Mutagenic structure/function analysis of the cytoplasmic cysteines of the insulin receptor

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Native human insulin receptor (hIR) has been reported to contain only one free thiol group proposed to lie near the ATP-binding domain of its β -subunit [Finn, Ridge and Hofmann (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 419–423]. The present study investigated the role of the six cytoplasmic cysteines of the β -subunit of the hIR using a mutagenic approach in which insulin receptors, mutated at each cytoplasmic cysteine (to alanine) in turn, were transfected into Chinese hamster ovary (CHO) cells. Cell lines expressing hIR mutation at high level were obtained which, by both flow-cytometric analysis towards an hIR-specific monoclonal antibody (83-7) and insulin-binding analysis, were similar to the well-characterized CHOT cell line which overexpresses native hIR. The ED_{50} and K_d values of the mutant receptors were the same as those of the wild-type hIR. Each of the mutant receptors signalled insulin action to stimulate receptor autophosphorylation and kinase activity as well as glucose utilization to levels appropriate for the receptor level expressed. In contrast, insulin-stimulated thymidine uptake and glucose-transport responses of two of the six mutant cell lines,

those expressing Cys981Ala and Cys1245Ala, were impaired compared with that of the native hIR-expressing cell line, CHOT. The β -subunits of each of the hIR cytoplasmic cysteine mutant cell lines could be alkylated specifically with *N*-[3 H]ethylmaleimide. The kinase activity of each receptor was inhibited by *N*-ethylmaleimide and stimulated by iodoacetamide, indicating that none of the cytoplasmic cysteines alone contributes the single free thiol group to the hIR structure. We conclude that the cytoplasmic cysteines of the hIR have a predominantly passive role in hIR activity although Cys-981 and Cys-1245 do affect mitogenic and glucose-transport responses of the receptor. Our findings indicate that the stoichiometry of a single free thiol group/mol of insulin-binding activity noted in previous studies is either spread fractionally over a number of the cytoplasmic cysteines or is one of the four cysteines in the ectodomain of the hIR β -subunit. Alternatively, the mutagenesis performed in the present study may enable differential exposure of a second titratable cysteine in wild-type and mutant receptors.

INTRODUCTION

The human insulin receptor (hIR) is a homodimer consisting of two α - and two β -subunits linked together by disulphide bonds, with only the β -subunits spanning the membrane and signalling insulin action via its intrinsic tyrosine kinase activity. The mechanism by which insulin activates this kinase and initiates downstream insulin signal transduction is currently unknown. The present study addresses the possibility that the cytoplasmic cysteines of the insulin receptor (IR) function in this activity.

Each $\alpha\beta$ monomer of the hIR contains 47 cysteines: 37 in the α -chain and 10 in the β -chain. Cys-524 has been shown to provide one of the α - α bonds holding the two α - β monomers together (Schäffer and Ljungqvist, 1992) and we have previously shown that additional cysteines are involved in dimer stabilization (Macauley et al., 1994). The β -subunits are linked only to the α -subunits through one or more disulphides in the ectodomain (Finn et al., 1990; Cheatham and Kahn, 1992; Schaffer and Ljungqvist, 1992). It has been reported that there are no free thiol groups in the α -chain and only one in the β -subunit of the hIR (Finn et al., 1990; Chiacchia, 1991). This free thiol is presumably localized to the cytoplasmic portion of the receptor which contains five (mouse IR) or six (hIR) cysteine residues (Ebina et al., 1985; Ullrich et al., 1985; Flores-Riveros et al., 1989). Marked effects of alkylation on receptor activity have been demonstrated, indicating a potential functional role for this

free thiol in insulin action. For example, alkylation of the receptor with iodoacetate has been shown to enhance insulin-stimulated receptor autophosphorylation and kinase activity (Wilden and Pessin, 1987; Li et al., 1991). *N*-Ethylmaleimide (NEM) alkylation by contrast has been shown to inhibit receptor autophosphorylation and kinase activity (Ridge et al., 1988; Finn et al., 1990). The latter studies suggested, furthermore, that the free thiol is located near the ATP-binding site of the receptor as NEM inhibition of receptor kinase activity could be partially blocked by co-incubation with MnATP. The epidermal growth factor receptor (EGFR) kinase is also inhibited by alkylation with NEM (Buhrow et al., 1982) suggesting that a free cysteine residue is functionally important in other tyrosine kinases.

The cytoplasmic domain of the hIR contains six cysteine residues: one in the juxtamembrane region (Cys-981), four in the catalytic domain (amino acids 1000–1271), at residues 1056, 1138, 1234 and 1245, and one in the C-terminal tail at residue 1308 (Ebina et al., 1985; Ullrich et al., 1985). The juxtamembrane cysteine residue is absent from IRs from rodent species, but the other five cytoplasmic cysteines are conserved (Flores-Riveros et al., 1989; Goldstein and Dudley, 1990). Comparison of cysteines of the kinase domain of the hIR across the whole tyrosine kinase family shows that Cys-1056 is not conserved, Cys-1138 is found in approx. 40% of the other tyrosine kinases and Cys-1234 and Cys-1245 are highly conserved, the latter being invariant in all but two of the tyrosine kinases (Hanks et al., 1988; Hanks, 1991).

Abbreviations used: hIR, human insulin receptor; NEM, *N*-ethylmaleimide; IAA, iodoacetamide; mAb, monoclonal antibody; CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; α -MEM, minimal essential medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; cAPK, cyclic AMP-dependent protein serine kinase.

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Mutagenesis of the cysteine (equivalent to hIR residue 1245) in the lymphocyte-specific tyrosine kinase p56^{lck} abolishes all catalytic activity (Veillette et al., 1993). Furthermore, functional involvement of cysteines in catalysis and/or substrate recognition has been demonstrated for other enzymes including phosphoenolpyruvate carboxykinase and protein tyrosine phosphatase (Gilbert, 1984; Guan and Dixon 1991; Lewis et al., 1989). Thus all of the available data taken together indicate that the cytoplasmic cysteines play functional and/or structural roles in insulin signal transduction.

In the present study we show that, in contrast with indications from these previous data, the cytoplasmic cysteines of the hIR have no effect on IR kinase activity or downstream insulin action in stimulating glucose utilization. However, insulin stimulation of thymidine uptake and glucose transport were impaired in cells expressing receptors containing the Cys981Ala or Cys1245Ala mutations. These studies are therefore indicative of a potential role for Cys-981 and Cys-1245 in these actions. Alkylation studies were unable to attribute the free thiol to any single cytoplasmic cysteine, indicating that the stoichiometry of alkylation (one free thiol/receptor monomer) is achieved by fractional alkylation of several cytoplasmic cysteines, by differential reactivity of specific cysteines in wild-type and mutant receptors, or by labelling one of the four cysteines in the ectodomain of the β -subunit of the hIR

MATERIALS AND METHODS

Site-directed mutagenesis

hIRs containing Cys981Ala, Cys1056Ala, Cys1138Ala, Cys1234Ala, Cys1245Ala or Cys1308Ala substitutions were generated by site-directed mutagenesis using the phagemid vector modification (McClary et al., 1989) of the *dut⁻ung⁻* procedure (Kunkel et al., 1987). A *Xba*I–*Bam*HI hIR fragment (gift from Dr. W. Rutter, UCSF, San Francisco, CA, U.S.A.) (Ebina et al., 1985), including the coding region for all six cytoplasmic cysteines, was cloned into the phagemid pGEM7Z (Promega, Madison, WI, U.S.A.) and transformed into *Escherichia coli* strain RZ1032. Single-stranded template was prepared and mutagenic primers (Table 1) were annealed to the DNA. Complementary strands were synthesized and the plasmids transformed into *E. coli* strain DH5 α (Gibco–BRL, Baltimore, MD, U.S.A.). Transformants were screened by colony hybridization using the mutagenic primers as probes, and the mutations were confirmed by DNA sequencing. The mutant *Xba*I–*Bam*HI fragments were then cloned back into the pECE expression vector containing the hIR DNA (gift from Dr. W. Rutter) (Ebina et al., 1985), which had been modified by deletion of the *Bam*HI site in the non-coding region of the vector.

Table 1 Mutagenic primers

Mutant	Mutagenic primers
Cys981Ala	5'-GATGTGTTTCCAGCCTCTGTGTACG-3'
Cys1056Ala	5'-GGGCTTACCCGCCATCAGTGG-3'
Cys1138Ala	5'-GCAGCGAGAAACGCCATGCTCGCC-3'
Cys1234Ala	5'-CCCGACAACGCTCCAGAGAG-3'
Cys1245Ala	5'-CATGCGCATGGCCTGGCAATTC-3'
Cys1308Ala	5'-CCTCGCAGCGCTCAGAGGGAG-3'

Cell culture and DNA transfection

Chinese hamster ovary (CHO) cells were maintained in minimal essential medium (α -MEM) containing 5% fetal calf serum (FCS) in an atmosphere of 5% CO₂ at 37 °C. Cell lines expressing the Cys981Ala, Cys1056Ala, Cys1138Ala, Cys1234Ala, Cys1245Ala or Cys1308Ala mutant receptors were obtained by co-transfection of 10 μ g of plasmid DNA and 2 μ g of pSVNeo DNA using a lipid-mediated transfection technique with Lipofectin (Gibco–BRL). Positive expressing clones were selected after limiting dilution by e.l.i.s.a. against biotinylated monoclonal antibody (mAb) α IR-1, an antibody to the hIR (Kull et al., 1982) obtained from the American Tissue Type Culture Collection, and by ¹²⁵I-insulin-binding analysis. High expressing cell lines were further selected by flow cytometry and sorted using a Coulter EPICS Elite flow cytometer. Briefly, cells were treated with another anti-hIR mAb, 83-7, kindly provided by Dr. Ken Siddle, Cambridge University, Cambridge, U.K. (Soos et al., 1986) followed by a fluorescein isothiocyanate (FITC)-labelled sheep anti-mouse secondary antibody (Selenius, Hawthorn, Vic., 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.

Metabolic labelling

Tran³⁵C-label (ICN Biomedicals) ([³⁵S]methionine/[³⁵S]cysteine approx. 7:3) was added to 80%-confluent cell monolayers in cysteine- and methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 0.5% FCS (CSL), and incubation was continued overnight (12–14 h). IRs were then prepared and analysed by SDS/PAGE after cell solubilization as detailed previously (Lammers et al., 1989; Macaulay et al., 1994) and summarized below.

Receptor extraction and immunoprecipitation

Cells in 55 cm² dishes were washed twice with PBS, pH 7.4, containing 100 μ M NaVO₄, then lysed by the addition (800 μ l/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 μ g/ml leupeptin, 1 μ g/ml pepstatin, 2 mM phenylmethanesulphonyl fluoride, 200 μ M NaVO₄ and hIR immunoprecipitated with mAb 83-7 essentially as detailed previously (Lammers et al., 1989; Macaulay et al., 1994). Receptors immunoprecipitates were used for (i) autophosphorylation and kinase assay, (ii) analysis by SDS/PAGE (metabolically labelled cells) or (iii) insulin-binding assay.

Receptor purification and alkylation with [³H]NEM

IRs from CHO or CHOT cells and each of the cytoplasmic cysteine mutant hIRs were purified by affinity chromatography on columns of anti-hIR mAb 83-14 (Soos et al., 1986) cross-linked to Protein A-agarose. The affinity resin was prepared utilizing a Pierce Immunopure IgG Orientation kit and affinity-purified mAb 83-14. Cell lysates prepared as described above from one 55 cm² dish for each mutant were mixed with 80 μ l of resin for 4 h at 4 °C. The resin was transferred to spin columns and washed three times with HNTG buffer (50 mM Hepes/150 mM NaCl/10% glycerol/0.1% Triton X-100, pH 7.2). IR was then eluted by the addition of 2 $\times$ 100 μ l washes of immunopure gentle elution buffer (Pierce). Alkylation was allowed to proceed for 10 min in the presence of 36 μ Ci of [³H]NEM (44.4 Ci/mmol), and then in the presence of 300 μ M unlabelled NEM for 30 min. Unlabelled NEM was then added to a

concentration of 12 mM and the receptor preparations were concentrated on 50 kDa cut-off Microsep centrifugal concentrators (Filtron). Alkylated hIR preparations were analysed by SDS/PAGE under reducing conditions followed by autoradiography after fluorography.

Receptor autophosphorylation and kinase activity

The effect of insulin on autophosphorylation and kinase activity was examined as detailed previously (Macaulay et al., 1994). 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. Where used, NEM or iodoacetamide (IAA) were added to 2 mM, 1 h after initiation of receptor immunoprecipitation, and then maintained throughout all washes and subsequent incubations. Autophosphorylation of hIR immunoprecipitates and kinase activities towards a synthetic 12-residue peptide were then determined in the same incubations. The peptide substrate used, RRDIFENDYFRK, was derived from the tyrosine-phosphorylation sequence (residues 1154–1165) of the hIR tyrosine kinase domain (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 NaVO_4 , 50 μM [γ - ^{32}P]ATP (8 $\mu\text{Ci}/\text{ml}$) and 0.28 mg/ml synthetic peptide in a final volume of 25 μl . 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 after SDS/PAGE in 7.5% acrylamide. Phosphorylated bands were visualized by autoradiography and excised and the level of autophosphorylation was quantified by Čerenkov counting in a liquid-scintillation counter. The supernatant was used to measure kinase activity towards the synthetic peptide substrate. Peptide was separated from the reaction mixture on P81 paper (Whatman), washed in 30% acetic acid/0.5% phosphoric acid, before Čerenkov counting.

Analysis of insulin-binding

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 $\mu\text{Ci}/\mu\text{g}$) 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 185–371 $\mu\text{Ci}/\mu\text{g}$; NEN–Du Pont) over a range of insulin concentrations using receptor bound to microtitre plates as previously described (Clark et al., 1991; Macaulay et al., 1994). Briefly, microtitre plates were precoated with 2 μg of rabbit anti-mouse IgG (DAKO), for 3 h at 22 °C, washed, blocked with 20 mg/ml BSA and then coated with 0.1 $\mu\text{g}/\text{well}$ anti-hIR mAb 83-7 (Soos et al., 1986) overnight at 4 °C. After being washed, 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 (40 000 c.p.m./well; 1 fmol–0.1 nmol/well) was added overnight in 50 μl at 4 °C. The wells were then washed and counted on a γ -counter. Data was analysed using the Ligand program (Munson and Rodbard, 1980).

Measurement of glucose utilization

This was determined as the conversion of [5 - ^3H]glucose into H_2O as described previously (Brown and Garratt, 1974; Macaulay and Larkins, 1990a,b; Macaulay et al., 1994). 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, pH 7.4, containing 0.5% BSA, 5 mM glucose, 2 mM pyruvate and 1.15 mM CaCl_2 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 - ^3H]glucose for an additional 30 min. $^3\text{H}_2\text{O}$ in the medium was then measured in aliquots after absorption into CaCl_2 .

Measurement of glucose transport

Glucose transport was measured as 2-deoxy[U - ^{14}C]glucose uptake as previously described (Macaulay et al., 1992, 1994). Cells prepared as described above for glucose-utilization studies were transferred to Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing 2 mM pyruvate, 0.5% BSA and 0.1 mM glucose, (instead of 4 mM glucose as used in utilization studies) and allowed to equilibrate for 2 h. Uptake of 5 mM 2-deoxy-[U - ^{14}C]glucose was measured over a 10 min time course, after insulin stimulation for 30 min over the concentration range 0.007–35 nM.

Measurement of thymidine incorporation

Assays were performed essentially as described previously (Chou et al., 1987). Confluent monolayers in 96-well plates were maintained in α -MEM/0.05% FCS for 18 h. Insulin was then added to wells in quadruplicate over a concentration range 0.07–700 nM in the same medium for the next 16 h before the addition of [6 - ^3H]thymidine (0.5 $\mu\text{Ci}/\text{well}$) in α -MEM for 2 h. Incubations were stopped by solubilization of the cells in 1% Triton X-100 and filtration on to glass-fibre filters with several washes with water using an Inotech Cell Harvester (Lansing, MI, U.S.A.).

Statistical analysis

Statistical analysis was performed using Student's t test. Results are expressed as means $\pm$ S.E.M. where appropriate. Statistical significance was determined at the 0.05 level.

RESULTS

Characteristics of the cell lines

Flow-cytometric analysis

Cell lines expressing receptors containing the Cys981Ala, Cys1056Ala, Cys1138Ala, Cys1234Ala, Cys1245Ala and Cys1308Ala mutations 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% expressing cells. These were sorted a further three times after which stable cell lines expressing each of the mutations were obtained. Figure 1 compares the fluorescence from the parental CHO cell line with that of the stable wild-type hIR-expressing cell line, CHOT, and each of the cytoplasmic cysteine mutants. The CHOT cell line, which is reported to express 2.5×10^5 – 2.5×10^6 hIRs/cell (Ellis et al., 1986), was produced by transfection with a construct in the same

Figure 1 Flow-cytometric analysis of cells expressing IR cytoplasmic cysteine mutants

CHO, CHOT and cell lines expressing IR cytoplasmic cysteine mutants were treated first with anti-hIR mAb 83-7 followed by FITC-conjugated sheep anti-mouse antibody and analysed by flow cytometry as described in the Materials and methods section.

Figure 2 Insulin-binding analysis

IRs from CHOT (+), Cys981Ala (X), Cys1056Ala (X), Cys1138Ala (◆), Cys1234Ala (*), Cys1245Ala (■) and Cys1308Ala (▲) mutant cell lines were immunoprecipitated in 96-well plates coated with anti-hIR mAb 83-7 and then incubated with 125 I-insulin (approx. 40000 c.p.m./well; 371 μ Ci/ μ g) in the presence of different concentrations of unlabelled insulin over the range 0.01 nM to 1 μ M. Each point represents the mean of three experiments in which triplicate determinations were made at each point within each experiment. S.E.M. values at each point were less than 5% and therefore not shown.

pECE vector as used in the present study. 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. Each of the cytoplasmic-cysteine-mutant cell lines displayed a similar shift (approx. 10-fold) in fluorescence compared with the parental line, indicating that the CHOT cell line and the mutant cell lines expressed comparable numbers of receptors/cell.

Insulin binding

The binding of 125 I-insulin to the wild-type and mutant receptors is shown in Figure 2. The concentration of unlabelled insulin required for half-maximal displacement of 125 I-insulin was approx. 0.5 nM for each of the mutant receptors as well as the

Table 2 Insulin-binding analysis

Data are derived from Scatchard analysis (Munson and Rodbard, 1980) of the number of experiments shown as described in Figure 2 and in the Materials and methods section.

Receptor	<i>n</i>	K_d1 (M)	K_d2 (M)	No. of receptors/cell
CHOT	5	$2.4(\pm 0.9) \times 10^{-10}$	$1.0(\pm 0.3) \times 10^{-8}$	$5.9(\pm 0.1) \times 10^5$
Cys981Ala	4	$2.5(\pm 0.4) \times 10^{-10}$	$1.1(\pm 0.3) \times 10^{-8}$	$7.0(\pm 0.1) \times 10^5$
Cys1056Ala	3	$3.2(\pm 1.8) \times 10^{-10}$	$1.5(\pm 0.5) \times 10^{-8}$	$7.3(\pm 2.2) \times 10^5$
Cys1138Ala	3	$2.8(\pm 1.0) \times 10^{-10}$	$2.2(\pm 1.5) \times 10^{-8}$	$9.8(\pm 3.8) \times 10^5$
Cys1234Ala	3	$2.3(\pm 1.0) \times 10^{-10}$	$2.9(\pm 2.1) \times 10^{-8}$	$5.2(\pm 0.8) \times 10^5$
Cys1245Ala	3	$1.9(\pm 0.1) \times 10^{-10}$	$1.1(\pm 0.3) \times 10^{-8}$	$9.2(\pm 2.1) \times 10^5$
Cys1308Ala	3	$2.4(\pm 0.7) \times 10^{-10}$	$1.3(\pm 0.5) \times 10^{-8}$	$9.4(\pm 2.7) \times 10^5$

Figure 3 [3 H]NEM alkylation of IR cytoplasmic cysteine mutants

IRs from CHO and CHOT and cell lines expressing each of the IR cytoplasmic cysteine mutants were purified by affinity chromatography on anti-hIR mAb 83-14 cross-linked agarose, and 36 μ Ci of [3 H]NEM was then added to each preparation initially in the absence and then in the presence of graded concentrations of unlabelled NEM over a 50 min period as detailed in the Materials and methods section. The receptor preparations were then concentrated on 50 kDa cutoff Microsep centrifugal concentrators and analysed by SDS/PAGE under reducing conditions followed by autoradiography after fluorography in two separate experiments. In (a) results for Cys1138Ala, Cys1234Ala, Cys1245Ala, Cys1308Ala, CHO and CHOT are shown. In (b) a separate experiment for Cys981Ala, Cys1056Ala, Cys1308Ala and CHOT are shown.

wild-type hIR (CHOT). At no insulin concentration did the displacement curves differ significantly from each other, although there was more variability at the lower concentrations. Analysis of the data by the method of Scatchard (Scatchard, 1949; Munson and Rodbard, 1980) (Table 2) yielded similar characteristic curvilinear plots that could be resolved into two classes of binding sites with similar K_d values and a similar number of binding sites/cell, supporting the data in Figure 1 from flow-cytometric analysis.

Figure 4 Effects of insulin and of alkylation with NEM or IAA on autophosphorylation of IR cytoplasmic cysteine mutants

IRs from CHOT and IR cytoplasmic cysteine mutant cell lines were immunoprecipitated with anti-hIR mAb 83-7 in six equivalent aliquots and incubated in the presence or absence of 0.7 μ M insulin to determine the effect of insulin on autophosphorylation and kinase activity as well as insulin binding level, as detailed in the Materials and methods section. In some experiments 2 mM NEM or 2 mM IAA was added for the final 60 min of immunoprecipitation and during incubation with [γ - 32 P]ATP, as indicated. Samples for autophosphorylation were matched for binding level and analysed by SDS/PAGE and autoradiography in the reduced state.

Receptor alkylation with [3 H]NEM

IRs from CHO, CHOT and each of the mutant cell lines were purified by affinity adsorption to anti-hIR mAb 83-14 affinity resin and high-salt elution before alkylation with [3 H]NEM as described in the Materials and methods section. Figure 3 shows two representative autoradiographs after SDS/PAGE of alkylated receptor preparations compared with native hIR metabolically labelled with 35 S from CHOT cells. The autoradiographs clearly show alkylation of bands of approx. 95 and 180–200 kDa in receptor preparations from CHOT cells and each of the mutant cell lines. No labelling of CHO receptor extracts (Figure 3a) was detected because mAb 83-14 is specific for hIR and does not affinity-purify the parental CHO receptors. This lane served as a non-specific control for the other preparations. The 95 and 180–200 kDa bands in each of the alkylated receptor preparations co-migrated with bands of similar molecular mass in native IR metabolically labelled with 35 S consistent with them being the β -subunit of the IR and proreceptor respectively. An additional band of slightly less than 180 kDa was apparent in the lane containing the Cys981Ala mutant receptor. This probably resulted from proteolytic cleavage of the proreceptor as it was not seen in other alkylations of this mutant receptor but was apparent in some of the other mutant receptors in other experiments (results not shown). The 135 kDa band seen in the native IR metabolically labelled with 35 S, the α -subunit of the IR, was not alkylated in any of the receptor preparations, consistent with previous studies that demonstrated alkylation of only the β -subunit under similar conditions (Finn et al., 1990). The experiments were repeated two (Cys981Ala) or three (CHOT and remaining hIR cytoplasmic cysteine mutants) times and similar results were obtained in each case. Thus, despite mutagenesis of each of the cytoplasmic cysteines of the hIR in turn, each mutant receptor was still capable of being alkylated by NEM specifically on the β -subunit.

Receptor autophosphorylation and kinase activity

The ability of insulin to stimulate receptor autophosphorylation and kinase activity, and the effects of alkylation with NEM or

Table 3 Effect of alkylation on insulin stimulation of receptor autophosphorylation *in vitro*

IR autophosphorylation was measured by Čerenkov counting of SDS/polyacrylamide gel slices after incubation as described in the legend to Figure 4. The number of experiments is given in parentheses; two or more determinations were made within each experiment.

Receptor	Insulin stimulation of receptor autophosphorylation (% of basal)	Alkylation effects on insulin-stimulated autophosphorylation (% of insulin-stimulated value)	
		NEM + insulin	IAA + insulin
CHOT	186 $\pm$ 22 (20)	65 $\pm$ 5 (9)	113 $\pm$ 3 (7)
Cys981Ala	201 $\pm$ 53 (5)	66 $\pm$ 5 (4)	111 $\pm$ 2 (3)
Cys1056Ala	203 $\pm$ 24 (12)	64 $\pm$ 2 (4)	109 $\pm$ 5 (4)
Cys1138Ala	182 $\pm$ 23 (12)	82 $\pm$ 6 (4)	111 $\pm$ 3 (3)
Cys1234Ala	205 $\pm$ 31 (11)	61 $\pm$ 10 (4)	119 $\pm$ 4 (3)
Cys1245Ala	194 $\pm$ 31 (13)	64 $\pm$ 6 (4)	108 $\pm$ 6 (4)
Cys1308Ala	198 $\pm$ 32 (12)	69 $\pm$ 3 (4)	111 $\pm$ 3 (3)

IAA on this activity, was assessed in immunoprecipitates obtained from cells expressing each of the cytoplasmic cysteine mutations. Figure 4 shows an autoradiograph of the CHOT and cysteine-mutant-cell immunoprecipitates after incubation in the absence of insulin, or in the presence of insulin with or without the alkylating agents NEM or IAA, followed by incubation with [γ - 32 P]ATP and analysis by SDS/PAGE. Insulin stimulated phosphorylation of two bands of apparent molecular mass 180 and 95 kDa and one band migrating with the front, which was probably the IR peptide substrate added for receptor kinase determination. The 95 kDa band we believe to be the hIR β -subunit and the 180 kDa band is probably the unprocessed proreceptor, similar to the studies shown in Figure 3. Over several experiments (Table 3), insulin elicited a 1.8–2-fold stimulation of receptor autophosphorylation *in vitro*. The ability of insulin to stimulate autophosphorylation of the 95 kDa β -subunit and phosphorylation of the other two bands was partially inhibited

Table 4 Effect of alkylation on insulin stimulation of cysteine-mutant-receptor kinase activity

Receptor kinase activity was measured in immunoprecipitated receptor preparations incubated in the presence or absence of 0.7 μ M insulin as the receptor was immunoprecipitated in the same experiments as receptor autophosphorylation (Figure 4). Basal kinase activity was 4234 $\pm$ 1316 c.p.m. for Cys981Ala, 2425 $\pm$ 644 c.p.m. for Cys1056Ala, 2786 $\pm$ 743 c.p.m. for Cys1138Ala, 3349 $\pm$ 1210 c.p.m. for Cys1234Ala, 4949 $\pm$ 1824 c.p.m. for Cys1245Ala, 4349 $\pm$ 1105 c.p.m. for Cys1308Ala and 4674 $\pm$ 1122 c.p.m. for CHOT.

Receptor	Insulin stimulation of receptor kinase (% of basal)	Alkylation effects on insulin-stimulated kinase (% of insulin stimulated value)	
		NEM + insulin	IAA + insulin
CHOT	393 $\pm$ 105 (22)	46 $\pm$ 5 (12)	171 $\pm$ 18 (13)
Cys981Ala	440 $\pm$ 34 (6)	47 $\pm$ 8 (6)	141 $\pm$ 8 (5)
Cys1056Ala	352 $\pm$ 57 (15)	39 $\pm$ 7 (8)	159 $\pm$ 12 (7)
Cys1138Ala	425 $\pm$ 74 (13)	32 $\pm$ 10 (6)	156 $\pm$ 20 (5)
Cys1234Ala	456 $\pm$ 95 (13)	42 $\pm$ 10 (5)	173 $\pm$ 25 (5)
Cys1245Ala	406 $\pm$ 56 (13)	48 $\pm$ 8 (6)	171 $\pm$ 22 (7)
Cys1308Ala	413 $\pm$ 69 (13)	40 $\pm$ 5 (7)	149 $\pm$ 13 (5)

by alkylation of the receptor immunoprecipitates with NEM, not only in the native IR as previously reported (Finn et al., 1990), but also in immunoprecipitates from each of the cytoplasmic-cysteine-mutant cell lines (Figure 4, Table 3). The level of inhibition was 18–39% of insulin-stimulated autophosphorylation (Table 3). Alkylation of receptor preparations with IAA enhanced slightly (8–19%) the ability of insulin to stimulate receptor autophosphorylation, although this did not reach statistical significance for the Cys1056Ala and Cys1245Ala mutants (Table 3).

The effects of alkylation of the cytoplasmic cysteine mutants on receptor kinase activity mirrored the results for receptor autophosphorylation (Table 4) except that the magnitude of the effects was greater. Thus insulin stimulated kinase activity 3.5–4.5-fold over basal and there was no significant difference between the stimulation seen with the native insulin receptor (CHOT cells) and any of the cytoplasmic-cysteine-mutant cell lines. NEM inhibited insulin-stimulated kinase activity by 52–68% and IAA augmented kinase activity by 41–73%. There was no significant difference between the effects of alkylation of the wild-type CHOT receptor and alkylation of the cytoplasmic cysteine mutant receptors. Basal kinase activity was variable from experiment to experiment, but within each experiment was similar for each of the cell lines investigated (see the legend to Table 4).

Effects of insulin on glucose utilization in cells expressing the hIR cytoplasmic cysteine mutations

A major action of insulin is to stimulate the uptake and storage of utilization of glucose in cells. The ability of insulin to stimulate glucose utilization in cells expressing each of the mutant receptors was compared with that in CHOT cells, expressing the wild-type hIR, and the parental CHO cell line, to assess the contribution of endogenous receptors (approx. 3000 per cell) to any effect seen (Figure 5). Insulin stimulated glucose utilization in the parental CHO cell line only at the highest concentration of insulin, 35 nM, tested. In contrast, the lowest insulin concentration examined, 0.007 nM, stimulated glucose utilization significantly

Figure 5 Effect of insulin on glucose utilization in cells expressing IR receptor cytoplasmic cysteine mutants

CHO, CHOT and cells expressing each of the IR cytoplasmic cysteine mutants were preincubated for 10 min in the presence or absence of the indicated concentrations of insulin. [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 means of eight to twelve experiments in which three independent cell incubations were performed within each experiment. All transfected cell lines showed significant ($P < 0.05$) stimulation by insulin above basal at insulin concentrations of 0.07 nM and above. The CHO cell response was significant only at 35 nM insulin ($P < 0.01$). S.E.M. values (not shown) were less than 20% of basal. Basal activities were 1290 $\pm$ 328 pmol/min per well ($n = 10$) for Cys981Ala ($\times$), 591 $\pm$ 213 pmol/min per well ($n = 8$) for Cys1056Ala ($\times$), 946 $\pm$ 429 pmol/min per well ($n = 8$) for Cys1138Ala ($\diamond$), 824 $\pm$ 216 pmol/min per well ($n = 6$) for Cys1234Ala ($*$), 751 $\pm$ 349 pmol/min per well ($n = 6$) for Cys1245Ala ($\square$), 734 $\pm$ 313 pmol/min per well ($n = 11$) for Cys1308Ala ($\blacktriangle$), 965 $\pm$ 326 pmol/min per well ($n = 10$) for CHOT ($+$) and 1014 $\pm$ 294 pmol/min per well ($n = 11$) for CHO ($\square$).

over basal in the native IR-expressing cell line, CHOT ($P < 0.05$), as well as two of the cytoplasmic-cysteine-mutant cell lines (Cys1056Ala and Cys1245Ala; $P < 0.001$ and $P < 0.05$ respectively). Insulin at the next highest concentration tested, 0.07 nM, significantly stimulated glucose utilization in all of the receptor mutants. Each mutant-expressing cell line approached maximal insulin-stimulated levels at 0.7 nM insulin which was similar to or higher than that of the cell line expressing native hIR CHOT. Basal glucose-utilization levels did not differ significantly from cell line to cell line (see legend to Figure 5). Thus none of the mutations markedly affected the insulin signal-transduction pathway to glucose utilization.

Effects of insulin on glucose transport in cells expressing hIR cytoplasmic cysteine mutations

The effects of insulin on glucose transport in each mutant-expressing cell line are shown in Figure 6. In the parental CHO cell line, a significant stimulation of only 12% ($P < 0.01$) was obtained only at the highest concentration of insulin, 35 nM. In contrast, significant stimulation ($P < 0.05$) was obtained at 0.07 nM insulin in the native-hIR-expressing cell line, CHOT, and each of the IR cytoplasmic cysteine mutants except for the Cys981Ala. In three of the cell lines, CHOT, Cys1138Ala and Cys1234Ala, significant stimulation ($P < 0.01$, $P < 0.01$ and $P < 0.05$ respectively) was achieved with the lowest concentration of insulin tested, 0.007 nM. The maximal response was also higher in the transfected cell lines than in the parental CHO cell line, and ranged between 27% (Cys981Ala) and 54% (Cys1056Ala), compared with 12% for the CHO cell line. The cell lines expressing Cys981Ala and Cys1245Ala both showed a significant impairment of insulin-stimulated glucose transport

Figure 6 Effect of insulin on glucose transport in cells expressing IR cytoplasmic cysteine mutants

CHO, CHOT and cells expressing each of the IR cytoplasmic cysteine mutants were preincubated for 30 min in the presence or absence of the indicated concentrations of insulin. 2-Deoxy[U- 14 C]glucose (0.1 mM; 0.25 μ Ci) was then added for the next 10 min, after which the reaction was stopped, the cells were washed and lysed, and samples were removed for determination of incorporation into cells as described in the Materials and methods section. Results, expressed as the percentage of basal glucose-transport rate, are given as means of three to ten experiments in which three independent cell incubations were performed within each experiment. All cell lines except the parental CHO and Cys981Ala showed significant ($P < 0.05$) stimulation by insulin concentrations of either 0.007 nM or 0.07 nM and above. The Cys981Ala cell response was significant only at 0.7 nM and 35 nM insulin ($P < 0.05$). The parental CHO response was significant only at 35 nM insulin ($P < 0.05$). S.E.M. values (not shown) were 12% of basal or less. Basal activities were 88 ± 18 pmol/min per well ($n = 4$) for Cys981Ala ($\square$), 33 ± 5 pmol/min per well ($n = 5$) for Cys1056Ala ($\times$), 36 ± 8 pmol/min per well ($n = 4$) for Cys1138Ala ($\blacklozenge$), 49 ± 13 pmol/min per well ($n = 4$) for Cys1234Ala ($\ast$), 77 ± 15 pmol/min per well ($n = 4$) for Cys1245Ala ($\blacksquare$), 48 ± 7 pmol/min per well ($n = 5$) for Cys1308Ala ($\blacktriangle$), 41 ± 7 pmol/min per well ($n = 12$) for CHOT ($+$) and 71 ± 8 pmol/min per well ($n = 15$) for CHO ($\square$).

compared with the CHOT cell line at all insulin concentrations tested ($P < 0.05$). Thus these mutations both affected the insulin signal-transduction pathway to glucose transport.

Effects of insulin on thymidine uptake in cells expressing the hIR cytoplasmic cysteine mutations

The effects of insulin on thymidine uptake in cells expressing hIR containing each of the cytoplasmic cysteine mutations were determined in order to assess potential effects of the mutations on mitogenic activity (Figure 7). Unlike the responses of glucose utilization and transport (Figures 5 and 6), the parental CHO cells demonstrated a significant stimulation of 24% at 0.7 nM insulin ($P < 0.05$) which increased to 69% at 700 nM insulin. The responses of the transfected cell lines fell into two groups. One group, those expressing Cys1056Ala, Cys1138Ala, Cys1234Ala and Cys1308Ala and CHOT, followed a similar response profile with significant insulin stimulation of thymidine uptake at 0.07 nM insulin (Cys1056Ala and CHOT) or 0.07 nM insulin (Cys1138Ala, Cys1234Ala and Cys1308Ala) and maximal stimulation of 108–155% of basal.

The cell lines expressing Cys981Ala and Cys1245Ala were different, following a profile similar to that of the parental CHO cell line. Stimulation of thymidine uptake was significant in the latter (25%; $P < 0.01$) at 0.07 nM insulin and in the former (45%; $P < 0.05$) at 0.7 nM insulin. Maximal insulin stimulation in this group was lower, at 52–69%. These data indicate that both the Cys981Ala and the Cys1245Ala mutation impair mitogenic signalling by the insulin receptor.

Figure 7 Effect of insulin on thymidine uptake in cells expressing IR cytoplasmic cysteine mutants

CHO, CHOT and cells expressing each of the IR cytoplasmic cysteine mutations were preincubated for 16 h in the presence or absence of the indicated concentrations of insulin before the addition of [3 H]thymidine (0.5 μ Ci/well) in α -MEM for 2 h. Incubations were stopped by solubilization of the cells in 1% Triton before cell harvesting on to glass-fibre filters and determination of incorporated label. Results expressed as the percentage of basal thymidine incorporation are given as means of four to ten experiments in which four independent cell incubations were performed within each experiment. Not all points were examined within all experiments. All cell lines except for those expressing the Cys981Ala mutant showed significant ($P < 0.05$) stimulation by insulin above basal at concentrations of insulin of 0.7 nM and above. The responses of cells expressing Cys981Ala were significant at the next highest concentration of insulin, 7 nM, ($P < 0.01$) and above. S.E.M. values (not shown) were 19% of basal or less. The symbols are the same as in Figures 5 and 6.

DISCUSSION

Cysteine residues have been shown in previous studies to have a functional as well as a structural role in enzyme activity. These roles include the formation of phosphate intermediates in phosphatase catalysis (Guan and Dixon, 1991), disulphide exchange (Gilbert, 1984) and the more obvious oxidation/reduction roles [see for example Landgraf et al. (1991)]. Thiols critical for activity have been identified in a number of enzymes, including phosphoenolpyruvate carboxykinase (Lewis et al., 1989) and lymphocyte-specific tyrosine kinase p56^{lck} (Veillette et al., 1993), using both mutagenic and chemical approaches. In the present study mutant hIRs were generated in which each cytoplasmic cysteine was replaced in turn with alanine in an attempt to identify critical hIR thiols. Two cysteines, Cys-981 and Cys-1245, appear to be important for mitogenic signalling and glucose uptake, as receptors expressing these mutations were less effective in transducing an insulin signal to stimulate uptake of thymidine and glucose than was the native receptor. These effects were observed in the face of normal IR autophosphorylation, IR kinase and glucose-utilization responses to insulin. Preliminary analysis of the effects of insulin on tyrosine-phosphorylation patterns of cell extracts of each mutant receptor line were indistinguishable from those of extracts of the native hIR-expressing cell line, CHOT (results not shown). Our data further imply that insulin stimulation of glucose transport is not rate-limiting for glucose utilization in these cell lines at 5 mM glucose, as impaired glucose-utilization responses in the cell lines expressing the Cys981Ala and Cys1245Ala mutations might otherwise have been expected in view of the impaired glucose-transport response seen in these lines.

Interpretation of these insulin effects on downstream signalling by the mutant IRs must be tempered with some caution. The magnitude of the insulin response in CHO cells, although similar to previous reports (i.e. Macaulay et al., 1992; Kato et al., 1993),

		αA	$\beta 1$	$\beta 2$	$\beta 3$	αB	
cAPK	1/	GNAAAAKGSEQESVKEFLAKAKEDFLKKWEDPSQNTAQDQDFRIKTLGTGSFGRVMLVKHKEG.....NHYAMKILDKQKVVKLKQ					
IR	953/	RKROPDGPLPLYASSNPEYLSASDVFPSCSVYVPDEWEVSREKILLRELGGSGFGMVYEGNARDIKGEAETRVAVKTVNESA..SLRE					
EGFR	645/	RRRHIVRKRTLRLRLQERELVEPLTPSGEAPNOALLRIKETEKKIKVLKSGAGFTVYKGLWIPEGEK.VKIPVAIKELREAT..SPKA					
			*	*	*	*	
		αC	$\beta 4$	$\beta 5$	αD	αE	$\beta 6$
cAPK	85/	IEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLYMVMEYVAGGEMFSLRRIGR.....FSEPHARFYAAQIVLTTEYLSLDLIY					
IR	1041/	RIEFLNEASVMKGFTCHHVRLLGVSQGPLVVMELMAHGDLKSYLRSLRPEAENNPGRPPPTLQEMIQMAAEIADGMAYLNAKKFMH					
EGFR	732/	NKEILD EAYVMASVDNPHVCRLLGLICTLSTVQLITQLMPFGCLLDYVREHKDN.....IGSQYLLNWCQVIAKGMNYLEDRLVH					
		*					
		$\beta 7$	$\beta 8$	$\beta 9$	αF	αG	
cAPK	165/	RDLKPENLLIDQGGYIQVTDGFAKRVKGR.....WTLCGTPEYLAPEIILSKGYNKAVDWMALGVLIYEMAA.GYPPFFADQPIQIYE					
IR	1131/	RDLAARNCMVAHDFTVKIGDFGMDRIYETDYRKGKGLLPVRWMAPESLKDCVFTTSSDMWSFGVVLWEITSLAEQPYQGLSNEQVLK					
EGFR	812/	RDLAARNVLTQPHVKITDFGLAKLLGAEKEYHAEGGKVPKWMALSTLHRIYTHQSDVWSYGVTVWELMTFGSKPYDGPPIASEISS					
		***	*	***	*	*	
		αH	αI				
cAPK	249/	KIVSG.KVRFPSHFSSDLKLLRNLLQVDTKRFGNLKDGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNDDYEEEEIRV					
IR	1221/	FVMDGGYLDQPDNCPERVTDLNRMCWQFNPKMR.PTFLEIVNLLKDDLHPSFPEVSFFHSEENKAP.....ESEELE.....MEFEDME					
EGFR	902/	ILEKGERLPQPPICTIDVYIMHVKCMIDADSR.PKPRELITIEFSKMARDPQRYLVIGQDERMHLP.....SPTDSNFYRALMDEEDMD					
			*				
cAPK	338/	SINEKCKGEFTEF					
IR	1299/	NVPLDRSSHQREEAGGRDGGSSLGFKRSYEEHIPYTHMNGGKKNGRIILTLPRSNPS					
EGFR	985/	DVVDAD EYLIPQGGFFSSPSTSRPTLLSSLATSNNSTVACIDRNLGQSCP IKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQSVF					
EGFR	1075/	KRPAGSVQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLVNTVQPTCVNSTFDSPAHWAAQKGSHQISLDNPDYQQDFFI'KEAKPNGI'FK					
EGFR	1165/	GSTAENAEYLRVAPQSSEFIGA					

Figure 8 Sequence alignment of the kinase domains of the hIR, cAPK and EGFR

The primary sequence of the hIR was aligned with the sequences of cAPK and EGFR by using invariant residues and the structural model of cAPK as modelled by Knighton et al. (1991, 1993a).

was small compared with that in normal insulin-responsive cell types such as adipocytes and skeletal muscle, in which the stimulation of insulin action is more typically 5–20-fold [see, for example, Macaulay and Larkins (1990a,b) and Wilson and Cushman (1994)]. Possible explanations for the lack of responsiveness in this cell type include, as previously suggested by Kato et al. (1993), the lack of GLUT4, effects of endogenous receptors, the formation of chimaeric receptors and/or defects in post-receptor signalling.

The finding of impaired receptor function in the Cys981Ala mutant cell line is surprising in view of the fact that this cysteine is replaced by serine in the rat and mouse IR sequences (Goldstein and Dudley, 1990; Flores-Riveros et al., 1989). However, this amino acid change may be considered functionally conservative as a recent study showed that mutagenesis of a cysteine that is normally palmitoylated in the transferrin receptor to serine resulted in a receptor palmitoylated on the serine when expressed in cells (Jing and Trowbridge, 1990). On the other hand, the second functionally significant mutation, Cys-1245, is invariant amongst all growth factor receptors and, with only two exceptions, across the entire known tyrosine kinase family (Hanks, 1991). However, the finding that the Cys1245Ala mutant was kinase-active was surprising in view of a recent report showing that the equivalent cysteine in the lymphocyte-specific tyrosine kinase, p56^{lck}, was critical for activity (Veillette et al., 1993). Interestingly, Cys-1138 and Cys-1234 are also highly conserved, albeit not to the extent of Cys-1245, and yet mutations at these cysteines produced hIRs that were apparently fully functional. Receptors expressing Cys1056Ala, which is histidine in most other tyrosine kinases, or Cys1308Ala, which lies in the unique C-terminal tail of the hIR, were also fully functional. Thus only two of six IR cytoplasmic cysteines, Cys-981 and Cys-1245, appear to be potential candidates for a role in IR signalling.

Previous alkylation studies (Ridge et al., 1988; Chiacchia, 1991) indicated that the hIR contains a single free thiol group within the β -subunit. Finn et al. (1990) showed that the effects of alkylation by NEM could be blocked to a large extent (approx. 70%) by preincubation of receptors with MnATP, indicating that the free thiol was located in the cytoplasmic region of the receptor, close to the ATP-binding domain, and could play a role in receptor function. We found, however, that each of the cytoplasmic cysteine mutant hIRs and the native hIR from CHOT cells could be labelled with [³H]NEM. This labelling was specific for free cysteine as, consistent with previous studies, only the β -subunit of the receptor was labelled in each case. Thus it is unlikely that any single cytoplasmic cysteine alone contributes the free thiol to receptor structure, as the appropriate mutant cell line would not be expected to be labelled with [³H]NEM.

These findings were supported by examination of the effects of alkylation on insulin stimulation of hIR autophosphorylation and kinase activity. NEM alkylation of the hIR has been shown previously to inhibit receptor activity, whereas IAA augments it (Shia et al., 1983; Pike et al., 1984; Wilden and Pessin, 1987; Li et al., 1991; Clark and Konstantopoulos, 1993). Mutagenesis of the hIR at the cysteine contributing the free thiol group would be expected to yield a receptor insensitive to the effects of alkylation. However, consistent with our direct [³H]NEM-labelling studies, we found no significant difference between the native hIR and each of the mutant hIRs with respect to their responses to NEM and IAA. In all cases, NEM inhibited insulin stimulation of receptor autophosphorylation and tyrosine kinase activity, whereas IAA augmented it, indicating again that no single hIR cytoplasmic cysteine alone contributes the single free thiol group to receptor structure.

There are at least three possible explanations for the stoichiometry seen in previous studies. The first, and most likely

alternative, is that the stoichiometry of 1 mol of NEM/mol of receptor-binding activity is afforded by fractional labelling of a number of the cytoplasmic cysteines. Fatty acid acylation of the hIR cysteines could provide a mechanism for this, as previous studies have shown that thioester linkages in other proteins, including the p56^{lck} tyrosine kinase (Paige et al., 1993) are metabolically labile [see Schmidt (1989) for review]. As a number of cysteines in the hIR are palmitoylated (Hedo et al., 1987; Magee and Siddle, 1988), fractional alkylation of more than one of the cytoplasmic cysteines might be expected. It is also possible that alkylation of one cysteine sterically hinders alkylation of a second free cysteine. A second possibility is that the differential accessibility and reactivity of cysteine residues changes between the mutant and wild-type receptors. A third possibility is that the single free thiol group is one of the four external β -subunit cysteines, although this latter possibility is unlikely for two reasons. First these residues are involved in α - β (Cheatam and Kahn, 1992) and intrachain β - β (Schäffer and Ljungqvist, 1992) disulphide bonds. Second, alkylation by NEM can be largely blocked by ATP (Finn et al., 1990), indicating that the free thiol lies close to the ATP-binding domain of the receptor kinase.

Our data cannot distinguish between these possibilities. However, implicit in the first alternative is that more than one cysteine is alkylatable and, to be consistent with the data of Finn et al. (1990), these residues must lie close to the ATP-binding domain of the receptor. Although there are no three-dimensional-structure data available for the hIR tyrosine kinase that allow us to demonstrate definitively which cysteines lie close to the ATP-binding domain and would therefore be potential free thiols, the hIR cytoplasmic sequence can be aligned with that of the catalytic domain of the cyclic AMP-dependent protein serine kinase (cAPK), the three-dimensional structure of which is known (Knighton et al., 1991; Taylor and Adams, 1992; Taylor et al., 1993). This form of homology modelling has recently been used to examine structural features of the epidermal growth factor receptor (EGFR) kinase domain (Knighton et al., 1993b).

The alignment of cAPK, EGFR and hIR is shown in Figure 8 and reveals the following features. The cAPK protein contains only two cysteine residues, Cys-199 and Cys-343, compared with the six in the cytoplasmic domain of the hIR. Both of the cAPK cysteines can be alkylated with iodoacetic acid and can be protected from alkylation by ATP, suggesting that they are close to the active site, although only alkylation of Cys-199 affects cAPK enzyme activity (Nelson and Taylor, 1983). None of the cysteines in the hIR cytoplasmic domain correspond to cyclic AMP residue 199, which is located in the T-loop involved in substrate interaction. In contrast, hIR residue 1308 is in a similar position to residue 343 of cAPK (Figure 8). Both are located 37 or 43 residues downstream of the true catalytic core domain, in similar glutamic acid-rich regions of the C-terminal tails of their respective proteins. In cAPK this tail is folded back over the ATP-binding site (Knighton et al., 1991, 1993a; Taylor and Adams, 1992).

The sequence alignments indicate that two of the hIR cysteine residues, Cys-1056 (in the loop connecting α C to β 4) and Cys-1138 (in the β -strand β 7) are homologous to residues near the ATP-binding region of cAPK and could therefore be prime candidates for the ATP-sensitive alkylation reported for IR. Although these residues are not conserved in the EGFR, it does have cysteine residues, Cys-751 and Cys-773, in the same ATP-binding cleft between the two lobes of the kinase domain. Finally, the alignments show that Cys-981 lies in the same region as the α A helix of the cAPK enzyme, an α -helix that spans the surface of both lobes of the catalytic domain (Veron et al., 1993).

It is therefore possible that Cys-981, Cys-1056, Cys-1138

and/or Cys-1308 lie in regions near the ATP-binding site that could be sensitive to alkylation. The two highly conserved cysteine residues at 1234 and 1256 are predicted to lie at either end of an α -helix, at the bottom of the second lobe and well away from the active site (Knighton et al., 1991, 1993a).

In conclusion, the present study demonstrates that none of the cytoplasmic cysteines alone contributes the free thiol group to the receptor structure; however, Cys-981 and Cys-1245 lie in regions of the receptor that are necessary for full functional activity. Description of the crystal structure of the kinase domain of the receptor may enable experiments to be designed that more fully elucidate the role of the cytoplasmic cysteines in IR function.

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