Thiol-Specific Biotinylation of the Insulin Receptor in Permeabilized Cells Enhances Receptor Function

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ABSTRACT: We examined the reactivity of insulin receptor sulfhydryls to biotinylation in Chinese hamster ovary cells that express high levels of human insulin receptors (CHO/HIRc cells). Following the biotinylation reaction, the insulin receptor was purified by immunoprecipitation, and resolved by SDS−polyacrylamide gel electrophoresis before electrotransfer to membranes. The use of enzyme-linked streptavidin in conjunction with a chemiluminescent technique allowed the detection of thiol-biotinylated receptor β-subunit, with no modification of the α-subunit. In cells expressing large numbers of IGF-1 receptors, the same technique enabled the detection of thiol-biotinylated IGF-1 receptors as well. Thiol-alkylation of intact CHO/HIRc cells with an impermeant reagent did not impair the ability of maleimidodibutyrylbiocytin (MBB) to biotinylate sulfhydryls on the receptor β-subunit after cell permeabilization with digitonin. In contrast, thiol-alkylation of digitonin-permeabilized cells prevented MBB-induced receptor biotinylation. The basal and insulin-activated insulin receptors exhibited a comparable reactivity to MBB. Furthermore, the use of affinity purification on monomeric avidin−agarose enabled us to learn that the biotinylation reaction was near-quantitative. MBB had no effect on insulin binding nor on receptor autophosphorylation and insulin-dependent receptor kinase activity. However, basal levels of receptor kinase activity were significantly elevated by thiol-biotinylation. Further, in the presence of vanadate, MBB retained the ability to enhance receptor kinase activity in permeabilized cells, consistent with the notion that this increased exogenous substrate phosphorylation was not accounted for by inactivation of protein tyrosine phosphatases. The dephosphorylation of thiol-biotinylated, ^{32}P-labeled insulin receptors by particulate protein tyrosine phosphatases was not affected. These results suggest that modification of reactive sulfhydryls located in the cytoplasmic domain of the insulin receptor β-subunit may play a critical role in insulin receptor function.

The insulin receptor is an oligomeric glycoprotein composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bridges. Upon insulin binding to the α-subunit, there is induction of autophosphorylation of the intrinsic protein tyrosine kinase within the β-subunit [reviewed by Rosen (1987)], resulting in the activation of the kinase-catalyzed phosphorylation of proteins and exogenous peptides [Araki et al., 1994; reviewed by White and Kahn (1994)]. It is widely believed that activation of the insulin receptor tyrosine kinase is essential for most (if not all) of the metabolic and growth-promoting actions of insulin.

Over the past several years, there has been increasing evidence for the role of sulfhydryl agents in insulin action. In particular, alkylating sulfhydryl reagents have been reported to inhibit various insulin responses [see Wilden et al. (1986) for references]. In cell-free systems, *N*-ethylmaleimide and iodoacetamide have been shown to have opposing effects on insulin receptor autophosphorylation and its associated kinase, although both should block free sulfhydryl groups (Shia et al., 1983; Zick et al., 1983; Pike et al., 1984; Fujita-Yamaguchi & Kathuria, 1985; Chen et al., 1986; Wilden & Pessin, 1987). Furthermore, it is suggested that unknown thiols released during tissue homogenization form mixed disulfides with reactive sulfhydryls on the insulin receptor (Li et al., 1991), thereby altering receptor function. The insulin receptor kinase activity is also sensitive to oxidizing agents. When added to intact cells, the insulinomimetic agent H_2O_2 (Czech et al., 1974; May & de Haen, 1979) enhances activation of the insulin receptor kinase (Kadota et al., 1987; Hayes & Lockwood, 1987; Wilden & Pessin, 1987). Furthermore, the capability of the reducing agent dithiothreitol (DTT)[1] to reverse these effects of H_2O_2 suggests that the action of H_2O_2 could be mediated by oxidation of critical sulfhydryls (Heffetz & Zick, 1989). Thus, it appears that some critical sulfhydryl group(s) on the insulin receptor is (are) susceptible to thiol and oxidizing agents. It implies that perturbation of the redox state of the cells could have profound effects on insulin receptor function.

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[1] Abbreviations: DTT, dithiothreitol; IGF-1, insulin-like growth factor 1; PVDF, poly(vinylidene difluoride); PAO, phenylarsine oxide; 4-PDS, 4,4′-dithiodipyridine; MBB, maleimidobutyrylbiocytin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); AMSP, 4-acetamido-4′-maleimidostilbene-2,2′-disulfonic acid; ECL, chemiluminescence; CHO/HIRc, Chinese hamster ovary cells overexpressing the human insulin receptor; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ATP, adenosine 5′-triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EDTA, ethylenediaminetetraacetic acid; PTPase, protein tyrosine phosphatase.

Glutathione is important in the regulation of redox state, and disturbances of glutathione metabolism seen in diabetes (McLennan et al., 1991) may indicate that the receptor tyrosine kinase activity could be modulated by changes in the level of intracellular thiols.

Very little is known about the presence of accessible sulfhydryl(s) on the insulin receptor in intact cells. Labeling studies using radioactive *N*-ethylmaleimide were performed on insulin receptor in cell-free systems (Wilden et al., 1986; Ridge et al., 1988; Chiacchia, 1991), which indicated that the receptor contains reactive thiol group(s). However, this may not be the case for cell surface insulin receptors. In order to investigate the presence of accessible sulfhydryl(s) in receptors embedded in the plasma membrane of cells, we have utilized a semipermeabilized cell model (Bernier et al., 1994) where much of the membrane architecture of the intact cell system is retained. In addition, we used a streptavidin blotting technique to enable sensitive detection of thiol-biotinylated insulin receptors. Although the α-subunit contains 3 times the number of cysteine residues in the primary amino acid sequence as compared to the β-subunit (Ebina et al., 1985; Ullrich et al., 1985), it appears that only sulfhydryl(s) in the intracellular domain of the receptor β-subunit is (are) susceptible to react with the biotinylating agent. In semipermeabilized cells, thiol-biotinylated insulin receptor retains the ability to undergo normal autophosphorylation, but has an enhanced insulin-independent (basal) kinase activity toward an exogenous substrate.

MATERIALS AND METHODS

Materials. Monoclonal anti-insulin receptor antibody, monoclonal anti-IGF-1 receptor antibody, and protein G-Plus/protein A−agarose were from Oncogene Science (Manhasset, NY). Precasted gradient 4−12% Tris−glycine gels, 10−20% Tricine gels, Tricine sample buffer, and poly(vinylidene difluoride) (PVDF) membranes were from Novex (San Diego, CA). Phenylarsine oxide and 4,4′-dithiodipyridine were from Aldrich (Milwaukee, WI), digitonin and maleimidodibutyrylbiocytin (MBB) from Calbiochem (La Jolla, CA), 5,5′-dithiobis(2-nitrobenzoic acid) and monomeric avidin−agarose from Pierce (Rockford, IL), wheat germ−agarose and horseradish peroxidase-conjugated streptavidin from Vector Laboratories, Inc. (Burlingame, CA), and 4-acetamido-4′-maleimidostilbene-2,2′-disulfonic acid (AMSP) from Molecular Probes, Inc. (Eugene, OR). Recombinant human IGF-1 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). [γ-^{32}P]ATP (~3000 Ci/mmol), goat anti-mouse IgG−horseradish peroxidase conjugate, and Enhanced Chemiluminescent (ECL) detection system were from Amersham Corp. (Arlington Heights, IL).

Cell Lines. Chinese hamster ovary clones that overexpress the wild-type insulin receptor (CHO/HIRc cells) and a mutant form of the receptor with a deletion of 43 amino acids at the C-terminus (CHO/ΔCT cells) were provided by Dr. Morris F. White (Joslin Diabetes Center, Boston, MA), while a NIH-3T3 mouse fibroblast clone that overexpresses the wild-type IGF-1 receptor (NWTb3 cells) was provided by Dr. Derek LeRoith (National Institutes of Health, Bethesda, MD). Cells were maintained in either Ham's F-12 medium (CHO/HIRc cells) or Dulbecco's modified Eagle's medium (DMEM) (NWTb3 cells) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Stably transfected NWTb3 cells were maintained in DMEM supplemented with 500 μg/mL G418 (Geneticin, Life Technologies, Inc.).

Thiol-Biotinylation in Permeabilized Cells. Confluent monolayers of cells in 3.5-cm dishes were incubated in serum-free medium for 3−5 h at 37 °C and then washed twice with Tris-buffered saline at pH 7.4. The permeabilization medium (20 mM HEPES, pH 7.5, 125 mM KCl, 5 mM NaCl, 11.1 mM glucose, and 35 μg/mL digitonin) was added for 20 min at room temperature after which the culture dishes were transferred to an aluminum cooling plate connected to a thermostatic water circulator. After 2 min on the plate at 6 °C, cells were incubated with or without 100 nM insulin (CHO/HIRc cells) or 10 nM IGF-1 (NWTb3 cells) for 15 min, and the phosphorylation reaction was initiated by the addition of 100 μM ATP (or 100 μM [γ-^{32}P]-ATP) and 4 mM $MnCl_2$. Reactions were carried out for up to 20 min at 6 °C and continued with the addition of MBB (0.2 mM) or dimethyl sulfoxide (1%) as vehicle. Thiol-biotinylation reaction was stopped after 10 min by the addition of 2 mM DTT. The cells were solubilized in lysis buffer [20 mM Tris-HCl, pH 7.5, containing 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, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 8 μg/mL aprotinin, and 2 μg/mL leupeptin]. Insoluble matter was removed by centrifugation for 20 min at 15 000 rpm in a microcentrifuge. The cell supernatants were added to a monoclonal antibody against the β-subunit of the insulin receptor (CHO/HIRc cells) or against the α-subunit of the IGF-1 receptor (NWTb3 cells), which has been prebound to protein G-Plus/protein A−agarose. After an overnight incubation at 4 °C, immunoadsorbed receptors were washed extensively by centrifugation with lysis buffer and once with 50 mM HEPES, pH 7.5, 0.1% Triton X-100 before addition of Laemmli sample buffer (Laemmli, 1970) to elute proteins and electrophoresis on 4−12% Tris−glycine gels under reducing conditions, and electrotransfer to PVDF membranes. Thiol-biotinylated receptor β-subunits were detected after blotting the membranes with enzyme-linked streptavidin. First, the membranes were incubated in blocking buffer composed of 1% polyvinylpyrrolidone (w/v) in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.6, and 0.1% Tween-20) for 1 h at room temperature, followed by the addition of horseradish peroxidase-conjugated streptavidin at a dilution of 1:200 000. Membranes were then washed extensively with TBS-T and developed using the ECL detection system.

Insulin Receptor Autophosphorylation in Permeabilized Cells. Confluent and then serum-deprived CHO/HIRc cells grown in 3.5-cm dishes were permeabilized, and incubated with or without 100 nM insulin for 15 min at 6 °C. The phosphorylation reaction was initiated by the addition of 100 μM [γ-^{32}P]ATP (1.7 μCi/nmol)/4 mM $MnCl_2$, and then allowed to continue for 5 min before the culture dishes were transferred to liquid nitrogen. The cells were solubilized in lysis buffer, and the ^{32}P-autophosphorylated receptor that had been recovered by immunoprecipitation (with a monoclonal anti-insulin receptor antibody) was separated by SDS−polyacrylamide gel electrophoresis under reducing conditions. The gel was dried and then autoradiographed at −70 °C on Amersham Hyperfilm-MP films with intensifying screens. Quantification of autoradiograms was performed

using ImageQuant software (version 3.3) on a Molecular Dynamic laser densitometer (Sunnyvale, CA).

Insulin Receptor Tyrosine Kinase Activity in Permeabilized Cells. Cell monolayers grown to confluence on 24-multiwell dishes (Costar) were permeabilized and incubated in the absence or presence of insulin for 15 min at 22 °C, and the insulin receptors were subsequently prephosphorylated by the addition of 100 μM unlabeled ATP/4 mM $MnCl_2$ for 15 min. The medium was removed, and substrate phosphorylation was initiated by the addition of 100 μL of permeabilization medium per well containing 20 μM [γ-^{32}P]ATP (4−5 μCi/nmol), 4 mM $MnCl_2$, and 27 μM peptide 983 as substrate. "Peptide 983" [SRGDYMTMQIG-amide], synthesized in our laboratory, was recently introduced as an exogenous substrate for the insulin receptor kinase (Bernier et al., 1994). Fifteen minutes later, the phosphorylation reactions were stopped by transferring an aliquot of the reaction mixture to a tube that contained 1 volume of 2× concentrated Tricine sample buffer. ^{32}P-Labeled peptide was resolved on 10−20% gradient Tricine−polyacrylamide gels under reducing conditions. After electrophoresis, the gels were fixed in a solution composed of 3.5% 5-sulfosalicylic acid (w/v) and 12% trichloroacetic acid (w/v) for 30 min, dried at 60 °C, and autoradiographed. The amount of ^{32}P-labeled substrate was quantified on dried gels with a Betascope 603 Blot Analyzer (Betagen; Waltham, MA; version 2.0 of the operating software) and/or Cerenkov radiation. The substrate phosphorylation reaction was linear up to 30 min at 22 °C.

Chromatography of Thiol-Biotinylated Insulin Receptor on Monomeric Avidin−Agarose. Confluent CHO/HIRc cells grown in 10-cm dishes were permeabilized and incubated in the permeabilization buffer in the presence of 0.2 mM MBB or 1% dimethyl sulfoxide as vehicle for 15 min at 6 °C, and the thiol-biotinylation reaction was terminated with the addition of 2 mM DTT. The cells were solubilized in lysis buffer for 60 min at 4 °C, and the solubilized proteins were separated from insoluble matter by centrifugation. The resulting supernatant was diluted in 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 200 mM NaCl, 1 mM orthovanadate, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 8 μg/mL aprotinin, and 2 μg/mL leupeptin (buffer A), and incubated batchwise with wheat germ−agarose overnight at 4 °C. The resin was washed with buffer A and then eluted with buffer A containing 0.3 M *N*-acetylglucosamine. The wheat germ−agarose eluate was incubated batchwise with preconditioned monomeric avidin−agarose at room temperature, according to the supplier's protocol. The breakthrough was recovered, and the monomeric avidin−agarose was washed extensively by centrifugation with 50 mM HEPES, pH 7.5, 0.1% Triton X-100. After washing, the monomeric avidin affinity resin was eluted with 1 volume of 2 mM *d*-biotin in 0.1 M sodium phosphate, pH 7.2, 0.15 M NaCl, and 0.1% Triton X-100. The amounts of insulin receptor present in the wheat germ−agarose eluate and in the monomeric avidin−agarose breakthrough and eluate were quantitated by Western blot analysis following SDS−polyacrylamide gel electrophoresis and electrotransfer of the proteins to PVDF membranes.

Assay of Dephosphorylation of Thiol-Biotinylated, ^{32}P-Labeled Insulin Receptors by Solubilized Particulate Protein Tyrosine Phosphatases. Confluent monolayers of CHO/HIRc cells in 10-cm dishes were permeabilized, and the thiol-biotinylation reaction with MBB was performed, with the exception that no phosphorylation reaction was done. The cells were lysed; the insulin receptors recovered in the wheat germ−agarose eluate were phosphorylated in 300-μL reaction volumes containing 100 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 0.1% Triton X-100, 1 mM DTT, and 40 μM [γ-^{32}P]-ATP (10 cpm/fmol), with 100 nM insulin. Reactions were carried out for up to 60 min at room temperature, and terminated by passing the reaction mixture through a G-25 column equilibrated with 25 mM HEPES, pH 7.4, and 0.1% Triton X-100. An aliquot of the each reaction mixture was saved, diluted in lysis buffer, and incubated for 16 h at 4 °C with monoclonal anti-insulin receptor antibody preadsorbed to protein G-Plus/protein A−agarose. Immunoadsorbed insulin receptor was washed extensively by centrifugation with lysis buffer and once with 50 mM HEPES, pH 7.5, 0.1% Triton X-100 before addition of Laemmli sample buffer and electrophoresis. Phosphorylated insulin receptor β-subunit was identified by autoradiography following SDS−polyacrylamide gel electrophoresis and electrotransfer of the proteins to PVDF membranes. The thiol-biotinylated insulin receptor was detected after blotting the membrane with enzyme-linked streptavidin.

Other aliquots of the reaction mixture were used in a dephosphorylation assay. Membranes were prepared from CHO/HIRc cells, essentially as described by Burke et al. (1994). These membranes were solubilized, and the soluble particulate fraction was used as the source of protein tyrosine phosphatases (Liotta et al., 1994). In brief, phosphorylated insulin receptors were incubated with solubilized membrane extracts (11 μg/assay) in 100-μL reaction volumes that contained 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin, 5 mM DTT, and 5 mM EDTA. Reactions were carried out for up to 5 min at 30 °C and terminated at various intervals by transferring an aliquot of the mixture to a tube that contained 1 volume of 2-fold-concentrated Laemmli sample buffer. Phosphorylated insulin receptor β-subunit was resolved following SDS−polyacrylamide gel electrophoresis under reducing conditions. The loss of ^{32}P from the receptor β-subunit was quantitated by Betagen counting of the fixed and dried gels.

Statistical Methods. The Scheffe's S analysis of variance was used for the statistical analyses. Data are presented as mean ± SEM of three or four experiments.

RESULTS

Biotinylation of Insulin Receptor β-Subunit. We meaured the extent of biotinylation of insulin receptor sulfhydryls with MBB in semipermeabilized cells, and compared it with *N*-[^{3}H]ethylmaleimide labeling of the receptor in cell-free systems. Permeabilized CHO/HIRc cells were autophosphorylated in the absence or presence of insulin followed by the addition of MBB; ^{32}P-labeled insulin receptors were then examined for their content of biotinylated thiols, as described under Materials and Methods. Immunoprecipitated ^{32}P-labeled insulin receptors show a 3−5-fold stimulation of receptor autophosphorylation by insulin (Figure 1A), which is in contrast to biotinylation where no difference was noted between basal and insulin-treated cells (Figure 1B). Quantitative analysis indicated that the extent of biotinylation of the receptor β-subunit was enhanced modestly by insulin over basal [(1.23 ± 0.15)-fold; mean ± SE, n = 6]. These

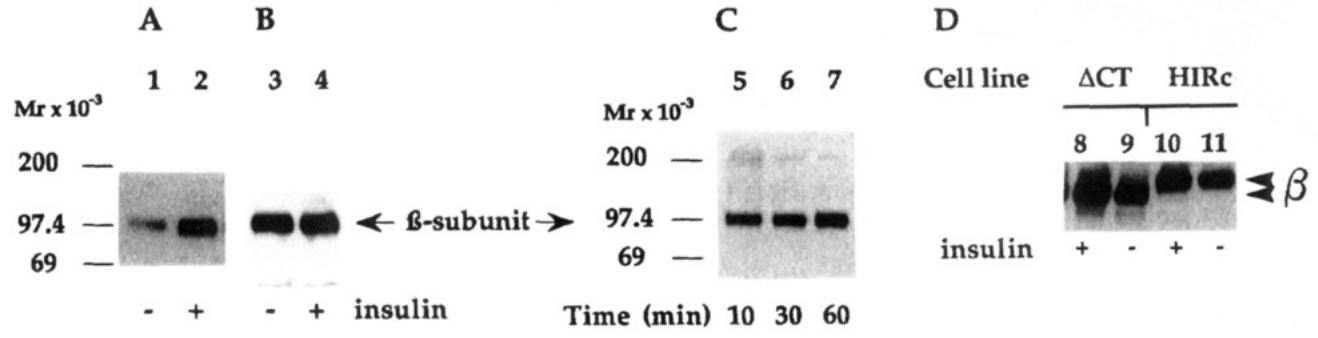

FIGURE 1: Thiol-biotinylation of phosphorylated human insulin receptor. Permeabilized CHO/HIRc cells were treated with or without insulin (100 nM) for 15 min, and the insulin receptors were subjected to autophosphorylation for 10 min at 6 °C. Thereafter, cells were incubated with 0.2 mM MBB for an additional 10 min and then solubilized. ^{32}P-Labeled insulin receptors were immunoprecipitated with a monoclonal anti-insulin receptor antibody. The immunoprecipitates were resolved by SDS−polyacrylamide gel electrophoresis, and transferred to PVDF membranes by electroblotting. The blot was autoradiographed (A) and then probed with horseradish peroxidase-conjugated streptavidin, and ECL was performed (B). A time-course of biotinylation was performed where MBB was added for periods up to 60 min at 6 °C (C: 10, 30, and 60 min in lanes 5−7, respectively). The relative molecular mass standards are shown × 10^{-3}. (D) Thiol-biotinylation of mutant (lanes 8, 9) and wild-type (lanes 10, 11) insulin receptors was performed in permeabilized CHO/ΔCT cells and CHO/HIRc cells that were preincubated with or without insulin, as indicated.

results suggest that there is no apparent difference in the reactivity of receptor sulfhydryls following cell stimulation with insulin. It can be seen that thiol-biotinylation occurred exclusively in the β-subunit of the native receptor with no modification of the receptor α-subunit sulfhydryls (Figure 1B). The use of a biotinylation reagent that reacts with α-amino groups (NHS-LC-biotin) resulted in rapid derivatization of both subunits (result not shown), in agreement with Levy-Toledano et al. (1993). A study of the time course for thiol-biotinylation of the receptor β-subunit revealed that biotin incorporation was rapid, with 75% labeling after 10 min incubation at 6 °C (Figure 1C). Pretreatment of MBB with L-cysteine but not L-glycine prevented biotinylation of the β-subunit, indicating the specificity of MBB as a thiol-modifying reagent (result not shown). Thiol-biotinylation was also observed in cells expressing large numbers of mutant insulin receptor from which the C-terminal 43 amino acids had been deleted (Figure 1D). Thus, it appears that this segment of the receptor is not likely to modulate the reactivity of the insulin receptor sulfhydryls to MBB.

Several sulfhydryl reagents were tested as probes for assessing the location of the accessible thiol(s) on the receptor as well as determining whether the reactive sulfhydryls are vicinals. Intact CHO/HIRc cells were incubated with the cell-impermeant maleimidostilbenedisulfonate (AMSD), washed to remove unreacted reagent, and permeabilized. Under these conditions, AMSD treatment had no effect on the ability of MBB to biotinylate the receptor β-subunit (Figure 2, lane 1). In contrast, preincubation of permeabilized cells with AMSD or disulfide exchange reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 4,4′-dithiodipyridine (4-PDS) totally prevented the receptor sulfhydryls from reacting with MBB (Figure 2, lanes 3, 4, 6). Our interpretation of these results is that reactive sulfhydryls are located in the cytoplasmic domain of the receptor β-subunit.

The possibility that MBB modifies reactive vicinal sulfhydryls on the receptor was explored with the use of phenylarsine oxide. This reagent, a trivalent arsenical compound that reacts with vicinal sulfhydryls, causes an accumulation of phosphorylated substrates in cells stimulated with insulin (Bernier et al., 1987; Frost et al., 1987; Levenson & Blackshear, 1989). When permeabilized cells were

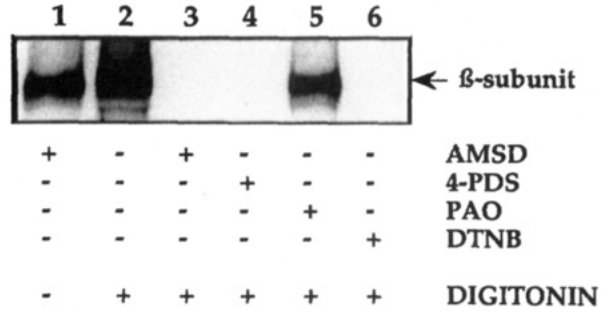

FIGURE 2: Inhibition of MBB labeling of cytoplasmic insulin receptor sulfhydryl(s) by thiol-modifying agents. The cells treated with AMSD (2 mM) for 15 min at room temperature (lane 1) or with buffer alone (lane 2) were washed to remove unreacted reagent, permeabilized, and incubated with 100 nM insulin for 15 min at 6 °C. Alternatively, the cells were permeabilized first and then incubated with insulin in the presence of either 2 mM AMSD, 2 mM 4-PDS, 0.7 mM PAO, or 2 mM DTNB (lanes 3−6). Thereafter, the insulin receptors were subjected to autophosphorylation for 10 min at 6 °C, and the cells were incubated with 0.2 mM MBB for an additional 10 min and then frozen in liquid nitrogen. After solubilization of the cells, the insulin receptors were immunoprecipitated with a monoclonal anti-insulin receptor antibody. The immunoprecipitates were electrophoresed in a SDS−polyacrylamide gel, and electrotransferred to PVDF membranes. The blot was probed with horseradish peroxidase-conjugated streptavidin. The data shown are representative of two separate experiments.

pretreated with phenylarsine oxide (PAO), the extent of receptor biotinylation by MBB was maintained, indicating a lack of reactive vicinal sulfhydryls in the receptor protein (Figure 2, lane 5).

To assess the efficiency of thiol-biotinylation by MBB, affinity purification of thiol-biotinylated receptors was performed using immobilized monomeric avidin. The monomeric form of avidin exhibits a binding affinity for biotin that is several orders of magnitude lower than that of the native tetrameric avidin (Wilchek & Bayer, 1988). This characteristic of monomeric avidin allows for gentler conditions of dissociation of the avidin−biotin complex and the recovery of biotinylated molecules. The starting material for purification was the 1% Triton X-100-solubilized preparation of permeabilized CHO/HIRc cells. Figure 3 demonstrates the relative distribution of the insulin receptor β-subunit from control and MBB-treated permeabilized cells

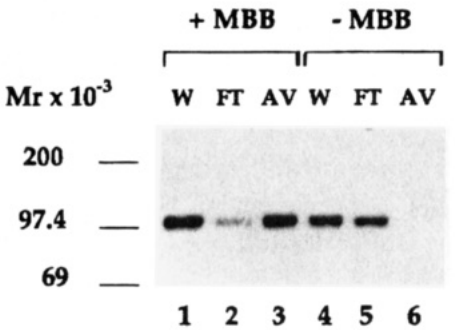

FIGURE 3: Distribution of thiol-biotinylated insulin receptors after monomeric avidin−agarose chromatography. Permeabilized CHO/HIRc cells were treated (lanes 1−3) or not treated (lanes 4−6) with 0.2 mM MBB, and solubilized in lysis buffer before chromatography of solubilized extracts on wheat germ lectin and monomeric avidin−agarose. Comparable aliquots of wheat germ lectin eluates (W) and monomeric avidin−agarose breakthrough (FT) and eluates (AV) were subjected to SDS−polyacrylamide gel electrophoresis under reducing conditions, and the proteins were electrotransferred to PVDF membranes. The blot was probed with a polyclonal antiserum raised against a peptide corresponding to residues 1293−1306 of the insulin receptor β-subunit.

after partial purification on wheat germ lectin (Figure 3, lanes 1 and 4) or after sequential wheat germ lectin and monomeric avidin−agarose affinity purification (Figure 3, lanes 2, 3 and lanes 5, 6). Western blot analysis reveals no receptor β-subunit in the monomeric avidin−agarose eluate from control cells (Figure 3, lane 6). However, more than 87% of the receptor β-subunit from MBB-treated cells after partial purification on wheat germ lectin was recovered in the monomeric avidin−agarose eluate (Figure 3, lane 3). Comparable results were obtained when measuring the ^{125}I-insulin binding activity present (data not shown).

Sulfhydryl Biotinylation of the IGF-1 Receptor. We investigated next the effect of MBB on wild-type IGF-1 receptors in NWTb3 cells. The insulin receptor and IGF-1 receptor are structurally similar; both receptor tyrosine kinase domains contain four cysteines that are highly conserved (Ullrich et al., 1986). However, the insulin receptor cytoplasmic domain contains cysteines at positions 969[2] and 1296 that are absent in the IGF-1 receptor. Therefore, a differential reactivity between CHO/HIRc cells and NWTb3 cells to MBB may give important information pertaining to the location of those critical sulfhydryls in the insulin receptor. NWTb3 cells were permeabilized, incubated with 10 nM IGF-1, and treated for 15 min with 0.2 mM MBB, and the extent of IGF-1 receptor biotinylation was compared with that of the insulin receptor from CHO/HIRc cells. As shown in Figure 4, treatment of NWTb3 cells with MBB resulted in thiol-biotinylation of the IGF-1 receptor β-subunit. Also, a characteristic decrease in mobility of the IGF-1 receptor β-subunit on SDS−polyacrylamide gel electrophoresis was observed (Goldfine, 1987). Thus, this finding may indicate that the same reactive thiols on both the insulin receptor and IGF-1 receptor are available for biotinylation by MBB.

Effect of MBB on Insulin Binding and Insulin Receptor Functions. Experiments were conducted to test whether MBB altered the catalytic properties of the insulin receptor. Treatment of intact cells with MBB had no significant effect

[2] The numbering system used represents that of the minus exon 11 variant receptor according to Ullrich et al. (1985).

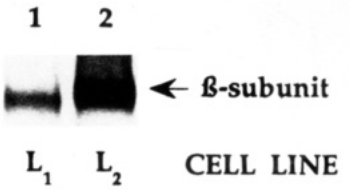

FIGURE 4: Thiol-biotinylation of the insulin receptor and IGF-1 receptor in permeabilized cells. Biotinylation of the insulin receptor (L_1) and IGF-1 receptor (L_2) in permeabilized CHO/HIRc cells and NWTb3 cells was performed as described in the legend of Figure 1. After solubilization of the cells, receptor proteins were immunoprecipitated with receptor-specific monoclonal antibodies. The immunoprecipitates were electrophoresed in a SDS−polyacrylamide gel and electrotransferred to PVDF membranes. The blot was probed with horseradish peroxidase-conjugated streptavidin.

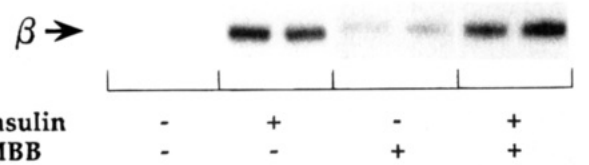

FIGURE 5: Effect of MBB on the autophosphorylation of insulin receptor β-subunit in permeabilized cells. Confluent monolayers of CHO/HIRc cells were serum-starved, permeabilized, and treated without or with 100 nM insulin in the absence or presence of 0.2 mM MBB for 15 min at 6 °C. The phosphorylation reaction was conducted with [γ-^{32}P]ATP/Mn^{2+} at 6 °C for 5 min. At the end of the reaction, the cells were immediately frozen in liquid nitrogen, solubilized, and further processed as described under Materials and Methods. ^{32}P-Labeled insulin receptor was immunoprecipitated with a monoclonal anti-insulin receptor antibody, and separated by SDS−polyacrylamide gel electrophoresis under reducing conditions. The gel was then processed for autoradiography.

on the insulin binding activity (data not shown), consistent with previous reports that indicate no change in insulin binding following the treatment of the receptors with thiol reagents (Wilden & Pessin, 1987; Clark & Konstantopoulos, 1993).

We have previously shown that insulin receptor autophosphorylation and exogenous substrate phosphorylation are activated severalfold over basal following insulin stimulation of digitonin-permeabilized cells (Bernier et al., 1994). The effects of MBB on insulin receptor autophosphorylation were examined. In confluent, serum-deprived CHO/HIRc cells that were semipermeabilized, insulin (100 nM) stimulated autophosphorylation by approximately 5-fold over the levels seen in the unstimulated cells; however, the extents of basal- and insulin-stimulated receptor autophosphorylation were not affected by MBB (Figure 5). It has been previously reported that sulfhydryl-modifying reagents can either stimulate or inhibit the intrinsic tyrosine kinase activity of the insulin receptor (Wilden et al., 1986; Wilden & Pessin, 1987; Li et al., 1991; Clark & Konstantopoulos, 1993), depending mostly on the nature of the alkylating group. Therefore, we examined the effects on the insulin receptor tyrosine kinase of MBB and selected sulfhydryl-modifying reagents that were shown to block MBB-induced thiol-biotinylation of the receptors.

Tyrosine kinase activity was examined in permeabilized cells using the synthetic peptide 983 as a substrate. As shown in Figure 6, substrate phosphorylation in control cells was increased up to 3-fold over basal levels by the presence of 100 nM insulin. The apparent stimulatory effect of MBB on insulin-dependent tyrosine phosphorylation of peptide 983

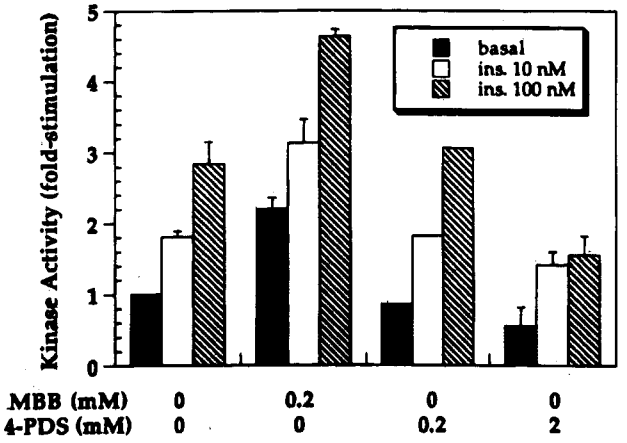

FIGURE 6: Effect of MBB and 4-PDS on insulin receptor kinase activity in permeabilized cells. CHO/HIRc cells were grown to confluence in 24-multiwell dishes. After permeabilization, cells were preincubated with 0, 10, or 100 nM insulin for 15 min at 25 °C, followed by the addition of vehicle alone, 0.2 mM MBB, or 0.2 or 2 mM 4-PDS for 5 min. Subsequently, the insulin receptors were allowed to phosphorylate peptide 983 for 15 min as described under Materials and Methods. The reaction was stopped by adding Tricine sample buffer to aliquots of the reaction mixture, and samples were electrophoresed in a 10−20% Tricine gradient gel. The gels were fixed and dried, and the ^{32}P incorporated in the peptide substrate was determined by Cerenkov counting. The activities have been plotted as the fold-stimulation over the basal activity without insulin or MBB/4-PDS treatment. The results represent the average ± SE for three to four independent experiments.

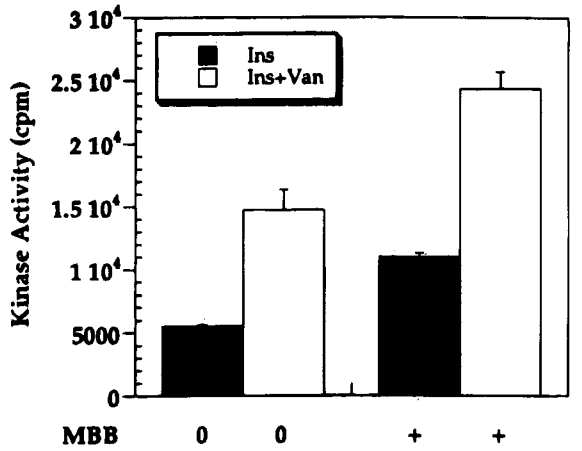

FIGURE 7: Effect of vanadate inhibition of protein tyrosine phosphatases on MBB-stimulated insulin receptor kinase. Permeabilized cells were preincubated with insulin (100 nM) in the absence or the presence of vanadate (200 μM) for 15 min, followed by the addition of vehicle or 0.2 mM MBB for 5 min. Substrate phosphorylation was initiated as described in the legend of Figure 6. Data are means ± total range of duplicate wells and are representative of two experiments.

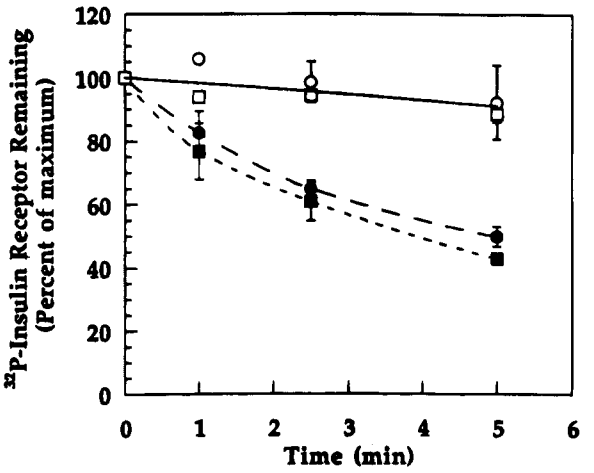

FIGURE 8: Dephosphorylation of ^{32}P-labeled, thiol-biotinylated insulin receptor by membrane-associated protein tyrosine phosphatases. Permeabilized cells were treated (squares) or untreated (circles) with 0.2 mM MBB, and the insulin receptors were partially purified on wheat germ−agarose and phosphorylated in the presence of insulin and 20 μM [γ-^{32}P]ATP/4 mM Mn^{2+}. The particulate fraction from CHO/HIRc cells was solubilized in Triton X-100-containing buffer and used as source of protein tyrosine phosphatases. Equivalent amounts of wheat germ−agarose-purified ^{32}P-labeled insulin receptors were incubated up to 5 min at 25 °C in a dephosphorylation buffer containing 0.1% Triton X-100 and 5 mM DTT in the presence (filled symbols) or absence (open symbols) of membrane extracts. Reactions were stopped by boiling in Laemmli sample buffer. Samples were loaded onto a SDS−polyacrylamide gel and electrophoresed under reducing conditions, and the radioactivity was associated with the β-subunit was determined by Betagen counting of the dried gels.

was due to a 2-fold increase in the basal levels of kinase activity. The receptor kinase was not affected by 0.2 mM 4-PDS, but 4-PDS at 2 mM had inhibitory effects on the enzyme activity (Figure 6). Thus, thiol-biotinylated receptors showed normal autophosphorylation and insulin-dependent kinase activity but increased insulin-independent (basal) kinase activity toward an exogenous substrate.

Pulse−chase experiments have clearly shown that vanadate inhibits all PTPases that dephosphorylate the insulin receptor in permeabilized cells (Bernier et al., 1994). To investigate whether the enhancing effect of MBB on the receptor kinase activity was the result of PTPase inhibition, permeabilized cells were preincubated with insulin and vanadate, followed by MBB treatment. Then, ^{32}P incorporation into peptide 983 was measured. As shown in Figure 7, inhibition of tyrosine dephosphorylation by vanadate caused a ~2.7-fold stimulation of insulin-stimulated kinase activity. Despite PTPase inhibition by vanadate, MBB increased further the rate of substrate phosphorylation by the receptor kinase. Together, these results suggest that the enhancement of the catalytic activity of the insulin receptor toward an artificial substrate in permeabilized cells by MBB occurs via a mechanism that does not involve PTPase inhibition, but rather is the consequence of thiol-biotinylation of the receptor.

Effect of MBB on the Dephosphorylation of Insulin Receptor β-Subunit in Vitro. The biotinylation of reactive thiol(s) may cause a conformational change in the receptor β-subunit, thereby reducing the accessibility of phosphotyrosine moieties of the receptor to cellular PTPases. To test this possibility, permeabilized CHO/HIRc cells were treated with or without 0.2 mM MBB; the insulin receptors were partially purified by wheat germ lectin chromatography, phosphorylated, and used as substrate in a dephosphorylation assay. The presence of thiol-biotinylated, ^{32}P-labeled receptor β-subunit was confirmed by streptavidin blotting (data not shown). Dephosphorylation of tyrosine-phosphorylated insulin receptor by PTPases contained in a Triton X-100-solubilized crude particulate fraction from CHO/HIRc cells was rapid (Figure 8) with less than 50% phosphorylated receptors remaining after a 5-min incubation. Dephosphorylation occurred at approximately equal rates whether the insulin receptor was thiol-biotinylated or not. No significant release of ^{32}P was observed when ^{32}P-labeled receptor was incubated in the absence of particulate PTPases.

DISCUSSION

A significant finding of this study is the use of enzyme-labeled streptavidin and chemiluminescence techniques to detect thiol-biotinylation of the insulin receptors. Insulin receptor has been shown to contain reactive cysteine residue(s) whose function remains unknown (Ridge et al., 1988). Because of the inherent limitations of studies with isolated receptors, we investigated the effects of a thiol-biotinylating reagent in digitonin-permeabilized cells, and found that MBB reacted only with the cytoplasmic portion of the receptor β-subunit under mild nondenaturing conditions that mimicks the intracellular milieu. The semipermeabilized cell system used in our studies offered the advantage of studying modulation of insulin receptor kinase activity to a degree that had been impossible with intact cells and yet retaining much of the cell membrane architecture of the *in vivo* system (Mooney & Anderson, 1989; Mooney & Bordwell, 1992; Bernier et al., 1994). We provide experimental evidence to show that the treatment of permeabilized cells with MBB caused near-quantitative thiol-specific biotinylation of reactive sulfhydryl(s) on the insulin receptors, along with an increase in insulin-independent (basal) receptor tyrosine kinase activity.

Labeling of purified human placental insulin receptor with *N*-[^{3}H]ethylmaleimide indicated the existence of only one free thiol group on the insulin receptor (Ridge et al., 1988; Chiacchia, 1991); Finn and co-workers (Finn et al., 1990) attempted to locate this reactive receptor thiol. They observed that ATP and a nonhydrolyzable ATP analog were capable of partially inhibiting alkylation of the isolated receptor. Thus, one possible explanation for the inhibition of thiol alkylation is that the reactive receptor cysteine is located near the ATP binding site. We were unable to confirm this observation. In fact, our results indicate that thiol-biotinylation in permeabilized cells was not reduced by the presence of exogenous ATP. Moreover, preactivation of the insulin receptor by autophosphorylation (in the absence or presence of insulin) did not affect the level of thiol-biotinylated receptors. Furthermore, it is unlikely that the conformational change in the carboxyl-terminal domain of the receptor β-subunit seen upon insulin binding (Perlman et al., 1989; Baron et al., 1990, 1991) will induce a differential reactivity/accessibility of receptor thiol(s) to MBB. This conclusion is supported by our data which indicate that MBB is capable of stimulating thiol-biotinylation of the insulin receptors in the CHO/ΔCT cells to the same extent as in the CHO/HIRc cells, even though the expressed mutant receptors are missing the carboxyl-terminal 43 amino acids (Myers et al., 1991). Thus, it would appear that insulin-dependent activation of insulin receptor functions does not involve any alteration in the total number of reactive sulfhydryl groups, as had been suggested (Wilden et al., 1986; Finn et al., 1990).

Our result show that thiol-biotinylation has an initial effect to enhance the basal level of insulin receptor kinase itself with no noticeable effect on receptor autophosphorylation. Studies on the activation of the insulin receptor kinase have shown that tris-phosphorylation of the 1146-kinase domain is needed for full tyrosine kinase activity, as substitution of any of the tyrosine residues in the kinase catalytic domain affects severely insulin receptor autophosphorylation and its associated tyrosine kinase (Ellis et al., 1986; Yonezawa & Roth, 1991; Zhang et al., 1991; Wilden et al., 1992). Interestingly, a naturally occurring mutation of a conserved arginine residue located immediately following tyrosine 1151 of the 1146-kinase domain in the insulin receptor has been found to cause a constitutive activation of the basal receptor kinase despite deficient autophosphorylation (Formisano et al., 1993). These data support our finding of an apparent dissociation between receptor autophosphorylation and kinase activation. The recently described crystal structure of the insulin receptor kinase domain (Hubbard et al., 1994) has provided a model to possibly explain our data. In the unphosphorylated state, tyrosine 1150 of the 1146-kinase domain is held in the catalytic site and blocks the binding of both ATP and substrate. Upon activation of the insulin receptor, tyrosine 1150 is disengaged and *trans*-autophosphorylated, allowing both binding sites to be accessible. The deactivation of the receptor kinase occurs when tyrosine 1150 returns to its autoinhibitory position (Hubbard et al., 1994). The increased tyrosine phosphorylation of exogenous peptide under basal (unstimulated) conditions could have resulted from the enhanced insulin receptor kinase activation, diminished receptor dephosphorylation, or a combination of both in MBB-treated permeabilized cells. One could speculate that biotinylation of reactive thiol(s) may have disengaged tyrosine 1150 from the catalytic site without *trans*-autophosphorylation reaction and increased the ability of exogenous substrate to bind to the insulin receptor. In this way, substrate phosphorylation can occur without significant tris-phosphorylation of the 1146-kinase domain. By promoting receptor autophosphorylation, insulin further stabilizes the noninhibiting conformation by specific electrostatic interactions involving the phosphorylated tyrosine 1150 (Hubbard et al., 1994).

Inhibition of PTPases by MBB could also result in an enhanced insulin receptor kinase activation. Dephosphorylation of the insulin receptor in permeabilized cell systems is catalyzed by membrane-associated PTPases (Mooney & Anderson, 1989; Mooney & Bordwell, 1992; Bernier et al., 1994). These enzymes require thiol reagents for maintaining PTPase activity (Walton & Dixon, 1993), making them likely targets for inactivation by thiol-specific modifying reagents. However, the fact that inhibition of PTPases by vanadate did not prevent the enhancing effect of MBB on the activity of the receptor kinase is suggestive of a novel mechanism of activation in which modification of critical thiol(s) on the insulin receptor is modulating its function. The present work provides evidence that the thiol-biotinylated ^{32}P-labeled receptor is dephosphorylated at a rate comparable to that of control receptor when incubated in the presence of a solubilized PTPase preparation. Thus, it appears that thiol-biotinylation does not induce conformational change in the insulin receptor structure that will reduce accessibility of phosphorylated tyrosines to PTPase action in cell-free systems; however, the possibility remains that such modified receptor is resistant to dephosphorylation in intact cells.

We took advantage of the overall similarity between the IGF-1 receptor and insulin receptor in an attempt to determine the location of the reactive receptor thiol(s). Treatment of permeabilized NWTb3 cells with MBB led to thiol-biotinylation of the IGF-1 receptor β-subunit in a similar fashion to that of the insulin receptor. Overall, this result demonstrates that the IGF-1 receptor contains also one or more accessible cysteine(s), and supports the possibility that

the highly conserved kinase catalytic region of both receptors contains the reactive thiol(s). The construction of receptor mutants with cysteine to serine substitution in the tyrosine kinase domain should provide insight into the role that such reactive cysteine(s) may play in the modulation of the insulin receptor function in intact cells.

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