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Membrane Topology of Insulin Receptors Reconstituted into Lipid Vesicles

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Abstract. Insulin receptors were incorporated into liposomes by two different procedures, one using dialysis and one using detergent removal by Bio-Beads. Receptor incorporation was analyzed by gradient centrifugation and electron microscopy. Reconstituted receptors projected up to 12 nm above the membrane and exhibited a T-shaped structure compatible with that previously described for the solubilized receptor. Insulin binding and autophosphorylation experiments indicated that approx. 50% of the receptors were incorporated right-side out. Such random orientation was confirmed by immunogold labeling of the α- and the β-subunit of the receptor. Immunogold labeling of the C-terminus of the β-subunit indicates that it resides about 6 nm off the membrane, while two α-subunit epitopes were labeled at about twice this distance, confirming that the α-subunit is harbored in the cross-bar of the T-structure.

Key words: Insulin receptor — Membrane reconstitution — Electron microscopy — Quaternary structure — Immunogold labeling

Introduction

Biochemical analysis of the insulin receptor, together with amino acid sequence data on the receptor precursor, derived from cloned cDNA, have established that the functional receptor is a disulfide-linked heterotetrameric integral membrane protein composed of two α-subunits (M_r 130,000) and two β-subunits (M_r 95,000) (Ebina et al., 1985; Ulrich et al., 1985; Goldfine, 1987). Insulin binding to the ectodomain of the receptor elicits a tyrosine kinase activity located at the intracellular domain of the receptor, considered to be an initiator of intracellular insulin effects. The ectodomain, especially the insulin binding region, as well as the intracellular domain, have been subject to extensive probing by monoclonal antibodies in order to assign the receptor functions to specific stretches of the amino acid sequence (Soos et al., 1986, 1989; Taylor et al., 1987; Prigent, Stanley & Siddle, 1990; Zhang & Roth, 1991).

We have previously determined the gross quaternary structure of the isolated, detergent-solubilized human placental insulin receptor by electron microscopy and deduced a model on the location of the receptor subunits (Christiansen et al., 1991). In the present study we have developed protocols for reincorporation of functionally intact, purified receptors into liposomal vesicles and determined the structure and membrane topology of the membrane-inserted receptor using high resolution electron microscopy and immunogold labeling of defined receptor epitopes.

Materials and Methods

Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin A, 1.10-phenanthroline, benzamidine hydrochloride, and *n*-octylglucoside were all obtained from Sigma (St. Louis, MO). Triton X-100 (Für electronische Zahlung von milchzellen) was from Merck, Darmstadt, Germany. Egg phosphatidylcholine, phosphatidylserine and cholesterol were from Serdary Research Laboratories (London, Canada). Porcine insulin and ^{125}I-(tyr-A14)-human insulin were a gift from Novo Nordisk, Bagsværd, Denmark. Triton X-100, (phenyl-^{3}H(N)) (1.0 mCi/ml) was obtained from NEN Research Products, Du Pont, Germany. 1.2-di (1-^{14}C) palmitoyl-L-3 phosphatidylcholine (113 mCi/mmol) and adenosine 5′[Y-^{32}P] triphosphate, triethylammonium salt (3,000 Ci/mmol) were from Amersham Int., UK. The molecular weight markers: cytochrome c, ovalbumin, bovine albumin, β-galactosidase, and cross-linked phosphorylase b were all from Sigma. Bio-Beads SM-2 were obtained from Bio-Rad.

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Purification of Insulin Receptors

Insulin receptors were isolated from human placenta according to Fujita-Yamaguchi et al. (1983) by sequential affinity chromatography on wheat germ agglutinin (WGA) and insulin coupled to Sepharose 4B. The details of this procedure with minor modifications were as described previously (Christiansen et al., 1991).

The dilute receptor preparation eluted from the insulin-Sepharose column in 0.05% Triton X-100 was applied directly to a small WGA-Sepharose column (0.2 ml) in order to concentrate the preparation. The column was washed with 10 ml of 50 mM HEPES buffer pH 7.4 containing 10% glycerol, 0.5 mM PMSF and 0.05% Triton X-100 at a flow rate of 2 ml/hr. The receptor was eluted with 2 ml of the same buffer containing 0.3 M *N*-acetylglucosamine. Further concentration of the receptor preparation was achieved by centrifugation in Centricon-30 microconcentrators (Amicon). The final volume was approx. 0.25 ml with a Triton X-100 concentration of about 0.3%.

Reconstitution of Insulin Receptors

Reconstitution of insulin receptors into liposomes composed of phosphatidylcholine:phosphatidylserine:cholesterol in a molar ratio of 70:10:20 was performed by two different methods.

n-Octylglucoside/Triton X-100 Dialysis

A dry film of 1.5 mg of the lipid mixture and a trace of [^{14}C]-phosphatidylcholine was solubilized in 800 μl of 50 mM HEPES buffer pH 7.6 containing 20% (w/w) glycerol, 0.5 M NaCl and 1.3% *n*-octylglucoside. To this solution was added 200 μl of the concentrated receptor preparation (80–120 μg of receptor) containing a trace of [^{3}H]-Triton X-100. After incubation overnight at 4°C, the mixture was dialyzed according to the following protocol: (1) For 1 hr against 10 ml of Buffer A: 50 mM HEPES buffer pH 7.6 containing 20% (w/w) glycerol and 0.4 MNaCl with addition of 0.6% *n*-octylglucoside; (2) for 4 hr against 10 ml Buffer A with addition of 0.3% *n*-octylglucoside; (3) for 24 hr against 50 ml Buffer A and (4) for 2 × 24 hr against 250 ml of Buffer A.

Triton X-100 Removal by Bio-Beads

A dry film of 1.65 mg of the lipid mixture containing a trace of [^{14}C]-phosphatidylcholine was solubilized in 800 μl of 50 mM HEPES buffer pH 7.6 containing 20% (w/w) glycerol, 0.5 M NaCl and 0.3% Triton X-100. To this solution was added 200 μl of the concentrated receptor preparation (80–120 μg of receptor) containing a trace of [^{3}H]-Triton X-100. If the lipid-/receptor mixture was turbid at this stage, a few microliters of 10% Triton X-100 were added. After incubation at 4°C overnight, 40 mg of thoroughly rinsed Bio-Beads (Holloway, 1973) was added. The mixture was slowly rotated for 3 hr at 4°C and another 40 mg portion of Bio-Beads was added and the mixture left slowly rotated for 60 hr at 4°C.

Gradient Centrifugation

To remove nonincorporated insulin receptors, approx. 700 μl of the crude reconstituate was layered on top of a linear 2–25% (w/w) sucrose gradient prepared in 50 mM HEPES buffer pH 7.6 containing 20% (w/w) glycerol and 0.4 M NaCl and centrifuged at 280,000 × *g* for 16 hr in a Beckman SW 40 Ti rotor at 4°C. Fractions of 0.65 ml were collected from the bottom of the tubes, and analyzed for lipid and Triton X-100 by scintillation counting and for receptor by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Insulin Binding Analysis

Insulin receptors were incubated for 16 hr at 4°C with 0.1 nM ^{125}I-insulin in a final volume of 0.1 ml of 50 mM Tris-HCl buffer pH 7.4 containing 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.1 mM PMSF. Free insulin was separated from bound hormone by addition of 25 μl of 0.4% bovine γ-globulin and 125 μl of 20% polyethyleneglycol 6000. After 10 min at 4°C, the samples were centrifuged at 12,000 × *g* for 5 min in a Beckman Microfuge. The pellet, suspended in 100 μl 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. Double determinations differed less than 15% in all experiments.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed in 0.1% SDS as described by Laemmli (1970) in either 6% gels (nonreducing conditions) or in 13% gels (reducing conditions). The samples were boiled for 15 min in 1.5% SDS in the absence or presence of 100 mM dithiothreitol. Gels were stained with silver (Heukeshoven & Dernick, 1985).

Autophosphorylation

Autophosphorylation experiments were performed by incubation of reconstituted insulin receptors in the absence or presence of 0.2% Triton X-100, with or without 0.5 μM insulin in 50 mM HEPES buffer pH 7.6 containing 10 mM $MgCl_2$ and 2 mM $MnCl_2$. After one-half hour at room temperature, 5 μCi of [γ-^{32}P]-ATP was added. The final ATP concentration was 50 μM. The reaction was stopped after 10 min by addition of sodium vanadate, sodium fluoride, ATP and EDTA at final concentrations of 1, 75, 3 and 30 mM, respectively, and the samples were analyzed by SDS-PAGE. The gels were stained with Coomassie Blue followed by autoradiography on Kodak XAR 5 film with Cronex Lightning Plus intensifying screens. The intensity of the bands was quantified with an LKB Ultrascan XL laser densitometer.

Immunogold Labeling

Two monoclonal mouse antibodies, 83-14 and 47-9, directed against different epitopes on the α-subunit (Soos et al., 1986) and one antibody, CT-1, directed against the C-terminus of the β-subunit (Prigent et al., 1990; Ganderton et al., 1992) of the receptor were generously provided by Dr. K. Siddle, Cambridge. The antibodies were conjugated to 3 nm colloidal gold particles by the procedure of Slot and Geuze (1985). The conjugates meet the following criteria for reactivity and specificity: (i) All three conjugates stained dots of the receptor preparation applied to nitrocellulose paper; (ii) the reactivity of CT-1 conjugate was inhibited by preincubation of the conjugate with a synthetic peptide (purchased from Kem-En-Tec, Copenhagen, Denmark) corresponding to the 10 C-terminal amino acid residues of the receptor β-subunit and (iii) the reactivity of the 83-14 and 47-9 conjugates was inhibited by preincubation of the conjugates with the receptor ectodomain (generously supplied by Dr. L. Schaefer, Novo-Nordisk (Andersen et al., 1990)).

Specimens for immunogold labeling were prepared by brief

(1–2 min) adsorption of the receptor preparations to EM grids as explained below. All maneuvers of washing and incubation of the grids were performed by floating the grids upside-down on drops lined up on dental wax plates. The grids were initially washed over six drops of 10 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl (TBS) followed by 5 min incubation on TBS-containing 1% cytochrome c. The specimens were subsequently incubated for 1 hr with the different gold conjugates, diluted to a mouse IgG concentration of about 2 μg/ml in TBS supplemented with 1% cytochrome c for blocking of unspecific adsorption of the gold conjugates to the specimens. After three washings on drops of TBS containing 1% cytochrome c and three washings on TBS, the specimens were negatively stained for EM as described below.

Electron Microscopy

The crude receptor reconstituate or the top fraction from the gradient was adsorbed to very thin carbon-coated Formvar films, carried on 400 mesh electron microscope grids, exposed to plasma glow before use. The preparation was negatively stained with 2% wt/vol sodium silicotungstate (pH 7.2) as described previously (Tranum-Jensen, 1988). The specimens were examined and photographed at 60–70,000× 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 using a low-dose exposure technique (35–50 $e^-/Å^2$ of specimen). Plates were developed in Kodak D-19 to an electron speed of 0.7 $\mu m^2/e^-$. Magnification of the microscope was calibrated using a Fullam SiO_2 grating replica no. 6002.

Distances between liposomal membrane surfaces to the core of the immunogold labels were measured directly on micrographs printed to a final magnification of 190,000× using a magnified ruler with scale divisions of 100 μm. Two fully independent preparations of the reconstituted receptor were labeled with all three immunogold labels. The measured distances for each of the probes did not differ significantly between experiments, and measurements were pooled.

Results

Reconstitution

The insulin receptor was reconstituted into lipid vesicles composed of phosphatidylcholine: phosphatidylserine: cholesterol in the molar ratio of 70:10:20. Two methods for reconstitution were used, one using lipid dissolved in *n*-octylglucoside to which was added the receptor in Triton X-100 followed by slow dialysis. The other method used Triton X-100 as the sole detergent and detergent removal by adsorption to Bio-Beads. By this method, an initial detergent to phospholipid ratio of 2:1 (w/w) and a Bio-Bead: detergent ratio of 12:1 (w/w), was chosen; i.e., close to the ratios used by Lévy et al. (1990).

The crude reconstituates were layered on top of linear sucrose density gradients and centrifuged to equilibrium. Representative gradient profiles are shown in Fig. 1. By both methods, reconstituted receptors were recovered in the top fraction together with the lipid. By the *n*-octylglucoside/Triton X-100 dialysis procedure, a small fraction of receptor banded at a density of 1.09. By both methods, residual Triton X-100 was present in the top fraction equivalent to a lipid to detergent molar ratio of 10:1.

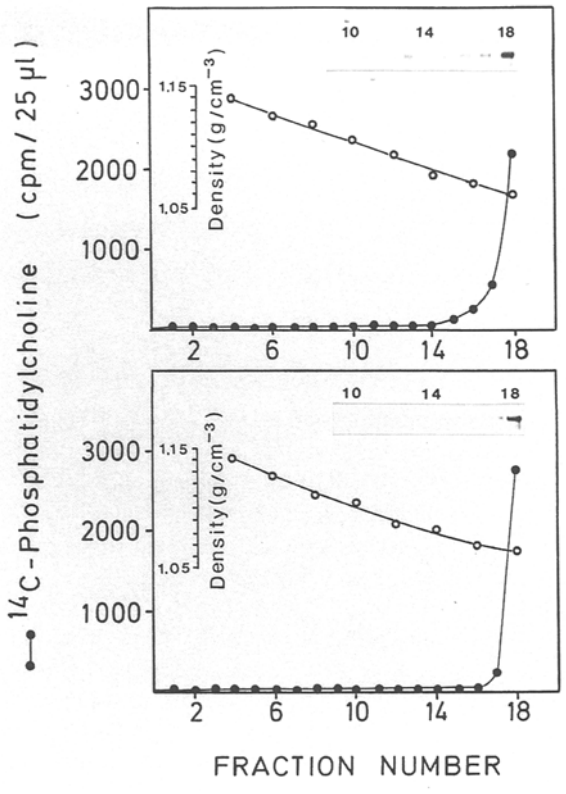

Fig. 1. Gradient centrifugation of insulin receptor-lipid vesicle reconstituates. The crude reconstituates obtained by *n*-octylglucoside/Triton X-100 dialysis (upper graph) or Triton X-100-Bio-Bead removal (lower graph) were layered on top of 2–25% (w/w) sucrose gradients in 50 mM HEPES buffer pH 7.6, containing 20% (w/w) glycerol and 0.4 M NaCl and centrifuged at 280,000 × *g* for 16 hr at 4°C. Fractions were collected from the bottom and analyzed for ^{14}C-phosphatidylcholine (●—●). Aliquots of each fraction were weighed to determine the specific gravity (○—○) and analyzed by SDS-PAGE in silver-stained 6% gels (inset) for evaluation of receptor incorporation.

Assay of Receptor Function

Insulin binding was measured in the top fractions from the density gradients with and without addition of a solubilizing amount of Triton X-100. For both reconstitution procedures, insulin binding was approximately doubled after addition of Triton X-100, indicating that about half of the reconstituted receptors was oriented right-side out (Table).

Autophosphorylation was performed on gradient

Table. Insulin binding to the reconstituted insulin receptor

Method	Exp.	−Triton X-100	+Triton X-100
		Insulin receptor (μg/ml)	
n-OG/TX	I	14	24
n-OG/TX	II	28	56
TX/Bio-Bead	I	28	56
TX/Bio-Bead	II	43	75

Gradient top fractions from both reconstitution methods were analyzed for insulin binding in the presence and absence of Triton X-100. Each figure is the mean of two determinations of insulin binding capacity.

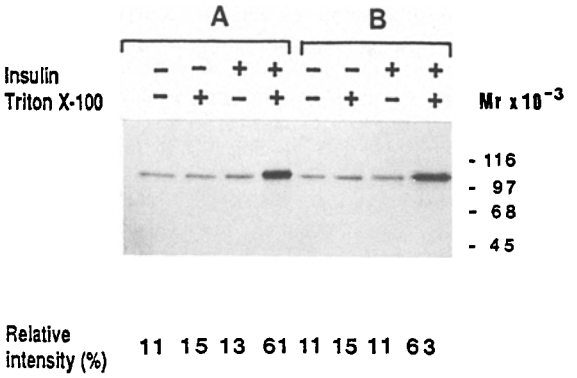

Fig. 2. Autophosphorylation of reconstituted insulin receptors recovered from density gradient top fractions. The autophosphorylation was evaluated by SDS-PAGE under reducing conditions in 13% gels. The gels were stained with Coomassie Blue followed by autoradiography. Densitometer readings are shown at the bottom of each lane of the autoradiogram. (*A*) Insulin receptor reconstituted by the *n*-octylglucoside/Triton X-100 dialysis method and (*B*) insulin receptor reconstituted by the Triton X-100 Bio-Bead method.

top fractions by addition of ATP to a concentration of 50 μM. As shown in Fig. 2, phosphorylation was detected only in the β-subunit of the receptor and the results were identical regardless of the reconstitution method: Without addition of insulin the low level of autophosphorylation observed increased slightly after addition of Triton X-100. Upon addition of insulin alone, autophosphorylation did not increase above basal level. In the presence of Triton X-100, autophosphorylation increased fourfold by addition of insulin. These observations lend support to the above interpretation of the insulin-binding experiments, and show in addition that the vesicles were impermeable towards small molecules like ATP.

Electron Microscopy

Electron micrographs of reconstituted insulin receptor, negatively stained with sodium silicotungstate, derived from four out of eight independent experiments, are shown in Fig. 3. No consistent morphological differences between the two methods of reconstitution were apparent. In the crude reconstituates, a substantial fraction of the receptor molecules formed disordered aggregates with low lipid content. The orderly reconstituted receptors were, as a general finding, nonuniformly distributed among the liposomes, some being heavily laden whereas others were devoid of receptors (Fig. 3*A*). Liposomes carrying no or a low number of receptors were collected in the top fraction of the density gradients (Fig. 3*B*); still in this fraction most of the incorporated receptors formed clusters. Details of individual receptor profiles were resolved on vesicles from the top fraction carrying a single or a few incorporated receptors. Panels *C, D* and *E* display the spectrum of profiles observed. A majority of profiles projected to a height of about 12 nm above the membrane, being composed of a mass carried on a stem (*C* and *D*). This mass often showed as a cross-bar forming a structure with the shape of letter T. Rarely, Y-shaped structures were seen (Panel *C*, frames *6* and *7*). Frames *1–3* of panel *E* display small knob-like structures projecting up to 6 nm from the membrane. Frames *4–7* of this panel show a selection of arch-shaped structures projecting 8–10 nm above the membrane. The arches represent a minority of the receptor profiles but were consistently observed.

Immunogold labeling experiments were performed utilizing two gold-conjugated monoclonal antibodies (83-14 and 47-9) directed against the α-subunit (Soos et al., 1986) and one gold-conjugated monoclonal antibody (CT-1) against the cytoplasmic domain of the β-subunit (Prigent et al., 1990; Ganderton et al., 1992). Figure 4 shows labeling of receptors with α-subunit antibody 83-14 (*A*) and with β-subunit antibody CT-1 (*B*). Prior addition of isolated receptor ectodomain to 83-14 and 47-9 conjugates completely abolished the labeling. Similarly, the specificity of the CT-1 conjugate was asserted by observation of a complete blockade after addition of a peptide corresponding to the 10 C-terminal amino acid residues of the β-subunit. To avoid unspecific adsorption of the label to the specimen, it was necessary to apply an appropriate protein as blocking agent. In initial experiments using albumin for this purpose, all fine structural details were obscured. Cytochrome c was found to be very effective as a blocking agent and rather less harmful to the structural resolution. Still, fine details of the labeled receptor structure could not be recognized (Fig. 4). The distances between the gold particle and the vesicle edge, however, could easily be measured and were found to depend on the conjugate used (*compare A* and *B* of Fig. 4). Results of measurements of the label to membrane gap with the three different conjugates are presented in Fig. 5*a*. For the α-subunit antibodies 83-14 and 47-9, the average label to membrane distance differed significant-

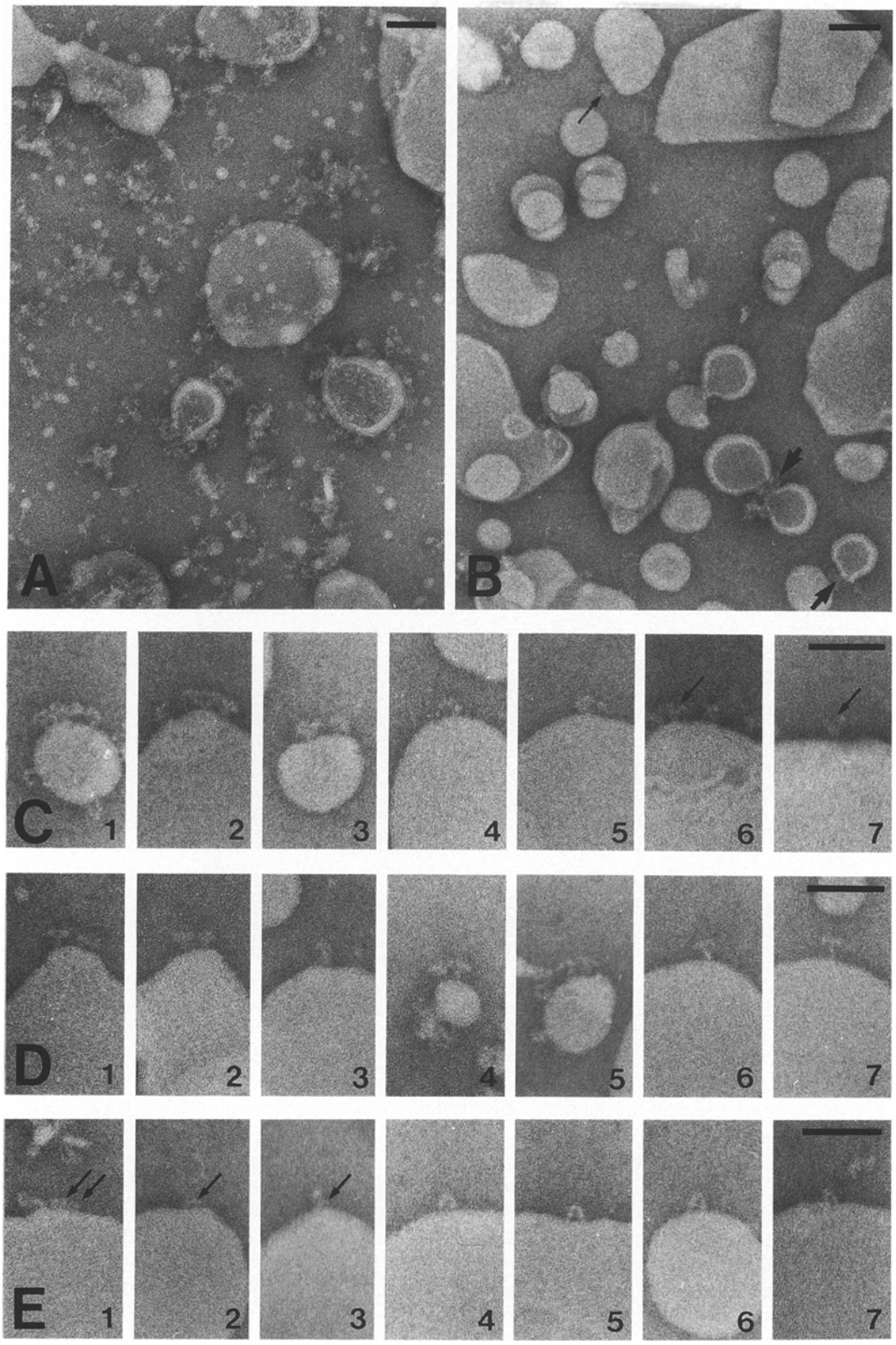

Fig. 3. Electron micrographs of negatively stained preparations of reconstituted insulin receptors. (*A*) Crude reconstituate composed of aggregated receptors with little associated lipid together with well-defined liposomes carrying varying numbers of receptors. (*B*) Top fraction from density gradient centrifugation. Liposomes carrying clustered receptors are indicated by bold arrows, solitary receptor by small arrow. (*C*) Frames *1–5* display receptors projecting to a height of 12 nm above the liposomal membrane. The individual receptor profiles are not distinctly resolved due to clustering. Frames *6–7* show the occasional Y-shaped receptor profiles. (*D*) Frames *1–7* display the characteristic T-shaped receptor profiles. (*E*) Frames *1–3* show the small knob-like structure interpreted to represent the intracellular domain of the receptor. Frames *4–7* display a collection of the arch-shaped structures consistently observed, but in low numbers. Another such arch may be seen in *C*, frame *1*. Scale bars indicate 50 nm.

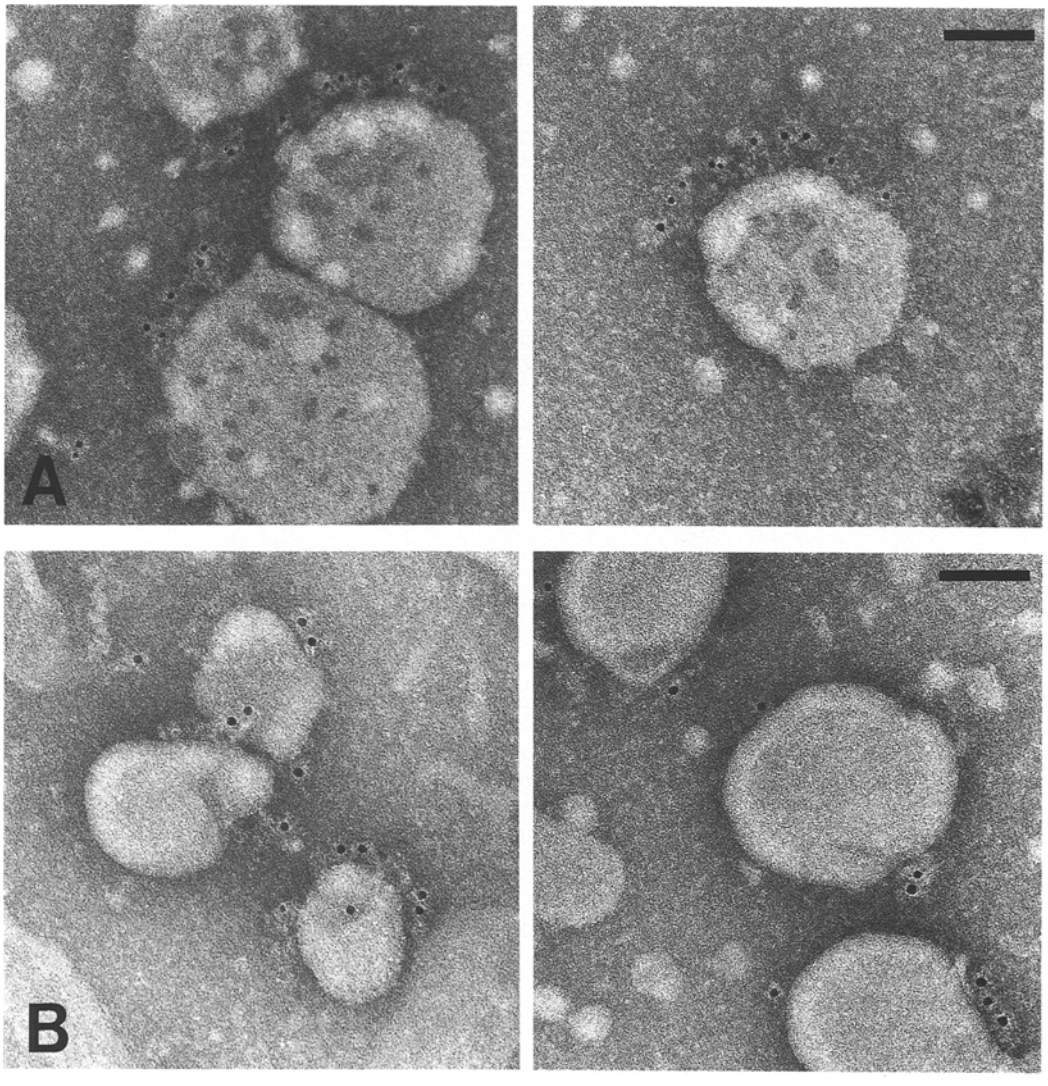

Fig. 4. Electron micrographs of reconstituted insulin receptors labeled with immunogold conjugates and negatively stained. Upper frames (*A*) show labeling of the α-subunit by the 83-14 conjugate. Lower frames (*B*) show labeling of the C-terminus of the β-subunit by the CT-1 conjugate. Scale bars indicate 50 nm.

ly being 11.5 and 15.5 nm, respectively, and for the β-subunit antibody CT-1, the average distance was 5.6 nm.

The distribution of distances to the membrane for the two labels with the shortest average distance (CT-1 and 83-14) shows a small accumulation close to the membrane. We interpret this as an effect coming up when the diameter of the conjugate exceeds the distance from the epitope to the bend edge of the liposomal membrane which acts as a low "wall" in the negatively stained specimens. This effect will produce a moderate positive bias in estimates of the true distance between the epitope and the membrane. This, together with small dimensional distorsions inherent in the electron microscopical techniques, warn against undue emphasis on the absolute values of the mean distances determined.

Discussion

In a previous study (Christiansen et al., 1991), we determined the gross quaternary structure of the detergent-solubilized human placental insulin receptor. The receptor was shown to have the shape of letter T with a stem of 24 nm and a cross-bar of 18 nm width, each with a thickness of 3–4 nm. Based on determination of molecular volumes in combination with established amino acid sequence data, the two α-subunits were assigned to the cross-bar and the two β-subunits to the stem.

The aim of this study was to determine the topology of insulin receptors after incorporation into lipid vesicles. Reconstitution of purified insulin receptors

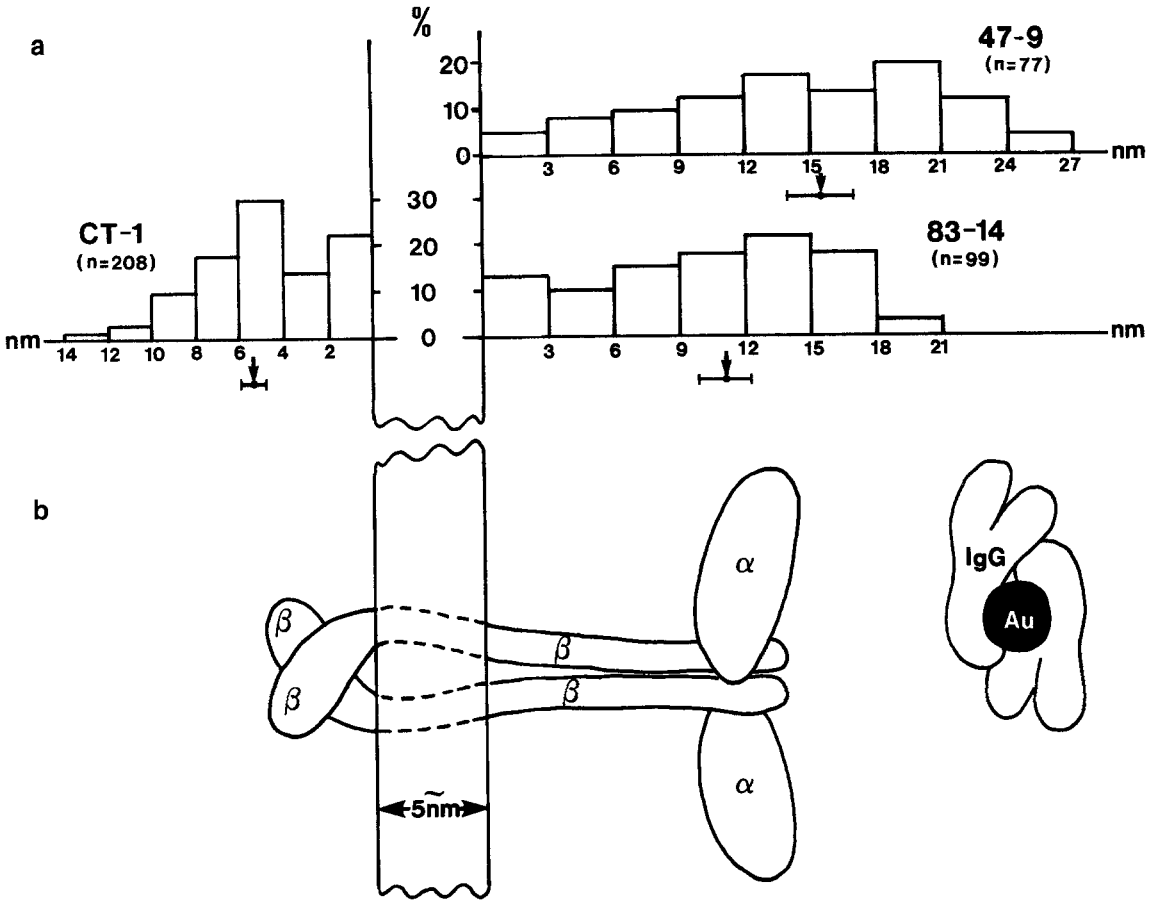

Fig. 5. (*a*) Diagrammatic presentation of the fractional distribution of measured distances between the edge of liposomal membranes and the core of the immunogold labels indicated. Symbols (⊢┴⊣) indicate mean values ± 2 × SEM. (*b*) Diagrammatic presentation of the model for the quaternary structure of the insulin receptor drawn to scale with a membrane of 5 nm which is the minimum observed thickness of liposomal membranes delineated by negative staining. The approximate dimensions of an immunogold conjugate with a 3 nm gold core are shown to the right for comparison. The model is drawn as a two-dimensional projection of the suggested receptor structure; i.e., that molecular volumes are related to linear dimensions by the third power. The junction of the α- and the β-subunits may well be more complex than shown.

had been performed previously by Sweet et al. (1985) using an *n*-octylglucoside dialysis procedure, and by Lewis and Czech (1987) using sodium cholate and Sephadex chromatography for detergent removal. In neither case have the reconstitution products been visualized by electron microscopy. In our hands, the *n*-octylglucoside reconstitution procedure did not result in receptor incorporation and the cholate method was reported to produce leaky vesicles (Lewis & Czech, 1987). Therefore, we sought improved protocols for reconstitution of insulin receptors with emphasis on microscopical verification. Two methods were found suitable. One uses a mixture of *n*-octylglucoside and Triton X-100 followed by dialysis. The other follows the method of Lévy et al. (1990) for reconstitution of bacteriorhodopsin, Ca^{2+}-ATPase and H^+-ATPase using Bio-Bead for removal of Triton X-100.

Using either method of reconstitution, insulin-binding assays showed a doubling of binding capacity upon addition of solubilizing concentrations of Triton X-100, indicating that half the amount of incorporated receptors was inaccessible to insulin; i.e., the sidedness of the receptor was random. Qualitative support for random sidedness of incorporated receptors was obtained by immunogold labeling. These results were supported and extended by receptor autophosphorylation experiments: In intact vesicles, receptor phosphorylation was not increased above background level by addition of insulin, indicating that vesicles prepared by both reconstitution methods were impermeable also towards Mn^{2+}/Mg^{2+}/ATP. In solubilized vesicles, insulin expectedly increased receptor phosphorylation. In the absence of insulin, receptor phosphorylation was slightly increased by solubilization. However, the doubling expected on the basis of a random orientation was not achieved, likely due to an inhibition of spontaneous phosphorylation by solubilizing concentrations of Triton X-100 (Sweet et al., 1985). It is noteworthy that the vesicles were functionally impermeable towards insulin as well as ATP despite the presence of residual Triton

X-100 in a molar fraction of 1:10 relative to the vesicle membrane lipids.

Immunogold labeling experiments utilizing two different antibodies against the α-subunit and one against the C-terminus of the β-subunit clearly showed that the extracellular domain of the receptor projected considerably higher above the plane of the membrane than the intracellular domain. Regrettably, the labeling technique did not allow fine details of the receptor structure to be resolved.

Microscopy of unlabeled vesicles carrying receptors in appropriately low numbers not to be aggregated revealed a spectrum of profiles projecting to a height of 12 nm above the membrane. These profiles had dimensions compatible with the expected 220 kD protein mass of the extracellular receptor domain deduced from amino acid sequence data (Ebina et al., 1985; Ullrich et al., 1985). Among these profiles, we regularly observed a T-shaped structure which conformed well to the model of the receptor previously deduced from microscopical observations on solubilized receptor preparations (Christiansen et al., 1991). Also, the projection height of the T-shaped structure is compatible with the distance between label and membrane found for the α-subunit specific immunogold conjugates.

The measured difference in label to membrane gap for the two antibodies against the α-subunit must indicate a difference in position of the epitopes in the folded protein. The antibody 83-14 reacting with amino acid 469-592 on the α-subunit has been shown to inhibit insulin binding and mimic insulin action both in isolated adipocytes (Taylor et al., 1987) and in transfected cells (Soos et al., 1989; Wilden et al., 1992), indicating that the amino acids 469-592 may contribute to the insulin binding site. Our data show that this epitope is located at a distance of about 11 nm from the membrane spanning domain of the receptor. The 47-9 antibody also inhibits insulin binding but does not mimic insulin action (Taylor et al., 1987), possibly by stabilizing a noninsulin binding conformation of the receptor. The epitope for the 47-9 antibody in a recent study by Zhang and Roth (1991) was found to be contained within the same amino acids (residues 450-601) as the epitope for the 83-14 antibody. Our results indicate that the epitope of the 47-9 antibody is localized at the most peripheral domain of the receptor. Thus, in spite of the fact that the two antibodies may recognize epitopes within the same stretch of about 150 amino acids, the present results show that the epitopes in the folded protein are approximately 4 nm apart. Furthermore, the above results of α-subunit labelings support the previous notion that this subunit is harbored in the cross-bar of the receptor structure.

It may be of significance that not all the observed receptor profiles are compatible with projected views of a single structural entity. Thus, we observed a few profiles which may relate to the Y-shaped conformation found in a recent study of the isolated receptor ectodomain (Schaefer et al., 1992). Besides the profiles described above, we observed regularly but in low numbers an arch-shaped structure which did not conform to the earlier suggested model. These profiles may just be pairs of degraded receptors or they may represent another conformation of the receptors. In addition to the results reported here, we have conducted initial experiments to disclose possible conformational changes induced by insulin but we have thus far not been able to detect consistent effects of insulin on the distribution of receptor profiles.

The mass of the intracellular receptor domain is close to 100 kD (Ebina et al., 1985; Ullrich et al., 1985). This is near to the limit of microscopic detection, and will expectedly be observed only when optimally located and stained on the edge of the vesicles. The observed knob-like structures projecting up to 6 nm from the membrane are likely to represent the intracellular receptor domain. Also, the height is compatible with the measured distance between the membrane and CT-1 gold conjugates.

In conclusion, we interpret our observations on the reconstituted receptor as diagramatically shown in Fig. 5*b*. This model is consistent with the structure previously reported for the solubilized receptor and establishes the localization of the membrane spanning domain relative to the quaternary structure of the receptor.

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