Cysteine-524 is not the only residue involved in the formation of disulphide-bonded dimers of the insulin receptor

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The human insulin receptor (hIR) is a member of the transmembrane tyrosine kinase receptor family. It is a disulphide-linked homodimer which can be reduced to two insulin-binding monomers by mild reduction of class-I disulphide bonds. The number of disulphide bonds between the α- and β-chains within the monomer or between the monomers in the dimer is not known, although one dimer bond involving hIR Cys-524 has recently been identified [Schaffer and Ljungqvist (1992) Biochem. Biophys. Res. Commun. **189**, 650–653]. In the present report hIR Cys-524 was converted into alanine by site-directed mutagenesis and expressed at high levels in Chinese hamster ovary (CHO) cells. The mutant receptor was processed normally and shown to bind insulin normally, with ED_{50} and K_D values not different from those of the wild-type hIR. It was still a disulphide-linked dimer as judged by SDS/PAGE, indicating that there are α–α-chain disulphide bonds additional to the Cys-524 linkage in the insulin receptor dimer. Insulin-stimulated receptor autophosphorylation and kinase activity of the mutated receptor were both impaired compared with that of the wild-type receptor by 49% and 53% respectively. CHO cells overexpressing the mutant receptor, however, did not show a reduced capacity to stimulate glucose utilization, indicative that the level of receptor expression was sufficient to saturate downstream insulin action. These findings indicate that α–α disulphides additional to that provided by Cys-524 hold the receptor dimer together and that mutagenesis of Cys-524 reduces the ability of the receptor to signal insulin action subsequent to hormone binding.

INTRODUCTION

The human insulin receptor (hIR) is a large transmembrane modular protein containing several functional domains and like other complex receptors appears to have evolved by exon shuffling and cassette assembly [see Olefsky (1990) for review]. It is a homodimer (not a heterotetramer as frequently described) consisting of two monomers held together by disulphide bonds. There are two classes of disulphide bonds in the IR (Massague et al., 1980), those that can be reduced under mild redox conditions to give α-β monomers (class I) and those (class II) that require stronger reducing conditions. This does not imply that all class-I disulphides are involved in dimer formation, only that all disulphides involved in dimer formation are class I. Only the β-chain spans the membrane and signals insulin action via its tyrosine kinase activity (Riedel et al., 1986).

While the primary sequence of the IR has been known for some time (Ebina et al., 1985; Ullrich et al., 1985), knowledge of the secondary and higher-order structures of the receptor is limited. The first major advance in understanding overall receptor structure was the model proposed by Bajaj et al. (1987) where the N-terminal half of the IR was shown to contain two homologous domains (L1, residues 1–119 and L2, residues 312–428) separated by the cysteine-rich region that itself was comprised of three repeating units each containing eight cysteine residues. The second major development was the observation that the C-terminal portion of the extracellular region of the IR contains two fibronectin type-III repeats, the first of which contains the insert domain that includes the α–β cleavage site and the alternatively spliced exon 11 (O'Bryan et al., 1991).

This information has been used in conjunction with electron microscopy (Christiansen et al., 1991; Schaefer et al., 1992) to propose a schematic Y-shaped representation of the IR ectodomain where the N-terminal L1/Cys-rich/L2 portions of the α-chains form two lobes that are splayed apart at the distal end of the receptor, with the fibronectin type-III repeats and adjacent upstream sequences forming close associations at the base of the receptor dimer (Schaefer et al., 1992). The importance of these associations is reflected in the C-terminal deletion studies of Schaefer et al. (1990) who found that the expressed ectodomain is a dimer but that the α-chain (which can still bind insulin) and several smaller constructs, when expressed on their own, were all monomers (Schaefer et al., 1990). This does not mean that there are no α-chain cysteines involved in dimer disulphide bond linkage in the native IR but merely reflects the failure of these smaller domains to associate and form dimers in the absence of the C-terminal fibronectin type-III repeats.

The disulphide bonds between IR monomers must be located upstream of the trypsin cleavage site at Lys-582 as the eight cysteine residues in the fibronectin type-III repeat and the insert regions are all contained in a 110 kDa monomeric fragment (residues 582–1355) obtained by tryptic digestion of the native receptor (Shoelson et al., 1988; Xu et al., 1990; Clark et al., 1991).

Recently, it has been shown by direct chemical analysis that the first cysteine residue (Cys-524) upstream of the fibronectin type-III repeat region is involved in one α–α interchain disulphide bond in the IR dimer, but as the authors point out it does not rule out the existence of additional dimer disulphide linkages (Schaffer and Ljungqvist, 1992). Chemical reactivity data of Finn et al. (1990) is consistent with the production of IR monomers by the reduction of a single α–α intermonomer disulphide linkage and one α–β intramonomer bond, while Chiacchia (1991), using a similar approach, concluded that there were two α–α interchain

Abbreviations used: hIR, human insulin receptor; CHO, Chinese hamster ovary; α-MEM, α-minimal essential medium; FCS, fetal-calf serum; MAb, monoclonal antibody; FITC, fluorescein isothiocyanate; DTT, dithiothreitol.

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disulphide bonds and no labile α–β class-I disulphides. Xu et al. (1990) presented evidence for dimer linkage involving a tryptic fragment (residues 122–270) that included most of the cysteine-rich region. In contrast, Waugh et al. (1989) concluded that there were no disulphide bonds between IR monomers in the L1/cysteine-rich region as they isolated, after chymotryptic digestion of heat-denatured IR, a 55 kDa fragment (residues 1 to approx. 310) which appeared to be a monomer.

Clearly, receptor dimerization is a key component of signalling mechanisms (Treadway et al., 1991) and the nature of the interactions between the hIR monomers in the preformed dimer are of importance to understanding IR function. In the present study we have mutated Cys-524 to Ala using site-directed mutagenesis in order to assess whether Cys-524 contributes the only α–α disulphide bond. The resultant receptor remains a disulphide-linked dimer that binds insulin normally, but cells overexpressing these mutant receptors show reduced insulin-stimulated kinase activity.

MATERIALS AND METHODS

Site-directed mutagenesis

$IR^{Cys\text{-}524 \rightarrow Ala}$, which contains a point mutation converting Cys-524 into Ala, was obtained by PCR-based mutagenesis. Two rounds of PCR were used to generate a mutagenic *Bam*HI–*Eco*RI fragment corresponding to the IR nucleotide sequence $IR_{994\text{–}2029}$. In the first round of PCR the mutagenic fragment $IR_{994\text{–}1802}$ was generated using a mutagenic primer (− strand) CTGTTGGA-ACCAGCCGCATCCTG (base changes underlined) and *Eco*RI (+ strand) primer CACGATGAATTCCAGCAAG. This was used as a primer for the second round of PCR that generated the fragment $IR_{994\text{–}2029}$ with the *Bam*HI (− strand) primer CTGAG-ATTGGATCCAGGGGCAC. This fragment, after digestion with *Bam*HI and *Eco*RI, was used to replace the corresponding IR fragment of the wild-type receptor in the pECE expression vector kindly provided by Dr. W. Rutter (Ellis et al., 1986) modified by deletion of a *Bam*HI site in the non-coding region. The sequence was checked by sequencing across the mutation and by restriction analysis with *Bam*HI and *Eco*RI to ensure there were no errors in the PCR fragment and its correct insertion into the plasmid.

Cell culture and DNA transfection

Chinese Hamster ovary (CHO) cells were maintained in α-minimal essential medium (α-MEM) containing 5% fetal-calf serum (FCS) in an atmosphere of 5% CO_2 at 37 °C. Cell lines expressing $IR^{Cys\text{-}524 \rightarrow Ala}$ were obtained by co-transfection of 10 μg of plasmid and 2 μg of pSVNeo using a lipid-mediated transfection technique with Lipofectin (Gibco BRL, MD, U.S.A). Positive expressing clones were selected after limiting dilution by e.l.i.s.a. against biotinylated MAb αIR-1 [a monoclonal antibody raised to the hIR (Kull et al., 1982) obtained from the American Tissue Type Culture Collection] and by ^{125}I-insulin-binding analysis. High-expressing cell lines were further selected by flow cytometry and sorted using a Coulter ERICS Elite flow cytometer. Briefly, cells were treated with another anti-hIR monoclonal antibody, MAb 83-7, kindly provided by Professor Ken Siddle, Cambridge University, Cambridge, U.K. (Soos et al., 1986) followed by a fluorescein isothiocyanate (FITC)-labelled sheep anti-(mouse IgG) second antibody (Selenius, Hawthorn, Australia). Flow cytometer excitation was with a 488 nm argon ion laser and FITC emission at 525 nm was analysed. Live cells were distinguished by negative red fluorescence from propidium iodide staining. Several clones were analysed. Although the predominant cell populations expressed the mutation (> 94%), a subpopulation of non-expressing cells was apparent despite selection by limiting dilution and four sorts selecting the top 5% of expressing cells.

Metabolic labelling

[^{35}S]Trans label (ICN Biomedicals, CA, U.S.A.) ([^{35}S]methionine/[^{35}S]cysteine; 7:3 approx.) was added to 80% confluent cell monolayers in cysteine- and methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 0.5% FCS (CSL, Australia), and incubation continued overnight (12–14 h). Insulin receptors were then prepared and analysed by SDS/PAGE following cell solubilization as detailed previously (Lammers et al., 1989) and summarized below.

Receptor extraction and immunoprecipitation

Cells in 55 cm^2 dishes were washed twice with PBS, pH 7.4, containing 100 μM sodium orthovanadate and then lysed by the addition of 800 μl/dish of 50 mM Hepes buffer, pH 7.2, containing 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 200 units/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM phenylmethanesulphonyl fluoride and 200 μM sodium orthovanadate essentially as detailed by Lammers et al. (1989). Dishes were incubated for 3 min on ice, the lysate collected, incubated a further 5 min on ice and then clarified by centrifugation at 12500 ***g*** for 2 min. The hIR in the supernatants were immunoprecipitated by diluting them 1:3 in 20 mM Hepes, pH 7.2, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol (HNTG buffer), and incubating with 3 μg/ml purified anti-hIR MAb 83-7 and 20 μl of Protein A–Sepharose (Sigma) slurry for 4 h at 4 °C. The Sepharose beads were pelleted by centrifugation, washed three times in 0.5 ml of HNTG buffer and receptor preparations used for: autophosphorylation and kinase assays; analysis by SDS/PAGE (metabolically labelled cells); or for insulin-binding assay.

Receptor autophosphorylation and kinase activity

The effect of insulin on receptor autophosphorylation and kinase activity was examined as follows. Receptor was immunoprecipitated from cell lysates in the presence or absence of 100 nM insulin and this insulin concentration was then maintained throughout all washes and subsequent incubations as appropriate. Autophosphorylation and kinase activities against a synthetic 12-residue peptide were then determined in the same incubation conditions. The peptide substrate used was RRDIFENDYFRK which is derived from the tyrosine phosphorylation sequence (residues 1154–1165) of the tyrosine kinase domain of the hIR (Ebina et al., 1985) modified so that only one phosphorylation site remained (modifications underlined). The reaction involved 10 min incubations of washed immunoprecipitates in the presence of 50 mM Hepes, pH 7.2, 150 mM NaCl, 0.2% (v/v) Triton X-100, 2 mM $MnCl_2$, 12 mM $MgCl_2$, 100 μM sodium orthovanadate, 50 μM [γ-^{32}P]ATP (8 μCi/ml) and 0.28 mg/ml of synthetic peptide in a final volume of 25 μl. Exceptions were experiments examining the kinetics of receptor kinase in which 3 min incubations at 30 °C were performed. All incubations were stopped by placing the samples on ice and the immunoprecipitated receptor was pelleted by pulse centrifugation. Receptor autophosphorylation was measured on the immunoprecipitate following SDS/PAGE, while the

supernatant was used to measure kinase activity against the synthetic peptide substrate. The extent of peptide phosphorylation was measured by taking 18 μl of supernatant and adding it to 18 μl of 5% trichloroacetic acid containing 4 μl of 10 mg/ml BSA and clearing the suspension by centrifugation, 12500 *g* for 5 min. Supernatants, 35 μl, were then spotted on to P81 paper (Whatman U.K.), washed three times for 10 min each wash in 30% acetic acid/0.5% phosphoric acid, and finally ethanol, before drying and counting in a scintillation counter.

The extent of receptor autophosphorylation was measured by SDS/PAGE following the addition of sample buffer to the immunoprecipitate after removal of the remaining supernatant. Autophosphorylation samples were boiled for 2 min and analysed by SDS/PAGE in 7.5% (w/v) acrylamide. Phosphorylated bands were visualized by autoradiography, excised, and the level of autophosphorylation quantified by Cerenkov counting in a liquid-scintillation counter.

Insulin-binding analysis

Insulin binding to immunoprecipitates was determined in HNTG buffer by incubation of equivalent samples to those used for the determination of tyrosine kinase and receptor autophosphorylation with ^{125}I-insulin (specific radioactivity 84–108 μCi/μg) overnight at 4 °C. Values obtained were used to adjust assays to similar binding levels within each experiment. For Scatchard analysis, binding was determined with monoiodinated ^{125}I-insulin (specific radioactivity 371 μCi/μg, NEN, Du Pont, U.S.A.) over a range of insulin concentrations using receptor bound to microtitre plates as previously described (Clark et al., 1991). Briefly, microtitre plates were precoated with 2 μg of rabbit anti-(mouse IgG) antibody (DAKO-immunoglobulins, Denmark), for 3 h at 22 °C, washed, blocked with 20 mg/ml BSA and then coated with 0.1 μg of anti-hIR MAb 83-7/well (Soos et al., 1986) overnight at 4 °C. After washing, the plates were coated with cell lysate prepared as described above, diluted 1:8 in PBS containing 0.002% sodium azide and incubated overnight at 4 °C. The number of cells contributing to the cell lysates were quantified by counting cells in duplicate plates. Microtitre plates, with the receptors adsorbed, were then washed and insulin (40000 c.p.m./well; 1 fmol–0.1 nmol/well) added overnight in 50 μl at 4 °C. The wells were then washed and counted on a gamma counter. Data analysis was performed using the Ligand program (Munson and Rodbard, 1980).

Glucose utilization

This was determined as the conversion of [5-^{3}H]glucose into ^3H_2O as described previously (Brown and Garratt, 1974; Macaulay and Larkins, 1990). Cells in 24-well plates were grown to near confluence and then incubated overnight in 0.05% FCS/α-MEM. Wells were washed twice in 750 μl of Krebs–Ringer bicarbonate Hepes buffer, 0.5% BSA, 5 mM glucose, 2 mM pyruvate, 1.15 mM $CaCl_2$, pH 7.4, and then allowed to equilibrate in the incubator for 60 min. Each well was then incubated in the presence or absence of insulin for 10 min followed by 1 μCi of [5-^{3}H]glucose for an additional 30 min. ^3H_2O in the medium was then measured in aliquots after absorption into $CaCl_2$.

Statistical analyses

Statistical analyses were performed using Student's *t* test. Results are expressed as the mean ± S.E.M. where appropriate. Statistical significance was determined at the 0.05 level and is indicated in the results.

RESULTS

Characteristics of the cell lines

(a) Flow cytometric analysis

Cell lines expressing receptors containing the Cys-524 → Ala substitution were selected as described in the Materials and methods section, initially by limiting dilution and analysis of insulin binding at tracer concentrations of insulin and by cell e.l.i.s.a. against biotinylated anti-hIR MAb αIR-1. High-expressing lines were then sorted by flow cytometry with anti-hIR MAb 83-7 selecting the top 5% of expressing cells. These were sorted a further three times. Figure 1 compares the fluorescence from the parental CHO cell line with that of the stable wild-type hIR-expressing cell line CHOT and the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant cell line. The CHOT line was originally also transfected in the pECE vector used in the present study and was reported to express 2.5×10^5–1×10^6 receptors/cell (Ellis et al., 1986). The shift in fluorescence was more than 10-fold and the symmetrical nature of the peak indicates a uniform population of cells expressing native hIR. The $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant cell line also displayed a shift to the right in fluorescence compared with the parental line of approx. 10-fold, similar to that of the CHOT cell line, indicating that both the CHOT cell line and the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant line expressed a similar number of receptors/cell. In contrast with the CHOT cell line, flow cytometric analysis of the

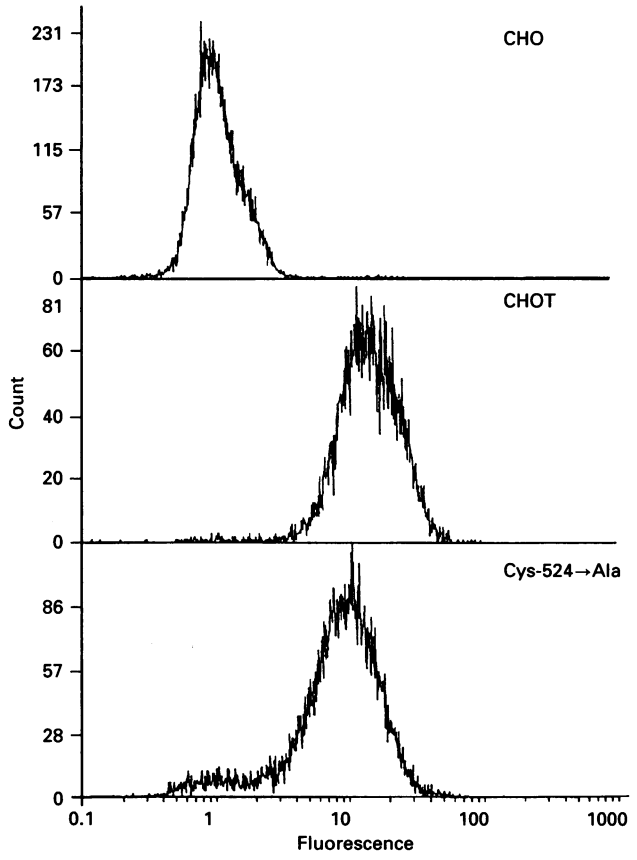

Figure 1 Flow cytometric analysis of insulin receptor expression

CHO (top), CHOT (middle) and $IR^{Cys\text{-}524 \rightarrow Ala}$ (bottom) cell lines were treated first with anti-hIR MAb 83-7 followed by FITC-conjugated sheep anti-(mouse IgG) antibody and flow cytometric analysis as described in the Materials and methods section.

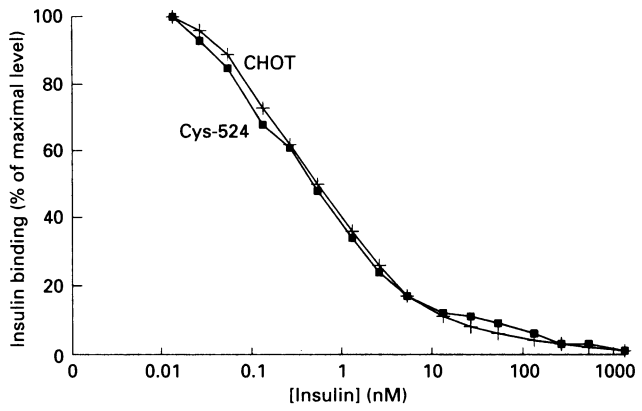

Figure 2 Insulin binding analysis

Insulin receptors from CHOT (+) and $IR^{Cys\text{-}524 \rightarrow Ala}$ (■) cell lines were immunoprecipitated in 96-well plates coated with anti-hIR MAb 83-7 and then incubated with ^{125}I-insulin (40000 c.p.m./well, 371 μCi/μg) in the presence of different concentrations of unlabelled insulin over the range 0.01 nM to 1 μM. Each point represents the mean of four experiments in which triplicate determinations were made at each point within each experiment. S.E.M. at each point were less than 5% and therefore not shown.

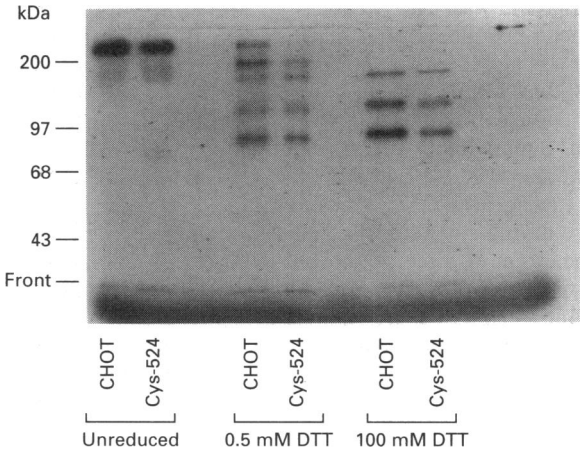

Figure 3 Effect of reduction on the mobility of ^{35}S-metabolically labelled insulin receptors

Cells were metabolically labelled with [^{35}S]methionine:[^{35}S]cysteine as described in the Materials and methods section and insulin receptors immunoprecipitated with anti-hIR MAb 83-7. Immunoprecipitates were solubilized with non-reducing Laemmli/SDS loading buffer or in the presence of 0.5 mM DTT or 100 mM DTT. Samples were analysed on 7.5% PAGE. Gels were incubated with Amplify (Amersham) before autoradiography.

$IR^{Cys\text{-}524 \rightarrow Ala}$ mutant line also showed a small population of cells, approx. 5% of the total, with fluorescence similar to the parental line despite extensive selection for high expression, indicating that the line was not completely stable.

(b) Insulin binding

The binding of ^{125}I-insulin to the wild-type and mutant receptors in a solid-phase assay in the presence of increasing concentrations of unlabelled insulin is shown in Figure 2. The concentration of unlabelled insulin required for half-maximal displacement of ^{125}I-insulin was similar for both receptors (ED_{50} $IR^{Cys\text{-}524 \rightarrow Ala}$ = 0.5 ± 0.1 nM, ED_{50} wild-type human IR 0.7 ± 0.2 nM, $n = 4$). At no concentration of insulin did the displacement curves differ significantly from each other. Furthermore, analysis of the data by the method of Scatchard (Scatchard, 1949; Munson and Rodbard, 1980) yielded characteristic curvilinear plots, that also did not differ and that could be resolved into two classes of binding sites with K_d values of $(2.9 \pm 1.0) \times 10^{-10}$ M and $(3.9 \pm 2.6) \times 10^{-8}$ M ($n = 4$) for wild-type hIR (CHOT); and K_d values of $(3.1 \pm 0.4) \times 10^{-10}$ M and $(7.7 \pm 5.7) \times 10^{-8}$ M ($n = 4$) for $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant hIR (results not shown). The number of binding sites/cell did not differ significantly, CHOT (wild-type hIR), $(504 \pm 160) \times 10^3$ sites/cell versus $IR^{Cys\text{-}524 \rightarrow Ala}$ $(312 \pm 120) \times 10^3$ sites/cell, although the CHOT cell line tended to be higher, confirming the data in Figure 1 from flow cytometric analysis.

Oligomeric structure of $IR^{Cys\text{-}524 \rightarrow Ala}$

The oligomeric structure of the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant receptor was examined in immunoprecipitates of metabolically labelled cells (Figure 3). Metabolically labelled protein from CHOT (wild-type hIR) and $IR^{Cys\text{-}524 \rightarrow Ala}$ cells was immunoprecipitated with MAb 83-7 and examined under non-reducing conditions, after reduction with 0.5 mM dithiothreitol (DTT) or 100 mM DTT, by SDS/PAGE on 7.5% gels. In the absence of DTT, a single high-molecular-mass band that only just entered the gel (approx. 400 kDa) was observed in both cases, as well as a lighter band of 180–200 kDa that was not well resolved. Following treatment of receptor immunoprecipitates with 0.5 mM DTT, the 400 kDa band collapsed to yield bands of approx. 180 kDa–200 kDa, 135 kDa and 95 kDa. Treatment of immunoprecipitates with 100 mM DTT resulted in the collapse of the 180–200 kDa band into the 135 kDa and 95 kDa bands. There was essentially no difference between the band sizes observed for the wild-type hIR from CHOT cells and the mutant receptor from the $IR^{Cys\text{-}524 \rightarrow Ala}$ cells. The band of approx. 400 kDa we interpret to be the dimeric receptor in both cases. The band of 180–200 kDa present only in the 0.5 mM DTT condition we interpret to be the α–β monomer which collapses under stronger reducing conditions to yield the 135 kDa α-subunit and the 95 kDa β-subunit. The unresolved band of 180–200 kDa in unreduced conditions that resolves upon reduction into a band of approx. 180 kDa we believe to be the unprocessed proreceptor, previously shown to be present in significant amounts in CHOT cells (Ellis et al., 1986). Thus the IR, despite mutagenesis of Cys-524 that removes one interchain α–α linkage (Schaffer and Ljungqvist, 1992), remains in dimeric association, indicating that additional cysteines, albeit with similar sensitivity to reduction, are involved in α–α disulphide linkage(s) in IR structure.

Effects of insulin on receptor autophosphorylation and kinase activity *in vitro*

The ability of insulin to stimulate $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant receptor autophosphorylation and kinase activity was assessed in immunoprecipitates. Figure 4(b) shows an autoradiograph of the CHOT and $IR^{Cys\text{-}524 \rightarrow Ala}$ cell immunoprecipitates after incubation with [γ-^{32}P]ATP followed by SDS/PAGE under both non-reducing and reducing (100 mM DTT) conditions and Western blotting. The blot was probed with MAb CT1 [directed towards the C-terminal 15 amino acids of the hIR (Ganderton et al., 1992); a gift from Professor Ken Siddle]; to confirm insulin-binding analysis of duplicate samples similar amounts of receptor were loaded on each lane (Figure 4a). Under non-reducing

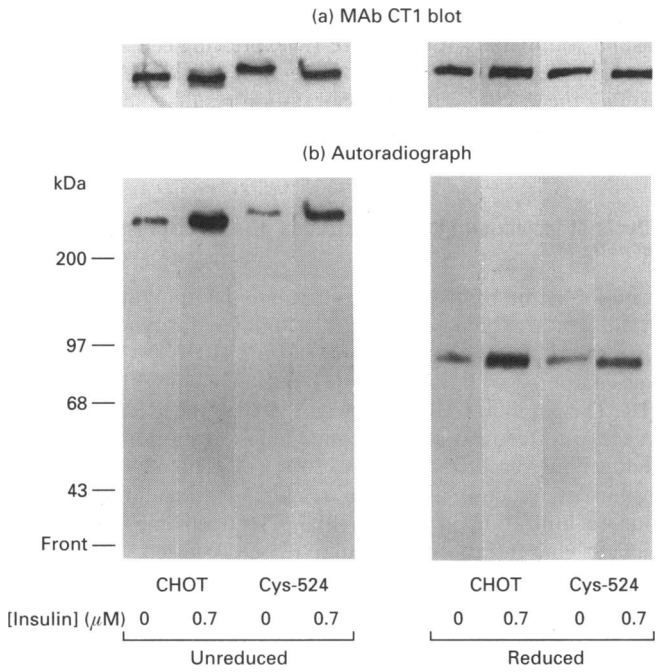

Figure 4 Effect of insulin on autophosphorylation of insulin receptors

Insulin receptors from CHOT and $IR^{Cys\text{-}524 \rightarrow Ala}$ cell lines were immunoprecipitated with anti-hIR MAb 83-7 in six equivalent aliquots and incubated in the presence or absence of insulin for determination of the effect of insulin on autophosphorylation and kinase activity (after incubation with [γ-^{32}P]ATP) as well as insulin-binding level as detailed in the Materials and methods section. Samples for autophosphorylation were matched for insulin-binding level of duplicate samples and analysed both in the reduced (100 mM DTT-treated) and unreduced state after separation on SDS/PAGE and Western blotting. Western blots were probed with MAb CT1 (to the IR β-subunit) and analysed with alkaline phosphatase detection. Only the IR portion of the blots are shown (**a**). The blots were then analysed by autoradiography (**b**).

conditions insulin stimulated phosphorylation of a band that only just entered the gel, consistent with it being the dimeric receptor in both CHOT and $IR^{Cys\text{-}524 \rightarrow Ala}$ cell immunoprecipitates. Since only the 95 kDa β-subunit of the IR is phosphorylated upon insulin stimulation, under reducing conditions the 135 kDa subunit was not visible. Figure 4 shows that insulin stimulated phosphorylation of the β-subunit of the $IR^{Cys\text{-}524 \rightarrow Ala}$ receptor, like the CHOT receptor, albeit to a slightly lesser extent, which is consistent with the results seen in the non-reduced lanes. Both the $IR^{Cys\text{-}524 \rightarrow Ala}$ and wild-type receptors ran at the same molecular mass as each other in reduced and non-reduced lanes, supporting the conclusions reached from the metabolic labelling studies that the $IR^{Cys\text{-}524 \rightarrow Ala}$ receptor is dimeric in nature. Over five experiments [in which determinations were carried out in triplicate and the receptors analysed by SDS/PAGE were matched for an equivalent insulin-binding level (assessed by binding analysis of parallel samples)], the magnitude of insulin-stimulated phosphorylation of the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant was less than that of the CHOT wild-type receptor. Insulin stimulation of receptor autophosphorylation above basal levels was $157 \pm 8\%$ (CHOT wild-type receptor) versus $128 \pm 6\%$ $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant ($P < 0.01$, $n = 5$, CHOT versus $IR^{Cys\text{-}524 \rightarrow Ala}$). The level of insulin stimulation of CHOT wild-type receptor was less than some reports in the literature [see for example Clark and Konstantopoulos (1993)]; first, because insulin stimulation was carried out *in vitro* and, secondly,

Table 1 Effect of insulin on kinase activity of insulin receptors

Receptor kinase activity was measured in immunoprecipitated receptor preparations incubated in the presence or absence of 0.7 μM insulin as the receptor was immunoprecipitated in the same experiments as receptor autophosphorylation (Figure 4). Data shown is from a representative experiment in which three determinations were made. Similar results were obtained in six separate experiments. *$P < 0.001$.

	Kinase activity (c.p.m./min)	
Cell line	−Insulin	+0.7 μM Insulin
CHOT	1146 ± 477 (3)	13137 ± 1496 (3)*
$IR^{Cys\text{-}524 \rightarrow Ala}$	725 ± 148 (3)	5625 ± 701 (3)*

because assays were performed in the presence of IR peptide for determination of kinase activity, which may compete for substrate. Similar effects have been seen by others (Dr. S. Clark and Ms. N. Konstantopoulos, personal communication).

Receptor kinase activity of the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant was also impaired (Table 1). Because differences in receptor amounts (^{125}I-insulin-binding levels) and differences in specific activity of the [γ-^{32}P]ATP between experiments made it difficult to pool data from different experiments, a representative experiment is shown in Table 1. Insulin stimulated the tyrosine kinase activity of the wild-type hIR in CHOT cells, in the experiment shown, by 13.5-fold above basal levels. Stimulation by insulin of $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant kinase activity in the same experiment was 7.9-fold. Similar results were obtained in five additional experiments in which duplicate or triplicate determinations were made. Overall, the level of kinase activity achieved by immunoprecipitates of $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant normalized for insulin binding to CHOT wild-type IR binding was only $47 \pm 8\%$ ($n = 6$, $P < 0.01$, CHOT versus $IR^{Cys\text{-}524 \rightarrow Ala}$) of that achieved by the CHOT wild-type receptor.

The kinetics of the impairment of insulin-stimulated receptor kinase were investigated. The K_m of $IR^{Cys\text{-}524 \rightarrow Ala}$ for ATP (56 ± 6 μM, $n = 3$) did not differ significantly from that of the native hIR from CHOT cells (74 ± 11 μM, $n = 3$). The effects of the mutation were manifested by a reduction in the $V_{max.}$ of the receptor for ATP (1.89 ± 0.30 pmol/min per well; $n = 3$), $IR^{Cys\text{-}524 \rightarrow Ala}$, versus ($4.85 \pm 1.29$ pmol/min per well; $n = 3$), CHOT, $P < 0.05$.

Effects of insulin on receptor autophosphorylation in intact cells

Insulin stimulation of hIR autophosphorylation in cells expressing $IR^{Cys\text{-}524 \rightarrow Ala}$ was compared with that in CHOT cells expressing the native hIR over a range of insulin concentrations (Figure 5). Following insulin stimulation, the level of receptor autophosphorylation in each cell line was assessed by Western-blot analysis of hIR immunoprecipitates with an anti-phosphotyrosine MAb (MAb αPtyr) (Figure 5, lower panels). Duplicate blots were probed with MAb CT1 to quantify receptor amounts and confirm that loadings were similar to that predicted from insulin-binding analysis of duplicate samples (Figure 5, upper panels). Three bands were apparent in MAb CT1 blots, at approx. 180 kDa (the pro-receptor), 50 kDa (the heavy chain of the immunoprecipitating antibody, MAb 83-7), as well as 95 kDa (the IR β-subunit, indicated). The density of the 95 kDa band in the MAb CT1 blot can be used to compare receptor amounts loaded and show that the receptor amount loaded on gels from the $IR^{Cys\text{-}524 \rightarrow Ala}$ cell line was similar, or slightly higher than that loaded from CHOT cells. Similar loadings were applied to blots

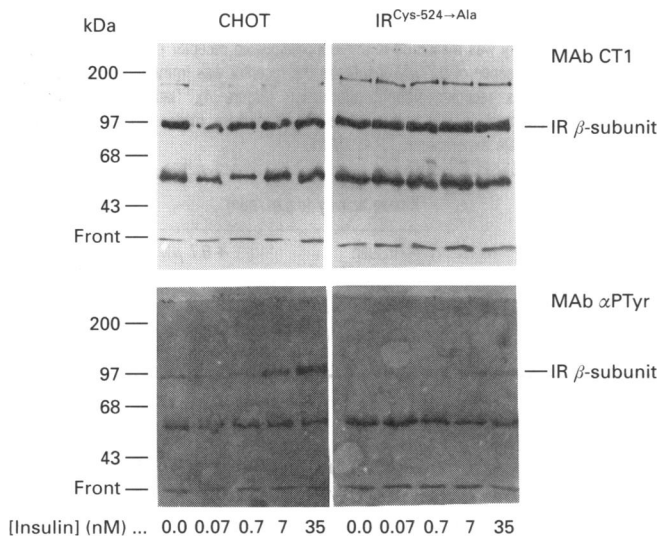

Figure 5 Effect of insulin on insulin receptor autophosphorylation in intact cells

Cells were preincubated with 0.1 mM pervanadate [prepared as previously described by Pronk et al. (1992)] in α-MEM containing 0.05% FCS for 5 min before incubation in the presence or absence of the indicated concentrations of insulin. Cell lysis was performed as described in the Materials and methods section except that cells were snap frozen in liquid N_2 before cell lysis. Receptors in cell lysates were immunoprecipitated with MAb 83-7 (to the hIR). Duplicate receptor immunoprecipitates were separated by SDS/PAGE and blotted to nitrocellulose. One blot was probed with MAb CT1 (to the hIR) and the other probed with an antiphosphotyrosine MAb (MAb αPTyr), prepared in our laboratory. Blots were analysed with the Renaissance system (Du Pont). The experiment was repeated twice with similar results.

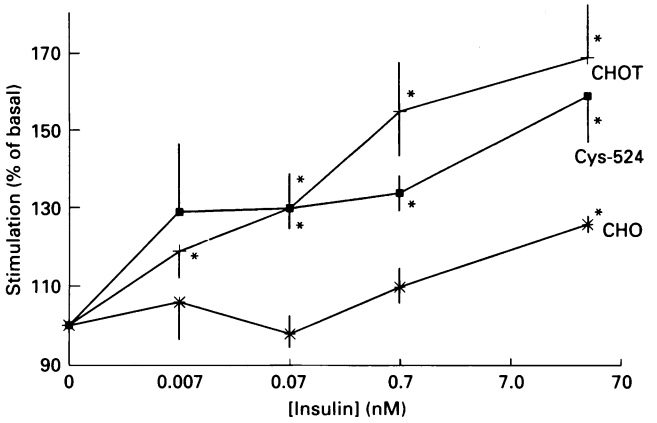

Figure 6 Effect of insulin on glucose utilization

CHO (×), CHOT (+) and $IR^{Cys\text{-}524 \rightarrow Ala}$ (■) cells were preincubated for 10 min in the presence or absence of the indicated concentrations of insulin. [5-^{3}H]Glucose was then added for the next 30 min, after which the medium was removed for the determination of glucose utilization as described in the Materials and methods section. Results expressed as the percentage of basal glucose utilization rate are given as the mean ± S.E.M. of 6–8 experiments in which three independent cell incubations were performed within each experiment. Significance from basal levels is indicated *

probed with MAb αPTyr. In this case, a concentration-dependent increase in density of the 95 kDa IR β-subunit band, and therefore tyrosine phosphorylation, was observed in both CHOT and $IR^{Cys\text{-}524 \rightarrow Ala}$ cell lines over the insulin concentration range tested (0–35 nM). The phosphorylation levels achieved following insulin stimulation of the native IR-expressing cell line, CHOT, were markedly higher than for the $IR^{Cys\text{-}524 \rightarrow Ala}$ cell line, confirming the *in vitro* autophosphorylation results and supporting the contention that the Cys-524 → Ala mutation impairs signalling to the receptor β-subunit.

Effects of insulin on glucose utilization of cells expressing $IR^{Cys\text{-}524 \rightarrow Ala}$

A major action of insulin is to stimulate the uptake and storage or utilization of glucose in cells. The ability of insulin to stimulate glucose utilization in cells expressing the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutation was compared with that in CHOT cells expressing the wild-type hIR. The parental CHO cell line, into which the hIR in each case was transfected, was also examined to assess the contribution of endogenous receptors (approx. 3000 per cell) on the effect of insulin on glucose utilization (Figure 6). Insulin stimulated glucose utilization in the parental CHO cell line only at the highest concentration of insulin (35 nM) tested. The lowest concentration of insulin, 0.007 nM, stimulated glucose utilization in the CHOT cell line by 19% ($P < 0.05$). Significant stimulation of 30% was achieved in both the CHOT and $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant cell lines at the next highest concentration of insulin tested, 0.07 nM, that was approximately half maximal. Maximal stimulation was achieved in the CHOT cell line at the next highest concentration of insulin tested, 0.7 nM, which although tending to be higher than that elicited in the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant cell line, did not reach statistical significance. The ability of insulin to stimulate glucose utilization in the $IR^{Cys\text{-}524 \rightarrow Ala}$ mutant cell line did not differ significantly from that of the CHOT cell line at any of the concentrations tested. Thus despite receptor kinase being impaired by mutagenesis of Cys-524, this was not reflected in insulin stimulation of glucose utilization.

DISCUSSION

The hIR is a homodimer consisting of two monomers held together by disulphide bonds. Attempts to identify the cysteines involved in disulphide bond formation have been difficult because each α–β monomer contains a total of 47 cysteine residues with 37 in the α-chain and 10 in the β-chain (four extracellular, six cytoplasmic). It is clear, however, that very few are involved in the disulphide bridges that hold the receptor monomers in the dimer (Finn et al., 1990; Chiacchia, 1991). In a preliminary analysis of the disulphide bond arrangements of the IR, Schaffer and Ljungqvist (1992) identified Cys-524 as one of the residues involved in covalent linkage of the two IR monomers. However, it was not clear whether this was the only disulphide bridge between the two monomeric units of the IR dimer as Xu et al. (1990) found evidence for disulphide linkage between the N-terminal domains (residues 122–270) of the α-chain and Chiacchia (1991) concluded that there are two α–α intermonomer linkages. The present study shows that Cys-524 is not the only cysteine involved in intermonomer disulphide linkage. A mutant $IR^{Cys\text{-}524 \rightarrow Ala}$ was produced by site-directed mutagenesis and transfected into CHO cells. The mutant receptor was expressed at high levels and appeared to be transported, glycosylated and proteolytically processed normally. It was still a disulphide-linked dimer, clearly indicating that there are additional α–α-chain disulphide bonds in the mature receptor.

While previous studies have demonstrated that treatment of cells with low concentrations of reducing agents (that presumably result in α–β monomer formation) activate the receptor (Fujita-Yamaguchi and Kathuria, 1985; Wilden et al., 1986; Clark and

Konstantopoulos, 1993), we found that substitution of Cys-524 for Ala reduced tyrosine kinase activity of the hIR by approx. 50%. Existence of this disulphide bond is therefore likely to be important for insulin signalling. This inhibition of receptor activity was not manifested in downstream insulin action, measured in the current investigation as glucose utilization. The inability to detect impaired glucose utilization in the face of impaired receptor activity probably reflects saturation of the pathway by overexpression of the receptor in CHO cells. Since the mutant receptor kinase activity was reduced compared with that of the native receptor, the results of the present study suggest the possibility that the activation effect of reduction seen in previous studies (Fujita-Yamaguchi and Kathuria, 1985; Wilden et al., 1986; Clark and Konstantopoulos, 1993) most likely results from reduction of another cysteine sensitive to low concentrations of reductant either in addition to, or independent of, Cys-524.

Since there are no free cysteines in the α-chain (Finn et al., 1990), our findings, together with previous data, indicate that there must be at least two additional interchain disulphide bonds in the IR dimer given that each α-chain contains an odd number (37) of cysteine residues. How can this be reconciled with the chemical reactivity data of Chiacchia (1991) who concluded that the production of IR monomers by the reduction of class-I disulphides only involved the reduction of two α–α disulphide linkages? Close inspection of the data of Chiacchia (1991) shows that the raw α-chain/β-chain alkylation ratios based on incorporated radioactivity into the native receptor, reduced dimer and free α–β monomer were 0.17/1, 2.1/2.0 and 3.8/2.2 respectively. This is equally consistent with the native receptor having one free cysteine, the reduced dimer resulting from the reduction of one intermonomer α–α linkage and one intramonomer α–β linkage and the production of free monomer involving the reduction of an additional two dimer α–α disulphides. When the raw data are corrected for estimated protein content as done by Chiacchia (1991), the relative ratios remain similar but the molar ratios of *N*-ethylmaleimide incorporated per mol of protein decrease by about 45% to 0.1/0.58, 1.1/1.0 and 1.87/1.0 for the native receptor, reduced dimer and free α–β monomer respectively. It is these ratios that led Chiacchia (1991) to conclude that only two α–α bonds were involved in dimer reduction, but it seems equally plausible that there may be a total of three interchain α–α disulphide linkages in the IR dimer and that class-I reduction involves these three bonds plus one intramonomer α–β disulphide linkage.

The three interchain disulphide linkages (α–α dimer linkages) must be located in the L1, L2 or cysteine-rich domains since, as discussed, they are not in the fibronectin type-III repeat/insert region. The recent demonstration that the L2 domain residues Cys-435 and Cys-468 are disulphide-bonded (Schaffer and Ljungqvist, 1992) suggests that their homologues in the L1 domain (residues 126 and 155) are also linked. Similarly, chemical analyses (Xu et al., 1990) and deletion expression studies (De Meyts et al., 1990) indicate that the L1 cysteines (Cys-8 and Cys-27) are disulphide-bonded, suggesting that their homologues (residues 312 and 333) in the L2 domain are also involved in an equivalent linkage. Thus it seems likely that the two extra disulphide bonds linking the IR monomers in the receptor dimer are present in the cysteine-rich region. Further direct chemical analysis coupled with confirmation using mutagenic approaches in this key region of the receptor will, at the least, further define receptor structure and may also provide new insights into receptor signalling. Our data indicate that Cys-524 is important for full functional activity of the hIR.

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