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Identification of the Cysteine Residues Involved in the Class I Disulfide Bonds of the Human Insulin Receptor: Properties of Insulin Receptor Monomers

Kuanghui Lu and Guido Guidotti*

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

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The cysteine residues involved in the class I disulfide bonds between the α subunits in the $(\alpha\beta)_2$ dimer of the human insulin receptor have been identified by labeling with *N*-ethylmaleimide and by site-directed mutagenesis. Both cysteine 524 and cysteine 682 form interchain disulfide bonds; their conversion to serine residues results in the absence of receptor dimers and the presence of $\alpha\beta$ monomers. The receptor monomers have a slightly lower affinity for insulin than the native receptor dimers. Insulin binding to the receptor monomers promotes their dimerization in the plasma membrane; at nanomolar concentrations of receptor, both unliganded and liganded receptors are monomers. Receptor monomers are stimulated by insulin to autophosphorylate and to phosphorylate exogenous subtrates with the same efficiency as the receptor dimers. The conclusion is that receptor dimerization is not required to activate the tyrosine kinase activity of the insulin receptor.

INTRODUCTION

The insulin and the epidermal growth factor (EGF) receptors are receptor tyrosine kinases with similar structures: a large extracellular ligand-binding domain, a single transmembrane region, and a cytoplasmic portion with a conserved protein tyrosine kinase domain (Ullrich and Schlessinger, 1990). In contrast to the EGF receptor, which is monomeric and dimerizes upon ligand binding (Yarden and Schlessinger, 1987; Ullrich and Schlessinger, 1990), the insulin receptor exists as a disulfide-linked dimer composed of two $\alpha\beta$ monomers. The two α chains are entirely extracellular and are held together by class I disulfide bonds, which are easily reduced by dithiothreitol (DTT). The β chains are linked to the α chains by class II disulfide bonds, which are highly resistant to reduction (Massague and Czech, 1982; White and Kahn, 1994). The significance of the covalent dimeric state of the insulin receptor remains unclear.

Binding of insulin to the extracellular α chains is thought to cause a conformational change within the quaternary structure of the receptor that leads to autophosphorylation of the β chains (White and Kahn, 1994). How the signal travels across the membrane to the cytoplasm through a single transmembrane domain is not yet understood. Ligand-induced receptor dimerization has been proposed to be a general mechanism for receptor activation (Schlessinger and Ullrich, 1992; Lemmon and Schlessinger, 1994). This model was initially proposed for activation of the EGF receptor (Schlessinger, 1988), and subsequently supported by the observations with other monomeric tyrosine kinase receptors, such as the platelet-derived growth factor (Ueno *et al.*, 1991) and the fibroblast growth factor (Amaya *et al.*, 1991) receptors. Whether or not the dimeric state of the insulin receptor is a prerequisite for the insulin-induced receptor kinase activation has been the subject of debate. Although some studies reported that it was necessary to have monomer-monomer interactions to form either covalently associated (Boni-Schnetzler *et al.*, 1986; Sweet *et al.*, 1987b; Morrison *et al.*, 1988) or noncovalent (Boni-Schnetzler *et al.*, 1988; Wilden *et al.*, 1989) dimers to measure kinase activation, other studies suggested that the monomers had tyrosine kinase activity (Mortensen *et al.*, 1991). The main reason for the contradictory observations is the difficulty in producing

* Corresponding author.

ideal functional monomers: all previous work involved receptor monomers obtained by mild reduction of the class I disulfides. These conditions are likely to produce a heterogeneous population of molecules with several other disulfides reduced in addition to the class I disulfides. Indeed, our earlier work has indicated that several other peptides of the α chain of the insulin receptor, in addition to those containing the class I cysteine residues, are labeled with [^{3}H]NEM under mild reducing conditions. To answer clearly the question as to whether the insulin receptor monomer has tyrosine kinase activity, it is necessary to identify the cysteine residues involved in the class I disulfide bonds and to replace them with similar amino acids that cannot link the monomers.

It is known that Cys 647 is involved in the class II disulfide bond, which links the α and β chains. When this residue was replaced with Ser, α_2 species were detected by affinity labeling (Cheatham and Kahn, 1992). In spite of several studies (Frias and Waugh, 1989; Waugh *et al.*, 1989; Xu *et al.*, 1990), the cysteine residues of the class I disulfides have not been identified, although a minimum of two cysteine residues per α chain are labeled under reducing conditions that generate receptor monomers, suggesting the existence of two class I disulfide bonds (Finn *et al.*, 1990; Chiacchia, 1991). We have identified the major tryptic peptide labeled with NEM under these conditions; it has the sequence EAPYQNVTEFDGQDACGSNSWTVVDIDPPLR, corresponding to that of residues 509–539 of the α chain, indicating that Cys 524 is likely to be one of the residues involved in the α-α linkage (our unpublished results). When expressed in Chinese hamster ovary cells, the mutated insulin receptor in which Cys 524 is changed to Ser is present as $(\alpha\beta)_2$ dimers, demonstrating that either Cys 524 is not part of the class I disulfides or Cys 524 and another cysteine residue make up the class I disulfides. The potential involvement of Cys 524 in the class I disulfides has also been reported by other groups (Scaffer and Ljungqvist, 1992; Bilan and Yip, 1994; Macaulay *et al.*, 1994).

In this work we show that Cys 524 and 682 are the two residues responsible for the class I disulfides. Receptors with both C524S and C682S mutations are present as receptor monomers on SDS-PAGE and bind insulin. The equilibrium between monomers and dimers in the membrane depends on the insulin concentration. At room temperature, receptor monomers have insulin-stimulated tyrosine kinase activity and the activity is independent of the receptor concentration.

MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Co (St. Louis, MO). [γ-^{32}P]ATP (6000 Ci/mmol), [^{125}I]Tyr^{A14}-insulin (porcine, 2000 Ci/mmol), and [^{125}I]-goat anti-rabbit immunoglobulin G (IgG) (8.4 μCi/μg) were obtained from DuPont-New England Nuclear (Boston, MA). Porcine insulin was from Calbiochem (La Jolla, CA). Anti-insulin antibodies were the generous gift of Dr. William Brennan (Pennsylvania State University College of Medicine, Hershey, PA).

Site-directed Mutagenesis

Site-directed mutants were constructed by the method of Kunkel *et al.* (1987). A 2.4-kb *Bam*HI–*Xba*I fragment from human insulin receptor (HIR) cDNA, contained in the pECE expression vector and designated as peT (Ellis *et al.*, 1986), was cloned into pBlueScript (pBS/KS+) phagemid (Stratagene, La Jolla, CA). Oligonucleotides complementary to the coding strand sequence were used as mismatched primers to introduce double Cys to Ser mutations (at Cys 682 and 683, with primer 5′-TGG ACA GGA GCT GCT TTC GCC GGC-3′, and at Cys 682 and 685, with primer 5′-CTT TGG ACA GGA GCA GCT TTC GCC-3′) or a single Cys to Ser mutation (at Cys 682, with primer 5′-ACA GGA GCA GCT TTC GCC GGC-3′). Phagemids carrying the mutations of interest were initially screened by the disappearance of a unique *Bsm*I site as a result of the first nucleotide change at codon 682 (T to A). The mutated sequence was confirmed by dideoxy sequencing of dsDNA. The *Bam*HI–*Xba*I fragment containing the mutations C682S and C683S, C682S and C685S, or C682S was ligated to a 2.0-kb *Hin*dIII–*Bam*HI fragment containing the C524S mutation, previously made here (our unpublished experiments). The 4.4-kb *Hin*dIII–*Xba*I full length mutant HIR cDNA was reintroduced into the pECE expression vector. The mutants were designated IR^{C524,682,683S}, IR^{C524,682,685S}, and IR^{C524,682S}.

Transfection and Cell Culture

pECE vectors containing wild-type or mutant insulin receptor cDNAs were initially introduced into Cos7 cells for transient expression using the DEAE-dextran transfection method. Cos7 cells (5 $\times$ 10^5/100 mm plate) were incubated with 1.6 ml of transfection mixture (0.8 ml of serum-free DMEM containing 8 μg of purified plasmid plus 0.8 ml of 1 mg/ml DEAE-dextran in 140 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 5 mM KCl) for 45 min at 37°C followed by a 3-h treatment with 100 μg/ml chloroquine in 10% fetal bovine serum. After 48–72 h, cells were analyzed for gene expression by immunoblot or insulin binding.

Cos7 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine in a humidified atmosphere of 5% CO_2 at 37°C.

Immunoblotting

Forty micrograms of crude membrane preparations were added to 3× Laemmli sample buffer containing 60 mM NEM and were boiled for 30 s before loading onto the gel. The samples were separated on 5% polyacrylamide gels, transferred to a nitrocellulose filter, and probed with an anti-β subunit antibody and [^{125}I]-anti-rabbit IgG.

Insulin Binding Assay

[^{125}I]-insulin binding to whole cells was done in a 6-well 35-mm culture plate. Cos7 cells expressing IR^{WT} or IR^{C524,682S} were washed three times with ice-cold phosphate-buffered saline (PBS). [^{125}I]-insulin (50 pM, 25,000 cpm/ml) was incubated with the cells for 90 min at 15°C with or without unlabeled insulin in binding buffer containing 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 10 mM glucose, 15 mM NaAc, and 0.1% bovine serum albumin (BSA). Cells were washed three times with ice-cold PBS, solubilized in 0.1% SDS, and counted in a gamma counter.

Insulin binding for the velocity sedimentation experiments was done with the following changes. Cos7 cells, grown on 100-mm plates, were washed three times with ice-cold PBS and incubated with different concentrations of unlabeled insulin (as indicated in the text) for 12 h at 4°C in the binding buffer. Cells were solubilized with the procedure indicated below (cell lysis).

The dissociation constant for insulin was measured with solubilized receptor. Cell lysate (2 μl) was added to 98 μl 50 mM HEPES buffer (pH 7.6) and incubated with increasing concentration of [^{125}I]-insulin from 50 pM to 1 nM for 1 h at 22°C in a buffer containing Triton X-100, 50 mM HEPES (pH 7.6), 10 mM $MgCl_2$, 5 mM EDTA, 0.1% BSA in a total volume of 200 μl (final Triton concentration, 0.06%). Nonspecific binding with each concentration of radioactive insulin was determined by the addition of 1 μM unlabeled insulin. The insulin-receptor complexes were precipitated by addition of 60 μl of 0.4% bovine gamma-globulin and 260 μl of 20% (w/v) polyethylene glycol, followed by incubation for 10 min on ice. After centrifugation for 10 min, the supernatants were discarded and the pellets were rinsed and counted in a gamma counter.

Cell Lysis and Membrane Protein Extraction

Cos7 cells expressing IR^{WT} or $IR^{C524,682S}$ in 100-mm plates were rinsed three times with ice-cold PBS. Cells were lysed by rocking the plates at 4°C for 20 min in 0.5 ml lysis buffer containing 1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 10 μg/ml chymostatin/pepstatin A, 0.5 μM leupeptin, and 1 μg/ml aprotinin. The lysates were centrifuged at 4°C for 10 min at maximum speed in a microfuge. The supernatants were used immediately or quick frozen in dry ice/acetone and stored at −70°. Protein concentration was determined by the method of Lowry *et al.* (1951).

Velocity Sedimentation

This procedure was performed as described (Aiyer, 1983) with some minor modifications. Briefly, two marker enzymes, catalase (final concentration, 500 U/ml) and lactate dehydrogenase (final concentration, 60 U/ml) were added to the cell lysate solubilized with 1% Triton X-100. An aliquot of 350 μl was layered onto 4.6 ml of a linear 5–20% (w/v) sucrose gradient containing 0.1% Triton, 85 mM Tris-sulfate (pH 7.8), 30 mM sodium sulfate, 10 mM glucose, and 1 mg/ml BSA. For experiments studying the distribution of insulin-bound receptors, insulin was added to the gradient at the same concentration as that used in the incubation with whole cells. The samples were centrifuged for 6 h at 48,000 rpm at 4°C in a Beckman SW 50.1 rotor (Fullerton, CA). Fractions of 200–250 μL were collected from the bottom of the tubes and analyzed for insulin binding by the polyethylene glycol precipitation method; the positions of the marker enzymes were determined by assay of their enzymatic activity according to the method of Clarke (1975).

Receptor Autophosphorylation

Diluted cell lysate, 30 μl, was added to 420 μl of binding buffer containing 50 mM HEPES, pH 7.6, 0.06% Triton X-100, 5 mM $MgCl_2$, and 2.5 mM EDTA and incubated with or without insulin (the concentration is indicated in the text) at 22°C for 30 min. Phosphorylation was started by adding 50 μl of reaction buffer to give a final concentration of 5 μM ATP containing 2–20 μCi of [^{32}P-γ]ATP (~0.4 or 8 Ci/mmol depending on the experiment), 2 mM $MnCl_2$, and 10 mM $MgCl_2$. After 10 min, the reaction was terminated by either precipitation with polyethylene glycol or with trichloroacetic acid (TCA).

Polyethylene glycol precipitation was done by addition of 150 μl of 0.4% bovine gamma-globulin and 650 μl of 20% (w/v) polyethylene glycol to the phosphorylation samples; after 10 min on ice, the samples were centrifuged for 10 min in a Beckman microfuge. The pellets were solubilized in 60 μl of Laemmli sample buffer containing 100 mM DTT. The samples were boiled and analyzed on 7.5% SDS-PAGE.

For TCA precipitation, 56 μl of ice-cold 50% TCA containing 10 mM ATP and 10 mM H_3PO_4 were added to the samples. After incubation on ice for 10 min, the samples were centrifuged in a Beckman microfuge for 5 min. The pellets were washed three times with 1 ml ice-cold 5% TCA and then dissolved in 2.5% SDS/1N NaOH and counted in liquid scintillation Formula 989.

For the kinetics of autophosphorylation, aliquots of cell lysates containing 160 fmol or 32 fmol of insulin receptors (insulin binding activity) were incubated with 100 nM insulin or buffer alone in a total volume of 90 μl for 30 min at room temperature, followed by addition of 10 μl of phosphorylation mixture. The reactions were stopped by addition of 50 μl of 3× Laemmli sample buffer containing 15% 2-mercaptoethanol. The samples were separated on 7.5% SDS-PAGE and examined by autoradiography. The regions of the gels corresponding to the 95-kDa band were excised, incubated in 1 ml of Solvable (Du Pont-New England Nuclear) at 55°C for 3 h, and counted with a liquid scintillation spectrophotometer.

RESULTS

Localization of Class I Disulfides

The identification of the cysteine residues involved in the class I disulfide bonds started with a comparison of the primary structures of the α chain of the HIR and the extracellular domain of the EGF receptor (Ullrich *et al.*, 1984; Ebina *et al.*, 1985). The supposition was that the most likely candidates for the relevant cysteine residues should be those that are uniquely present in the α chain of the HIR. Of the 37 cysteine residues in the α chain, those at positions 524, 647, 682, 683, and 685 are not present at similar positions in the EGF receptor. Because Cys 647 has been shown to be responsible for the class II disulfide bond between the α and β chains (Cheatham and Kahn, 1992), we were left with a choice of one or more of four remaining cysteine residues, all of which are also present in the primary structure of the insulin growth factor-1 receptor, which also exists as disulfide-linked $(\alpha\beta)_2$ dimers (Ullrich *et al.*, 1986).

In other work (our unpublished results), we have identified Cys524 as the residue principally labeled with NEM after mild reduction of the HIR with DTT. Accordingly, we expressed the mutant HIR C524S in Cos7 cells and found that the IR is present as $(\alpha\beta)_2$ dimers, indicating that Cys 524 is not the only residue involved in the class I disulfides. A similar result was also reported by Schaffer and Ljungqvist (1992), Macaulay *et al.* (1994), and Bilan and Yip (1994).

Because Cheatam and Kahn (1992) had demonstrated that the triple mutant $IR^{C682S,C683S,C685S}$ was still an $(\alpha\beta)2$ dimer under nonreducing conditions, we concluded that the class I disulfides were most likely a combination of Cys524 and one or more of Cys682, Cys 683, and Cys 685. Accordingly, two triple mutants were then constructed, $IR^{C524S,C682S,C683S}$ and $IR^{C524S,C682S,C685S}$. The two mutants share the C524S and C682S mutations, and each has an additional and different cysteine substitution. Expression of the mutant IRs in Cos7 cells was examined by nonreducing SDS-PAGE done on the crude membranes followed by

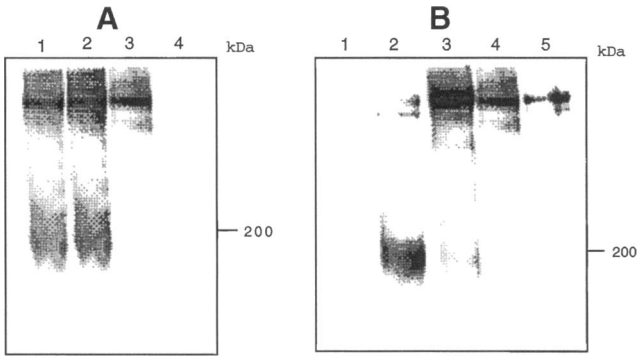

Figure 1. Immunoblots of insulin receptors. Mutant and wild-type insulin receptors were transiently expressed in Cos7 cells. Crude membrane preparations were separated on 5% nonreducing SDS-PAGE, transferred onto nitrocellulose, and probed with rabbit anti-IR β subunit antibodies followed by goat anti-rabbit [^{125}I]IgG. (A) $IR^{C524S,C682S,C685S}$ (lane 1), $IR^{C524S,C682S,C683S}$ (lane 2), IR^{WT} (lane 3), and untransfected Cos7 cells (lane 4). (B) Untransfected Cos7 cells (lane 1), $IR^{C524S,C682S}$ (lane 2), IR^{C682S} (lane 3), IR^{C524S} (lane 4), and IR^{WT} (lane 5).

immunoblotting with an anti-β subunit antibody (Figure 1A, lanes 1 and 2). Two bands with apparent masses of 350 kDa and 195 kDa, corresponding to the sizes of the $(\alpha\beta)_2$ dimer and $\alpha\beta$ monomer, respectively, are present in each lane. In contrast, when the normal IR is expressed only the 350-kDa band is obtained (Figure 1A, lane 3), while there is no IR visible in the untransfected cells (Figure 1A, lane 4). The presence of abundant $\alpha\beta$ monomers in the cells expressing the two receptors with triple mutations pointed to Cys524 and Cys682, mutated in both mutants, as forming the class I disulfides. The presence of $(\alpha\beta)_2$ dimers in the two triple mutant preparations can be explained by the subversion of Cys683 and Cys685 from intrachain disulfide bonds to an interchain disulfide bond when the intrachain partner is replaced by Ser.

On the basis of these considerations, we constructed the C524S and C682S double mutant and the C682S single mutant cDNAs and transfected them into Cos7 cells. As can be seen in Figure 1B, the $IR^{C5245,C682S}$ mutant (Figure 1B, lane 2) is present principally as an $\alpha\beta$ monomer, whereas the two single mutants IR^{C682S} (Figure 1B, lane 3) and IR^{C524S} (Figure 1B, lane 4) mainly are $(\alpha\beta)_2$ dimers as is the IR^{WT} (Figure 1B, lane 5). These results indicate that both Cys524 and Cys682 are involved in the class I disulfides; mutation of only one of the cysteine residues does not result in monomer formation. There are small amounts of receptor dimer in the $IR^{C5245,C682S}$ mutant preparation and of receptor monomer in the IR^{C682S} preparation; we attribute the presence of these forms of the receptors to inappropriate processing of the mutated receptors allowing the formation of intermonomer disulfide bonds between alternative cysteine residues in one case and the formation of intrachain disulfide bonds with Cys524 in the other case.

Insulin Affinity

A central question is the insulin affinity of the receptor monomers compared with that of the covalent dimers.

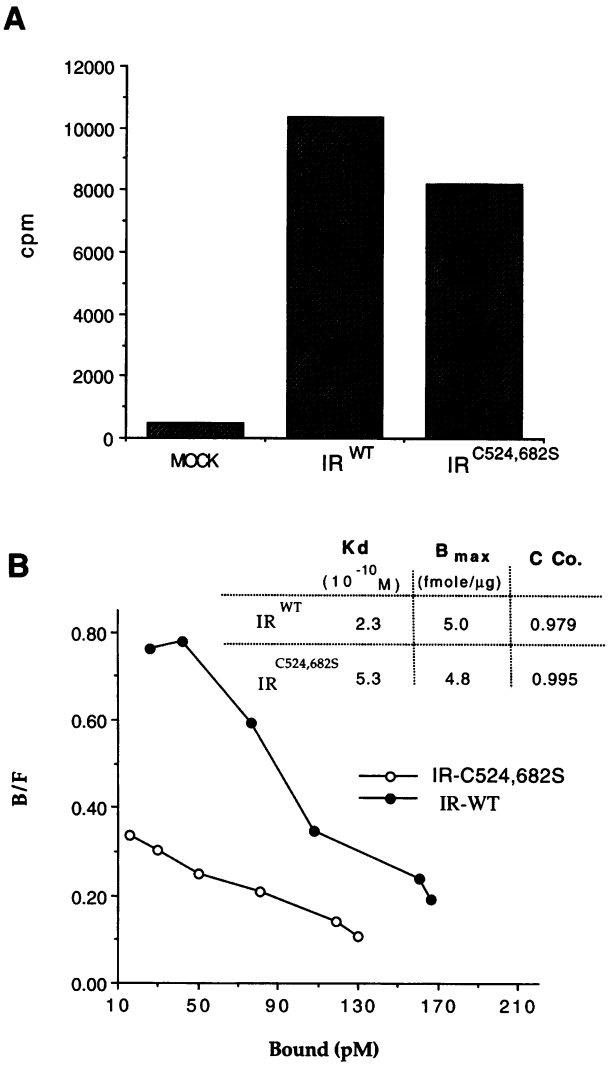

Figure 2. (A) [^{125}I]-insulin binding to Cos7 cells. Cos7 cells expressing IR^{WT} or $IR^{C524S,C682S}$ were incubated with 50 pM [^{125}I]-insulin in the presence and the absence of 1 μM unlabeled insulin for 90 min at 15°C. The cells were washed and then solubilized with 1% SDS; the radioactivity of the solution was measured in a gamma counter. (B) Scatchard analysis of insulin binding. Cells expressing IR^{WT} or $IR^{C524S,C682S}$ were solubilized with 1% Triton X-100 and the solution was diluted 100-fold with 0.06% Triton X-100. Duplicate aliquots were incubated with increasing concentrations of [^{125}I]-insulin from 50 pM to 1 nM for 1 h at room temperature followed by precipitation of the receptor with polyethylene glycol. Specific binding at each concentration of [^{125}I]-insulin was determined by subtraction of the radioactivity in companion tubes with 1 μM unlabeled insulin. The data were analyzed by the LIGAND program (Munson and Rodbard, 1980; McPherson, 1985).

Figure 2A shows that cells expressing IR^{WT} and $IR^{C524S,C682S}$ bind similar amounts of insulin (within 80% of one another) when incubated with 50 pM [^{125}I]-insulin, indicating that the mutant receptors are expressed at the cell surface and bind insulin.

The affinity of the receptors for insulin was determined with solubilized receptors rather than with cells because of the variability in the efficiency of transient expression in Cos cells. Figure 2B shows the results of insulin binding to solubilized IR^{WT} and $IR^{C524S,C682S}$ in 0.06% Triton X-100 expressed as Scatchard plots. Although the dissociation constant for insulin is slightly greater for the mutant receptor, K_d = 0.53 nM, as compared with that for the wild-type receptor, K_d = 0.23 nM, the number of binding sites per μg of protein (~5 fmol/μg) is the same for both receptors. There is no evidence of cooperativity or of heterogeneity of binding sites in these plots. The measured concentration of IR in this experiment (200 pM) indicates that the concentration of IR in the Cos cells is 200 nM (the cell pellet was dissolved in a 10-fold volume of 1% Triton X-100 and diluted 100-fold in 0.06% Triton X-100 buffer). Assuming that all the receptors are in the plasma membrane and that the plasma membrane protein is 1% of the total protein of the cell, the receptor concentration in the plasma membrane is 1200 molecules per μm^2. The average distance between receptor molecules in the plane of the membrane is about 30 nm, which corresponds to that between molecules in solution (three dimensions) when the concentration is 20 μM.

Triton X-100 was used at a concentration of 0.06% because higher concentrations inhibit insulin binding to the receptor, and have a much greater effect on the mutant $IR^{C524S,C682S}$ than on the IR^{WT}. Although 0.18% Triton X-100 causes only a small reduction in insulin binding, 0.3% detergent causes an 80% loss in binding to $IR^{C524S,C682S}$ (Figure 3). The deleterious effect of Triton X-100 on the mutant IR may be a consequence of the monomeric state of this receptor.

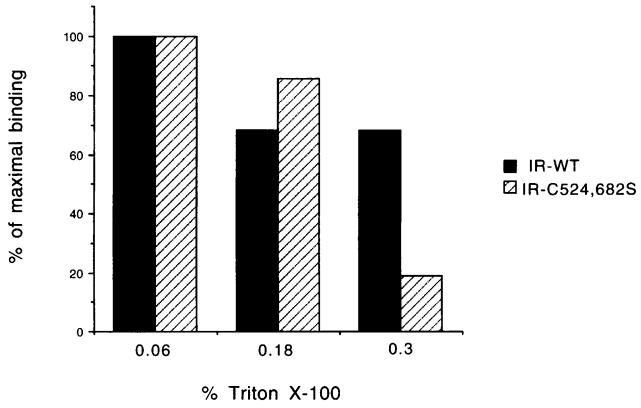

Figure 3. Effect of the concentration of Triton X-100 on insulin binding to solubilized insulin receptors. Solubilized IR^{WT} and $IR^{C524S,C682S}$ were incubated with 100 pM [^{125}I]-insulin for 1 h at room temperature in the binding buffer containing the indicated concentrations of Triton X-100, followed by precipitation of the insulin receptor-insulin complex with polyethylene glycol. Specific binding was determined by subtraction of the radioactivity in companion tubes containing 1 μM unlabeled insulin.

Interactions between Mutant Receptor Monomers

The $IR^{C524S,C682S}$ mutant, incapable of intermolecular covalent interactions, now resembles other monomeric receptors with a single transmembrane domain such as the EGF receptor (Yarden and Schlessinger, 1987), the platelet-derived growth factor receptor (Heldin *et al.*, 1989), the fibroblast growth factor receptor (Amaya *et al.*, 1991), and the human growth hormone receptor (Cunningham *et al.*, 1991), which have been shown to undergo ligand-induced receptor dimerization. The question here is whether or not insulin binding causes dimerization of $IR^{C524S,C682S}$. Accordingly, we examined the sedimentation properties of the receptors in sucrose density gradients; Aiyer (1983) has shown that the dimeric and monomeric IR are easily identified as peaks with sedimentation coefficients of 10.2S and 6.6S, respectively. This method was used because Aiyer (1983) showed that receptor monomers and dimers cannot be separated by gel filtration.

Insulin Induces Noncovalent Membrane-bound Receptor Dimerization

First, we studied the effect of insulin binding to the membrane-bound receptors; under this condition the receptor concentration is approximately 20 μM in the membrane and there is no detergent during the binding reaction. Cos7 cells expressing $IR^{C524S,C682S}$ or IR^{WT} were incubated with various concentrations of insulin overnight at 4C° in the binding buffer. The cells were solubilized with a 10-fold volume of 1% Triton X-100 and the monomers and dimers of the IR at a total concentration of 20 nM were separated by sucrose density gradient centrifugation. The location of the IR in the gradient was determined by [^{125}I]-insulin binding.

In cells transfected with IR^{WT} there is a single peak at the receptor dimer position whether or not the cells have been incubated with insulin (Figure 4A for the free IR^{WT}; the same pattern was obtained for the insulin-bound receptor). On the other hand, in the absence of insulin most of the mutant receptor is present as the monomer (Figure 4B). The small peak at the dimer position is mainly the endogenous insulin receptor from Cos7 cells. When cells expressing the mutant receptor were incubated with 10 nM insulin before solubilization, most of the receptor became dimeric (Figure 4C). Figure 4D shows the effect of the

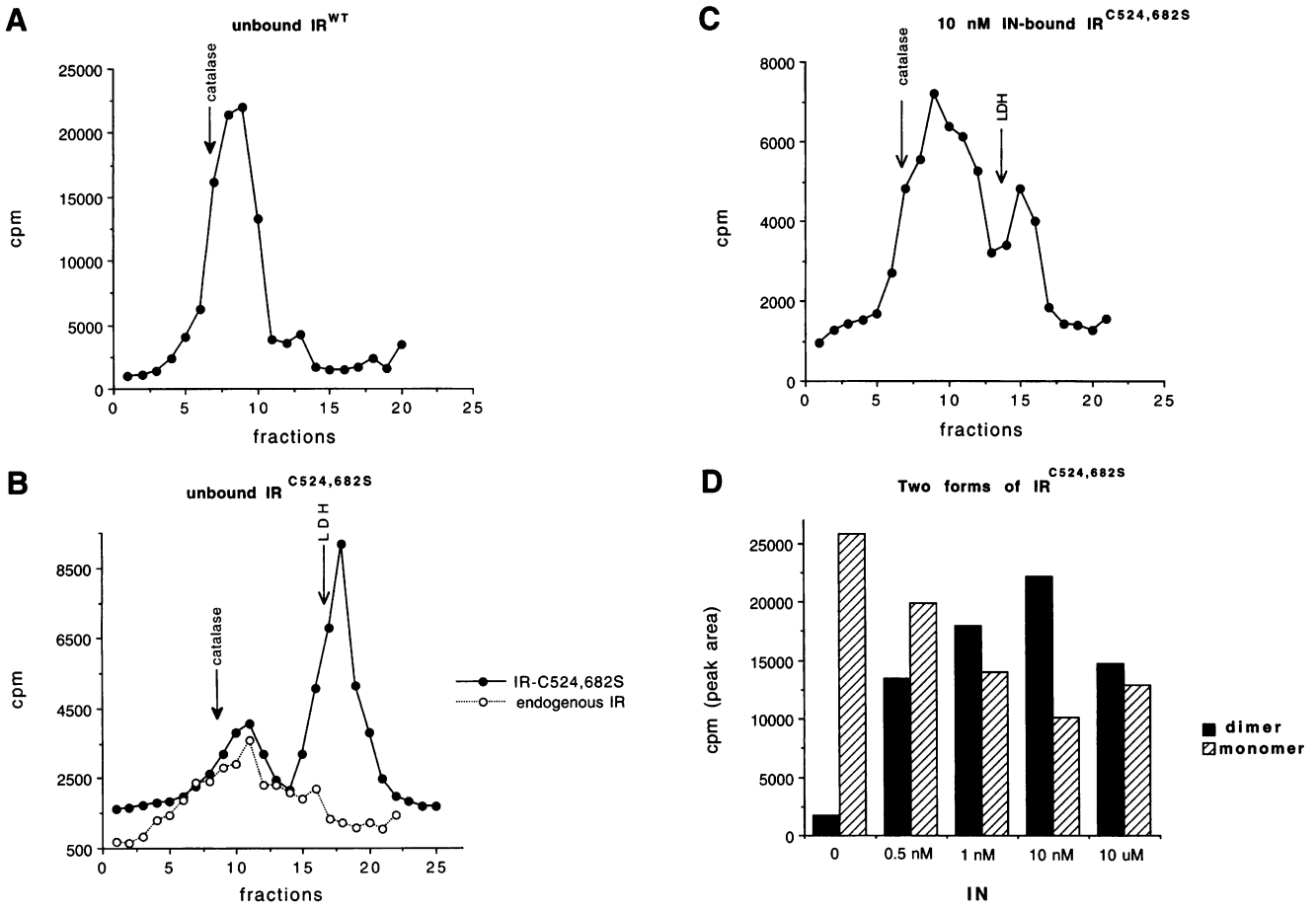

Figure 4. Velocity sedimentation of insulin receptors in Triton X-100. Cos7 cells grown in 100-mm plates were transfected with the cDNAs for IR^{WT} or $IR^{C524S,C682S}$. Forty-eight hours later, the cells were washed with cold PBS and incubated with the indicated concentrations of insulin overnight at 4°C in binding buffer. The cell lysates, made by solubilizing the cells with 1% Triton X-100, were separated on sucrose density gradients as described under MATERIALS AND METHODS. Each fraction was assayed for its ability to bind 200 pM [^{125}I]-insulin and for the presence of the marker enzyme activities. (A) Insulin-free IR^{WT}; (B) insulin-free $IR^{C524S,C682S}$ (closed circles) and endogenous IR of Cos 7 cells (open circles); (C) $IR^{C524S,C682S}$ incubated with 10 nM insulin; and (D) amounts of receptor dimers and monomers present after incubation of cells expressing $IR^{C524S,C682S}$ with different insulin concentrations. These data were obtained by adding the radioactivity present in the dimer and monomer peaks. The value for the endogenous IR of Cos7 cells has been subtracted from each dimer peak.

insulin concentration on the distribution of the IR between monomers and dimers in the membrane. The fraction of insulin binding sites present as dimers is 0.07 in the absence of insulin, 0.4 at 0.5 nM insulin, 0.56 at 1 nM insulin, 0.7 at 10 nM insulin, and 0.54 at 10 μM insulin.

This result supports the view that insulin induces dimerization of the IR (Boni-Schnetzler *et al.*, 1988; Chiacchia, 1988; Morrison *et al.*, 1988). Surprisingly, however, at the highest concentration of insulin a substantial fraction of the IR is still present as monomers, suggesting that the value of the dimer-monomer dissociation constant for the liganded receptors is close to 20 μM, the equivalent receptor concentration in the plasma membrane. One can reach the latter conclusion because in all the experiments the dimer and monomer peaks are distinct with no evidence of movement toward one another, indicating that the rate of equilibration between monomers and dimers is much slower, indeed negligible, compared with that of the separation in the centrifugal field. It appears that at 4°C, solvents containing Triton X-100 solubilize the receptor dimers and monomers present in the membrane and prevent further redistribution; hence, the pattern seen after sucrose density centrifugation reflects the situation in the membrane at the moment of solubilization.

To test this hypothesis, the solution of liganded receptors at a concentration of 20 nM, obtained from the cells incubated with 10 nM insulin, was diluted 20-fold to a concentration of 1 nM liganded receptor;

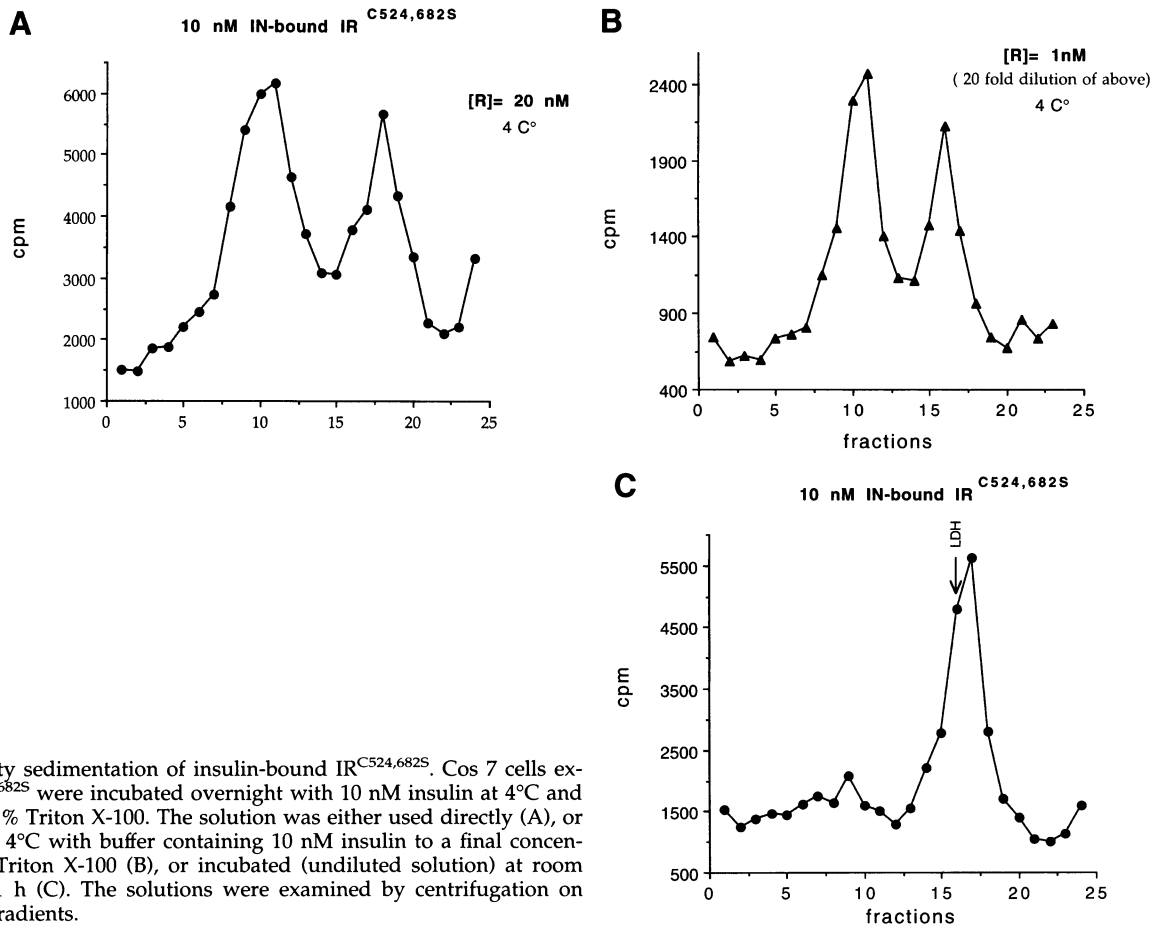

Figure 5. Velocity sedimentation of insulin-bound $IR^{C524,682S}$. Cos 7 cells expressing $IR^{C524S,C682S}$ were incubated overnight with 10 nM insulin at 4°C and solubilized with 1% Triton X-100. The solution was either used directly (A), or diluted 20-fold at 4°C with buffer containing 10 nM insulin to a final concentration of 0.06% Triton X-100 (B), or incubated (undiluted solution) at room temperature for 1 h (C). The solutions were examined by centrifugation on sucrose density gradients.

both solutions were examined by sucrose density centrifugation. Figure 5 shows that the distribution of receptor dimers and monomers is the same at receptor concentrations of 20 nM (Figure 5A) and 1 nM (Figure 5B), suggesting that the equilibrium is frozen in solutions of Triton X-100 at 4°C.

Receptor Dimers Dissociate to Monomers at Receptor Concentrations Less than 20 nM

If the monomer-dimer dissociation constant has a value equivalent to 20 μM, as indicated by the results in Figure 4D, only receptor monomers should be present at a receptor concentration of 20 nM under conditions where equilibration is not prevented. Accordingly, the solution containing receptor dimers (Figure 5A) was incubated at room temperature for 1 h in a buffer containing 10 nM insulin, to allow monomer-dimer equilibration, and then examined by sucrose density centrifugation. Only receptor monomers are present (Figure 5C), indicating that, indeed, insulin-bound receptor dimers at a concentration of 20 nM receptor become monomeric at a temperature that allows subunit equilibration in Triton X-100; thus, the K_d for the dissociation must be greater than 20 nM.

To confirm the proposition that after solubilization receptor monomers remain monomeric whether or not they are bound to insulin, receptors solubilized from insulin-free cells were diluted with 0.06% Triton X-100 to a concentration of 1 nM receptor before incubation with insulin. Figure 6, A and B, shows the patterns obtained with ligand-bound and ligand-free receptors, respectively, at 4°C; in both cases, only receptor monomers are present. Furthermore, liganded receptors remain monomeric at 25°C (Figure 6C) and also when incubated with the phosphorylation mixture containing ATP, $MgCl_2$, and $MnCl_2$ (Figure 6D). These results are particularly relevant, because they indicate that the binding curve shown in Figure 2B and the phosphorylation results presented below represent the properties of receptor monomers.

Monomeric Receptors Autophosphorylate

One of the central questions in this study is the kinase activity of the monomeric receptor. Because the mu-

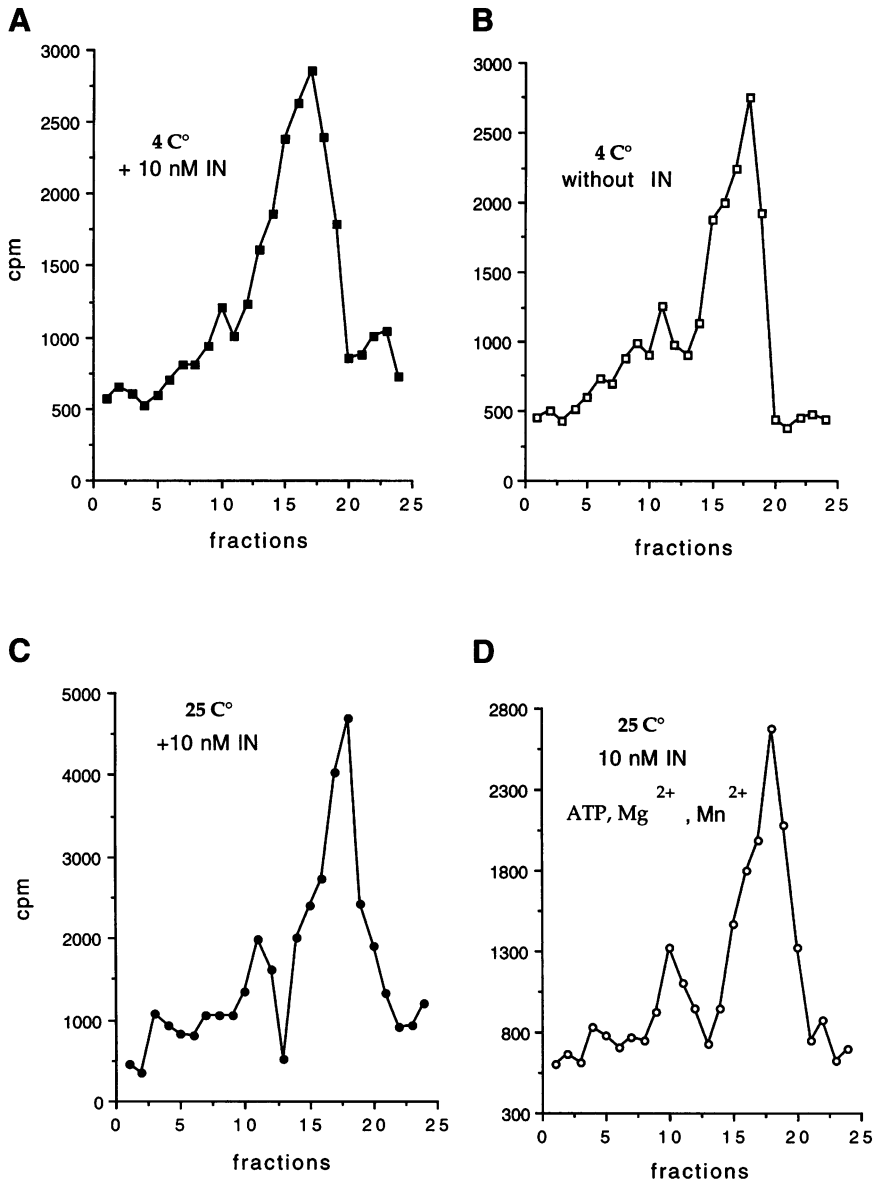

Figure 6. Velocity sedimentation of diluted insulin receptors. (A and B) Thirty microliters of the cell lysate containing $IR^{C524S,C682S}$ were diluted to a total volume of 500 μl (final Triton X-100 concentration of 0.06%) and incubated with 10 nM insulin (A) or buffer only (B) overnight at 4°C. The solutions were examined by sucrose density centrifugation. (C and D) Thirty microliters of the cell lysate containing $IR^{C524S,C682S}$ were diluted to a total volume of 500 μl (final Triton X-100 concentration of 0.06%) and incubated with 10 nM insulin (C) or with 10 nM insulin, 5 μM ATP, 10 mM $MgCl_2$, and 2 mM $MnCl_2$ (phosphorylation mixture) (D) for 1 h at 25°C. The solutions were examined by sucrose density centrifugation.

tant $IR^{C524S,C682S}$ is monomeric under phosphorylation conditions (Figure 6D), its ability to carry out insulin-dependent autophosphorylation was determined.

Solubilized insulin receptors, 0.32 nM, in 0.06% Triton X-100 were incubated with various insulin concentrations for 30 min at 22°C, and then the phosphorylation mixture containing [γ-^{32}P]ATP was added for 10 min. The radioactivity of the β chain was determined after reducing SDS-PAGE and is shown in Figure 7. Insulin stimulates dose-dependent receptor phosphorylation with both IR^{WT} (Figure 7A) and $IR^{C524S,C682S}$ (Figure 7B) with the maximal effect at 100 nM insulin, a concentration that causes greater than threefold stimulation of autophosphorylation. Higher concentrations of insulin (1 and 10 μM) caused a decrease in the extent of phosphorylation in both the normal and the mutant receptor. This result suggests that the monomeric mutant receptor has insulin-stimulated tyrosine kinase activity.

It might be argued that even though the mutant receptor is monomeric under the conditions of the assay, it has kinase activity only when the monomers transiently associate into dimers. In this case, the kinase activity of the mutant receptor should depend on the receptor concentration. Accordingly, we measured the rate of autophosphorylation with two different concentrations of IR^{WT} and $IR^{S524,682}$. Solubilized insulin receptors at concentrations of 1.6 and 0.32 nM were incubated with or without 100 nM insulin for 30 min at 22°C followed by autophosphorylation for various

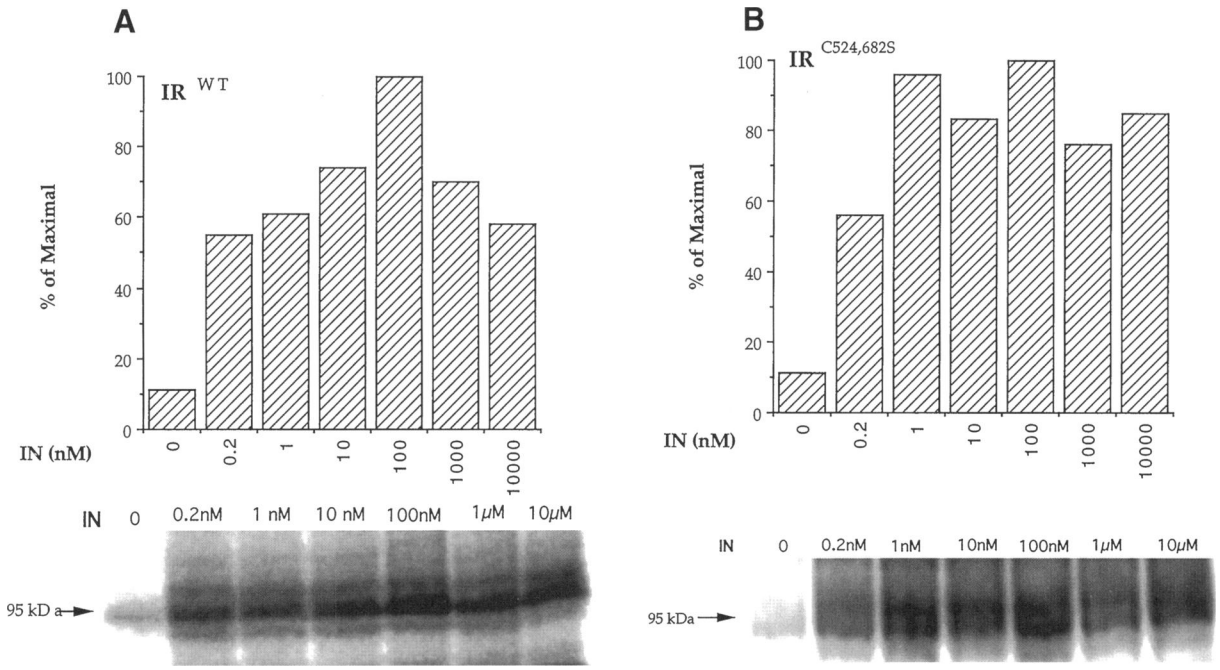

Figure 7. Autophosphorylation of solubilized IR^{WT} and $IR^{C524S,C682S}$. Diluted cell lysate (30 μl) was incubated with insulin at the indicated concentration for 30 min at room temperature followed by autophosphorylation performed as described in MATERIALS AND METHODS. The samples, precipitated with polyethylene glycol, were separated on reducing 7.5% SDS-PAGE. The radioactive image was produced by a BAS 2000 Bio-Imaging Analyzer; quantitation of the radioactivity of the 95-kDa region was done with a PDI scanner using the program Quantity One. (A) IR^{WT}; and (B) $IR^{C524S,C682S}$.

times. The results are shown in Figure 8; the rate of autophosphorylation is shown as the slope of each curve.

It is evident that a fivefold dilution of the receptor concentration has no effect on the specific rate of autophosphorylation of either the normal receptor (Figure 8, A and B) or the mutant receptor (Figure 8, C and D): the insulin-dependent rates of autophosphorylation are 49 and 10 fmol Pi/min for the normal receptor and 38 and 9.4 fmol Pi/min for the mutant receptor at concentrations of 1.6 and 0.32 nM, respectively. These rates correspond to addition of ~3 mol of phosphate per mole of receptor in 10 min in all situations. Although this result is expected with the normal receptor because the monomers are held together by the class I disulfides, the independence of the specific autophosphorylation rate of the $IR^{C524S,C682S}$ on the receptor concentration clearly indicates that the receptor monomer is fully active as a kinase.

To examine further the effect of receptor concentration on the behavior of the mutant receptor, we measured the rate of insulin-dependent exogenous phosphorylation as a function of receptor concentration. In these experiments, the rate of incorporation of phosphate in all the proteins in the cell extract was measured at receptor concentrations of 200 and 40 pM (Figure 9). It can be seen that the specific activity of the insulin-dependent phosphate incorporation is approximately the same for the mutant (Figure 9, A and B) and the normal (Figure 9, C and D) receptors at both concentrations of receptor. The insulin-dependent rate of phosphate incorporation here is 30 fmol Pi/per fmol receptor in 10 min. Clearly, there is no effect of receptor concentration on the rate of exogenous phosphorylation.

DISCUSSION

The central findings in this work are the identification of Cys 524 and 682 of the α chain of the insulin receptor as the residues involved in the class I disulfide bonds between αβ monomers and the demonstration that the αβ monomers are able to bind insulin and autophosphorylate as monomers.

Comparison of the primary structures of the insulin, insulin growth factor-1, and EGF receptors revealed that the most likely candidates for the class I disulfides were Cys 524, 682, 683, and 685, inasmuch as Cys 647 had been shown to be involved in the class II disulfide linking the α and β chains (Cheatam and Kahn, 1992). We first mutated Cys 524 alone, because it was the principal cysteine residue of the gently reduced receptor labeled with [^{3}H]NEM; the receptor was still present as a dimer, as is shown in Figure 1B, lane 4.

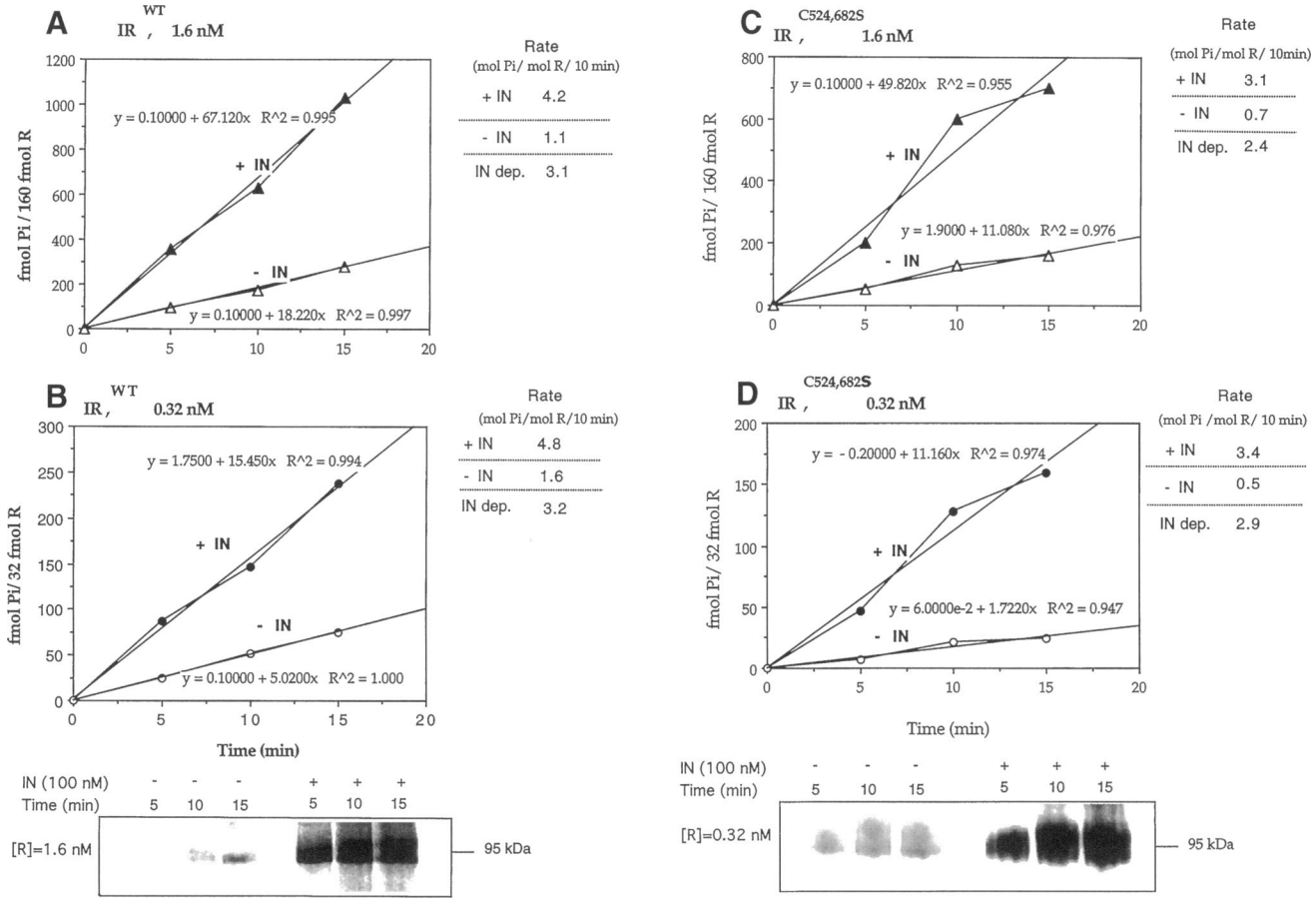

Figure 8. Kinetics of autophosphorylation of $IR^{C524S,C682S}$ and IR^{WT}. Volumes of cell lysates containing 160 fmol or 32 fmol of insulin receptors (insulin binding activity) were incubated with 100 nM insulin or buffer alone in a total volume of 90 μl for 30 min at room temperature, followed by addition of 10 μl of phosphorylation mixture. The reactions were stopped at the indicated times with 5× Laemmli sample buffer. The samples were subjected to 7.5% SDS-PAGE under reducing conditions and analyzed by autoradiography. The region of the gel corresponding to the 95-kDa band was excised and its radioactivity determined with a liquid scintillation spectrophotometer. (A and B) IR^{WT}, 160 fmol and 32 fmol, respectively; (C and D) $IR^{C524,682S}$, 160 fmol and 32 fmol, respectively. The slopes of the curves are in the units of fmol Pi/160 or 32 fmol receptor/min. The rate of phosphorylation is expressed as mol Pi/mol R/10 min. At the bottom of panels B and D are shown the autoradiographs corresponding to the data in panels A and D, respectively.

Accordingly, we tackled the three cysteine residues at positions 682, 683, and 685 (Figure 1A). Mutation of Cys 524 and 682 to Ser produced a monomeric receptor (Figure 1B, lane 2) that is expressed at the cell surface (Figure 2A) in amounts comparable to those of the wild-type receptor.

An unusual feature of this monomeric receptor, at a concentration of 200 pM, is its sensitivity to the concentration of Triton X-100; as can be seen in Figure 3, at detergent concentrations of 0.3% or greater there is nearly complete loss of insulin binding at 22°C. Although this phenomenon is not clearly rationalized, it is important to realize its existence to conduct experiments under the proper conditions.

The insulin affinity of the mutated monomeric receptor is approximately one-half of that of the wild-type receptor dimer (Figure 2B). This result is comparable to the observation of Boni-Schnetzler *et al.* (1986, 1987) that monomers obtained by reduction have one-third to one-sixth the affinity of the native receptor, and contrary to the finding of Sweet *et al.* (1987a) that the monomer has a 20-fold greater insulin affinity compared with that of the dimer.

In the plasma membrane, where the total receptor monomer concentration is equivalent to approximately 20 μM, insulin receptors are principally monomeric (Figure 4B); insulin binding promotes receptor dimerization (Figure 4C) and at saturation with ligand approximately half the receptor monomers have dimerized (Figure 4D). After solubilization, temperature and the detergent Triton X-100, in addition to the receptor concentration, affect the equilibrium between receptor monomers and dimers. At 4°C, the receptor monomers and dimers can associate and dissociate in

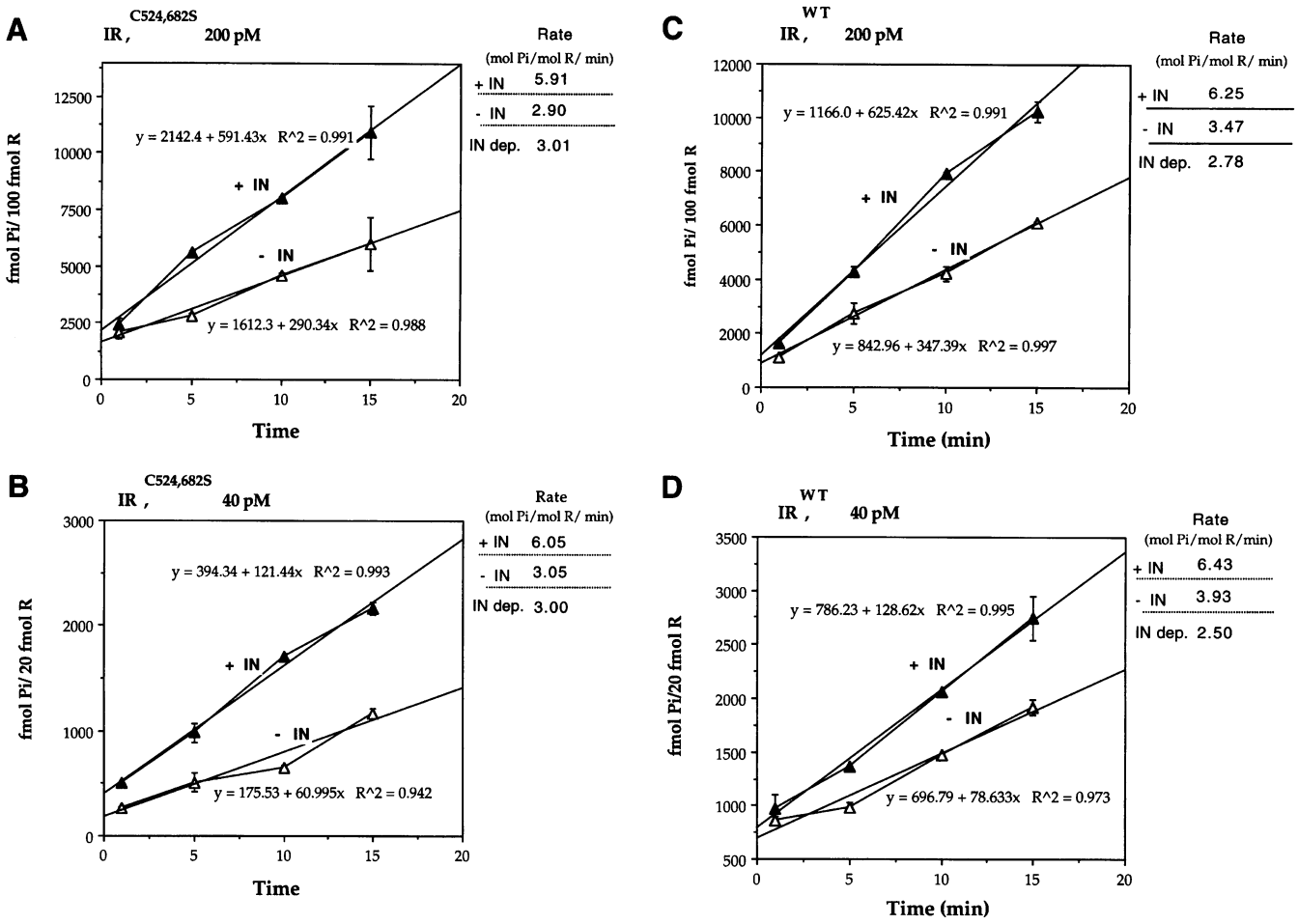

Figure 9. Kinetics of exogenous phosphorylation by $IR^{C524S,C682S}$ and IR^{WT}. Volumes of cell lysates containing 20 μg or 4 μg of insulin receptors were incubated with 100 nM insulin (filled triangles) or buffer alone (open triangles) in a total volume of 500 μl for 30 min at room temperature, followed by addition of the phosphorylation mixture. Samples were taken at the indicated times, the protein was precipitated with 5% TCA, and the radioactivity of the solubilized precipitate was determined with a liquid scintillation spectrophotometer. (A and B) $IR^{C524S,C682S}$, 200 pM and 40 pM, respectively. (C and D) IR^{WT}, 200 pM and 40 pM, respectively. The slopes of the curves are in the units of fmol Pi/100 fmol or 20 fmol receptor/min. The rates of phosphorylation are expressed as mol Pi/mol receptor/min. The results are the mean ± SE of three separate experiments performed in duplicates.

the membrane (Figure 4, B–D); once solubilized in Triton X-100 at 4°C, no further equilibration is possible (Figure 5, A and B). On the other hand, if the temperature of the solubilized receptors is increased to 22°C, subunit equilibration is established (Figure 5C). With this knowledge, it is reasonable to conclude that the receptor dimer-monomer dissociation constant for the mutated insulin receptor has a value of approximately 20 μM and that below 1 μM only receptor monomers are present (Figure 6). Because it is likely that the value of the dissociation constant for the native receptor is similar, we conclude that noncovalent dimers were not present in any of the experiments described by the groups of Pessin (Wilden *et al.*, 1989) and Pilch (Boni-Schnetzler *et al.*, 1988), who claimed that noncovalent dimerization of the reduced insulin receptor, at nM concentrations, is a prerequisite for the tyrosine kinase activity of the receptors under these conditions.

Another central result of this work is the clear evidence that receptor monomers are capable of autophosphorylation (Figure 7B), because at the receptor concentration used in this assay only receptor monomers are present (Figure 6, C and D) and furthermore there is no effect of the concentration of receptor on the kinase activity (Figure 8, C and D; Figure 9, A and B). The evidence for insulin-stimulated kinase activity of receptor monomers is readily compatible with the finding that isolated β chains (Villalba *et al.*, 1989) and truncated receptors (Shoelson *et al.*, 1991) also have kinase activity as monomers. This result, however, does not support the notion derived from examination of the crystal structure of the tyrosine kinase domain

that the binding of ATP and of "self" Tyr 1162 in the active site are mutually exclusive (Hubbard *et al.*, 1994). Clearly, insulin binding to the α chain can activate the kinase activity of the β chain in the $\alpha\beta$ monomer and receptor dimerization is not required for kinase activity.

These results support the only report in the literature that receptor monomers are capable of kinase activity (Mortensen *et al.*, 1991), while they are contrary to the voluminous information indicating that only receptor dimers have kinase activity (Boni-Schnetzler *et al.*, 1986, 1988; Sweet *et al.*, 1987b; Morrison *et al.*, 1988; Wilden *et al.*, 1989; Shoelson *et al.*, 1991). Our interpretation of this discrepancy is that in all the other studies, except for that of Mortensen *et al.* (1991), there were several problems as follows: 1) receptor monomers were compromised by the reduction reaction required to obtain monomers; 2) estimation of the presence of monomers and dimers was done by gel filtration, which does not resolve these species (Sweet *et al.*, 1987b; Morrison *et al.*, 1988; Wilden *et al.*, 1989); and 3) in cases where receptor monomers were found to have kinase activity (Boni-Schnetzler *et al.*, 1988; Wilden *et al.*, 1989), the presence of receptor dimers was implicated through the erroneous assumption that monomers do not have kinase activity.

What is the explanation, then, of the experiments with native receptor dimers composed of different monomers suggesting that intramolecular transphosphorylation of the β chains takes place predominantly (Frattali *et al.*, 1992; Frattali and Pessin, 1993; Lee *et al.*, 1993; Taouis *et al.*, 1994)? Our interpretation of these results, in light of the evidence that the receptor monomer is fully capable of insulin-stimulated kinase activity, is that in the receptor $(\alpha\beta)_2$ dimer the contacts between adjacent β chains inhibit *cis-* but allow *trans*phosphorylation. According to this view, insulin receptor dimers are not required to elicit the kinase activity of the β chains, rather they alter the kinase reaction from intrachain to interchain, probably as a consequence of steric effects.

It is not obvious what the advantage of receptor dimers for the signal transduction process might be. One possibility is that the interactions with other proteins either for signal transduction or for receptor internalization are facilitated by having two copies of the monomers close together. It does seem apparent, however, that in the case of the insulin receptor, the interactions between monomers are sufficiently weak, as suggested by Chiacchia (1988), so that insulin binding cannot promote dimerization at a normal receptor concentration in the membrane. Hence, the necessity to use the device of covalent dimer formation.

One wonders whether the necessity of receptor dimerization for kinase activation of other growth hormone receptors has also been overemphasized.

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