Structural Studies of the Detergent-solubilized and Vesicle-reconstituted Insulin Receptor*

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Christine N. Woldin‡, Frederick S. Hing‡§, Jongsoon Lee¶, Paul F. Pilch, and G. Graham Shipley¶

From the Departments of Biophysics and Biochemistry, Center for Advanced Biomedical Research, Boston University School of Medicine, Boston, Massachusetts 02118

Insulin binding to the insulin receptor initiates a cascade of cellular events that are responsible for regulating cell metabolism, proliferation, and growth. We have investigated the structure of the purified, functionally active, human insulin receptor using negative stain and cryo-electron microscopy. Visualization of the detergent-solubilized and vesicle-reconstituted receptor shows the αβ heterotetrameric insulin receptor to be a three-armed pinwheel-like complex that exhibits considerable variability among individual receptors. The α-subunit of the receptor was labeled with an insulin analogue-streptavidin-gold conjugate, which facilitated the identification of the receptor arm responsible for insulin binding. The gold label was localized to the tip of a single receptor arm of the three-armed complex. The β-subunit of the insulin receptor was labeled with a maleimide-gold conjugate, which allowed orientation of the receptor complex in the membrane bilayer. The model derived from electron microscopic studies displays a "Y"-like morphology representing the predominant species identified in the reconstituted receptor images. The insulin receptor dimensions are approximately 12.2 nm by 20.0 nm, extending 9.7 nm above the membrane surface. The β-subunit-containing arm is approximately 13.9 nm, and each α-subunit-containing arm is 8.6 nm in length. The model presented is the first description of the insulin receptor visualized in a fully hydrated state using cryo-electron microscopy.

The insulin receptor is a well-known transmembrane protein that has been the focus of extensive scientific study for over 3 decades. It is through this receptor that the peptide hormone insulin regulates a multitude of cellular processes including glucose transport and metabolism, fatty acid metabolism, DNA and protein synthesis, amino acid transport, and mitogenesis. Insulin binding to the extracellular domain of the insulin receptor results in a conformational change that is coincident with the autophosphorylation of the β-subunit of the receptor (6). Autophosphorylation is an intramolecular process (7, 8) that occurs in trans (9, 10) such that one β-subunit phosphorylates the tyrosine residues on the other β-subunit in the heterotetramer. A subdomain organization of the insulin receptor ectodomain has been proposed based on sequence comparison with the epidermal growth factor receptor (11), growth hormone receptor and tenasin (12), and the tumor necrosis factor receptor-1 (13). The ectodomain is composed of two large homologous domains, L1 (residues 1–119) and L2 (residues 313–428), separated by several cysteine-rich domains (residues 155–312) (11). Secondary structural predictions suggest that each large homologous domain is composed of α-helix, β-strand, turn, β-strand motif (11). The cysteine-rich regions of the insulin receptor may adopt one or two loop configurations similar to those identified in the tumor necrosis factor receptor-1 (13). The carboxyl-terminal segment of the insulin receptor extracellular domain contains two potential fibronectin type III re-

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§ Current address: Dept. of Psychiatry, King Drew Medical Center, Los Angeles, CA 90059.

¶ Current address: Joslin Diabetes Center, Boston, MA 02215.

The abbreviations used are: IRS, insulin receptor substrate; IGF-1, insulin-like growth factor-1; BSA, bovine serum albumin; PBST, phosphate-buffered saline with Tween 20; HRP, horseradish peroxidase; BBpa insulin, B25-t-benzylphenylalanine, B29-biotinyl insulin; biotin-BMCC, 1-biotinamido-4-(maleimidomethyl)cyclohexane carboxamido] butane; DTT, dithiothreitol; eggPC, egg phosphatidylcholine; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis.
peats, each consisting of a seven-stranded β-sheet structure. This structural information relies heavily on sequence based predictions. A segment of the IGF-1 receptor extracellular domain containing the L1-cystine-rich-L2 regions has recently been crystallized (14) and solved (15). The crystal structure exhibits this region of the monomeric α-subunit fragment as a relatively extended segment (40 × 48 × 105 Å) (15). This fragment is the first successfully crystallized extracellular segment of the insulin receptor class of proteins, and the information obtained begins to provide insight into the extracellular organization of this class of proteins.

Segments of the cytoplasmic domain of the insulin receptor have also been crystallized. A peptide fragment of the insulin receptor juxtamembrane region has been crystallized complexed with the IRS-1 phosphotyrosine binding domain (16). The resulting structure demonstrated the importance of specific structural features, including a key phosphotyrosine residue, in this interaction (16). The tyrosine kinase domain of the insulin receptor has also been crystallized in both the dephosphorylated (17) and phosphorylated (18) forms. The crystal structure of the kinase region enabled identification of key residues involved in the specificity of the family of tyrosine kinases. The phosphorylated form of the kinase region demonstrated the conformational changes that take place in the activated receptor exposing the nucleotide binding site and positioning the catalytic loop for substrate interactions. These structures cannot, however, be used to address the importance of interactions between the two β-subunits of the heterotetrameric complex, nor can they address the role that insulin binding plays in receptor activation.

Electron microscopic studies allow one to study the holoreceptor and the domain organization that so far cannot be captured in a crystalline state. Negative stain electron microscopic studies of the purified placental αβ2 heterotetrameric insulin receptor have identified "Y"-like and "T"-like conformations in the detergent-solubilized state (19). These studies are supported by images of the extracellular domain of the receptor identifying short Y-like and "V"-like structures (12). Images of the reconstituted insulin receptor visualized using negative stain electron microscopy also show Y-like, V-like, T-like, and stalklike projections from the vesicle surface (20). All of the microscopic studies to date are limited by the use of heavy metals for preservation and contrast. These heavy metals potentially distort structural detail, in addition to requiring a certain degree of sample drying.

Cryo-electron microscopy enables visualization of a protein in a fully hydrated state, free of heavy metals and the associated artifacts. Macromolecular complexes are preserved in a non-crystalline, or vitreous ice layer and visualized under low electron dose conditions to minimize radiation damage (21). The relative background noise is higher, and the contrast between protein and background is lower than in stain; however, the structural detail of a biological sample is more accurately preserved. We have applied the technique of cryo-electron microscopy to study the insulin receptor structure and visualized the insulin receptor in both the detergent-solubilized and vesicle-reconstituted forms. Using specific labeling techniques, we have also been able to identify distinct subdomains of the heterotetrameric complex under cryo conditions. These studies demonstrate the contribution that electron microscopy can make to understanding the entirety of a complex and structurally challenging protein such as the insulin receptor.

**MATERIALS AND METHODS**

**Insulin Receptor Purification**

**Purification Protocol**—Fifteen dishes (500 cm²) (Vanguard, Neptune, NJ) of NIH-3T3 cells overexpressing the human insulin receptor cDNA (1502 cells, kindly provided by Dr. Simeon Taylor, National Institutes of Health, Bethesda, MD) were grown to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and 0.1 g/liter Geneticin (Life Technologies, Inc.). Cells were washed twice with 50 ml of phosphate-buffered saline containing 10 mM HEPES buffer of 10 μM leupentin (Roche Molecular Biochemicals), 0.1 TIU/ml aprotinin (American Bioanalytical, Natick, MA), 1 mM pepstatin (Sigma), 1 mM α-phenanthrolin (Sigma), 1 mM phenylmethylsulfonyl fluoride (American Bioanalytical), 25 mM benzamidine-HCl (Sigma), and 5 mM EDTA. Cells were then lysed with 50 ml of 10 mM HEPES also containing the proteinase inhibitor mixture. This first lysis solution was poured off and discarded before a second 50 ml of 10 mM HEPES with the protease inhibitor mixture was added. While in the second lysis buffer, the remaining adherent cellular components were scrapped from the plates and poured into 250-ml centrifuge tubes on ice. The membranes in lysis buffer were centrifuged in a GSA rotor for 1 h at 21,520 × g at 4 °C. The supernatant was discarded, and the pellet was stored (for no longer than 1 month) at −80 °C.

Frozen membranes were thawed on ice. The membranes were resuspended in 30 ml HEPES with the protease inhibitor mixture. The resuspended membranes were transferred to a 50-ml conical tube. 10% Triton X-100 (Roche Molecular Biochemicals) was added to a final concentration of 2%, and the membranes were incubated with gentle agitation at 4 °C for 1 h. The detergent-solubilized membranes were centrifuged in a T70 rotor for 75 min at 148,600 × g at 4 °C. The supernatant was collected and filtered through several 0.45-μm syringe filters (Nalgene, Rochester, NY).

The filtered supernatant was loaded onto a 1.6-liter bed volume Sephacryl 400 (Amersham Pharmacia Biotech) column using a 50-ml maximum volume Superloop (Amersham Pharmacia Biotech). The column was controlled by fast pressure liquid chromatography at a rate of 1.33 ml/min, and 12-ml fractions were collected between 650 and 1650 ml. The first 80 fractions were assayed for protein amount using the Bradford-based Bio-Rad protein assay. An insulin binding assay was performed on 10 μl from every odd fraction in the region immediately preceding the protein peak and including the first few fractions of the protein peak itself.

The 125I insulin solution for the insulin binding assay (adapted from Refs. 22 and 23) consisted of 125I insulin (NEN Life Science Products) in 3 ml of 1 mg/ml solution of BSA (Sigma) in 30 mM HEPES until the cpm of 90 μl was between 15,000 and 20,000 cpm. 90 μl of 125I insulin solution was added to 10 μl from each chosen fraction, and incubated at room temperature for 45 min. 250 μl of 1.25 mg/ml γ-globulins (Sigma) in 30 mM NaPO4, pH 7.4, and 250 μl of 25% polyethylene glycol (PEG 8000) in 30 mM NaPO4, pH 7.4, were added and incubated at 4 °C for 45 min. The samples were centrifuged at 15,000 × g at 4 °C for 20 min. The supernatant was aspirated, and the remaining pellet was counted in a γ-counter. Results were expressed as the percentage of total insulin binding (cpm in the pellet/total cpm in 90 μl of 125I BSA solution)×100 and graphed along with the protein profile. Fraction corresponding to the insulin binding peak were pooled and applied to the next column.

Pooled Sephacryl 400 fractions were placed over a 5-ml bed volume wheat germ agglutinin (EY Laboratories, San Mateo, CA) column (24) equilibrated with 500 ml of 30 mM HEPES, 0.1% Triton X-100, 0.02% NaNO3, and protease inhibitor mixture using a perisaltic pump until at least three times the applied volume had been passed over the wheat germ agglutinin column. The column was then washed with a minimum volume of 500 ml of 30 mM HEPES, 0.1% Triton X-100, 0.02% NaNO3, and protease inhibitor mixture. The protein was eluted with 40 ml of 0.3 M N-acetyl glucosamine (Sigma) in 20 ml Triton with 0.1% Triton X-100 without protease inhibitors over a period of 1–2 h.

The entire wheat germ agglutinin column eluant was loaded onto a 1-ml MonoQ column (HR 5/5; Amersham Pharmacia Biotech) equilibrated with 20 ml of 20 mM Tris and 0.1% Triton X-100 using a Superloop, and controlled by fast pressure liquid chromatography at a rate of 1 ml/min. The column was then washed with 20 ml of 20 mM Tris and 0.1% Triton X-100, and eluted with a 120-ml linear gradient from 0% to 50% of 1 M NaCl. 1-ml fractions were collected through the entire gradient and assayed for protein content. The insulin binding assay was carried out as described above, on 2 μl from the fractions that constitute the protein peak. Fractions from the MonoQ column that centered around the second insulin binding peak, corresponding to αβ2 holoreceptor (data not shown), were pooled. The first insulin binding peak, identified as αβ half-receptor, was pooled independently and further purified in a procedure identical to that for the αβ2 holoreceptor. The pooled fractions were loaded onto a DEAE (Fluka, Milwaukee, WI) column for concentration.
The pooled MonoQ fractions were diluted 3-fold with 20 mM Tris, 0.1% Triton X-100 before being continuously loaded onto a 500-μl bed volume DEAE column that had been equilibrated with 20 mM Tris and 0.1% Triton X-100. After at least three times the applied volume was allowed to pass over the column, the DEAE column was washed with 50 ml of 20 mM Tris. This 20 mM Tris wash was then followed by a layering 500 μl of 20 mM Tris, 0.1% Triton X-100, 0.5 mM NaCl onto the column and allowing it to incubate for approximately 5 min before collecting the eluant. This was repeated a total of six times to ensure maximal protein recovery. 2 μl from each of the six elutions were assayed for protein amount, and the first three fractions were pooled for the final gel filtration step.

The Superose 6 (Pharmacia) column consisted of two 100-ml bed volume columns connected in series and was equilibrated with 600 ml of 30 mM HEPES, 0.1% Triton X-100, 0.02% NaN₃, and 150 mM NaCl. The pooled fractions from the DEAE column were loaded onto the Superose 6 column using a 2-ml sample loop, and controlled by fast pressure liquid chromatography at a rate of 0.33 ml/min. 1-ml fractions were collected between 60 and 200 ml. As described above, 20 μl of every buffer change were collected and assayed for protein amount, and the fractions surrounding and including the protein peak were assayed for insulin binding activity. The fractions containing the highest amount of both insulin binding activity and protein amount were pooled.

The pooled Superose 6 fractions were diluted 3-fold with 20 mM Tris and 0.1% Triton X-100, and loaded onto a new DEAE column (100 μl bed volume) for concentration and detergent exchange. The diluted fractions were continuously loaded onto the DEAE column with 20 mM Tris, and 0.1% Triton X-100 until at least three times the diluted volume had passed over the column. The column was washed with 5 ml of 20 mM Tris, and 0.6% β-ocyt-glucoside (Phaststeil Laboratories, Waukegan, IL) and eluted by layering 100 μl of 20 mM Tris, 0.6% β-ocyt-glucoside, 10% glycerol, and 0.5 mM NaCl onto the column and allowing it to incubate for approximately 10 min before collecting the eluant. The elution procedure was repeated a total of six times to ensure maximal protein recovery. 1 μl of each fraction was assayed for protein amount, and those containing more than 200 μg/ml protein were aliquoted for storage at −80 °C. Normal protein yields were approximately 200 μg/purification.

**SDS-PAGE**—The relative protein composition and insulin receptor purity at each step of the purification was determined by SDS-PAGE. The protein amounts from each step were loaded to show relatively constant amounts of insulin receptor through each stage of the purification procedure. Samples were run on a 3–10% gradient gel under non-reducing conditions using the Laemmli buffer system (25). The gel was immediately transferred to a fixative solution (40% methanol and 10% acetic acid) and silver stained (Bio-Rad).

**Western Blotting**—A second gel was run under the same conditions as above, using the same samples, and subjected to Western blotting. After electrotransfer (Transblot, BioRad, Richmond, CA) to Immobilon (Bedford, MA) by wet blotting (1250 total milliamps), the membrane was blocked in a 10% milk solution in 30 mM HEPES, 150 mM NaCl, 0.01% NaN₃, pH 7.4. Biotin-BMCC was added to a final concentration of 1 mM and incubated at room temperature for 2 h. The membrane was washed three times with 200 μl of 20 mM Tris and 0.6% β-ocyt-glucoside for 10 min. After blotting the membrane, it was blocked for 2 h at room temperature with a blocking solution composed of 20 mM Tris, 0.6% β-ocyt-glucoside, and 0.5 mM NaCl.

**Labeling of the Insulin Receptor**

Streptavidin-Nanogold—30 μg of purified insulin receptor in 20 ml Tris and 0.1% Triton X-100 was incubated with 10⁻⁶ M B25-l-benzoyl-phenylalanine, B29-biotinyl insulin (BBpa insulin) (26) in a total volume of 100 μl overnight, in the dark, at 4 °C. The BBpa insulin-receptor solution was exposed to 3 amps of UV light at a wavelength of 345 nm for 1 h on ice. Streptavidin-Nanogold (NanoProbes, Stony Brook, NY) was added to a 5-fold molar excess over BBpa insulin and incubated at 4 °C for 1 h.

300 μl of wheat germ agglutinin-agarose beads (50% beads υυυυ) equilibrated in 30 mM HEPES, 0.1% Triton X-100, 0.02% NaN₃, and the protease inhibitor mixture (see purification protocol) were added to the cross-linked BBpa insulin-receptor complex and incubated with gentle agitation overnight at 4 °C. The beads were settled with a 3-s pulse in a bechtop microcentrifuge at 4 °C, and the supernatant containing unbound streptavidin-Nanogold and uncross-linked BBpa insulin was removed. The wheat germ agglutinin-agarose beads were washed three times with 500 μl of 20 mM Tris and 0.1% Triton X-100 without protease inhibitors for 10 min. At each wash, the beads were settled with a 3-s pulse in a bechtop microcentrifuge at 4 °C and the supernatant was removed. The wheat germ agglutinin-agarose beads were washed three times with 500 μl of 20 mM Tris and 0.6% β-ocyt-glucoside for 15 min. At each wash, the beads were settled with a 3-s pulse in a bechtop microcentrifuge at 4 °C, and the supernatant was removed. The Q-Sepharose beads were washed three times with 500 μl of 20 mM Tris and 0.6% β-ocyt-glucoside for 15 min. At each wash, the beads were settled with a 3-s pulse in a bechtop microcentrifuge at 4 °C, and the supernatant was removed. The Q-Sepharose was eluted by incubating the beads with 100 μl of 20 mM Tris, 0.7% β-ocyt-glucoside, and 1 mM NaCl for 30 min. The beads were settled, and the supernatant was removed. The beads were eluted two additional times by incubating 100 μl of 20 mM Tris, 0.6% β-ocyt-glucoside, and 1 mM NaCl for 15 min before settling the beads and removing the supernatant. The three elutions were pooled and concentrated.

The gold-labeled insulin receptor was concentrated, and the salt was removed using a Microcon-50 microconcentrator (Amicon) blocked in 1% milk overnight. 400 μl of the Q-Sepharose pools were added to the Microcon-50, diluted to 500 μl, and centrifuged at 12,700 × g for 10 min. 200 μl of 20 mM Tris, 0.6% β-ocyt-glucoside, 100 mM NaCl, and 0.01% NaN₃ were added, and the Microcon was spun again for 13 min. 50 μl of 20 mM Tris, 0.6% β-ocyt-glucoside, 100 mM NaCl, and 0.01% NaN₃ was added to the Microcon, which was then inverted and pulsed. Gold-labeled insulin receptor was visualized in a 10% acrylamide gel by wet blotting. The gel was stained with Coomassie Blue, visualized by silver staining (Bio-Rad).

**Butane (Biotin-BMCC)—Biotin-BMCC (Pierce) was suspended in 30 mM HEPES, 0.1% Triton X-100, 0.02% NaN₃. The membrane was incubated with a polyclonal anti-insulin receptor β-subunit peptide antibody (R1064) in 10% milk solution in 30 mM HEPES, 0.02% NaN₃, pH 7.4. Biotin-BMCC was added to a final concentration of 1 mM and incubated at room temperature for 1 h. Samples were split into two tubes, each containing 9 μl of the reaction mix. To one of these tubes, sample buffer containing DTT was added to a final concentration of 150 mM DTT, heated at 100 °C for 5 min, and allowed to cool.

Samples were run on a 7% polyacrylamide mini-gel using the Laemmli buffer system and electrophoretically transferred to Immobilon-P membranes. The membrane was blocked for 2 h at room temperature in a 10% milk solution in 30 mM HEPES, 150 mM NaCl, and 0.02% NaN₃. The membrane was incubated with streptavidin-HRP (Amersham Pharmacia Biotech) for 1 h at 37 °C and washed three times for 15 min with PBST. The signal was detected by using an enhanced chemiluminescence system and exposing the membrane to film. The peroxidase enzyme was inactivated by incubating the membrane for 30 min at room temperature. The membranes were blocked in phosphate-buffered saline, followed by three 10-min washes in PBST. The insulin receptor β-subunit was incubated with a polyclonal anti-β subunit peptide antibody (R1064) in 2% BSA in PBST for 2 h at 37 °C and washed three times for 10 min at 37 °C in PBST. Protein A/G HRP (diluted 1:10,000 in 2% BSA in PBST) was incubated with the membrane for 1 h at 37 °C. The membrane was washed three times for 10 min at 37 °C in PBST, and the signal was detected as described above. The α-subunit was identified by incubating

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the same membrane with an anti-α-subunit antibody (Upstate Biotechnology, Inc.) in 2% BSA in PBST overnight at 4 °C, washing three times for 10 min with PBST, followed by incubation with goat anti-rabbit IgG-HRP in 2% BSA in PBST for 1 h at 37 °C. The blot was washed, and the signal was detected as previously described.

Monomaleimide-Nanogold—9.2 μg of purified insulin receptor was dialyzed using a dialysis membrane molecular weight cut-off of 50,000 (Spectrum, Houston, TX) at 4 °C against 100 ml of 30 mM HEPES, 0.6% β-ocetylglucoside, and 50 mM NaCl, without NaN3, for 1.5 h followed by dialysis against 350 ml of fresh buffer for 4 h. Six nanomoles of monomaleimide-Nanogold was suspended in 20 μl of isopropanol and brought to a final volume of 200 μl with distilled water, as suggested by Nanoprobes, immediately prior to use. Assuming that the cysteine of each β subunit in the αβ2 heterotrimer was available for labeling, a 10-fold molar excess or 20 μl of the suspended monomaleimide-Nanogold was added to the 9.2 μg of purified receptor and incubated at 4 °C in the dark overnight. The insulin receptor-monomaleimide-Nanogold complex was dialyzed in a 50-μl dialysis button using a 50,000 molecular weight cut-off membrane against 200 ml of 30 mM HEPES, 0.6% β-ocetylglucoside, 50 mM NaCl for 7.5 h at 4 °C. The dialysate was replaced with 400 ml of the same buffer and dialyzed overnight at 4 °C, replaced again with 300 ml of the same buffer, and dialyzed for 9 h at 4 °C. Maleimide-gold-labeled insulin receptor was prepared for cryo-electron microscopy or receptor reconstitution.

**Reconstitution**

**Formation of Large Unilamellar Vesicles**—The concentration of egg phosphatidylcholine (eggPC) (Avanti Polar Lipids Inc., Alabaster, AL) in chloroform was calculated by dry weight using a Cahn Balance. The volume corresponding to 1 mg of eggPC was added to a 25 ml-round-bottomed flask, and the chloroform was evaporated using a Rotovac. Approximately 5 ml of 2:1 chloroform:methanol were added to the round bottom flask, the lipid was redissolved, and the solvent was then evaporated using a Rotovac. The addition and evaporation of 5 ml of 2:1 chloroform:methanol were repeated two more times until a thin, even lipid film lined the inside of the flask. The dried eggPC film was then lyophilized, at room temperature, in the dark, overnight.

The lyophilized lipid film was resuspended in 500 μl of 30 mM HEPES, 50 mM NaCl, and 0.01% NaN3 to a final concentration of 2 mg/ml. After the solution appeared opaque, it was transferred to a 1.5-ml microcentrifuge tube for the freeze/thaw procedure. The vesicle solution was repeatedly placed in a liquid N2 bath for 30 s and then immediately placed in a 50–60 °C water bath until thawed for a total of six times. The resultant particulate vesicle solution was extruded through two 0.1-μm filters at room temperature for a total of 10 times to produce large unilamellar vesicles (27).

**Reconstitution Dialysis—**4 μg of purified αβ2 insulin receptor was added to 27.5 μl of large unilamellar vesicles (2 mg/ml) in a total volume of 55.0 μl and dialyzed in 50-μl dialysis buttons against 10 ml of 30 mM HEPES, 100 mM NaCl, 0.6% β-ocetylglucoside, and 0.01% NaN3 overnight at 4 °C. The sample was then sequentially dialyzed at 4 °C against 10 ml of 30 mM HEPES, 100 mM NaCl, 0.3% β-ocetylglucoside, and 0.01% NaN3 for 3 h; 10 ml of 30 mM HEPES, 50 mM NaCl, 0.15% β-ocetylglucoside, and 0.01% NaN3 for 3 h; 20 ml of 30 mM HEPES, 25 mM NaCl, and 0.01% NaN3 without β-ocetylglucoside for 3 h. The dialysis continued at 4 °C against 100 ml of 30 mM HEPES, 100 mM NaCl, and 0.01% NaN3 overnight, followed by 250 ml of the fresh buffer for 7 h, and finally against 500 ml of 30 mM HEPES, 100 mM NaCl, and 0.01% NaN3 overnight. The reconstituted sample was prepared for electron microscopy.

**Electron Microscopy**

**Negative Stain Electron Microscopy**—The samples were diluted to the appropriate concentration using buffer that contained the lowest possible amounts of salt and detergent. Control vesicles and reconstitutions were diluted to a lipid concentration of 0.1 mg/ml with 30 mM HEPES, 25 mM NaCl, and 0.01% NaN3. Detergent-solubilized receptor was diluted to a protein concentration of 5 μg/ml with 30 mM HEPES, 50 mM NaCl, 0.6% β-ocetylglucoside, and 0.01% NaN3. Carbon-coated copper grids (400-mesh Gilder grids) (Ted Pella Inc., Redding, CA) were glow-charged in 300–500 nm underfocus immediately prior to use. 4 μl of diluted sample were incubated on a grid for 2 min and rinsed with 8–12 drops of water. 4 drops of 1% uranyl acetate (Electron Microscopy Sciences, Fort Washington, PA) were then applied to the grid, and the final drop was allowed to incubate for 30 s before blotting off the excess stain.

**RESULTS**

**Insulin Receptor Purification**

Insulin receptors were purified from NIH-3T3 cells expressing the human insulin receptor cDNA. Isolated cell membranes were solubilized in Triton X-100, and the resultant protein solution was subjected to conventional chromatographic separation including gel filtration, affinity chromatography, and ion exchange chromatography. The presence of the insulin receptor was monitored throughout the purification using protein and insulin binding assays (data not shown). The final product from the purification is a single protein of approximately 350 kDa under non-reducing conditions (Fig. 1A, lane negative).
6) confirmed as the heterotetrameric insulin receptor by Western blotting (Fig. 1B, lane 6). Despite the absence of reducing agent, a distinct band of approximately 200 kDa was often present during the purification, and this was identified as αβ half-receptor by Western blotting (Fig. 1B, lanes 3–5). The half-receptor could be separated from the heterotetrameric receptor by ion-exchange chromatography (data not shown) and is not present in the purified product corresponding to 350 kDa is αβ heterotetrameric insulin receptor. The band corresponding to 200 kDa in lanes 3–6 is β-subunit. The final purification product corresponding to 200 kDa in lanes 3–6 is αβ half-receptor. C, autophosphorylation assay. In the presence of insulin, phosphotyrosine content of the fully purified receptor is comparable to that of control wheat germ-purified insulin receptor. Lane 1, membranes; lane 2, Triton-solubilized proteins; lane 3, Sephacryl 400 pools; lane 4, wheat germ agglutinin-agarose eluant; lane 5, MonoQ pools; lane 6, Superose 6 pools.

Detergent-solubilized Insulin Receptor

The purified, functionally active, heterotetrameric insulin receptor was visualized in the detergent-solubilized form using negative stain electron microscopy under low electron dose conditions. Several stains were used including sodium phosphotungstate and methylamine tungstate (data not shown); however, uranyl acetate provided the highest contrast and the most reproducible results. There were no large protein aggregates evident in the stained samples; however, occasionally, small clusters containing two or three receptors were seen.

Regions with light, even stain were optimal for visualization of individual receptor particles with minimal detergent artifact. Selected receptors from the resulting micrographs are shown in Fig. 2. The negatively stained insulin receptors take on the same Y- or T-shaped appearance reported previously (19). As illustrated in Fig. 2A, there is considerable variation among individual receptors. Some appear as a perfect Y (Fig. 2A, 1), while others appear as a perfect T (Fig. 2A, 15); however, most of the receptors do not conform precisely to either shape and demonstrate “characteristics” of these two shapes (Fig. 2A, 7, 11, 12, 17, 18, 20, and 22). Purified receptors were incubated in the presence of 100-fold molar excess of insulin prior to staining. The presence of insulin did not appear to affect the general staining characteristics of the insulin receptor. Unlike the results reported by Johnson et al. (30), insulin did not appear to induce aggregation of the purified holoreceptors. Fig. 2B displays selected individual insulin receptors in the presence of insulin. These receptors show the same Y- and T-like characteristics observed in the absence of insulin (Fig. 2A). There are receptors that appear like perfect Y (Fig. 2B, 4) and T (Fig. 2B, 12) shapes, although the majority of the receptors appear to be variations on these themes. These data demonstrate that insulin receptors exhibit the same general multiformity with and without insulin. In the presence of insulin, the receptors appear to be slightly more globular and less variable in structure. Quantitative measurements, however, show that insulin does not affect the overall length of individual receptor arms, nor does it affect the distribution of arm lengths (data not shown). Insulin receptors in the presence of insulin appear to be slightly larger in width than insulin receptors without insulin (data not shown); however, the limited resolution of these images prohibits the conclusion that this difference represents an insulin-induced conformational change.
In order to visualize the insulin receptor in a fully hydrated state, free of heavy metals and staining artifacts, we used the technique of cryo-electron microscopy. When preserved in vitreous ice and visualized using cryo-electron microscopy, the insulin receptor retains the Y- or T-shaped appearance identified above in negative stain. Individual receptors from this field, and others, have been selected and displayed in Fig. 3B. In the selected receptors, the Y-like characteristics of the insulin receptor (Fig. 3B, 4, 10, 12, 23, 25, etc.) seem to dominate over the T-like characteristics (Fig. 3B, 20 and 32), although both are evident. For many of the receptors, the Y-like or T-like appearance depends greatly on the perception of the individual observer. Of the 48 representative receptors selected and displayed in Fig. 3B, no 2 are identical, differing in arm length, separation angles, or both. Because of the individual receptor variability, and lack of orienting features, overall receptor dimensions were not determined. The cryo-electron microscopy data do, however, confirm the gross insulin receptor structure identified in negative stain. As illustrated by Fig. 3B, the four individual subunits of the insulin receptor come together to form a three-armed macromolecular complex.

Gold Labeling

Streptavidin-Nanogold—To address the issue of where insulin binds to the three-armed pinwheel complex identified using cryo-electron microscopy, we have employed an insulin analogue, BBpa insulin (26) and streptavidin-Nanogold. BBpa insulin is a photoactivatable insulin analogue that is covalently cross-linked to the insulin receptor when exposed to UV light (26). Residue B29 of this analogue has a biotin moiety that is accessible to streptavidin binding (26). Using a streptavidin-Nanogold conjugate, we have been able to label the insulin binding site with a 1.4-nm gold particle and visualize the insulin receptor-BBpa-insulin-streptavidin-Nanogold complex by cryo-electron microscopy.

Insulin receptors in the presence of the BBpa-insulin-streptavidin-Nanogold complex exhibit the same characteristics that were evident in the detergent-solubilized insulin receptor alone (Fig. 4). The receptors are identifiable, three-armed complexes (Fig. 4A, 1, 4, 7, 10, 14, and 15). Only one gold particle is seen per receptor, suggesting that only one insulin...
analogue binds per receptor, confirming the studies reported by Lee et al. (9). The Nanogold particles were localized to the tip of one arm, and the identity of the gold was confirmed by defocus pairs (data not shown). Because insulin binds to the a-subunit of the insulin receptor, the presence of a gold particle at the tip of one of the arms identifies that arm as containing the a-subunit. Identifying one of the a-subunits does not assist in orienting the receptor subunit composition with respect to the two-dimensional projection, as this does not distinguish the identity of the other two arms. The location of the gold particle does, however, confirm that insulin binds to that arm and suggests that the insulin binding site is not itself at the arm tip. Because streptavidin was used as a linker between the gold particle and the insulin analogue, the true insulin binding site is at least 5 nm away from the gold particle. Additionally, the lack of distinct density attributable to the streptavidin molecule in the gold-labeled receptors suggests that the streptavidin density is concealed by the receptor density. Taking into account the streptavidin spacer and the effect of projecting the receptor complex in two dimensions, it is possible that the insulin binding site lies toward the center of the receptor molecule in a manner similar to that of the interaction between growth hormone and the growth hormone receptor (31).

**Biotin-Maleimide Labeling**—Previous studies on the extracellular domain of the insulin receptor report a V-like or short T-like structure (12). This suggests that two of the arms of our three-armed structure contain the a-subunits, and the third arm contains both b-subunits. Identifying the b-subunit enables us to orient the receptor because the identity of all three arms would be determined. The b-subunit of the insulin receptor can be distinguished from the a-subunit based on its free sulfhydryl content (32–34). We incubated purified insulin receptor with a biotin-maleimide conjugate and detected this conjugate with streptavidin-linked horseradish peroxidase. Fig. 5 shows that in the absence of reducing agent, the heterotetrameric insulin receptor is labeled by the maleimide conjugate. Complete reduction of the holoreceptor into its component subunits demonstrates that greater than 95% of the labeling of the αβ2 receptor is located in the β-subunit with a trace corresponding to incorporation into the a-subunit (Fig. 5A, lane 4). Compared with the extremely strong β-subunit band, this labeling is inconsequential and is most likely a result of some small degree of disulfide reduction. The identity of the individual subunits was confirmed with anti-insulin receptor a-subunit antibody and anti-insulin receptor b-subunit antibody (Fig. 5B, lane 4). These data confirm the previous reports that only the β-subunit is accessible to N-ethylmaleimide (NEM) labeling in the absence of reducing agents (32–34).

**Maleimide-Gold Labeling**—Using monomaleimide-Nanogold, we specifically labeled the β-subunit of the insulin receptor for visualization using cryo-electron microscopy. Unlike the streptavidin-Nanogold used to label the α-subunit of the insulin receptor, monomaleimide-Nanogold does not have a large protein spacer. When the maleimide moiety is covalently attached to the sulfhydryl of a protein, the center of the gold particle is only 2 nm from the cysteine side chain (35). This property enables more direct labeling of the insulin receptor than the insulin-biotin-streptavidin complex used to label the α-subunit.

Fig. 6 shows representative maleimide-Nanogold-labeled insulin receptors visualized using cryo-electron microscopy. A, representative receptors were selected and oriented to position the gold-labeled β-subunit at the 6 o'clock position using the SPIDER image analysis software package (29). Displayed images were recorded at 1.7 μm underfocus. Defocus pairs (not shown) were recorded 500 nm underfocus (see “Materials and Methods”). Protein and gold are black. B, key depicting individual maleimide-gold-labeled receptors seen in A.
suggest that either only one sulfhydryl in the heterotetrameric receptor is accessible to maleimide labeling, or that there is steric interference from the first gold particle preventing the binding to a second maleimide-gold label.

**Insulin Receptor Reconstitution Visualized Using Negative Stain Electron Microscopy**

Detergent-solubilized forms of isolated membrane proteins frequently lose some of the functional activity present in the membrane bound forms (reviewed in Refs. 36 and 37). This loss of function can be indicative of minor structural changes that result from the absence of membrane interactions. Reconstituting the insulin receptor into pre-formed phospholipid vesicles provided two important experimental advances. First, it enabled us to study the insulin receptor in a model membrane environment that more closely approximates the physiologic state of the receptor. Second, like the maleimide-gold labeling, reconstituting the receptor enabled us to orient the receptor in a two-dimensional plane by identifying the β-subunit-containing arm. Sequence analysis has identified the β-subunit as the only subunit that crosses the membrane (38, 39). We have created a population of large unilamellar egg phosphatidylcholine (eggPC) vesicles by extrusion (27) (Figs. 7A and 8A) and used these vesicles for insulin receptor reconstitution. The pre-formed unilamellar eggPC vesicles were incubated with detergent-solubilized purified insulin receptor and 20 mM β-oc-
yl glucoside prior to detergent removal over a 48-h period using stepwise dialysis. The resulting reconstituted receptors were visualized by both negative stain and cryo-electron microscopy.

Reconstituted insulin receptors were negatively stained in 1% uranyl acetate and visualized under low electron dose conditions. These samples did not stain as uniformly and reproducibly as the detergent-solubilized receptors; however, there were consistently regions of light, even staining that enabled adequate visualization of the reconstituted receptors (Fig. 7B). Individual reconstituted receptors were selected from representative micrographs and displayed in Fig. 7C. These receptors are more difficult to see in negative stain than are their detergent-solubilized counterparts (cf. Fig. 2). Fig. 7C (1) demonstrates the typical Y-like structure protruding from the vesicle surface, and several other receptors demonstrate the two armed V-like structure that results when one of the Y arms is hidden (Fig. 7C, 15, 19, and 20). Some regions clearly contain more than one reconstituted receptor per vesicle (Fig. 7C, 12). Other regions are highly suggestive of two adjacent receptors per vesicle; however, both receptors are not clearly demarcated (Fig. 7C, 5 and 14). Most of the negatively stained reconstituted receptors are seen as stain-excluding regions extending from the vesicle surface providing minimal structural detail.

**Insulin Receptor Reconstitution Visualized Using Cryo-electron Microscopy**

Reconstituted insulin receptors preserved in vitreous ice were visualized by cryo-electron microscopy under low electron dose conditions (Fig. 8B). Cryo-electron microscopy overcomes many of the limitations of negative stain, and the reconstituted receptor images provide much more detail (Fig. 8C) than the negatively stained images (Fig. 7C). The original vesicle population was fairly heterogeneous; however, the reconstitution procedure seemingly resulted in an increase in the proportion of multilamellar vesicles (data not shown). Free protein was occasionally evident in the background. The criterion we used for selecting reconstituted receptors required that there be an evident membrane perturbation at the site corresponding to the receptor’s transmembrane region. Use of this strict crite-

![Fig. 7. Vesicle-reconstituted insulin receptors preserved in 1% uranyl acetate and visualized under low electron dose conditions. Micrographs were recorded 300–500 nm underfocus. Protein and lipid are white. A, egg phosphatidylcholine vesicles (without protein) prepared by extrusion. B, representative electron micrographs exhibiting vesicle-reconstituted insulin receptors (circled). Not all receptors are circled. C, individual reconstituted receptors were selected from micrographs such as that in B. Receptors were oriented and displayed using the SPIDER image analysis software package (29). Most panels contain one reconstituted receptor; however, 5 and 14 are examples of adjacent receptors incorporated into a single vesicle. D, key outlining reconstituted receptors and vesicle membranes seen in C.](http://www.jbc.org/Downloaded_from)
Vitreous ice and visualized using cryo-electron microscopy. Micrographs were recorded at 1.7 μm and low pass-filtered for contrast enhancement. The resultant images are displayed in Fig. 8C.

Fig. 8C shows the reconstituted insulin receptors as the same three-armed complex that we have identified for the detergent-solubilized insulin receptors (Fig. 3B). The reconstituted insulin receptors demonstrate one of the arms traversing the vesicle bilayer and the other two arms removed from the vesicle surface (Fig. 8C). Most of the reconstituted receptors exhibit more Y-like characteristics (Fig. 8C, 1, 2, 5, and 11); however, there are still some receptors that exhibit more T-like characteristics (Fig. 8C, 45 and 47). As with the detergent-solubilized receptors, the reconstituted receptors still display extremely variable structures. Anchoring the receptor transmembrane domain in a model membrane does not appear to limit the receptororientational variability to any great extent. Of the 48 reconstituted receptors in Fig. 8C, no two images are identical. These receptor images do not contain any distinct globular domains in contrast to the report by Tranum Jensen et al. (20), identifying globular domains at the arm tips of negatively stained insulin receptors. The images displayed in Fig. 8C each contain one clear reconstituted receptor; however, there are some views that do contain additional receptors in the fields (Fig. 8C, 5, 11, and 24). These “single” images were selected to illustrate clear receptor structure. More than one receptor, however, can be present on a single vesicle, and the presence of these multiple receptors in close proximity does not appear to limit the variability of the resulting images (data not shown).

Reconstituted β-Subunit-labeled Insulin Receptors

We have oriented the detergent-solubilized three-armed receptor complex with respect to subunit composition by gold labeling the β-subunit as described above. The reconstituted insulin receptors were already orientationally defined based on identification of the β-subunit-containing arm as the only arm able to cross the membrane (38, 39). Our reconstituted αβ2 heterotetrameric receptor images, however, did not clearly define the intravesicular component of the receptor. We applied a β-subunit labeling technique, similar to that used for the detergent-solubilized insulin receptor, to the reconstituted αβ2 heterotetrameric receptor to identify the intravesicular component of the receptor.

The β-subunit of purified insulin receptor was labeled with monomaleimide-Nanogold and reconstituted into pre-formed unilamellar eggPC vesicles. Because the receptor was gold-labeled prior to reconstitution, the gold-labeled β-subunit could be either intravesicular or extravesicular. The reconstituted labeled receptor was visualized by cryo-electron microscopy, and data were recorded in defocus pairs (−1.7 μm and −0.5 μm) to confirm the presence of gold attached to the receptor. The criteria used for identification and selection of the reconstituted gold-labeled receptor required that the receptor have clear protein at a defocus of −1.7 μm and clear corresponding gold at a defocus of −0.5 μm. Both components, however, did not have to be visible at a single defocus value. There also had to be detectable membrane disturbance at the region of receptor insertion. Individual receptors meeting these criteria were selected and displayed in Fig. 9.
The gold-labeled reconstituted insulin receptors displayed in Fig. 9 demonstrate the same three-armed complex described for detergent-solubilized and reconstituted insulin receptors. The gold particle (seen as a black dot in the primed numbers) can easily be identified at the −0.5 μm defocus value (Fig. 9A). The gold particle is attached to the one receptor arm that is part of the membrane-spanning arm, confirming that this is the β-subunit-containing arm. The position of the gold particle with respect to the vesicle membrane is not constant. For example, the gold particle in Fig. 9A (I’) is 2.4 nm from the vesicle membrane, while the gold particle in Fig. 9A (3’) is flush against the membrane, and the gold particle in Fig. 9A (4’) appears to be within the lipid bilayer. The precise cysteine residue that is labeled by maleimide conjugates has yet to be identified. It has been proposed that the accessible cysteine on the β-subunit lies near the ATP binding site because NEM inhibits insulin receptor kinase activity (40) and binding of nucleotides to the insulin receptor decreases NEM labeling (32). By assuming that the gold particle covers at least the kinase region of the receptor and potentially part of the C terminus, we have been able to approximate the length of the intravesicular component. The positioning of the gold particle on the reconstituted images must be considered in terms of the resultant two-dimensional projection of a three-dimensional object. Just as the true receptor arm length is represented by the longest projection, the true distance between membrane and gold particle is represented by the longest projection. The longest projection, represented by Fig. 9A (7’), is 4.3 nm when measured from the vesicle membrane surface through the entire gold particle.

**DISCUSSION**

Insulin binding to the insulin receptor initiates a cascade of cellular events that are responsible for regulating cell metabolism, proliferation, and growth (reviewed in Refs. 2 and 41). We have been using the tools of electron microscopy to investigate the structure of the insulin receptor. Negative stain and cryo-electron microscopy both show the detergent-solubilized insulin receptor as a three-armed pinwheel structure. The different orientations and subtle variations are consistently represented by both techniques. Cryo-electron microscopy, however, provides more structural detail and a more accurate representation of the receptor in an aqueous environment. The gross structural appearance of the detergent-solubilized αβ2 heterotetrameric receptor correlates with the previously reported Y-like or T-like appearance of the insulin receptor extracellular domain (12) and holoreceptor (19), respectively. The individual images in Figs. 2 and 3C exemplify the considerable variability that the insulin receptor demonstrates when visualized by electron microscopy.

Electron microscopy involves reducing a three-dimensional object (the insulin receptor) to a two-dimensional projection (the resulting micrograph). The variability seen among receptor images can be explained in one of two ways. The first explanation is that there is inherent flexibility within the protein itself, allowing it to freely adopt multiple conformations. The second explanation involves the orientation of the receptor in the ice layer. Because the ice layer thickness of a sample viewed by cryo-electron microscopy is on the order of 100 nm (much larger than the dimensions of the receptor), the detergent-solubilized insulin receptor has a full 360° of rotational freedom in that ice layer. This rotational freedom enables the insulin receptor to exist in innumerable different orientations in the ice layer. Without an appropriate orientational reference, the cause of the apparent flexibility cannot be determined. By using gold labels visible under the conditions of cryo-electron microscopy, we were able to identify the subunits composing the three-armed receptor complex in an effort to elicit an orientational reference.

We have compiled the data from our cryo-electron microscopic studies of the insulin receptor to construct a two-dimensional model (Fig. 10A). Several assumptions were made in composing this model. First, the insulin receptor is presented with Y-like characteristics, as this is the predominant species observed in our reconstituted samples. The α-subunit-containing arms are represented at 90° angles to each other and 45° angles to the normal plane. Second, the vesicle membrane is depicted as a bilayer traversed by the receptor. The bilayer thickness is assumed to be 6 nm from the extracellular membrane surface to the intracellular membrane surface. Third, the width of the β-subunit-containing arm determined for the detergent-solubilized and reconstituted receptors was applied to the intravesicular segment, a region for which no width measurements were obtained. Finally, the overall dimensions of 12.2 nm × 20.0 nm were mathematically determined based on the individual receptor arm lengths and the 90° angle separating the α-subunit-containing arms. As a result, the numerical values assigned to the receptor dimensions must be considered only as approximations.

The four subunits of the insulin receptor (two α-subunits and two β-subunits) come together to form a three-armed pinwheel-like complex. The dimensions for this complex, represented as a Y in Fig. 10A, are 12.2 nm × 20.0 nm. One of the arms, the β-subunit-containing arm, crosses the vesicle membrane. The
total length of this single arm is 13.9 nm, 4.3 nm of which is in the intravesicular compartment. The other two arms of the three-armed pinwheel-like complex are the α-subunit-containing arms and are each 8.6 nm long. Based on a 6-nm-wide membrane, the β-subunit-containing arm of the receptor projects 3.6 nm above the surface of the membrane, so the entire extracellular segment of the receptor extends 9.7 nm above the membrane surface. The individual receptor arms are each approximately 1.5 nm wide.

The organization of the two α-subunits and two β-subunits into the αβ2 heterotetrameric receptor is schematically presented in Fig. 10B. The individual subunits in this cartoon are in close enough proximity to allow for the known intersubunit interactions. The longer arm of the three-armed receptor complex, identified using electron microscopy, is represented as containing both β-subunits and the more C-terminal region of both α-subunits. The two receptor arms that are not in direct contact with the membrane are each represented as containing the majority of a single α-subunit. The interchain disulfide linkages, two connecting the α-subunits and one connecting each β-subunit to the respective α-subunit, are represented in the extracellular portion of the longest arm. Fig. 10B incorporates the general features of the insulin receptor identified microscopically and the subunit interactions identified biochemically (42–44). The resultant schematic suggests a potential organization for the individual subunits in the Y-shaped insulin receptor.

Construction of our two-dimensional model enables us to compare our results with those of others who have studied the structure of the insulin receptor. Schaefer et al. (12), studied the insulin receptor extracellular domain using rotary shadowing and negative stain electron microscopy. Using rotary shadowing, they saw structures that they describe as "X" or Y structures. They measure the length of two arms, end to end through the midpoint, as being 20 nm. Applying the same measurement criteria to our model, we calculate a value of 15.2 nm. Their negatively stained images of this same sample produce dimensions of 10 nm × 15 nm, while our cryo model produces smaller dimensions for this same region, 9.7 nm × 12.2 nm.

Christiansen et al. (19) have studied the full-length αβ2 heterotetrameric insulin receptor using negative stain electron microscopy. They report images that are predominantly T-like with dimensions of 18 nm wide and 24 nm long, which are also larger than the dimensions of our receptor (12.2 nm wide and 20.0 nm long) visualized under cryo conditions. Using gold-labeled antibodies, Tranum-Jensen et al. (20) have detected the β-subunit approximately 5.6 nm away from the membrane, and two different regions of the α-subunit 15.5 and 11.5 nm away from the membrane. As with our own measurements, these measurements can only be considered as approximations. Unlike our data, these measurement values have an antibody linker and a 3-nm colloidal gold particle, so the dimensions are expected to be considerably larger than our own.

The tyrosine kinase region of the cytoplasmic portion of the insulin receptor has been crystallized in both the dephosphorylated and phosphorylated forms (17, 18). We calculated the estimated diameter of the kinase region of a single β-subunit from their data and obtained an approximate value of 4.5 nm for both instances. Juxtaposing two β-subunits produces dimensions of 9.0 nm × 4.5 nm for the kinase region of the intracellular domain of the heterotetrameric receptor. This is extremely large, even compared with the negatively stained images of the insulin receptor. Clearly, the three techniques of x-ray crystallography, cryo-electron microscopy, and negative stain electron microscopy are producing similar results, yet are not in perfect agreement.

There are several reasons why there appears to be such variability among the measurements produced by these techniques. First, the resolution of electron microscopy without image analysis is limited. Results from applying quantitative analysis to images generated by these techniques can vary substantially depending on the individual gathering the data. The small variations that occur when two people measure the
same object are amplified when working with such small images. Even in our own images of the insulin receptor printed at magnification of approximately 530,000, a measured difference in interpretation of 0.1 mm can translate into 0.5-nm difference in the true object. As a result, nanometer differences between different groups measurements still correspond to agreement between the data. Second, each technique focuses on a different element of the protein. Rotary shadowing focuses on the metal outline of the protein, as does negative stain. In both of these cases, the protein is adhered to a flat surface. In the case of negative stain, the protein is dried in the presence of a heavy metal, which can induce flattening and a considerable amount of distortion. The insulin receptor may be very susceptible to this flattening, resulting in the images generated appearing larger than the true protein size. Cryo-electron microscopy relies on the protein itself to provide contrast. If the protein is not very well ordered and the edges are not very well defined, the amount of contrast may be lower and boundaries more difficult to identify. As a result, the quantitative measurements determined under cryo conditions may represent an underestimate of true receptor size. We suspect that this may, in part, explain the calculated 1.5-nm receptor arm width, which is most likely an underestimate of true receptor arm width. Finally, x-ray crystallography studies pertaining to the insulin receptor have been limited to a fragment of a single β-subunit (17, 18), and the extracellular domain of the related IGF-1 receptor (15). The subunit interactions and quaternary organization of the insulin receptor in its entirety cannot be derived from these data. Structural analysis of the heterotetrameric insulin receptor in a more native state, using techniques such as cryo-electron microscopy, is necessary to complement the atomic resolution data obtained from crystallographic studies.

Cryo-electron microscopy provides structural detail that most closely approximates the insulin receptor in its native environment. Of the four techniques used to study the insulin receptor, negative stain electron microscopy, rotary shadowing, cryo-electron microscopy, and x-ray crystallography, the cryo-electron microscopic data should be considered most representative of the fully hydrated, heterotetrameric insulin receptor structure. It is the only one of these techniques that enables visualization of a biological sample in its entirety in a fully hydrated state. The added benefit of being able to visualize detail of the insulin receptor inserted into a model membrane system provides insights not possible with other techniques.

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Structural Studies of the Detergent-solubilized and Vesicle-reconstituted Insulin Receptor
Christine N. Woldin, Frederick S. Hing, Jongsoo Lee, Paul F. Pilch and G. Graham Shipley

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