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Cysteine 981 of the Human Insulin Receptor Is Required for Covalent Cross-Linking between β-Subunit and a Thiol-Reactive Membrane-Associated Protein

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ABSTRACT: The cytoplasmic domain of the insulin receptor (IR) β-subunit contains cysteine (Cys) residues whose reactivity and function remain uncertain. In this study, we examined the ability of the bifunctional cross-linking reagent 1,6-bismaleimidohexane (BMH) to covalently link IR with interacting proteins that possess reactive thiols. Transfected Chinese hamster ovary cells expressing either the wild-type human IR, C-terminally truncated receptors, or mutant receptors with Cys → Ala substitutions and mouse 3T3-L1 adipocytes were used to compare the BMH effect. The results showed the formation of a large complex between the wild-type human receptor β-subunit and molecule X, a thiol-reactive membrane-associated protein, in both intact and semipermeabilized cells in response to BMH. Prior cell stimulation with insulin had only a modest effect in this process. Western blot analysis revealed that the receptor α-subunit was not present in the β−X complex. The BMH cross-linking did not inhibit in vitro tyrosine phosphorylation of the receptor complexed with molecule X. Both the human IR Cys981Ala mutant and murine IR, that lacks the equivalent of human Cys^{981}, failed to react with BMH. Finally, no covalent association between IR β-subunit and IRS-1, the protein tyrosine phosphatase LAR or SHP-2 was observed in BMH-treated cells expressing the wild-type human IR. These results demonstrate a striking difference in reactivity among the cytoplasmic IR β-subunit thiols and clearly show that Cys^{981} of human IR β-subunit is in close proximity to a thiol-reactive membrane-associated protein under basal and insulin-stimulated conditions.

After binding to the insulin receptor (IR)[1] α-subunit, insulin triggers activation of the intrinsic tyrosine kinase function of the receptor β-subunit cytoplasmic domain, which, in turn, phosphorylates cellular substrates [e.g., insulin receptor substrates (IRS) 1−4 and Shc proteins] with subsequent activation of downstream signal transduction molecules, including phosphatidylinositol 3-kinase and p21Ras/ mitogen-activated protein (MAP) kinase (*1, 2*). The human insulin receptor is a homodimeric disulfide-linked glycoprotein composed of two αβ monomers each of which contains 37 cysteine residues in the α-subunit and only 10 sulfhydryls in the β-subunit (*3, 4*). Alkylation of IR with ^{3}H-labeled *N*-ethylmaleimide (NEM) suggests that it has one free thiol group per αβ monomer located in the β-chain (*5, 6*). The reactive thiol appears to be near the ATP-binding domain because alkylation is partially inhibited by ATP•Mn^{2+} complex (*7*). Derivatization of β-subunit sulfhydryl groups has had opposite effects on the intrinsic tyrosine kinase activity of IR depending on the alkylating agent used. For example, addition of the bulky NEM to solubilized and purified receptor preparation attenuates insulin-stimulated receptor autophosphorylation and kinase activity toward exogenous substrates (*7, 8*), whereas the smaller alkylating agent iodoacetamide can potentiate these events (*9, 10*). Four of the six intracellular cysteine residues in the human IR β-subunit are highly conserved among members of the insulin receptor kinase family (*11*).

It is generally accepted that intracellular reduction/oxidation (redox) potential plays an important role in the modulation of insulin action. For example, partial inhibition of gluthatione metabolism in intact cells stimulates insulin receptor tyrosine phosphorylation when measured in vitro (*12*). A similar increase in in vitro IR kinase activity is observed following cell treatment with hydrogen peroxide (*13, 14*). Moreover, it has been reported that insulin can stimulate the formation of hydrogen peroxide in a Gαi2-dependent manner (*15*). Because protein tyrosine phosphatases are sensitive to oxidative inactivation (*16, 17*), their inhibition may partly explain why an increase in kinase activity occurs with solubilized and purified IR from oxidant-treated cells. However, oxidation of critical cysteine residues in the receptor kinase domain may result also in an increase in its intrinsic activity (*18*).

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[1] Abbreviations: hIR, human insulin receptor; CHO, Chinese hamster ovary cells; MBB, maleimidobutyrylbiocytin; BMH, 1,6-bismaleimidohexane; PAO, phenylarsine oxide; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; PVP, poly(vinylpyrrolidone); DTT, dithiothreitol; NEM, *N*-ethylmaleimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ECL, chemiluminescence; Δ43 and Δ69, human insulin receptor mutant with a C-terminal truncation of 43 or 69 amino acid residues in the β-subunit; NPEY, Asn-Pro-Glu-Tyr; IRS, insulin receptor substrate.

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A number of cytosolic Src homology 2 (SH2) domain proteins and membrane-associated glycoproteins can regulate IR function by interacting with different regions of this receptor (*19−22*), raising the possibility that the interaction between the insulin receptor and associated proteins may be affected by the reactivity of the receptor cytoplasmic thiol(s). In the present study, we have investigated the ability of IR to interact with neighboring proteins in Chinese hamster ovary (CHO) cells expressing human insulin receptors and in differentiated mouse 3T3-L1 adipocytes using 1,6-bismaleimidohexane (BMH), an irreversible homobifunctional cross-linking reagent highly specific for thiols (*23, 24*). Furthermore, we examined the role of various receptor domains and specific IR cytoplasmic cysteines in this association.

EXPERIMENTAL PROCEDURES

Materials. Monoclonal anti-insulin receptor antibody (clone 29B4) and protein G-plus/protein A−agarose were purchased from Oncogene Science (Manhasset, NY); horseradish peroxidase- (HRP-) conjugated monoclonal anti-phosphotyrosine antibody and polyclonal antibodies against insulin receptor α-subunit (sc-710) and SHP-2 (sc-280) were from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-conjugated streptavidin was from Vector Laboratories, Inc. (Burlingame, CA). HRP-modified donkey anti-rabbit antibody, ^{125}I-Insulin (2000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and the enhanced chemiluminescent (ECL) detection system were from Amersham Corp. (Arlington Heights, IL), whereas murine anti-LAR antibody was from Transduction Laboratory (Lexington, KY). Tran^{35}S-label was purchased from ICN (Irvine, CA), G418 sulfate (Geneticin) was from Life Technologies, Inc. (Gaithersburg, MD), and maleimidobutyrylbiocytin (MBB), manumycin, and insulin were from Calbiochem (La Jolla, CA). Sodium orthovanadate, L-cysteine, 2-mercaptoethanol, dithiothreitol (DTT), biotin-labeled insulin, and poly(vinylpyrrolidone)-360 (PVP) were from Sigma Chemical Co. (St. Louis, MO); 2-nitro-5-thiocyanatobenzoic acid (NTCB) and phenylarsine oxide (PAO) were from Aldrich Chemical Co. (Milwaukee, WI); BMH was from Pierce Chemical Co. (Rockford, IL), and TPCK-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ). Blotting-grade blocker nonfat dry milk was purchased from Bio-Rad Laboratories (Hercules, CA), whereas Pronase and Pefabloc-SC were from Boehringer-Mannheim (Indianapolis, IN). Precast 4−12% Tris-glycine gels, 10−20% Tricine gels, poly(vinylidene difluoride) (PVDF) membrane, Tris-glycine SDS sample buffer, and Tricine sample buffer were purchased from Novex Corp. (San Diego, CA). Dulbecco's modified Eagle's medium, Ham's F-12 medium, and serum were obtained from Cellgro (Herndon, VA).

Cell Culture and DNA Transfections. Chinese hamster ovary (CHO) cells stably transfected with a plasmid containing neomycin resistance gene driven by SV40 promoter without (neo) or with a plasmid encoding the wild-type exon 11 minus variant of the human insulin receptor (HIRc) have been described (*25*). CHO cells expressing IR with a deletion of the tetrameric amino acid sequence Asn-Pro-Glu-Tyr in the cytoplasmic juxtamembrane domain (ΔNPEY), and a C-terminal 43- (Δ43) or 69- (Δ69) amino acid truncation in the β-subunit were gifts from Drs. Paulo Berhanu (University of Colorado Health Sciences Center, Denver, CO), Morris F. White (Joslin Diabetes Center, Boston, MA), and Jeremy M. Tavaré (University of Bristol, Bristol, U.K.), respectively. CHO cells overexpressing IR mutants with a single Cys → Ala substitution at residue 981[2] (C981A), 1056 (C1056A), or 1234 (C1234A) were obtained following electroporation of parental CHO-K1 cells with 25 μg of pECE plasmids encoding mutated IR cDNA and 2.5 μg of pSVNeo at 340 V and 960 μF. All pECE plasmids used in this study were generously provided by Dr. S. Lance Macaulay (CSIRO Division of Bimolecular Engineering, Victoria, Australia) (*26*). After a 2-week selection with G418 sulfate, independent clones were expanded and grown to confluency in Ham's F12 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. High-expressing cell lines were selected by ^{125}I-insulin binding analysis performed as described (*27*). In other instances, cells in suspension were incubated with 10 nM biotinylated insulin for 1 h at 4 °C followed by the addition of 1:10 dilution *R*-phycoerythrin-labeled streptavidin (Becton-Dickinson Immunochemistry Systems, San Jose, CA) in order to assess receptor expression on a FACScan flow cytometer (Becton-Dickinson, Cockeysville, MD). 3T3-L1 fibroblasts were grown and differentiated into adipocytes as described previously (*28*).

DNA Sequencing of the IR Mutants with Single Cys → Ala Substitutions. Yields of 50−100 μg of pECE plasmids encoding hIR mutants with single Cys → Ala point mutations were obtained from a Qiagen plasmid midi kit. Further purification of these plasmids was achieved by extraction with an organic mixture composed of phenol:chloroform: isoamyl alcohol (25:24:1). The primers used for sequencing the various Cys → Ala point mutations were as follows: C981A, 5′-GCCCCCTCATCTTTGTCTTTC-3′; C1056A, 5′-GTGGCGGTGAAGACGGTCAAC-3′; and C1234A, 5′-CCAAGGCCTGTCTAATGAAC-3′. DNA sequencing was performed with the U.S. Biochemical Corp. Sequenase Version 2.0 sequencing kit according to the manufacturer's instructions. The samples were run on a 6% polyacrylamide gel, which was then dried and autoradiographed.

Metabolic Labeling and Chemical Cross-Linking. For metabolic labeling with [^{35}S]methionine, confluent monolayers of cells were incubated for 16 h with 60 μCi/mL Tran^{35}S-label in methionine- and cysteine-free RPMI 1640 medium containing 3% fetal calf serum. Cells were washed three times in Hepes-buffered saline, pH 7.5, before semipermeabilization with digitonin as previously described (*25*). Intact as well as semipermeabilized cells were prepared for chemical cross-linking by addition of 200 μM BMH or dimethyl sulfoxide as vehicle. The cross-linking reaction was allowed to proceed for 10 min at 6 °C, unless otherwise noted, and was quenched by incubation with 4 mM L-cysteine for an additional 5 min before lysis.

Pronase Digestion of Cell Surface Proteins. Cells were prepared for Pronase digestion by washing twice in PBS without Ca^{2+} and Mg^{2+} and then incubated in PBS containing 0.05% (w/v) Pronase. Reaction was allowed to proceed for 10 min at 6 °C and was quenched by the addition of 8

[2] According to the numbering system of Ullrich et al. (*3*).

volumes of 10% serum. Cells were then washed twice in PBS. Cell lysis and IR immunoprecipitation were performed as summarized below.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Both intact and semipermeabilized cells were solubilized in immune precipitation buffer [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM orthovanadate, 100 mM NaF, 0.1% SDS, 0.5% (w/v) deoxycholate, 1% (w/v) Triton X-100, 0.02% NaN_3, 0.25 mM Pefabloc-SC, 1 mM benzamidine, 8 μg/mL aprotinin, and 2 μg/mL leupeptin] and the cell lysates were centrifuged (16000*g* for 20 min at 4 °C) to remove insoluble materials. The clarified lysates from CHO cells were incubated with a monoclonal anti-IR antibody (clone 29B4) whereas 3T3-L1 adipocyte lysates were incubated with the polyclonal αCT-IR antibody, which was raised against a peptide derived from the IR carboxyl terminus (*29*). After the addition of protein G-plus/protein A−agarose resin, the immunocomplexes were incubated for 16 h at 4 °C and then washed twice with immune precipitation buffer and two times with washing buffer (50 mM HEPES, pH 7.5, and 0.1% Triton X-100). The washed immunocomplexes were denatured in Laemmli sample buffer containing 5% 2-mercaptoethanol and resolved by SDS−polyacrylamide gel electrophoresis (PAGE) (*30*). For Western blot analysis of whole cell lysates, cells were lysed directly in 1.5-fold concentrated Laemmli sample buffer containing 7.5% 2-mercaptoethanol and 1 mM orthovanadate, and resolved by SDS−PAGE. Gels were electrotransferred to PVDF membrane. To assess the extent of thiolbiotinylation of the IR β-subunit, the membranes were incubated with blocking buffer I [1% PVP in 50 mM Tris-buffered saline, pH 7.6, containing 0.1% Triton X-100 (TBST)] for 1 h and then probed with a 1:100 000 dilution of HRP-linked streptavidin in blocking buffer I. For immunoblotting, the membranes were incubated with blocking buffer II [5% (w/v) nonfat dried milk in TBST] and probed with specified antibodies. The blots were then incubated with HRP-conjugated secondary antibody followed by chemiluminescence detection, as suggested by the manufacturer (Amersham). Band intensities were quantitated by laser densitometry with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Insulin Receptor Autophosphorylation. Aliquots of anti-IR immunoprecipitates from control and BMH-treated cells were incubated in 100 mM Hepes, pH 7.5, 0.1% Triton X-100, 200 μM orthovanadate, 10 mM $MgCl_2$, and 5 mM $MnCl_2$ for 30 min at room temperature. The phosphorylation reaction was initiated by the addition of 3 μM [γ-^{32}P]ATP (3 cpm/fmol); 30 min later, 1 volume of 3-fold concentrated Laemmli sample buffer containing 15% 2-mercaptoethanol was added to quench the reaction. The proteins were resolved by SDS−PAGE and the gels were fixed, dried, and autoradiographed.

Phosphopeptide and Phosphoamino Acid Analyses. The ^{32}P-labeled monomeric and cross-linked IR β-subunit complexes were located on the dried gels by autoradiography, excised, and rehydrated in water for several hours. The gel pieces were minced and incubated in 50 mM ammonium bicarbonate containing 50 μg/mL TPCK-trypsin for 7 h, followed by a second addition of trypsin and further hydrolysis for 14 h at room temperature. The gel residue was removed by filtration and the filtrate was lyophilized. Two-dimensional phosphopeptide mapping of the tryptic digests was performed on 20 × 20 cm cellulose thin-layer plates (Eastman Kodak Co., Rochester, NY) essentially as described (*31*). Phosphoamino acid analysis was performed on PVDF membrane by hydrolysis of immobilized ^{32}P-labeled proteins with constant boiling 6 N HCl (Pierce) at 110 °C for 1 h. The liberated phosphoamino acids were separated by one-dimensional electrophoresis at 10 °C in the presence of unlabeled standards on cellulose thin-layer plates (EM Separations, Gibbstown, NJ) for 1 h at 1000 V in pH 2.5 buffer [5.9% (v/v) acetic acid, 0.8% (v/v) formic acid, 0.3% (v/v) pyridine, and 0.3 mM EDTA] (*32*).

Chemical Cleavage of Phosphoproteins on Nitrocellulose. The ^{32}P-labeled proteins electrotransferred to nitrocellulose (0.45 μm) were located by autoradiography, excised, and soaked in 1.0% PVP in 100 mM acetic acid for 30 min at 37 °C to block protein binding sites on the membrane. After a series of washes in deionized water, the membranes were incubated for 16 h at 37 °C with 50 mM ammonium bicarbonate containing 6 M urea, 0.5 M KCl, 3 mM EDTA, 0.1% SDS, and 1 mM NTCB, followed by a second addition of NTCB and further hydrolysis for 24 h. After spinning in a microcentrifuge tube, the liquid was transferred to a new tube. Usually 85−90% of the ^{32}P radioactivity was released from the membrane. One volume of 1% acetic acid and 10 μg of BSA were added to the reaction mixtures and applied onto 0.05 μm nitrocellulose with a slot blot apparatus. Peptides were eluted off the membrane by the addition of 50% pyridine and 100 mM sodium acetate and lyophilized before their separation by SDS−PAGE.

RESULTS

Thiol-Specific Cross-Linking of the IR β-Subunit in Cultured Cells. Recent studies have identified proteins that are specifically associated with the insulin receptor. Such molecules might regulate the enzymatic activity or modulate the physical proximity of the receptor with its specific substrates (*33, 34*). To investigate whether cellular proteins with reactive sulfhydryl groups interact with the IR β-subunit cytoplasmic domain, metabolically labeled CHO/HIRc cells were treated without or with 100 nM insulin followed by the addition of the bifunctional chemical cross-linking reagent BMH. For cross-linking to occur, reactive thiol groups must be at a distance fixed by the length of the cross-linking agent. Total cell lysates were subjected to immunoprecipitation with a monoclonal α-IR antibody followed by reduction of the immune complexes and denaturing gel electrophoresis. Autoradiography of the dried gel was then performed. Because it contains more methionines, the 97 kDa β-subunit was labeled more intensely than the 130 kDa α-subunit (Figure 1A, lanes 3 and 4). Radioactive bands were quantified and the β-subunit/α-subunit ratio was calculated. A decrease in the ratio would be indicative of a selective reduction in the recovery of monomeric β-subunit. In the absence of insulin, we found that treatment of semipermeabilized cells with BMH led to a 40% loss in the amount of IR β-subunit as evidenced by the fact that the radioactivity ratio between the β-subunit and α-subunit passed from 1.6 ± 0.1 to 1.0 ± 0.1 in the absence and presence of BMH, respectively ($P < 0.001$, $n = 7$) (Figure 1B). The loss in β-subunit was concomitant with the appearance of a ^{35}S-labeled high molecular weight complex (Figure 1A, compare lanes 1 and 3). Western blot analysis indicated that the protein complex

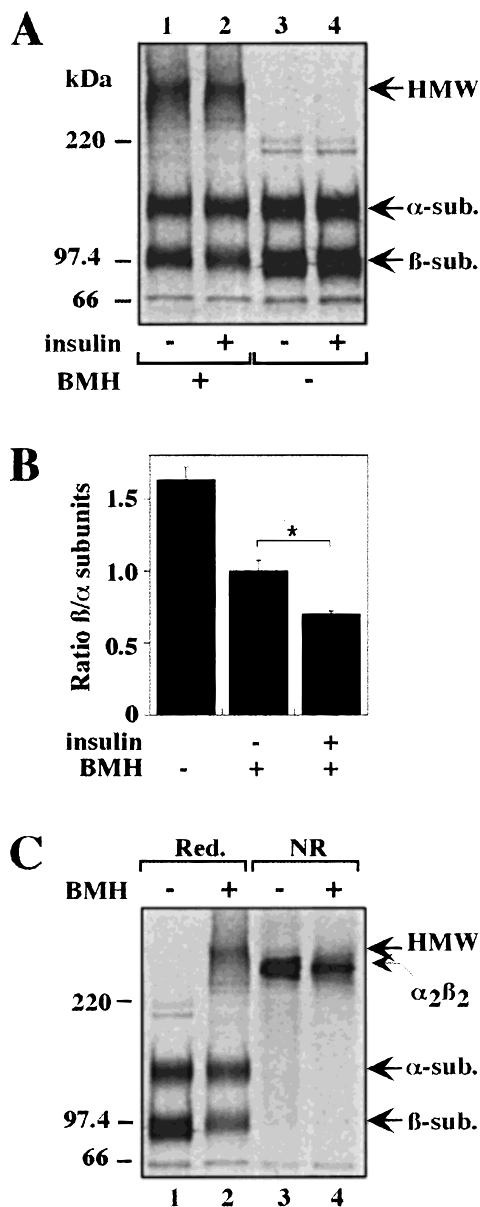


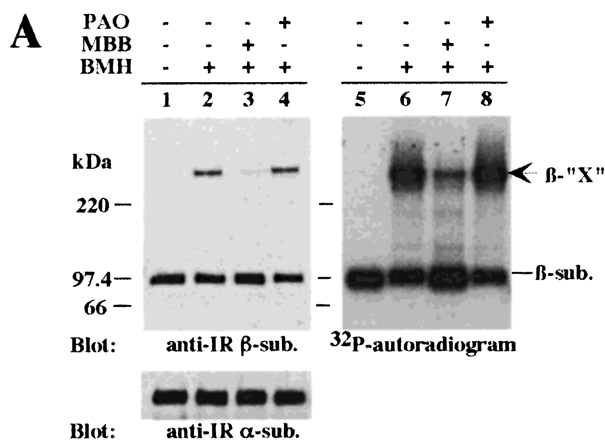
FIGURE 1: Effect of *bis*maleimidohexane on the insulin receptor. Confluent CHO/HIRc cells were metabolically labeled in methionine/cysteine-free medium with 60 μCi/mL [^{35}S]methionine and cysteine for 16 h. After washing, the cells were semipermeabilized and incubated in the absence or presence of 100 nM insulin for 15 min at 6 °C followed by the addition of 100 μM ATP/4 mM Mn^{2+}. Cells were then exposed to DMSO (vehicle) or 200 μM BMH for 10 min at 6 °C. IR immunoprecipitates were denatured in Laemmli sample buffer and separated by SDS−PAGE under reducing (panels A, B, and C, Red.) or nonreducing (panel C, NR) conditions. Shown are autoradiograms of the Enlightning-treated dried gels. The positions of prestained molecular weight markers (in kilodaltons) are indicated. HMW denotes a high molecular weight complex. (B) The level of radioactivity associated with both receptor subunits was quantitated by electronic autoradiography and expressed as a ratio of β-subunit/α-subunit. Data are the means ± SEM ($n = 5-7$). *, $P < 0.05$. Comparison between groups were made by Anova coupled to Fisher's PLSD post-hoc test.

contained the IR β-subunit but not α-subunit (see below). Similar findings were obtained in cells expressing IR mutants with a specific deletion of the NPEY motif in the cytoplasmic juxtamembrane domain (ΔNPEY) or a 43-amino acid truncation at the receptor C-terminus (Δ43) (data not shown). As shown in Figure 1B, relative to unstimulated cells, insulin had a small but significant effect at reducing further the amount of β-subunit recovered in response to BMH treatment, with a β-subunit/α-subunit ratio of 0.7 ± 0.02 ($P < 0.05$, $n = 5$).

Using gel electrophoresis under nonreducing conditions, we determined that the immunoprecipitates from BMH-treated cells exhibited a ~2.5-fold decrease in the level of disulfide-linked $\alpha_2\beta_2$ heterotetrameric IR species when compared with control cells (Figure 1C, lane 4 vs lane 3). Addition of 2-mercaptoethanol to these samples allowed the reduction of IR disulfides with concomitant detection of monomeric α- and β-subunits and of a ^{35}S-labeled complex whose size was greater than that of the tetrameric receptor (Figure 1C, lane 2 vs lanes 3 and 4). The complex could not be detected under nondenaturing conditions. This is consistent with the notion that the native receptor may be covalently linked to a large protein in response to BMH, giving rise to a protein complex that poorly enters the gel.

Next, intact CHO/HIRc cells were stimulated with insulin and then exposed to BMH. Cellular extracts were incubated with α-IR antibody, and the immunoprecipitates were subjected to SDS−PAGE under reducing conditions and immunoblotting with antibodies specific for the IR α- and β-subunits (Figure 2A). In agreement with our results shown above, there was a striking difference between the electrophoretic patterns of IR β-subunit from control and BMH-treated cells. Treatment with BMH yielded two immunoreactive bands corresponding to the monomeric 97 kDa β-subunit and a large β-subunit-containing protein complex (Figure 2A, left upper panel). In the absence of BMH, no β−X complex was detected. The decrease in monomeric receptor β-subunit in immunoprecipitates from BMH-treated cells was not accompanied by a loss in IR α-subunit levels (Figure 2A, left lower panel). Cells that were pretreated with the thiol-reactive reagent MBB prior to BMH showed a marked reduction in the formation of β−X complex, demonstrating that both reagents recognize the same reactive IR β-subunit sulfhydryls (Figure 2A, lane 3). The effect of PAO, a trivalent arsenical compound that complexes vicinal dithiols of proteins, was then evaluated. PAO has been previously shown to be an inhibitor of insulin action by interrupting signal transmission between the insulin receptor and the glucose transport system (*35, 36*). In the study herein, no inhibition in the formation of β−X complex was observed in cells that were pretreated with 70 μM PAO (Figure 2*A*, lane 4), strongly suggesting that vicinal dithiols do not participate in the cross-linking reaction.

In view of the above results, we investigated whether the β−X complex is catalytically active by undergoing phosphorylation in vitro. To this end, IR immunoprecipitates from insulin-stimulated cells treated or not with BMH were assayed in the presence of [γ-^{32}P]ATP and divalent cations. The pretreatment of cells with MBB but not PAO led to a significant decrease in the detection of phosphorylated β−X complex concomitant with elevated phosphorylation of the monomeric β-subunit (Figure 2A, right panel). The autoradiograms of the dried gels from independent experiments were quantitated by electronic autoradiography (Figure 2B). Taken together, these results indicate that, in both intact and semipermeabilized cells, the IR β-subunit contains reactive thiol group(s) that is(are) accessible to the cross-linker to produce a complex with an interacting protein harboring



B
120
100
80
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40
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0
Percent of Total
β-sub
β-X
PAO - - - +
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BMH - + + +

FIGURE 2: Reactivity of the IR β-subunit thiols toward BMH in intact cells. Serum-starved CHO/HIRc cells were treated for 5 min with 200 μM orthovanadate and 100 nM insulin followed by the addition of 100 μM MBB, 70 μM PAO, or vehicle. Ten minutes later, 100 μM BMH was added for 10 min where indicated and the cross-linking reaction was terminated by the addition of 4 mM L-Cys. (A) After cell lysis, IR immunoprecipitates were divided into two aliquots. (Left panels) Western blot analysis with αCT-IR antibody was performed. The same membrane was then reprobed with IR α-subunit antibody. (Right panel) In vitro phosphorylation of IR immunoprecipitates was carried out in the presence of [γ-^{32}P]-ATP and divalent cations followed by electrophoresis on an SDS−polyacrylamide gel and autoradiography. The positions of prestained molecular weight markers (in kilodaltons) are indicated on the left. β−X denotes formation of a high molecular weight complex that contains IR β-subunit. (B) Percent incorporation of radioactivity into the receptor β-subunit (solid bars) and β−X complex (open bars). Data are the means ± SD (n = 4).

reactive sulfhydryl moieties. Furthermore, the immunoprecipitated complex exhibits the ability to be phosphorylated in vitro.

Initial Characterization of the β−X Complex. Cell treatment with proteases may enable investigation of topology of the proteins interacting with the insulin receptor. Semipermeabilized (Figure 3, lanes 1 and 2) and intact (lanes 3−6) CHO/HIRc cells were pretreated with insulin and then incubated in the absence or presence of BMH before Pronase digestion. Cells were lysed, followed by immunoprecipitation of the solubilized insulin receptors and analysis either by Western blotting with anti-IR α- and β-subunits (Figure 3, left panels) or by in vitro phosphorylation with [γ-^{32}P]ATP (Figure 3, right panel). There were no receptor subunits detectable following Pronase treatment of semipermeabilized cells. In contrast, the addition of Pronase to intact cells yielded a β-subunit species that migrated slightly faster than the IR β-subunit from control, untreated cells (Figure 3, lane 3 vs lane 5). Under these conditions, the α-subunit was undetectable, demonstrating its exquisite sensitivity to Pronase. A slightly faster β−X species that originated from BMH-treated cells incubated with Pronase was detected (Figure 3, lane 4 vs lane 6), consistent with the notion that the receptor α-subunit is not present in the complex and that the receptor-associated protein may contain a rather small external domain.

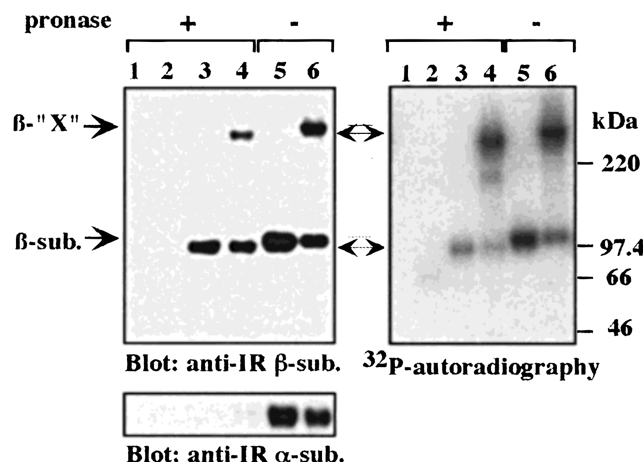

FIGURE 3: Effect of cell treatment with Pronase on the integrity of the β−X complex. CHO/HIRc cells were treated with 200 μM vanadate and 100 nM insulin for 10 min at 37 °C followed by semipermeabilization with digitonin (lanes 1 and 2) or not (lanes 3−6). Cells were then incubated in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of 200 μM BMH for 10 min at 6 °C, followed by a series of PBS washes and incubation with 0.05% (w/v) Pronase. After cell lysis, IR immunoprecipitates were divided into two aliquots. (Left panels) Western blot analysis with αCT-IR antibody was performed. The same membrane was then reprobed with IR α-subunit antibody. (Right panel) In vitro phosphorylation of IR immunoprecipitates was carried out in the presence of [γ-^{32}P]ATP followed by gel electrophoresis and autoradiography. The positions of prestained molecular weight markers (in kilodaltons) are indicated on the right. The double-headed arrows denote the faster-migrating species after cell treatment with Pronase.

Digestion and Phosphopeptide Mapping of the Phosphorylated β−X Complex. The phosphorylation of the β−X complex in vitro could involve the well-known ability of the IR β-subunit to autophosphorylate. Following electrophoresis and transfer onto nitrocellulose membrane, ^{32}P-labeled β−X complex and monomeric β-subunit were excised from the membrane (Figure 4A), put in separate tubes, and treated with the chemical agent NTCB under strong denaturing conditions. Treatment with NTCB results in cyanylation of cysteines on polypeptide chains, inducing a cleavage N-terminal to the cyanylated cysteines (*37*). The resulting fragments were resolved by gel electrophoresis and visualized by autoradiography (Figure 4B). The pattern of phosphopeptides in these two samples was strikingly similar; however, digestion of the β−X sample produced an ∼11 kDa fragment that was heavily phosphorylated. One-dimensional phosphoamino acid analysis revealed that phosphorylation occurred exclusively on tyrosine residues (Figure 4C). To assess whether catalytically active IR β-subunit is present in the β−X complex, another strategy was used whereby the distribution of the major insulin receptor autophosphorylation sites was studied. It involves trypsin digestion of ^{32}P-labeled samples followed by two-dimensional phosphopeptide mapping (*31*). The results presented

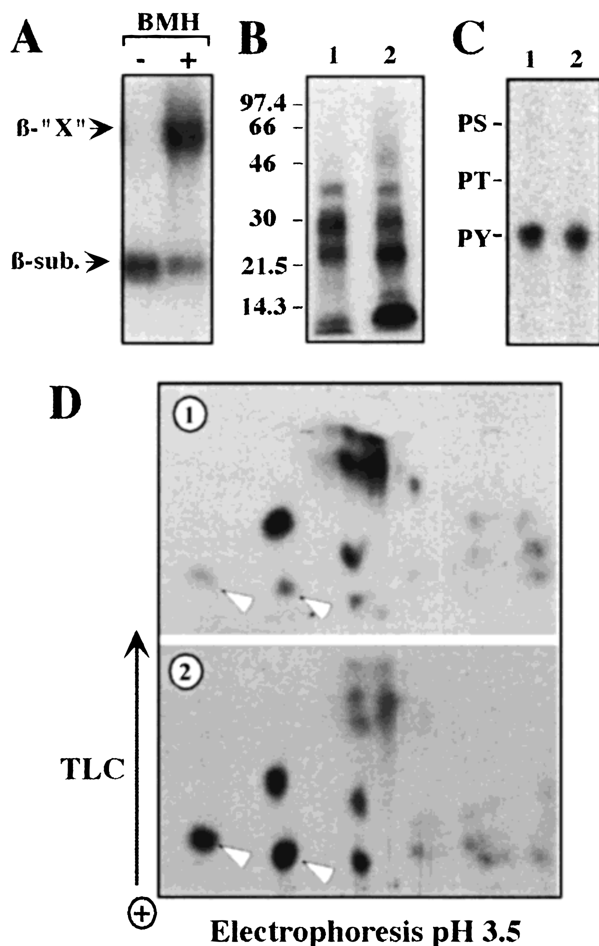

FIGURE 4: β−X complex contains catalytically active IR β-subunit. Insulin-treated CHO/HIRc cells were incubated in the absence (−) or the presence (+) of 200 μM BMH. IR immunoprecipitates were phosphorylated in vitro with [γ-^{32}P]ATP followed by electrophoresis on an SDS−polyacrylamide gel, transfer onto nitrocellulose membrane, and autoradiography (panel A). (B) ^{32}P-Labeled receptor β-subunit (lane 1) and β−X complex (lane 2) were excised from nitrocellulose and subjected to NTCB-mediated cleavage as described under Experimental Procedures. The resulting fragments were resolved by gel electrophoresis, transferred onto PVDF membrane and visualized by autoradiography. (C) After hydrolysis in 6 N HCl, the phosphoamino acids liberated from the β-subunit (lane 1) and β−X complex (lane 2) were analyzed by high-voltage electrophoresis on thin-layer cellulose plates in the presence of unlabeled standards followed by autoradiography. PY, phosphotyrosine; PT, phosphothreonine; PS, phosphoserine. (D) Phosphopeptide analysis of ^{32}P-labeled β-subunit (upper panel) and β−X (lower panel) was carried out as described under Experimental Procedures. Shown are the autoradiographs of the thin-layer cellulose plates. The white arrowheads depict the locations of two phosphotyrosine-containing peptides that are enriched in the β−X complex.

in Figure 4D clearly show that the β−X digest was characterized by the presence of two radiolabeled peptides (panel 2, white arrowheads) that were barely detectable in the monomeric β-subunit digest (panel 1). The identity of both spots is well-known (*38*); they correspond to the phosphopeptides derived from the tris-phosphorylated form of the IR kinase domain. Altogether, our results suggest that steric hindrance in the receptor autocatalytic domain does not appear to have occurred following the cross-linking reaction.

Effect of a 69 Amino Acid Deletion from the C-Terminal Domain on IR Thiol Reactivity. The importance of Cys[1308] in the overall IR thiol reactivity was assessed in CHO cells overexpressing a truncated receptor mutant that lacks this residue (Δ69). The results shown in Figure 5A indicate that the receptor mutant contained comparable amounts of reactive thiols as that of the wild-type HIRc receptor in response to the thiol-specific biotinylating agent MBB. Furthermore, the β subunit of the truncated receptor was able to react with BMH and form a cross-linked β−X complex (Figure 5B), strongly suggesting that Cys[1308] is not a reactive thiol. Although it would appear that the formation of β−X complex is increased in CHO/Δ69 cells in the particular experiment presented in Figure 5B, the results of several experiments indicate that the extent of BMH cross-linking was not significantly different when one compared CHO cells expressing wild-type IR and truncated receptor mutants.

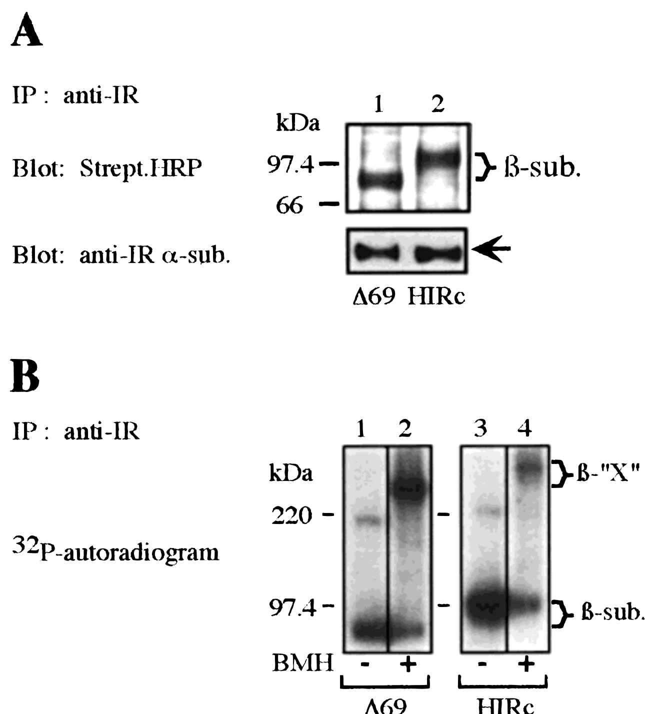

FIGURE 5: Effect of a C-terminal 69-amino acid deletion on IR thiol reactivity. (A) Serum-starved CHO/Δ69 and CHO/HIRc cells were semipermeabilized with digitonin and treated with 100 μM MBB for 10 min at 6 °C, and the IR was immunoprecipitated. The immune pellets were resolved by SDS−PAGE and immunoblotted with HRP-conjugated streptavidin (upper panel). The membrane was then reprobed with IR α-subunit antibody (lower panel). (B) After incubation with vanadate and insulin, cells were treated in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of BMH as indicated in the legend of Figure 4. IR immunoprecipitates were phosphorylated with [γ-^{32}P]ATP in vitro and then electrophoresed. Shown in a representative autoradiogram of the dried gel.

Effects of Cysteine-to-Alanine Substitutions on IR Thiol Reactivity. Six cysteines are present in the cytoplasmic domain of the human IR β-subunit (*3, 4*). In addition to the C-terminal Cys[1308], one of these residues (Cys[981]) is located close to the juxtamembrane region and four others (e.g., 1056, 1138, 1234, and 1245) are located in the kinase domain where only Cys[1056] and Cys[1234] are exposed to solvent (*39*). CHO cells were stably transfected with plasmids encoding hIR mutant with single Cys → Ala substitution at 981, 1056, or 1234. Cell lines expressing neo alone or in combination with wild-type HIRc were also generated as controls. Positive clones expressing high number of receptors were chosen to

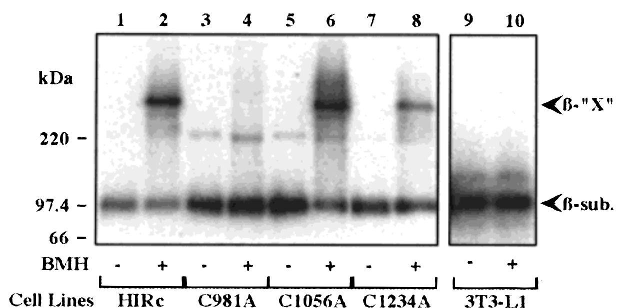

FIGURE 6: Effects of single Cys → Ala substitution on IR β-subunit thiol reactivity. Serum-starved CHO cells expressing HIRc or hIR with single Cys → Ala point mutations (C981A, C1056A, and C1234A) and 3T3-L1 adipocytes were treated with 200 μM vanadate and 100 nM insulin for 15 min at 37 °C, followed by an incubation in the absence (−) or the presence (+) of 100 μM BMH for 10 min at 6 °C. IR immunoprecipitates were phosphorylated in vitro and then resolved by SDS−PAGE. A representative autoradiogram of three separate experiments is shown.

examine thiol reactivity of the IR β-subunit in the presence of BMH. The CHO/HIRc cells and cells expressing Cys1056Ala and Cys1234Ala receptor mutants showed the formation of β−X complex after treatment with the thiol-specific cross-linker, whereas Cys981Ala-expressing cells failed to do so (Figure 6). Similar results were obtained in three separate experiments. To independently verify that Cys[981] may influence receptor cross-linking, we determined the extent to which BMH could stimulate β−X formation in fully differentiated 3T3-L1 adipocytes, a mouse cellular model known for its high responsiveness to insulin. The murine IR lacks a cysteine residue equivalent to human IR Cys[981] (*40*). Exposure of 3T3-L1 adipocytes to BMH did not result in the detection of β−X. These data suggest that a difference in reactivity exists among the IR cytoplasmic cysteines and that IR Cys[981] may have the potential to confer signaling advantage by virtue of its close interaction with molecule X, a membrane-associated thiol-reactive protein.

Effects of Cysteine-to-Alanine Substitutions on IR Autophosphorylation and Kinase Activity in Intact Cells. To investigate whether Cys → Ala point mutations have a direct effect on IR autophosphorylation, cells were serum-starved for 4 h and then incubated in the absence or the presence of 100 nM insulin for 5 min. As shown by Western blot analysis of IR immunoprecipitates probed with anti-phosphotyrosine specific antibody, each mutant-expressing cell line was responsive to insulin (Figure 7A). Note the comparable levels of expression of wild-type and receptor mutants (Figure 7A, lower panel). Thus, it appears that none of the mutations affected the ability of insulin to promote IR autophosphorylation. Next, proximal events in insulin signaling were investigated. When total cell lysates were probed with either anti-IRS-1 or anti-phosphotyrosine antibody, no inhibitory effect on tyrosine phosphorylation of IRS-1 was noted in insulin-treated CHO cells expressing HIRc or mutated receptors (Figure 7B). The upper panel in Figure 7B shows that the slower migration of IRS-1 in cells treated with insulin (with the exception of CHO/neo cells) likely results from increased tyrosine phosphorylation. Reprobing the membrane with a phosphospecific anti-ERK1/ERK2 antibody also revealed a large increase in ERK activation in response to insulin in all the cell lines studied (data not shown).

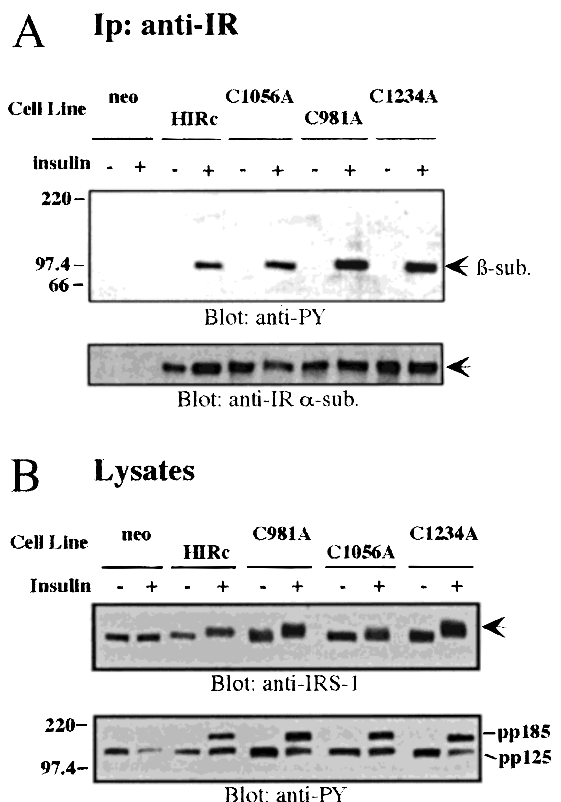

FIGURE 7: Effects of Cys → Ala substitution on IR β-subunit autophosphorylation and kinase activity in intact cells. Serum-starved cells were treated in the absence (−) or the presence (+) of 100 nM insulin for 5 min at 37 °C. (A) IR immunoprecipitates were resolved by SDS−PAGE and immunoblotted with HRP-conjugated antiphosphotyrosine (PY) (upper panel) followed by IR α-subunit antibody (lower panel). (B) Whole-cell extracts were loaded on gels, and the proteins were resolved by SDS−PAGE and immunoblotted with either IRS-1 (upper panel) or antiphosphotyrosine (lower panel) antibodies. The slower migrating IRS-1 species in insulin-treated cells is denoted by an arrow. Phosphotyrosyl-containing proteins of 185 and 125 kDa are detected in the lower panel. Comparable findings were obtained in three other independent experiments.

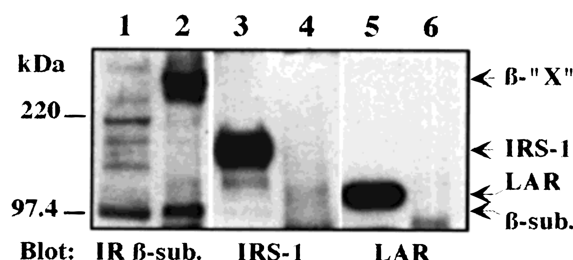

FIGURE 8: IRS-1 or LAR is not present in the β−X complex. After incubation with vanadate and insulin, serum-starved CHO/HIRc cells were semipermeabilized with digitonin and then immediately lysed in sample lysis buffer (lanes 1, 3, and 5) or incubated with 100 μM BMH for 10 min at 6 °C (lanes 2, 4, and 6). Whole-cell extracts (odd lanes) and IR immunoprecipitates (even lanes) were resolved by SDS−PAGE and immunoblotted with antibodies against either IR β-subunit (lanes 1 and 2), IRS-1 (lanes 3 and 4) or LAR (lanes 5 and 6).

Initial Characterization of Molecule X. We next questioned what the identity of molecule X was by immunoblotting IR immunoprecipitates from BMH-treated CHO/HIRc cells with antibodies against known IR-associated proteins. As shown in Figure 8, probing the blot with an antibody specific against the IR β-subunit allowed the detection of the β−X complex,

which sharply contrasted with the lack of signal when similar blots were probed with antibodies against IRS-1, the transmembrane protein tyrosine phosphatase LAR (Figure 8, lanes 4 and 6), or the membrane-associated SHP-2 (data not shown).

DISCUSSION

The human IR cytoplasmic domain contains six cysteines whose reactivity and function remain uncertain. We have shown here that Cys^{981} of the IR β-subunit is reactive and within proximity of a thiol-reactive membrane-associated protein, termed molecule X. Our experiment with the thiol-specific homobifunctional cross-linking agent, BMH, indicates that the β−X complex migrates with an apparent molecular mass larger than the ~420 kDa tetrameric receptor species, thus ruling out the possibility that it is composed of homodimers of β-subunit (~190 kDa). Hence, the possibility that BMH may increase aggregation of IR subunits, as has been shown with cross-linking agents specific for amino groups (*41, 42*), can be excluded. Independent observations suggest that this BMH-mediated process depends on cytoplasmic rather than extracellular cysteine residue(s). First, the addition of Pronase to intact cells was accompanied by a relatively modest alteration in the integrity of the β−X complex while causing its proteolytic degradation upon incubation of semipermeabilized BMH-treated cells with Pronase. Second, substitution of Cys^{981} in the cytoplasmic juxtamembrane region of the receptor totally abolished the formation of the β−X adduct. Of interest, the extent of BMH-induced cross-linking was somewhat higher in cells pretreated with insulin. The possibility of small insulin-induced alterations in affinity of the hIR for molecule X therefore cannot be excluded due to the observed decrease in the ratio of β-subunit/α-subunit in treated vs untreated cells. Earlier observations indicated that the reactivity of the hIR thiols does not differ between basal and activated forms of the receptor (*25*). Thus, one can infer that molecule X and the IR β-subunit cytoplasmic domain are in close proximity in both basal and insulin-stimulated cells.

An interesting observation in our studies is that the covalent cross-linking of the IR β-subunit with molecule X did not alter the receptor's ability to autophosphorylate the three critical tyrosine residues in the activation loop in vitro. This result was obtained by a two-dimensional phosphopeptide mapping technique originally developed by Tavaré and Denton (*38*) that allows unequivocal identification of the phosphorylated fragments generated following tryptic cleavage of radiolabeled IR β-subunit. Thus, it appears that BMH-mediated cross-linking has no detrimental effect on the native conformation of the autocatalytic domain of the hIR while exerting minimal steric hindrance near the ATP binding site and/or activation loop. An increase in the tris-phosphorylated form of the IR kinase domain occurs upon exposure of BMH-treated cells to insulin. We speculate that BMH, a thiol-reactive agent, has the ability to prevent the dephosphorylation of the activation loop of IR by cellular tyrosine phosphatases. Indeed, the catalytic activity of these enzymes, which depends on a critical free cysteine residue, is markedly inhibited upon exposure to sulfhydryl-modifying molecules (*43*).

Our studies also provide insights as to the identification of Cys^{981} as a reactive receptor thiol. Earlier works have speculated that a nucleophilic cysteine residue was located near the ATP-binding site, on the basis of the observation that pretreatment of solubilized IR with ATP-bound complex blocked subsequent thiol alkylation of the receptor (*5, 7*). Crystallographic data indicate that the ATP binding site of the nonphosphorylated IR is hindered by its activation loop (*44*). Upon autophosphorylation, there is rearrangement of the insulin receptor activation loop, which facilitates a reorientation of the N- and C-terminal lobes of the kinase, thereby allowing productive ATP binding and, consequently, substrate phosphorylation (*39*). Thus, modification of Cys^{981} by bulky alkylating agents (e.g., NEM) may impede lobe rotation and efficient binding of ATP by the receptor kinase, whereas small alkylating agents (e.g., iodoacetamide) or Cys → Ala substitution at position 981 may be permitted without altering conformational change needed for full receptor activation in response to insulin. One could argue that the position of the IR activation loop under basal conditions was not altered in CHO cells expressing Cys → Ala hIR mutants (at position 981, 1056, or 1234), because of the lack of detectable autophosphorylation in unstimulated cells.

Our finding that Cys^{981} in the hIR β-subunit is a reactive nucleophile is in sharp contrast with a report of Macaulay et al. (*26*) showing that there was no particular reactive thiol within the cytoplasmic domain of this receptor. These authors surveyed receptor alkylation with tritiated NEM in IR immunoprecipitates, whereas our experiments examining the effect of single Cys → Ala substitutions were performed in intact cells. Differences in experimental procedures could certainly explain some of the discrepancies, as the behavior of IR is known to be markedly affected following tissue homogeneization, whereby cellular thiols that are released during solubilization of the cells can form mixed disulfides with the IR (*9, 45*).

Upon addition of insulin, CHO cells expressing hIR with various Cys → Ala substitutions, including Cys981Ala, exhibit insulin binding properties (ED_{50} and K_d values) that are similar to those of the wild-type hIR (*26*). Similarly, we found a normal level of receptor autophosphorylation, IRS-1 phosphorylation, and increase in ERK activation in all the cell lines studied, as assessed by sensitive immunoblot techniques. These observations suggest that the cytoplasmic receptor thiols are not implicated in downstream biological responses of insulin. However, it has been recently shown that CHO/Cys981Ala cells have an abrogation of thymidine incorporation in response to insulin (*26*), which may indicate the existence of an alternate mitogenic signaling pathway that is dependent on Cys^{981}. This hypothesis could not explain the fact that thymidine incorporation is increased upon insulin addition in 3T3-L1 adipocytes (*46*), an insulin-responsive cell line expressing the murine IR that lacks the equivalent of the human Cys^{981}. Whether molecule X is involved in insulin signal transduction is unclear. It is plausible that this thiol-reactive membrane-associated protein interacts with IR at several points of contact.

In response to insulin, several proteins such as IRS-1, IRS-2, Grb14, Grb10, and SHP-2 interact with one or more of the phosphorylated tyrosine residues present in the activation loop (Tyr^{1158} and $pTyr^{1162}$) of the insulin receptor (*19, 21, 22, 33, 47*−*50*). Furthermore, the IR β-subunit contains the juxtamembrane NPEY motif and C-terminal domain that are

known to associate with a number of cytosolic molecules, including IRS, Shc, SH2 domain-containing proteins, and MAD2 (*51*). In addition, the receptor-type protein tyrosine phosphatase, LAR, the glycoprotein PC-1, and the major histocompatibility complex (MHC) class I antigens are transmembrane proteins with large extracellular domains that have been implicated in the regulation of IR by interacting specifically with the receptor (*52*−*54*). Hence, the possibility existed that one or many of these signaling intermediates may be responsible for the formation of the β−X complex. However, in the studies herein we found that the BMH-mediated covalent association of the IR β-subunit with protein X occurred in cells that overexpress receptor mutants with either a discrete deletion of the NPEY motif, truncation of the C-terminal region, or kinase-dead function (our unpublished data). Moreover, the treatment of intact cells with Pronase slightly decreased the size of the cross-linked β−X complex, suggesting that the interacting protein has a rather small extracellular domain. Consistent with these observations, IRS-1, LAR, or SHP-2 was not found associated with the IR β-subunit in the β−X complex.

In summary, we have shown here that Cys^{981} of the human IR β-subunit is essential for covalently cross-linking the β-subunit to a thiol-reactive membrane-associated protein, termed molecule X. This association does not cause significant change in IR kinase activity but appears to be somewhat increased upon insulin treatment. Furthermore, in insulin-responsive 3T3-L1 adipocytes, the murine IR does not bind to the molecule X, which is explained by the lack of the Cys residue equivalent to human IR Cys^{981}. Known signaling intermediates such as IRS-1, LAR, or SHP-2 are not part of the β-X complex. Efforts are underway to characterize molecule X and establish the biological significance of such interaction between the hIR β-subunit and molecule X.

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