1*F*). EM of such partially reduced preparations confirmed the lowering in average molecular size of the material. Most interesting was the appearance of thin rod-like structures, not present in the intact $\alpha_2\beta_2$ preparations, the width and length of these rods being compatible with a genesis by cleavage of the T profiles down along their stem (Fig. 1*G*).

By incubation of the partially reduced receptors in the phosphorylation medium, the material did reassociate into forms with apparent molecular weight like that of $\alpha_2\beta_2$ heterotetramers, as clearly demonstrated in SDS/PAGE (Fig. 1*F*, lane 3). However, the gel also showed some staining above the apparent M_r of 350,000, which could represent forms with higher molecular weights than corresponding to the heterotetramers. EM of this reassociated material show that most material had reassociated in an apparently less

ordered fashion, forming aggregates of a larger molecular volume than the $\alpha_2\beta_2$ species (Fig. 1*H*). Only a minor fraction of this reassociated material would conform to solitary T-shaped structures found in the native receptor preparations.

DISCUSSION

In spite of great interest in the structure and function of the insulin receptor, only a few attempts have been made to study its quaternary structure by EM. Kopp *et al.* (6) tried but failed to visualize the detergent-solubilized receptor by negative staining. Johnson *et al.* (7) reported on the appearance of the ectodomain of the insulin receptor after negative staining as discrete globular structures, which after incubation with insulin formed loops or branched and folded linear assemblies. However, the structure and reactivity of the isolated ectodomain may differ from that of the intact receptor, and comparisons cannot be made on the basis of the limited data yet available.

The T-shaped structure shown in Fig. 1 *D* and *E 4–10* are constantly present in our preparations with a frequency that leaves no doubt that they represent one projected view of the receptor. Assuming that the stem and crossbar of the T can be approximated by cylindrical rods, we can calculate a molecular volume of 420 nm^3, which, assuming a partial specific volume of 0.75 cm^3/g, corresponds to a molecular mass of 340 kDa of which ≈200 kDa reside in the crossbar. These are reasonable figures because measurements at this small-dimensional scale are beset with uncertainty due to irradiation damage to negatively stained specimens during imaging. Further, the size of the carbohydrate moiety of the receptor is uncertain (16), as well as its contribution to the molecular volume seen in negatively stained specimens. After insulin-stimulated phosphorylation of the solubilized receptor we have not, within the limits of resolution in the electron microscope, been able to detect alterations in the T-shaped structure. However, this does not exclude that structural changes may accompany ligand binding and phosphorylation of the membrane-inserted receptor.

The insulin receptor, when subjected to limited reduction, has been reported to dissociate into $\alpha\beta$ heterodimers that can reassociate into $\alpha_2\beta_2$ heterotetramers upon phosphorylation (9). Our electron microscopical observations on such reduced preparations did show emergence of elongated structures of a size and shape compatible with half the T structure of $\alpha_2\beta_2$. After phosphorylation of these cleavage products, the material tended to aggregate, and only a few solitary copies of the T-shaped structure was seen.

The insulin receptor is often pictured as shown in Fig. 2*A*. This model is based on experiments done on whole cells and on the purified receptor. Thus, labeling experiments indicate that the α subunit carries the insulin-binding site, and phosphorylation studies show that the β subunit is an insulin-sensitive protein kinase. The amino acid sequence of the insulin-receptor precursor (4, 5) substantiates and further extends this model of the insulin receptor. Thus, the α subunit of 719 or 731 amino acid residues contains no hydrophobic sequences of a length sufficient to span the membrane, and this subunit is, therefore, probably entirely extracellular. The amino acid sequence of the 620 amino acid-long β subunit can be divided into three domains: an amino-terminal 194-residue-long domain, a 23–26 stretch of hydrophobic amino acids, which probably represents a single transmembrane domain, and a 403-amino acid carboxyl-terminal cytoplasmic domain, in which a consensus sequence of an ATP-binding site is found. Thus, the extracellular and the intracellular amino acid domains of the heterotetrameric

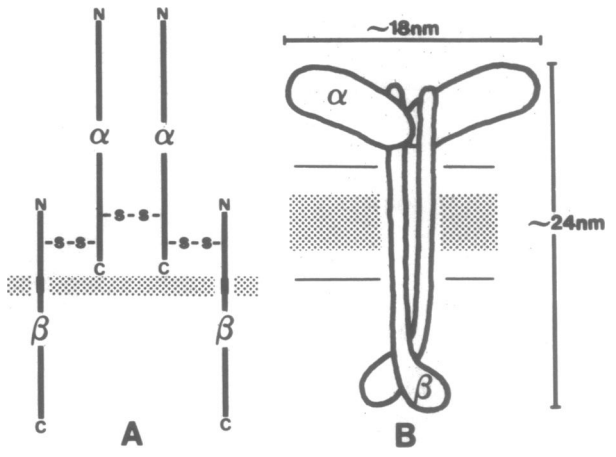

FIG. 2. Models of the insulin receptor. (*A*) Basic subunit composition of the $\alpha_2\beta_2$ heterotetrameric insulin receptor. (*B*) Model of the heterotetrameric insulin receptor based on EM data, drawn to scale for a cell membrane of 4-nm hydrophobic core (shaded).

receptor represent molecular weights of ≈220,000 and 100,000, respectively—i.e., a ratio of ≈2:1.

Assuming that the structure of the heterotetrameric receptor is symmetrical relative to an axis perpendicular to the membrane, the stem of the T-shaped structure must carry the transmembrane domain. The above ratio of 2:1 of extracellular-to-intracellular mass can only be reconciled with the EM data by assigning the two α subunits to the crossbar, which has dimensions sufficient to harbor this mass. Consequently, the intracellular domain of the two β subunits must be located at the base of the stem, which is too small to represent the receptor ectodomain. In Fig. 2*B*, these interpretations are depicted.

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