Construction and Characterization of a Monomeric Insulin Receptor

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A mutant insulin receptor was constructed by replacing cysteine residues Cys524, Cys682, Cys683, and Cys685 with serine. The mutant was expressed in COS7 and Chinese hamster ovary cells, did not form covalently linked dimers, and was present at the cell surface. There was half as much insulin binding activity at the cell surface in cells expressing the mutant compared with that in cells expressing the wild type receptor. The intracellular processing of the mutant receptor was affected, since its β-subunit migrated more slowly than that of the wild type receptor on SDS-PAGE. The mutant was capable of insulin-dependent autophosphorylation and phosphorylation of insulin receptor substrate-1 in vivo and could be cross-linked into receptor dimers when membrane-bound. The amount of insulin-dependent autophosphorylation of the mutant receptor was half that of the wild type receptor. However, after solubilization the monomeric insulin receptor had minimal autophosphorylation activity, and, unlike the naturally occurring monomeric receptor tyrosine kinases, the solubilized monomeric insulin receptor did not dimerize in response to insulin binding as determined by sucrose density gradient centrifugation.

The insulin receptor (IR) differs from most other receptor tyrosine kinases because it is present in the plasma membrane as a disulfide-linked dimer of two αβ monomers (1). The β chains are linked to the α chains by class II disulfide bonds, which are resistant to reduction; Cys647 is the α chain residue involved in the class II disulfides (2). The two α chains are extracellular and are linked by class I disulfide bonds, which are easily reduced by dithiothreitol (3). At least two α chain cysteine residues are involved in the class I disulfide bonds (4, 5), one of which is cysteine 524 (6–8).

In a previous work, we reported that the cysteine residues involved in the class I disulfides between the α chains were Cys524 and Cys682 (9). Whereas the involvement of Cys524 in the class I disulfides had been suggested by other investigators (6–8), evidence that at least one of the cysteine residues of the triplet at positions 682, 683, and 685 formed an additional disulfide bond was presented subsequently by Sparrow et al. (10). Further support was provided by the report that an α-subunit fragment, in which amino acids 469–703 and 718–729 had been deleted, is a monomer and binds insulin (11).

A curious finding of the previous work was that when cells expressing the IR/C524S,C683S,C685S mutant were incubated with insulin before solubilization of the receptors, most of the receptors dimerized. We became concerned that the dimerization might be caused by formation of disulfide bonds between Cys683 and Cys685. To investigate this possibility, we constructed another insulin receptor by replacing Cys683 and Cys685 in addition to Cys524 and Cys682, with serine. The new receptors did not form covalent dimers, were expressed on the cell surface, and underwent autophosphorylation in response to insulin while membrane-bound. However, in the monomeric state the insulin receptors had decreased insulin-dependent kinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials—** Dulbecco’s modified Eagle’s medium and nutrient mixture F-12 (Ham’s F-12), LipofectAMINE, penicillin/streptomycin/glutamine, and fetal bovine serum were purchased from Invitrogen. Vector plasmid pcDNA3.1His/V5 and V5 antibody were purchased from Invitrogen. Insulin receptor antibodies Ab-1 and Ab-4 were purchased from Oncogene Research Products (Cambridge, MA). Antibodies against insulin receptor β chain (294), insulin receptor α chain (N-20), phosphotyrosine (PY-99), and protein G-plus were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-insulin receptor substrate-1 (IRS-1) antibody was obtained from Upstate Biotechnologies, Inc. (Lake Placid NY). [γ-32P]ATP and [35S]insulin were purchased from PerkinElmer Life Sciences. SuperSignal West Pico Chemiluminescent Substrate and GelCode Blue Stain Reagent were from Pierce. Ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EGS) was purchased from Sigma.

**Cell Culture and Transient Transfection—** COS7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml glutatione at 37 °C in 5% CO2. CHO cells were maintained in F-12 medium with 10% fetal bovine serum at 37 °C in 5% CO2. To transiently express the insulin receptors, subconfluent cells were transfected with plasmid DNA and LipofectAMINE according to the manufacturer’s instructions. The ratio of DNA to LipofectAMINE was 1:3.

**Construction of Expression Plasmids—** The BamHIXbaI fragment of pCEC-C524S,C682S,C685S, which contains serine mutations at residues 524, 682, and 685 (9), was inserted in pBluescript KS(+) to make plasmid pJW5. In order to introduce a BstBI site at the C terminus of the β-subunit, a mutagenesis PCR was carried out with T3 primer and primer R1 (CGGTAGGCACTGTTcGaAAGGATTGGAC), and pJW5 as template. The BstBI site is underlined, and the lowercase letters represent the changes from the original bases in the primer R1. In this way, the last amino acid of the human insulin receptor was changed from serine to phenylalanine. The stop codon was changed to glutamic acid. The PCR product was cloned into the BamHI/BstBI site of Lip-128 (New England BioLabs, Inc., Beverly, MA) to make pJW6. The 3.5-kb HindIII/PmlI fragment of pECE (12) (from William J. Rutter, University of California, San Francisco, CA) was cloned into the HindIII/PmlI site of pJW6 to make pJW17, which contains the whole human IR gene. The HindIII/BstBI fragment from pJW17 was inserted into pcDNA3.1-V5/His (version A) to make pJW64, which encodes the human insulin receptor with a V5/His epitope tag. Mutations of the C terminus of the insulin receptor (Ser to Phe, stop to Glu) and the...
introduction of the V5/His epitope tag did not affect its basic biological functions (see “Results”). The Ser^524^ mutation was imported from pECE-C524S,C682S,C685S by replacing the HindIII/BamHI region of pJW64 with the corresponding fragment of pECE-C524S,C682S,C685S.

**TABLE I**

| Binding sites/cell | IR^WT | IR(C524S,C682S,C683S,C685S) | IR(C524S) | IR(C682S,C683S,C685S) | LacZ | Not transfected |
|--------------------|-------|---------------------------|-----------|------------------------|------|----------------|
|                    | 1.1 × 10^6 (3.7 × 10^5) | 4.2 × 10^5 (7.6 × 10^4) | 8.1 × 10^5 (1.6 × 10^4) | 1.3 × 10^6 (4.6 × 10^4) | 3.9 × 10^4 (6.8 × 10^4) | 3.9 × 10^4 (6.8 × 10^4) |

To introduce mutations in the region of Cys^682^ to Cys^685^, the following primers were used: S682, ATGAGGATTCGGCCGGCGAATcCTGCTCCTGTCCAA; S682,3, ATGAGGATTCGGCCGGCGAAaGCaGCTCCTcTCCAA; S683,5, ATGAGGATTCGGCCGGCGAATGCaGCTCCaGTCCTAA; and S682,3, ATGAGGATTCGGCCGGCGAAaGCaGCTCCTGTCCTAA. The mutated bases are shown in lowercase type. All of the modified regions of the IR cDNA were sequenced to confirm the mutations and verify that no other mutation occurred.

**SDS-PAGE and Immunoblotting** — The samples in Laemmli sample buffer (reducing or nonreducing depending upon the experiments) were heated at 95 °C for 5 min before subjecting them to SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose (300 mA for 75 min). The nitrocellulose was blocked with 5% nonfat milk prior to incubation with primary antibody. The primary and secondary antibodies were dissolved in TBST (25 mM Tris, 0.02% KCl, 0.8% NaCl, 0.1% Tween 20, pH 8.0) with 1% nonfat milk. Immune complexes were detected by enhanced chemiluminescence.

**Insulin Binding Assay** — CHO cells (70–80% confluent) in 100-mm plates were transfected with 5 μg of plasmid containing the cDNA for the wild type and mutant insulin receptors indicated in Table I. After serum starvation for 4 h, the cells were incubated with 50 pM 125I-insulin overnight at 4 °C. The cells were washed with cold binding buffer three times and then solubilized with 0.1% SDS. The radioactivity of the solutions was determined with a γ-counter. Nonspecific binding was determined by including 10 μM nonradioactive insulin in the incubation. The results are means of two independent experiments; the deviations are in parentheses.

![Immunoblot](https://example.com/immunoblot.png)

**FIG. 1. Immunoblots of mutant insulin receptors.** A, COS7 cells were transfected with plasmids containing the cDNA for the insulin receptors; after 48 h, crude membranes were solubilized with Laemmli buffer containing 50 mM N-ethylmaleimide followed by SDS-PAGE (3% stacking gel and 5% separating gel) and immunoblotting with anti-V5 antibody. Lane 1, IR(C524S,C682S,C683S,C685S); lane 2, IR(C524S,C682S,C685S); lane 3, IR(C524S,C682S,C683S,C685S); lane 4, IR(C524S,C682S,C683S); lane 5, IR(C524S); and lane 6, IR(C524S,C683S,C685S) with V5 epitope, pJW65 (IR(C524S,C682S,C683S,C685S) with V5 epitope), and pET3 (IR(C524S,C682S,C683S) without V5 epitope) were subjected to SDS-PAGE (4–15% gradient gel) under nonreducing or reducing conditions and immunoblotted with anti-insulin receptor antibody Ab-4. Lanes 1 and 4, IR^WT^ with V5 epitope; lanes 2 and 5, IR(C524S,C682S,C683S,C685S) with V5 epitope; lanes 3 and 6, IR^WT^ without V5 epitope.

![Immunoblot](https://example.com/immunoblot2.png)

**B** crude membranes from COS7 cells transfected with pJW64 (IR^WT^ with V5 epitope), pJW65 (IR(C524S,C682S,C683S,C685S) with V5 epitope), and pET3 (IR(C524S,C682S,C683S) without V5 epitope) were subjected to SDS-PAGE (4–15% gradient gel) under nonreducing or reducing conditions and immunoblotted with anti-insulin receptor antibody Ab-4. Lanes 1 and 4, IR^WT^ with V5 epitope; lanes 2 and 5, IR(C524S,C682S,C683S,C685S) with V5 epitope; lanes 3 and 6, IR^WT^ without V5 epitope.
FIG. 2. **Scatchard analyses of insulin binding to the dimeric and monomeric insulin receptors.** The crude membranes from the COS7 cells expressing dimeric or monomeric insulin receptors were incubated with 50 pM $^{125}$I-insulin and increasing concentrations of cold insulin at 4 °C overnight. The membranes were separated from the solution by centrifugation, washed, and then assayed for radioactivity with a γ-counter. No significant binding could be detected to the crude membranes from the COS7 cells expressing LacZ (data not shown). The dissociation constants are $5.6 \times 10^{-10}$ and $5.1 \times 10^{-10}$ M for wild type and mutant IRs, respectively, and the maximal amounts of insulin binding are $1.58 \times 10^{-11}$ and $1.12 \times 10^{-11}$ mol/μg of membrane, respectively.

FIG. 3. **Insulin binding does not cause dimerization of the monomeric insulin receptor.** 80 μg of crude membranes from COS7 cells expressing dimeric or monomeric insulin receptors were solubilized with 1% Triton X-100; the solutions were diluted to 0.1% Triton X-100 with binding buffer (see "Experimental Procedures") and centrifuged at 40,000 rpm for 30 min in a Beckman Ti70.1 rotor. The supernatants were incubated without or with 20 nM insulin at 4 °C overnight. The samples were layered onto 4.6 ml of linear 5–20% (w/v) sucrose gradients containing 0.1% Triton X-100 and 20 nM insulin or no insulin and centrifuged at 48,000 rpm for 6 h at 4 °C in a Beckman SW50.1 rotor. Fractions of 200 μl were collected from the top of the tube, and the location of the insulin receptor in the fractions was determined by its ability to bind $^{125}$I-insulin. Catalase and aldolase were used to calibrate the gradients, and their positions were determined by SDS-PAGE.
After incubation, the cells were washed three times with ice-cold phosphate-buffered saline, solubilized in 0.1% SDS, and counted in a 

\(^{125}\)I-Insulin binding to crude membranes was done by incubating 30 g of crude membranes with 50 pM \(^{125}\)I-insulin and increasing concentrations of porcine insulin (0–50 nM) in 50 mM HEPES, pH 7.4, with 0.1% BSA for 12 h at 4 °C. The membrane proteins were collected by centrifugation for 30 min at 4 °C. No specific binding could be detected from the membranes of COS7 cells transfected with cDNA3.1-LacZ.

**Sucrose Density Gradient Sedimentation**—This was performed as described by Aiyer (12) and Lu and Guidotti (9). Briefly, 80 μg of crude membranes from COS7 cells expressing dimeric or monomeric insulin receptors were solubilized with 1% Triton X-100; the solutions were diluted to 0.1% Triton X-100 and centrifuged at 40,000 rpm for 30 min in a Beckman Ti70.1 rotor. The supernatants were collected and incubated with or without 20 nM insulin at 4 °C overnight. The solutions were then layered onto 4.6 ml of linear 5–20% (w/v) sucrose gradients containing 0.1% Triton X-100 and 20 nM insulin or no insulin. The samples were centrifuged for 6 h at 48,000 rpm at 4 °C in a Beckman SW50.1 rotor. Fractions of 200 μl were collected from the top of the tubes. Equivalent amounts of solubilized dimeric or monomeric insulin receptors were separated under identical conditions except without insulin in the gradient. To locate the insulin receptor, fractions were assayed for their ability to bind \(^{125}\)I-insulin. Catalase and aldolase were loaded on the sucrose gradients with the samples. The location of the two enzymes was determined by SDS-PAGE, followed by staining with GelCode Blue Stain Reagent (Pierce).

**Immunoprecipitation**—Cells were solubilized with lysis buffer (0.5%
Nonidet P-40 in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 100 mM NaF, 0.2 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin. After centrifugation at 13,000 rpm to remove cell debris, 800 μg of cell lysate protein were incubated with appropriate antibodies for 2–4 h, followed by the addition of 10–20 μl of protein G-agarose and incubation for 1–3 h. The beads were collected by centrifugation and washed with lysis buffer three times. The immunoprecipitated proteins were released from the bead by Laemmli buffer containing 100 mM dithiothreitol and analyzed by SDS-PAGE followed by immunoblotting.

Receptor Autophosphorylation—Cells, 48 h after transfection, were incubated in a medium without serum for 2–4 h (as indicated), treated with 100 nM insulin in a medium containing 1% BSA for 5 min. Insulin stimulation was stopped by washing with ice-cold PBS three times. A, cells were solubilized with 0.5% Nonidet P-40 and immunoprecipitated with anti-phosphotyrosine antibody (PY20) and protein G-agarose. The precipitate was examined by SDS-PAGE and immunoblotting with anti-V5 antibody. B, cells were solubilized with Laemmli loading buffer and examined by SDS-PAGE and immunoblotting with anti-V5 antibody. The columns in each panel show the relative intensities of the bands compared with those of the dimeric insulin receptors in the presence of insulin. The panels show one of two independent experiments.

**RESULTS**

Construction of a Monomeric Insulin Receptor—Previously, we identified Cys524 and Cys682 as the residues involved in the class I disulfide bonds between the α chains (9). The IR(C524A,C682A) mutant, however, was able to dimerize in the presence of insulin. To overcome this problem, we constructed IR mutants in which Cys524 and Cys682 were both changed to Asp or Arg to prevent monomer-monomer interactions by electrostatic repulsion. We found, however, that the IR(C524D,C682D) and IR(C524R,C682R) mutants were a mixture of monomers and dimers when examined by nonreducing SDS-PAGE (data not shown).
IRC524S, C682S, C683S, C685S mutant compared with the wild type

tions 682, 683, and 685 are involved in the class I disulfides. This supposition was shown to be correct; necessary to mutate all four Cys residues at positions 524, 682, 683, and 685 to produce the mutant C524S, C682S, C683S, C685S and compared them with C524S, C682S, C685S and C524S, C683S, C685S and compared them with C524S, C682S, C683S, C685S. Since the two mutants, C524S, C682S, C685S, and C524S, C682S, C683S, are expressed as dimers (2), we focused our attention on the mutants C524S, C682S, C685S, and C524S, C682S, C683S, and C524S, C683S, C685S and compared them with C524S, C682S. As can be seen in Fig. 1A, all four mutants were present as dimers and monomers (lanes 2, 3, 4, and 5, respectively) when examined by nonreducing SDS-PAGE. The result suggested that it may be necessary to mutate all four Cys residues at positions 524, 682, 683, and 685 in order to obtain monomeric insulin receptors. This supposition was shown to be correct; lane 1 of Fig. 1A shows that in the mutant C524S, C682S, C683S, C685S there was only the monomeric receptor. Notice that in all lanes the proreceptor, composed of the fused and unproteolyzed αβ chain, was visible.

Fig. 1B shows a more detailed analysis of the IR(C524S,C682S,C683S,C685S) mutant compared with the wild type IR (IRWT). Under nonreducing conditions, only receptor monomers were present after SDS-PAGE (lane 2), whereas IRWT was dimeric (lanes 1 and 3). Under reducing conditions, only the β subunit and the proreceptor were visible (lanes 4–6); the addition of the epitope tag to the C terminus of the IR made the β subunit migrate more slowly than the native β subunit (lane 4 compared with lane 6). In addition, the β subunit of the mutant IR migrated more slowly than that of the wild type (lane 5 compared with lane 4). The difference in mobility was also observed for the mutant β chain expressed in CHO cells (see Fig. 4A).

We conclude that Cys524 and all three Cys residues at positions 682, 683, and 685 are involved in the class I disulfides.

Monomeric IR Is Expressed on the Cell Surface—In order to determine whether the IR(C524S,C682S,C683S,C685S) mutant is present at the cell surface, varying amounts of the plasmids containing the wild type and mutant IR cDNAs were used to transfect CHO cells. After 48 h, the cells were assayed for insulin binding, and the results for the maximum binding are shown in Table I. The cells expressing IRWT had approximately twice the amount of surface IR compared with those expressing IR(C524S,C682S,C683S,C685S).

Two possibilities for the lower amount of insulin binding to the cells expressing mutant IR are impairment in transport of the receptor to the cell surface and lower insulin affinity of the receptor. In order to study the latter question, binding of 125I-insulin to crude membranes with the wild type and mutant receptors was analyzed by the method of Scatchard (Fig. 2). For wild type and mutant IRs, the dissociation constants were 5.6 × 10⁻⁹ M and 5.1 × 10⁻⁹ M, respectively, and the maximal insulin binding was 1.58 × 10⁻¹¹ and 1.12 × 10⁻¹¹ mol/μg of membrane, respectively. Interestingly, the plots for both receptors were curvilinear.

We conclude that the IR(C524S,C682S,C683S,C685S) mutant has a normal affinity for insulin but is defective in its ability to be delivered to the plasma membrane.

Insulin Binding Is Not Associated with Dimerization of IR(C524S,C682S,C683S,C685S) Monomers—There is considerable evidence that monomeric receptor tyrosine kinases dimerize in response to ligand binding (for reviews, see Refs. 14 and 15). Since the IR(C524S,C682S,C683S,C685S) mutant does not form covalent dimers, we determined the size of the receptor in the absence and the presence of insulin by its sedimentation behavior in a sucrose density gradient (9, 12). Crude membranes from COS7 cells expressing dimeric or monomeric insulin receptors were incubated with or without 20 nM insulin overnight at 4 °C after solubilization, followed by centrifugation at 48,000 rpm for 6 h in a linear 5–20% (w/v) sucrose gradient containing 0.1% Triton X-100 and 20 nM or no insulin. Fractions were collected and assayed for insulin binding to locate the insulin receptor. The results (Fig. 3) clearly show that the IRWT traveled at the position of the receptor dimer, whereas the mutant had the size of a receptor monomer. Insulin binding caused a slight increase in the sedimentation rate of both IRWT and IR(C524S,C682S,C683S,C685S) mutant, indicative of an insulin-dependent change in the shape of the receptors, consistent with
the results of Florke et al. (16). Insulin binding did not induce the dimerization of monomeric insulin receptors under these conditions.

**Autophosphorylation of the Insulin Receptor**—To determine whether the monomeric insulin receptor autophosphorylates in response to insulin, we treated intact CHO cells expressing the wild type and the mutant IR with insulin and measured the amount of phosphate bound to the β subunit. The level of expression of receptor dimers and monomers was approximately the same (Fig. 4A). To measure receptor phosphorylation, intact cells were incubated with insulin; then, the cell extracts were either directly separated by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 4B) or first immunoprecipitated with anti-phosphotyrosine antibody, and the precipitates were separated by SDS-PAGE and immunoblotted with anti-V5 antibody, which recognizes the V5 chain (Fig. 4C). The results clearly show that the receptor monomer had insulin-dependent phosphorylation and that the total amount of receptor dimer phosphorylation was twice that of the receptor monomer. This result is again compatible with the surface expression of IRWT and IRWT/C524S,C682S,C683S,C685S (Table I). It is also apparent that the amount of phosphorylation of the receptor monomer in the absence of insulin was significant.

This insulin-independent activation of the insulin receptor monomer could be a consequence of the monomorphic state or of a structural change of the receptor brought about by the mutations. We tried to answer this question by comparison of insulin-dependent autophosphorylation of the receptor dimers and monomers with that of receptors lacking only one set of disulfide bonds. Insulin receptor dimers are held together by two sets of disulfide bonds, one at position 524 and the other set at positions 682, 683, and 684. Hence, we constructed the mutants IRWT/C524S and IRWT/C682S,C683S,C685S and measured the amount of phosphate incorporated in intact cells by immunoprecipitation with anti-phosphotyrosine antibody followed by quantitation with anti-V5 antibody. The results (Fig. 5A) indicate that all of the mutants had considerable phosphorylation of the β chain in the absence of insulin, that there was insulin-independent phosphorylation in all cases, and that the stimulation by insulin was greatest for the IRWT, followed by IRWT/C524S, IRWT/C682S,C683S,C685S, and IRWT/C682S,C683S,C685S in that order. It is also important to notice that the total amount of receptor present in the cell (Fig. 5B) was not proportional to the amount of insulin binding on the cell surface (Table I), indicating that some of the mutants are impaired in their delivery to the plasma membrane. The conclusion is that the cysteine to serine mutations alter the structure of the insulin receptor so that
with Laemmli buffer containing 1% cDNA3.1-V5/His-LacZ control. The experiment is one of two independ-
ent experiments.

There is some insulin-independent activation of the receptor.

**Kinase Activity of the Insulin Receptor**—To determine whether the insulin receptor monomer has exogenous kinase activity, we asked whether it is capable of phosphorylating IRS-1, a substrate of the insulin receptor kinase (17). A plasmid containing the cDNA for IRS-1 (17) was cotransfected with plasmids containing the cDNA for IRWT or IRC524S,C682S,C683S,C685S into CHO cells. After 48 h, the insulin-dependent phosphorylation of IRS-1 was assayed. Fig. 6 shows that IRS-1 underwent insulin-dependent phosphorylation by both insulin receptor dimer and monomer. The level of phosphorylation by the receptor dimer was approximately twice that by the receptor monomer, as was seen for receptor auto-phosphorylation. In addition, there was phosphorylation of IRS-1 even in the absence of insulin, confirming the finding that some of the expressed insulin receptor is present in the activated state even in the absence of insulin.

**Autophosphorylation of the Solubilized Insulin Receptor**—In order to determine whether the insulin receptor monomer has kinase activity when it is in the monomeric state, the membranes were solubilized with Triton X-100. Under these conditions, the insulin receptor monomers are monomeric (Fig. 3). As is shown in Fig. 7 for receptors expressed in CHO cells, the extent of autophosphorylation of the insulin receptor monomer in the monomeric state was greatly reduced compared with the level obtained when the insulin receptor monomer is in the membrane. The amount of autophosphorylation was approximately one-fourth to one-sixth of that of the insulin receptor dimer. The bottom panel of Fig. 7 shows that the amounts of β chain and proreceptor in each of the samples are equivalent. This result suggests that insulin receptor monomers in the monomeric state have reduced kinase activity compared with that of the native insulin receptor dimers as well as compared with that of the insulin receptor monomers that are in the membrane. Presumably, membrane-bound insulin receptor monomers are present at a sufficiently high concentration to form noncovalent dimers.

To test this hypothesis, we determined whether membrane-bound receptor monomers could be cross-linked to dimers in the presence of insulin. The membranes from cells expressing dimeric or monomeric insulin receptors were incubated with 125I-insulin overnight in the absence or presence of excess unlabeled insulin. The membranes were then exposed to EGS to bring about insulin-α chain and α-chain-α chain cross-linking (13). The membranes were then subjected to SDS-PAGE under reducing conditions to obtain α chain monomers and dimers, and the gel was analyzed with a Bio-Rad imager. As can be seen in Fig. 8, α-chain monomers and dimers bound to 125I-insulin (lanes 1 and 3) are present in both receptor dimer and monomer lanes. A greater percentage of the α chain can be covalently dimerized in the receptor dimer than in the receptor monomer (62 versus 29%), as might be expected since in the dimer the α chains are kept close to one another by the class I disulfide bonds. Nevertheless, even the receptor monomers are capable of forming noncovalent dimers at the concentrations present in the membranes during the overexpression protocol.

We conclude that the kinase activity of the receptor monomer in the monomeric state is substantially lower than that of the receptor dimer.

**DISCUSSION**

The results shown in Fig. 1 clearly indicate that only the insulin receptor in which all four Cys residues, 524, 682, 683, and 685, are mutated to Ser is present exclusively as a monomer. All of the other mutants with only three mutations of these residues are present as a mixture of dimers and monomers, as is the IRC524S,C682S/C683S,C685S mutant previously described. We conclude that, in addition to Cys524, all three of the vicinal Cys residues in the 682–685 region are involved in the class I disulfides.

N-Ethylmaleimide labeling of receptor monomers produced by mild reduction of native insulin receptor with dithiothreitol indicated that at least two cysteine residues are involved in class I disulfides (4, 5). One of them was identified as Cys524 (6), whereas the other residue(s) could not be identified by labeling but was localized to the triplet at positions 682, 683, and 685 (10). Since it is apparent now that only mutation of all four Cys residues produces receptor monomers, it remains unclear why the labeling results were different. Possibly, the vicinity of the cysteine residues at positions 682, 683, and 685 allowed intra-chain disulfide bond formation so that only one of the three remained free to react with N-ethylmaleimide. It is also apparent how receptor monomers are formed in the partial mutants; possibly, the disulfide bonds between Cys682, Cys683, and Cys685 are not between homologous residues.

One clear result is the decrease in the surface expression of the IR524S,C682S,C683S,C685S mutant compared with IRWT (Table 1). Two possible explanations are that the receptor monomer is hindered in its passage from the endoplasmic reticulum to the plasma membrane by the quality control system and that the receptor is toxic to the cell. The latter possibility is supported in part by the observation that even in the absence of insulin there is partial activation of the receptor monomer (Figs. 4–7).

Since it is evident that the IR524S,C682S,C683S,C685S mutant is a monomer when examined by SDS-PAGE (Fig. 1A) and by velocity sedimentation after solubilization by detergent (Fig. 3), the curvilinear Scatchard plot (Fig. 2) of the mutant, resembling that of the IRWT, presents a problem. If the IR524S,C682S,C683S,C685S mutant is monomeric in the membrane, the two binding components represent some heterogeneity. If instead the receptor monomer is dimeric in the membrane, the curvilinear plot may represent the same negative cooperativity of the native dimeric receptor (18). Negative cooperativity is not easily understood in light of the structural...
The importance of dimerization for the activation of monomeric receptor tyrosine kinases (e.g. the EGF receptor) has been extensively described (14, 15). Strangely, the insulin receptor exists as a dimer in which the monomers are covalently linked by disulfide bonds. One possibility for this arrangement is that insulin receptor monomers are incapable of dimerization even in the presence of insulin. Another possibility is that the monomer-monomer interactions are sufficiently weak so that at the concentration of native insulin receptor in the membrane, only a small fraction of the receptor monomers dimerize. The second possibility is supported by the evidence that the solubilized IR<sub>C682S,C685S</sub> mutant is defective in insulin-dependent autophosphorylation (Fig. 7) when it is monomeric (Fig. 3), whereas it is capable of insulin-dependent autophosphorylation and exogenous kinase activity on IRS-1 in cells (Figs. 4–7), where it is partially dimeric (Fig. 8).

We conclude that insulin receptor monomers are incapable of autophosphorylation in the monomeric state; however, noncovalent dimerization and autophosphorylation can take place in the plasma membrane when the concentration of receptor monomers is sufficiently high.

It seems apparent now that the results in our previous paper on the dimerization of the IR<sup>C524S,C682S</sup> mutant after incubation with insulin (9) may have been obtained by redistribution of the remaining disulfide bonds to produce disulfide-linked dimers. This is another reason that we believe that all three cysteine residues at positions 682, 683, and 685 are involved in the class I disulfide bonds.

The conclusion that the insulin receptor monomer in the monomeric state has negligible kinase activity supports considerable work, all done with dithiothreitol-reduced insulin receptors, by the groups of Pilch and Pessin (20–25). The Pilch group showed that receptor monomers could be separated from receptor dimers by sedimentation in a density gradient and that the monomers had reduced autophosphorylation activity compared with the native dimers (20). They further demonstrated that insulin receptor monomers could undergo autophosphorylation at a receptor concentration sufficient to allow dimerization (21). Although the dimerization was not shown, its involvement was suggested by studies on the concentration dependence of insulin receptor autophosphorylation (22). On the other hand, the group of Pessin presented studies on insulin receptor monomers which were interpreted as showing that the monomers only had autophosphorylation activity if they formed covalently associated (23, 24) or noncovalently associated receptor dimers (25). These studies were not straightforward, however, because the putative size of the insulin receptors was determined by gel filtration, which does not separate monomers and dimers. In any event, the conclusion reached by both groups was that insulin receptor monomers are not as competent to autophosphorylate as are receptor dimers.

The conclusions of the earlier work by Pilch and Pessin and of that presented here, that insulin receptor dimers are required for insulin-dependent receptor autophosphorylation, are supported by the finding that even the soluble insulin receptor cDNA, and members of the laboratory, especially Alison Grinthal, for discussions.
Construction and Characterization of a Monomeric Insulin Receptor
James Jianping Wu and Guido Guidotti

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