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Receptors for Insulin and Epidermal Growth Factor: Interaction With Organomercurial Agarose

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The receptor for both insulin and epidermal growth factor (EGF) from human placental membranes, after crosslink labeling with ^{125}I-labeled insulin and EGF, can be adsorbed to an organomercurial-agarose derivative (Affi-Gel 501) and can be recovered from the gel by elution with dithiothreitol (DTT). Pretreatment of crosslink-labeled membranes with N-ethylmaleimide (NEM) blocks the ability of the receptor to react with the organomercurial column. NEM also abolishes the protein kinase activity of both receptors. Under appropriate conditions, insulin can promote the reaction of the insulin receptor with the organomercurial-agarose derivative. For both the insulin and EGF receptors, our results provide an avenue for the isolation of the sulfhydryl-containing receptor domains that may play a role in the control of receptor function.

Key words: insulin, EGF, sulfhydryls, NEM, Affi-Gel 501

We have been interested for some time in two hydrodynamic forms of the insulin receptor (R_I and R_{II}) that can be detected in electrophoretic [1–4], chromatographic [5–8], and sucrose gradient systems. In recent work [8] we have observed that the insulin-mediated conversion of the receptor from the R_I to the R_{II} form, which appears to correspond to a hydrodynamically smaller receptor species, is accompanied by the exposure of a receptor group (most likely a sulfhydryl) that reacts with N-ethylmaleimide (NEM). Further, we have observed that the R_{II} form of the receptor can be converted back to the R_I form with the use of oxidized glutathione [8] Our work [8,9], in keeping with the observations of others [10–13], suggests that disulfide-sulfhydryl exchange reactions may play an important role in terms of insulin receptor structure and function. Little is known about the presence or absence of reactive sulfhydryl groups in the receptors for insulin and epidermal growth factor (EGF). Thus, in view of our interest in identifying the receptor domains that might be involved in disulfide-sulfhydryl exchange reactions, we have, in the work de-

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scribed here, explored the interaction of the receptors for insulin and EGF with sulfhydryl affinity columns (Affi-Gel 501 and 401 and cysteamine-agarose). Further, we have examined the ability of insulin to promote the interaction of the insulin receptor with an organomercurial-agarose gel (Affi-Gel 501). Finally, we studied briefly the effect of NEM on the protein kinase activity of the partially purified insulin receptor.

MATERIALS AND METHODS

Chemicals

Affi-Gel derivatives (501 and 401), the dye for protein determinations, and Triton X-100 were from Bio-Rad (Rockville Center, NY). Prior to use, Triton X-100 was purified and stored as suggested by Chang and Bock [14]. N-ethylmaleimide (NEM) and disuccinimidyl suberate (DSS) were from Pierce (Rockford, IL). Wheat germ agglutinin (WGA) agarose (6.3 mg lectin/ml packed gel) and cysteamine-agarose were from PL Biochemicals (Milwaukee, WI). Carrier-free ^{125}Iodide was from Amersham (Arlington Heights, IL). Mouse EGF was isolated from male mouse submaxillary glands, as described [15].

Preparation of Placental Membranes

A crude "microsomal" membrane fraction was prepared according to previously described methods [16,17] from fresh, full-term, human placental tissue, obtained at cesarean section. Phenylmethylsufonyl fluoride (PMSF; 1 mM) was used in the homogenization and washing buffers to minimize proteolysis of receptor proteins.

Iodination of Insulin and EGF

Both peptides were iodinated by a modification of the chloramine-T method, essentially as described previously [18], to specific activities of 100-150 μCi/μg. Radiolabeled peptide was separated from unreacted ^{125}Iodide by gel filtration on Sephadex G-15 or G-25.

Preparation of Soluble Crosslink-Labeled Receptors

Freshly prepared placental membranes (adjusted to a final concentration of 1.6 mg/ml protein) were incubated with ^{125}I-labeled insulin (40 ng/ml) or EGF (0.5 ng/ml) in the presence or absence of excess (5 μg) unlabeled insulin or EGF in 1 ml of Krebs-Ringer-phosphate (KRP) buffer (pH 7.4). Equilibration was allowed to proceed for 1 hr at room temperature. Membranes were then washed three times by centrifugation at 37,000g for 20 min at 4°C and were resuspended in 1 ml of ice-cold Krebs-Ringer phosphate buffer (pH 7.4). Crosslinking was achieved essentially as previously described [19] by adding DSS (dissolved in dimethylsulfoxide [DMSO] at 100 mM) to the washed membrane suspension at a final nominal concentration of 1 mM. The reaction mixture was incubated for 15 min at 0°C and was then quenched by the addition of 0.5 ml of 0.5 mM TRIS-HCl buffer (pH 7.4). Crosslink-labeled membranes were washed three times with 10 mM TRIS-HCl buffer (pH 7.4) and the final membrane pellets were extracted for 24 hr at 4°C in (1 ml volume for 1.6 mg protein) 10 mM TRIS-HCl buffer containing 2% v/v Triton X-100. The extract was clarified by centrifugation (250,000g for 1 hr at 4°C). The crosslink-labeled receptor in the clarified supernatant either was applied directly to the organomercurial column or was first partially purified by adsorption to 2.0-ml columns of WGA-agarose and

eluted with 0.2M N-acetyl D-glucosamine in 10 mM TRIS-HCl buffer, pH 7.4, containing 0.1% v/v Triton X-100.

Treatment of Membranes With NEM

In experiments where membranes were treated with NEM, this reagent was added either before or after the crosslink-labeling reaction. NEM dissolved in DMSO was added to the membrane suspension ($\simeq$2 mg/ml protein in 10 mM TRIS-HCl buffer, pH 7.0) to yield a final NEM concentration of either 5 or 50 mM; the final concentration of DMSO was 2% v/v. The reaction mixture was incubated for 30 min at 24°C and membranes either were solubilized directly in 2% Triton X-100 for application to the Affi-Gel 501 column or were washed three times by centrifugation with 40 volumes of 10 mM TRIS-HCl buffer prior to either solubilization or use in an affinity crosslinking reaction. Pretreatment of membranes with NEM did not alter either the binding of radioligand or the crosslinking of insulin and EGF to their receptors (data not shown). In the experiment designed to assess the ability of insulin to promote the attachment of the receptor to the organomercurial column, the following sequence of treatments with NEM was used: 1) Membranes (1.6 mg/ml in 10 mM TRIS-HCl, pH 7.0) were reacted with 50 mM NEM in 2% DMSO for 30 min at 4°C as described above. 2) The washed NEM-treated membranes were divided into four aliquots and were crosslink-labeled with 40 ng/ml ^{125}I-insulin, as outlined above; two aliquots were crosslink-labeled in the simultaneous presence of 10 μg/ml unlabeled insulin. 3) One set (ie, two of the aliquots, one crosslinked in the presence of and the other in the absence of excess unlabeled insulin) of the ^{125}I-insulin–crosslinked membranes were again treated with 25 mM NEM, as outlined above; the second set of crosslink-labeled membranes was treated with 2% DMSO alone. All four sets of membranes were solubilized in Triton X-100 as outlined above and were applied to the Affi-Gel 501 column.

Sulfhydryl Affinity Chromatography

The sulfhydryl affinity gels (Affi-Gel 501 and 401) were used essentially as recommended by the manufacturer (Bio-Rad). Affi-Gel 501 was activated with 50 mM sodium acetate, pH 5, followed by washing the column (0.2 to 0.5 ml bed volume in a 0.6 cm ID pasteur pipette) with 10 volumes (about 2 ml) of the sodium acetate solution containing 4 mM mercuric acetate. Prior to applying the sample (about 0.2 to 0.5 ml of Triton X-100 extract, 0.2 mg/ml protein), the column was washed with 10 mM TRIS-HCl buffer, pH 7.5, containing 0.1% v/v Triton X-100. A sample (0.2–0.5 ml) was placed on the gel and applied to the column over a 30-min period at room temperature. The gel was then washed with the TRIS-HCl–0.1% Triton X-100 buffer (about 50 volumes at 12 ml/hr) and the sample was eluted at the same flow rate in the same buffer containing 10 mM dithiothreitol (DTT). Fractions of about 0.25 ml were collected to monitor the eluted radioactivity by crystal scintillation counting (efficiency about 82%). Protein was quantitated by the method of Bradford [20] using an appropriate buffer blank. Samples containing DTT were dialyzed against buffer (50 mM TRIS-HCl buffer, pH 7.5, containing 0.1% v/v Triton X-100) prior to the estimate of protein.

Gel Electrophoresis and Autoradiography

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate-containing gels (SDS-PAGE) and autoradiography were performed essentially as previously described

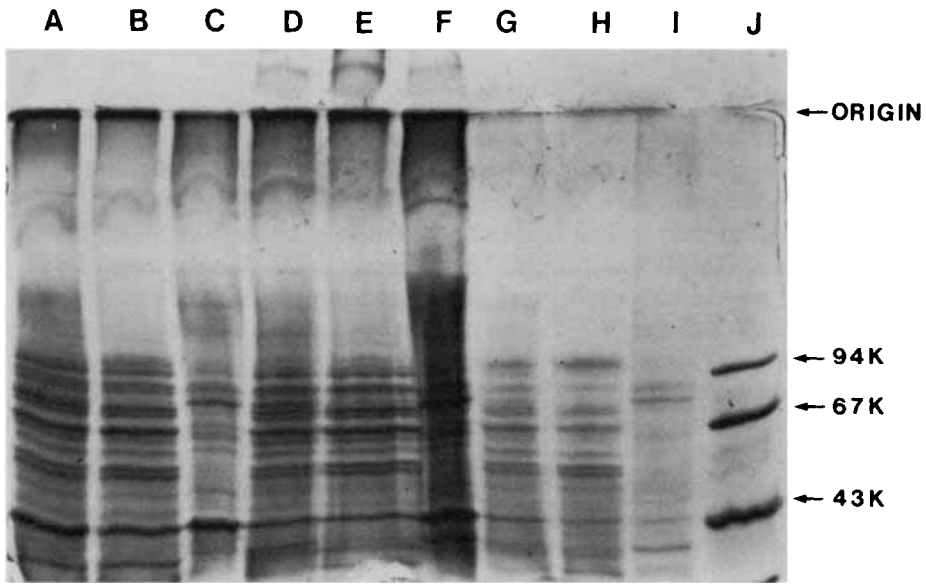

Fig. 1. Electrophoretic analysis of soluble placental membranes isolated by Affi-Gel 501 chromatography. The Triton X-100–solubilized protein fraction was applied to the Affi-Gel 501 column (protein concentration ≤ 0.2 mg/ml) either before (lane A) or after (lanes B, C) partial purification on WGA-agarose. Protein that either passed through the WGA-agarose column (lane B) or adsorbed to the WGA column (lane C) was applied to the Affi-Gel 501 column. Samples that either passed through the Affi-Gel column (lanes D–F) or were eluted from the column with DTT (lanes G–I) were analyzed by SDS-PAGE (5–10% linear gradient gels) in the presence of mercaptoethanol. Lane A) initial extract; lane B) material not adsorbed by WGA; lane C) proteins eluted with N-acetylglucosamine from WGA; lane D) proteins from lane A that passed through the Affi-Gel 501 column; lane E) proteins from lane B that passed through the Affi-Gel column; lane F) proteins from lane C that passed through the Affi-Gel column; lanes G–I) proteins from lanes A to C that were eluted from the Affi-Gel column with 10 mM DTT; lane J) standard proteins, with molecular weights indicated by arrows.

[21] using the buffer system of Laemmli [22] and either linear gradient (5–15%) or 7.5% gels. Either human erythrocyte membranes [23] or standard proteins (Sigma Chemical Co., St. Louis, MO) were used as molecular weight markers to calibrate the gels. Autoradiographic exposures at −70°C using Kodak X-Omat R film with Cronex lightening plus intensifying screens took from 2 to 6 weeks to visualize the radiolabeled receptor bands.

RESULTS

Interaction of Placental Membrane Proteins With Organomercurial Agarose (Affi-Gel 501)

The protocols developed for the use of the organomercurial column were designed primarily to determine if free sulfhydryl groups were present in the receptors for EGF and insulin, so as to permit the adsorption of the crosslink-labeled receptors to the affinity column and elution with DTT. Thus, conditions were sought to minimize the adsorption of nonspecifically crosslink-labeled protein (see below), essentially without regard to the degree of receptor purification that might be achieved.

TABLE I. Adsorption of Crosslink-Labeled Insulin Receptor to Organomercurial Agarose*

Sample	Total volume (ml)	Total receptor (ΔCPM)	Total protein (mg)	Specific activity	Receptor purification (-fold)
TX-100	3	216,000	9.0	2.42×10^4	1.0
WGA	4	196,000	0.64	30.6×10^4	13
Affi-Gel 501	1	189,000	0.05	378×10^4	156

*Identical membrane aliquots were crosslink-labeled with ^{125}I-insulin (7 ng/ml) either in the presence or absence of an excess (50 μg/ml) of unlabeled insulin. Aliquots were solubilized (4 hr at 24°C) in buffer containing 1% Triton X-100 (TX-100) and the clarified (250,000 g for 90 min at 4°C) extracts of each aliquot (3 ml total volume) were first purified in parallel on columns (1 ml bed volume) of wheat germ agglutinin-agarose (WGA), equilibrated with TRIS-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.1% v/v Triton X-100. Fractions eluted with N-acetyl glucosamine (10% w/v) were pooled (total volume, 4 ml) and portions (3.5 ml) of the pooled samples from each membrane aliquot were applied in parallel to identical columns of Affi-Gel 501 (0.5 ml bed volume) equilibrated with 50 mM TRIS-HCl buffer containing 0.1% TX-100. After washing the Affi-Gel columns with 20 ml of buffer, the protein adsorbing to the column was eluted with 1 ml buffer containing 10 mM DTT. The total protein content [21] and radioactivity (CPM) were determined for all samples. For identical aliquots, obtained from membranes labeled in the absence or presence of unlabeled insulin and run in parallel on the columns, the difference in radioactivity (ΔCPM) reflected the amount of specifically crosslink-labeled receptor solubilized from the membranes; this value (ΔCPM) was used to estimate the specific activity, in terms of receptor content per milligram, for the initial Triton X-100 extract (TX-100) and for the samples recovered from the wheat germ agglutinin (WGA) and Affi-Gel columns. The calculated specific activities were used to estimate the degree (-fold) of receptor purification.

Nonetheless, it was of interest to evaluate the interaction with the organomercurial column of some of the other proteins in the soluble membrane preparation (Fig. 1) and to determine the approximate degree of purification (Table I), both in terms of total membrane protein and in terms of receptor (as estimated for the insulin receptor). The mercurial column was evaluated in conjunction with the use of WGA-agarose. The protein fractions obtained were subjected to electrophoretic analysis (Fig. 1). Both the fraction that was adsorbed to the WGA-agarose column (Fig. 1, lane C) (this fraction contains both the insulin and EGF receptors, along with other membrane glycoproteins) and the fractions that did not adsorb to the lectin column (Fig. 1, lane B) were applied to the Affi-Gel column. Proteins that either passed through the mercurial column (Fig. 1, lanes D–F) or that were adsorbed to the column and eluted with DTT (Fig. 1, lanes G to I) were analyzed by polyacrylamide gel electrophoresis under reducing conditions in the presence of sodium dodecyl sulfate (SDS-PAGE). A number of the soluble membrane glycoproteins, purified about 14-fold from the initial membrane extract by the lectin-adsorption step, were capable of reacting with the mercurial column so as to be recovered by DTT elution (eg, compare lanes C and I of Fig. 1). It was not possible to obtain an accurate estimate of the degree of purification of the various constituents eluted with DTT from the Affi-Gel 501 column because of the uncertainties concerning the identities of the numerous proteins recovered and the uncertainties related to the efficiencies with which the various proteins reacted with the organomercurial column. Nonetheless, as will be seen below, the procedure led to the recovery, upon elution with DTT, of both the insulin and EGF receptors from the organomercurial column. In terms of overall protein, the degree of purification provided by the mercurial column was about 180-fold (Table I) relative to the protein present in the initial membrane extract. These preliminary

experiments illustrated the general feasibility of isolating a variety of membrane proteins by this approach and demonstrated that the protein fraction eluted from the organomercurial column did not contain major amounts of protein constituents that migrated in electrophoretic gels in the region of the receptors for insulin (α subunit $\simeq$ 130 K) and EGF (180 K and 160 K).

Adsorption of Insulin and EGF Receptors to Organomercurial Agarose

Since the binding of EGF by the solubilized placenta receptor is difficult to quantitate, we first evaluated the interaction of the solubilized insulin receptor with the mercurial column. In a pilot experiment, virtually all (99%) of the insulin binding activity present in an aliquot (0.98 mg protein in 0.5 ml) of solubilized insulin receptor could be adsorbed to a column (0.25 ml bed volume) of Affi-Gel 501; approximately 1% of the binding activity (polyethylene glycol precipitation assay) was recovered in the protein fraction that did not adsorb to the column. Unfortunately, the conditions of elution with DTT (10 mm), which destroys the receptor's ability to bind insulin, did not permit an accurate estimate of the amount of insulin receptor that could be recovered from the column. To detect the presence of either the EGF or the insulin receptor after the chromatographic steps (using either WGA columns or Affi-Gel 501 columns), it was necessary to use crosslink-labeled receptor preparations. The electrophoretic profiles of the soluble receptors could be readily analyzed, as illustrated in the autoradiograms (lane A of Figs. 2 and 3); the electrophoretic profiles of the crosslink-labeled receptors were not altered by the treatment of membranes with NEM either before or after crosslink labeling with the radiolabeled peptides. Membranes were crosslink-labeled using DSS with either ^{125}I-labeled insulin or EGF, both in the presence and absence of a large excess (100- to 1000-fold) of the corresponding unlabeled peptide. As is the case with such crosslink-labeling experiments, an appreciable amount of radioactivity does become crosslinked to protein (so-called nonspecific labeling), even in the presence of a large excess of unlabeled ligand. Although this nonspecifically crosslinked radioactivity is not incorporated into the receptor (eg, see lanes A and B of Figs. 2 and 3), the ^{125}I-label appears to be incorporated "nonspecifically" into components that can adsorb along with the receptors to the organomercurial columns. When equal aliquots of specifically and nonspecifically labeled membrane protein were first purified using wheat germ agglutinin-agarose and were then applied to the mercurial columns, it was possible to obtain a reasonable degree of receptor purification, as documented for the insulin receptor in Table I. Because of the appreciable amount of nonspecific receptor labeling (especially for insulin), in the experiments with the organomercurial columns to be described below, it was of importance to evaluate critically the behavior of both the specifically labeled (ie, receptor) and "nonspecifically" labeled (ie, nonreceptor) constituents. The degree of nonspecific crosslinking was much greater for insulin (up to $\simeq$ 40%) than for EGF (up to 10%). To evaluate the binding to the mercurial columns of both specifically labeled and nonspecifically labeled material, equivalent amounts of radioactivity were routinely applied to the columns; thus, in terms of the absolute amounts of protein applied to the columns, much greater amounts were present in the samples of "nonspecifically" labeled material (ie, about 2.5-fold more for protein nonspecifically labeled with insulin and about 10-fold more protein nonspecifically labeled with EGF).

As illustrated in Figures 4A and 5A, elution of the Affi-Gel 501 column with DTT yielded radiolabeled protein from membranes that were crosslink-labeled either

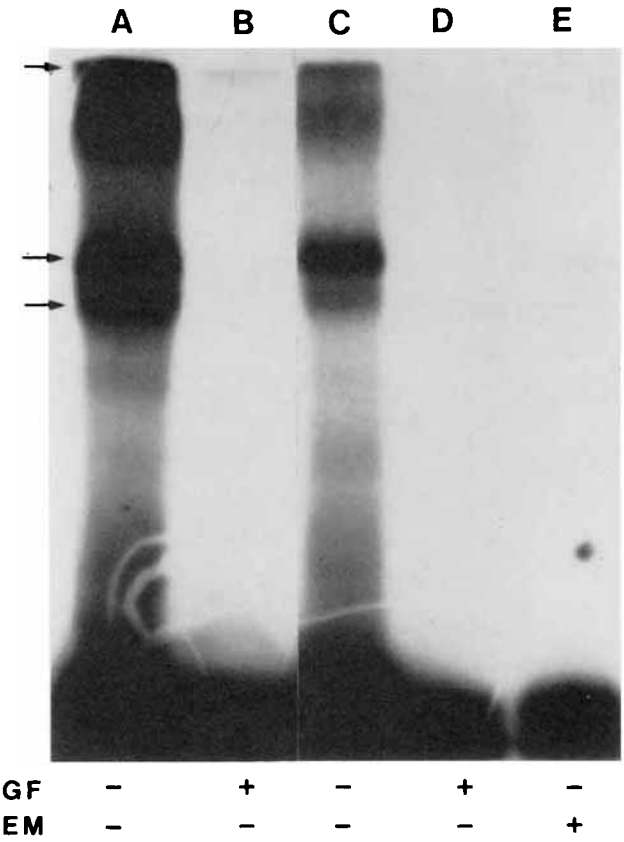

Fig. 2. Electrophoretic analysis and autoradiography of crosslink-labeled EGF receptor. Membranes, crosslink-labeled with ^{125}I-EGF either without (lanes A, C, E) or with (lanes B and D) excess unlabeled EGF were solubilized and analyzed by SDS-PAGE (7.5% gels) in the presence of mercaptoethanol either before (lanes A, B) or after (lanes C–E) elution with DTT from Affi-Gel 501. One preparation crosslink-labeled in the absence of unlabeled EGF was pretreated with NEM prior to chromatography (lane E). Radioactivity in the peak tubes eluted with DTT (Fig. 4, arrows) was analyzed. Aliquots of the DTT-eluted samples were mixed with an appropriate amount of electrophoresis sample buffer, heated (100°C, 10 min), and analyzed directly. Lanes A, B) solubilized membranes crosslink-labeled in the absence (lane A) or presence (lane B) of excess unlabeled EGF; lane C) material from lane A eluted from Affi-Gel 501 with DTT; lane D) material from lane B eluted with DTT; lane E) membranes labeled as in lane A, but pretreated with NEM prior to adsorption to Affi-Gel and elution with DTT. Prior to chromatography, NEM-treated membranes crosslinked in the absence of unlabeled EGF appeared as in lane (A). The arrows indicate the specifically labeled receptor. Equal amounts of radioactivity were analyzed for the DTT-eluted samples ($\simeq$5,000 cpm). The arrows in the middle of the gel indicate the receptor components of molecular weights 180 (upper) and 160 (lower arrow) kilodaltons; the top arrow denotes the start of the separating gel.

in the absence (closed circles) or presence (open circles) of unlabeled peptide. In the second peak in Figures 4A and 5A, the difference in radioactivity (ie, closed circle value minus the corresponding value for open circles) reflects the specifically labeled receptor that was bound to the column. The difference plot (squares, Fig. 4A and 5A) provides an indication of the elution profile for the majority of the specifically labeled protein (ie, receptor) that was bound to the mercurial column and eluted with DTT. The EGF receptor was eluted by DTT as a sharp peak, as would be expected according to its single-chain structure, whereas the insulin receptor was eluted by

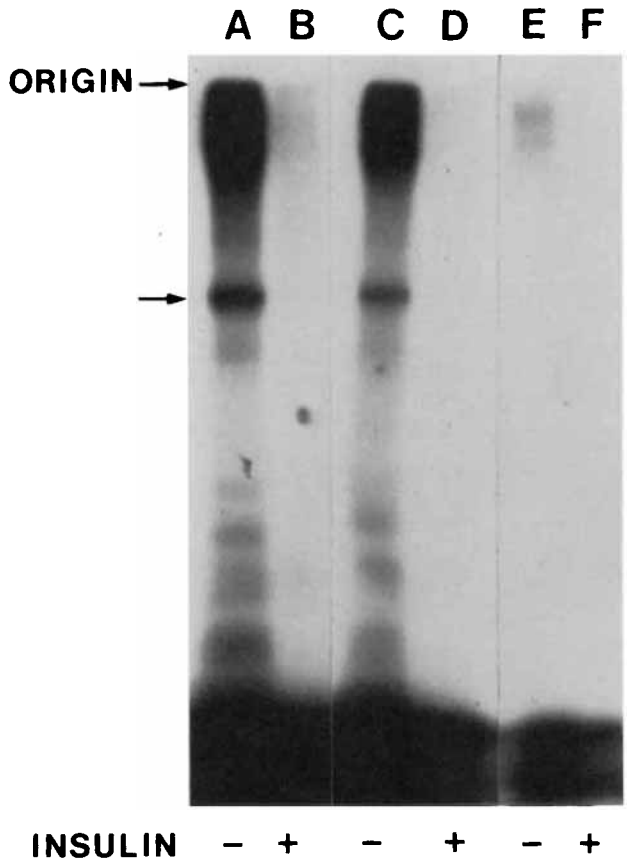

Fig. 3. Electrophoretic analysis and autoradiography of crosslink-labeled insulin receptor. Membranes crosslink labeled with ^{125}I-insulin either without (lanes A, C, E) or with (lanes B, D, F) excess unlabeled insulin were solubilized in Triton X-100 either before (lanes E, F) or after (lanes E, F) treatment with 5mM NEM. Solubilized material was applied to the Affi-Gel 501 column, and radioactive material eluted with DTT (Fig. 5, arrows) was analyzed by SDS-PAGE in the presence of mercaptoethanol. Samples for electrophoresis were mixed with appropriate amounts of electrophoresis sample buffer, heated (10 min, 100°C), and analyzed directly. Lanes A, B) solubilized membranes crosslink-labeled in the absence (lane A) or presence (lane B) of excess unlabeled insulin; lane C) material from lane A eluted from Affi-Gel 501 with DTT; lane D) material from lane B eluted with DTT from Affi-Gel; lane E) membranes as in lane A but pretreated with NEM prior to adsorption to Affi-Gel and elution with DTT; lane F) material from B pretreated with NEM prior to adsorption to and elution from Affi-Gel 501 with DTT. The arrow denotes the insulin receptor subunit of about 130 kilodaltons, described previously [19]. For the DTT-eluted samples, equal amounts of radioactivity ($\simeq$5,000 cpm) were applied to the gels.

DTT as a broader peak of radioactivity, as might be expected on the basis of its multichain disulfide-linked structure; some radioactivity from the insulin receptor would also be expected to come from free A or B chains that might be released upon treatment with DTT. The adsorption to the mercurial column of the constitutents nonspecifically labeled with insulin could be minimized either by reducing the concentration of protein applied to the mercurial column (optimal concentration $\simeq$ 0.2 mg/ml); or by using crosslink-labeled material that was first purified using WGA-

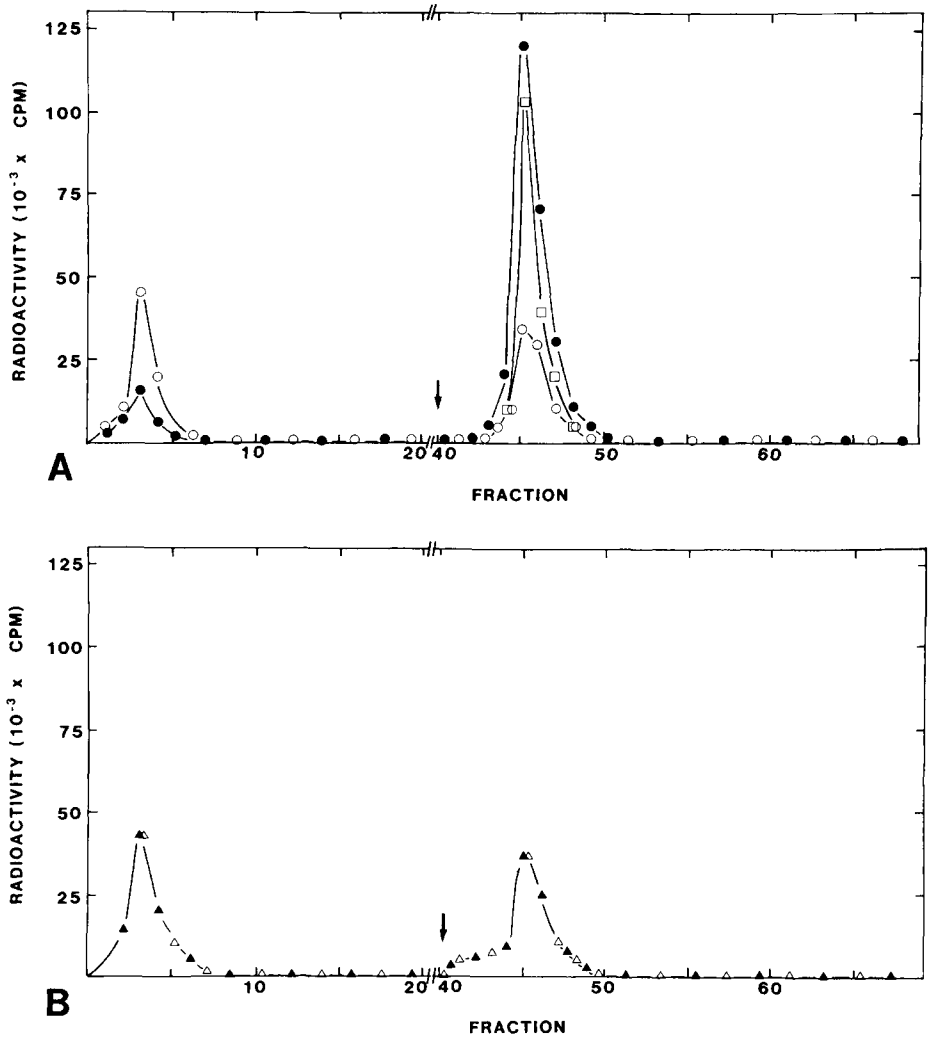

Fig. 4. Adsorption of crosslink-labeled EGF receptor to Affi-Gel 501 and effect of NEM. A) Membranes were crosslink-labeled with ^{125}I-EGF either in the absence (closed circles) or presence (open circles) of an excess of unlabeled EGF. Membranes from both crosslinking reactions were solubilized, equal amounts of radioactivity from both preparations were applied to the organomercurial column, and fractions (0.25 ml) were collected until radioactivity stopped eluting from the column. At this point (arrow), elution with 10 mM DTT was begun. Difference curve (open squares) indicates the elution of specifically labeled receptor. B) After crosslink-labeling in the absence (closed triangles) or presence (open triangles) of excess unlabeled EGF, membranes were treated with 5 mM NEM prior to solubilization and chromatography as in (A). NEM-treated membranes were crosslink-labeled with ^{125}I-EGF either without (closed triangles) or with (open triangles) excess unlabeled EGF. The arrow denotes elution with 10 mM DTT.

agarose. Since for the nonspecifically labeled samples much greater amounts of protein (but equal amounts of radioactivity) were applied to the column, the difference plots shown in Figures 4A and 5A underestimate the degree of purification yielded by the mercurial columns.

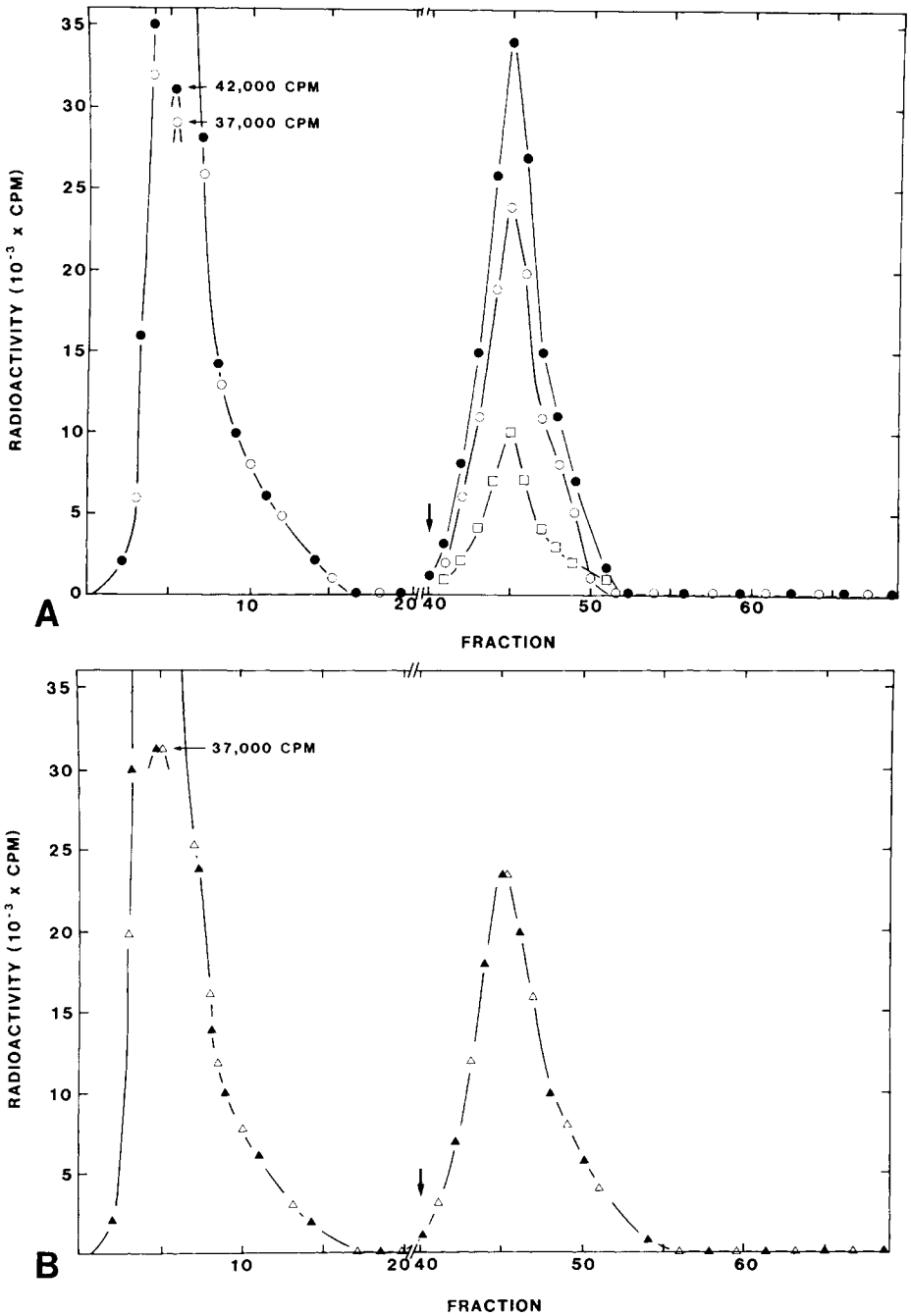

Fig. 5. Adsorption of crosslink-labeled insulin receptor to Affi-Gel 501 and effect of NEM. A) Membranes were crosslink-labeled with ^{125}I-insulin either in the absence (closed circles) or presence (open circles) of excess unlabeled insulin. Membranes were solubilized, partially purified using WGA-agarose, and then chromatographed on the Affi-Gel column with DTT elution (arrow) as outlined in Figure 1. Difference curve (open squares) indicates the elution of specifically labeled receptor. B) After crosslink labeling with ^{125}I-insulin in the absence or presence of excess unlabeled insulin, membranes were treated with 5 mM NEM prior to solubilization and chromatography as in (A). NEM-treated membranes were crosslink-labeled with ^{125}I-insulin either without (closed triangles) or with (open triangles) excess unlabeled insulin. The arrow denotes elution with DTT.

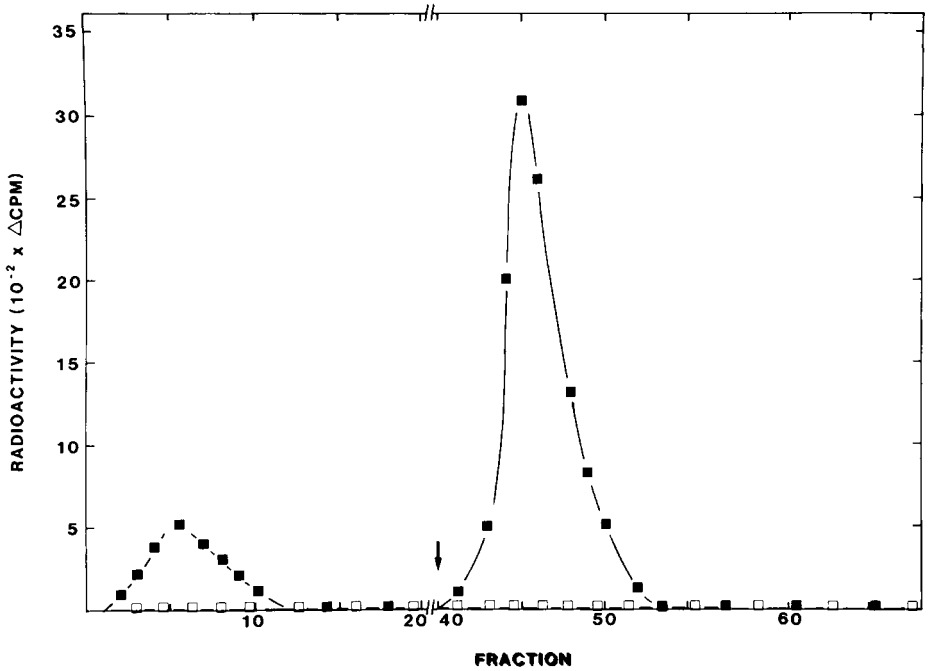

Fig. 6. Insulin-promoted adsorption of the insulin receptor to Affi-Gel 501. As outlined in Materials and Methods, membranes were treated with NEM (50 mM) prior to crosslink labeling with ^{125}I-insulin, in the presence or absence of excess unlabeled insulin. After crosslink labeling, one set of membrane aliquots were again treated with 25 mM NEM. All aliquots were then solubilized and applied to the Affi-Gel 501 column as outlined in Figures 4 and 5. A difference plot is shown, reflecting the elution with DTT of receptor-associated radioactivity (ie, total crosslinked radioactivity minus radioactivity cross-linked in the presence of an excess of unlabeled insulin). NEM treatment after crosslink labeling markedly reduced the adsorption of specifically labeled receptor eluted with DTT from the column (fractions 41 to 52). Closed squares, material treated with NEM only before crosslink-labeling; open squares, material treated with NEM both before and after crosslink-labeling.

The identity of the radioactively labeled components eluted by DTT from the Affi-Gel 501 column was confirmed by SDS-PAGE and autoradiography (Figs. 2 and 3). Clearly, the specifically crosslink-labeled material that was eluted with DTT from the column contained radiolabeled components of the receptors for either EGF (Fig. 2) or insulin (Fig. 3). Because of the complexity of the mixture of radiolabeled constituents present in the unadsorbed fraction, we did not analyze this fraction further; rather, we focused our attention solely on those components bound to the Affi-Gel column. The majority of the nonspecifically labeled material eluted from the column with DTT migrated in the electrophoretic gels either as low-molecular weight proteins or as high-molecular weight substituents that barely entered the gel (Figs. 2 and 3).

If membranes were treated with NEM after crosslink-labeling but before solubilization with Triton X-100, neither the EGF nor the insulin receptor was adsorbed to the mercurial column. This result for NEM-treated receptor preparations was confirmed both by the elution with DTT of the same amount of radioactivity from preparations crosslink-labeled in either the presence or absence of an excess of unlabeled ligand (Figs. 4B and 5B) and by the absence in the autoradiograms of

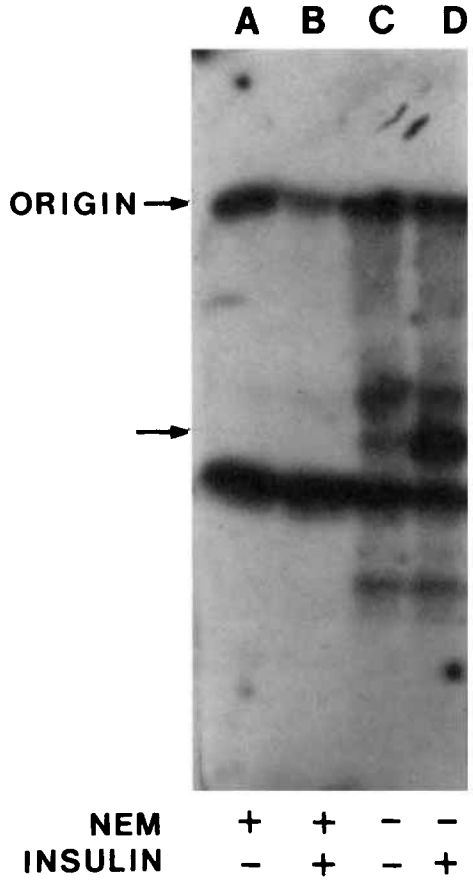

Fig. 7. Effects of NEM on insulin-stimulated kinase. Placental membranes (2 mg/ml) were solubilized with 2% v/v Triton X-100 and a partially purified glycoprotein fraction was obtained by WGA-agarose affinity chromatograhpy. The ability of insulin to enhance phosphorylation of the soluble protein from the lectin column was assayed in the presence and absence of 10 mM NEM. Protein (60 μg was preincubated for 20 min at room temperature with or without insulin 4 μg in 80 μl final volume) in the presence or absence of 10 mM NEM. The reaction buffer (80 μl final volume) contained 20 mM TRIS-HCl, pH 7.4, 10 mM $MgCl_2$, and 0.1% v/v Triton X-100. After the preincubation for 120 min, γ-ATP^{32} (15 μCi; final concentration 20 μM) was added and the incubation was continued for an additional 10 min at 24°C. The reaction was terminated by the addition of 20 μl of fivefold concentrated electrophoresis sample buffer, followed by boiling for 2 min. Samples were anlayzed by SDS-PAGE in 7.5% gels in the presence of mercaptoethanol and radiolabeled bands were visualized by autoradiography after 48-hr film exposure. The arrow indicates the 90-kilodalton labeled protein thought to represent the insulin receptor β subunit. Samples were incubated with NEM in the absence (lane A) or presence (lane B) of insulin or samples were incubated in the absence of NEM without (lane C) or with (lane D) insulin.

labeled receptor bands in the material eluted from the columns with DTT (Figs. 2 and 3, lane E).

Insulin-Mediated Interaction of the Insulin Receptor With the Organomercurial Column

Since our previous work [8] demonstrated that the binding of insulin to its receptor resulted in the exposure of an NEM-reactive substituent, presumed to be a receptor sulfhydryl, we were interested to determine if insulin could promote the

interaction of the receptor with the organomercurial column. Thus, reactive membrane sulfhydryls were first masked with NEM prior to the crosslink-labeling reaction with ^{125}I-labeled insulin. This pretreatment with NEM did not interfere with the crosslinking reaction for either insulin or EGF. The electrophoretic analysis of the crosslink-labeled receptors for insulin and EGF was the same (lane A, Figs. 2 and 3) for NEM-treated membranes as for untreated membranes. After the addition of insulin, the ability of crosslink-labeled receptor to adsorb to the organomercurial column was again evaluated either before or after a repeat treatment with NEM.

In the experiments depicted in Figure 6, pairs of aliquots containing equal amounts of radioactivity were obtained from membranes crosslink-labeled with ^{125}I-insulin either in the absence or presence (ie, nonspecific crosslink-labeling) of an excess of unlabeled insulin. Both pairs of aliquots were applied to the Affi-Gel 501 column. The aliquots derived from membranes treated with NEM only prior to the crosslinking reaction were analyzed first. For each aliquot, some radioactivity passed through the column; the difference plot (ie, radioactivity from the aliquot crosslink-labeled in the absence of insulin minus the radioactivity from the nonspecifically labeled sample) indicated that in this experiment some specifically labeled receptor failed to bind to the column. Nonetheless, elution with DTT yielded more radioactivity from the membrane aliquot labeled with ^{125}I-insulin alone, compared with the nonspecifically labeled sample. The difference plot (solid symbols, second peak in Fig. 6) reflected the elution of specifically labeled receptor. The second set of aliquots, containing equal amounts of radioactivity, was obtained from membranes treated with NEM both before and after the crosslinking reaction. When this second pair of aliquots was applied to the Affi-Gel 501 column, a different result was obtained. The amount of radioactivity passing through the column was the same for each aliquot (ie, the nonspecifically labeled protein as well as the companion aliquot). Thus, the difference plot for the unretarded fractions was near zero (open symbols, Fig. 6). When the column was subsequently eluted with DTT (arrow, Fig. 6), the same amount of radioactivity was released for both the nonspecifically labeled aliquot and its counterpart. Thus, the difference plot, reflecting the presence of specifically labeled receptor, was close to zero. The difference plot (open and closed squares, Fig. 6) indicated that the second treatment with NEM had markedly reduced the amount of specifically labeled receptor that could be adsorbed to the column and eluted in the second peak with DTT.

Evaluation of Other Sulfhydryl Affinity Columns

The ability of both crosslink-labeled receptors to react with either Affi-Gel 401 (containing a terminal sulfhydryl) or cysteamine-agarose was also evaluated (data not shown). The crosslink-labeled EGF receptor readily adsorbed to Affi-Gel 401; however, the crosslink-labeled insulin receptor failed to react with this support. Nonetheless, insulin receptor purified by insulin-agarose affinity chromatography and subsequently radiolabeled with ^{125}I was bound by the Affi-Gel 401 column. Neither receptor appeared capable of binding to the cysteamine-agarose derivative. Thus, the mercurial column used for the experiments described above appeared to be best suited for experiments dealing simultaneously with both the EGF and insulin receptors.

Effect of NEM on Insulin-Stimulated Receptor Kinase

In view of our interest in receptor sulfhydryl groups and because of apparently conflicting data relating to the effects of sulfhydryl reagents on the kinase activity of

the insulin receptor [24–26], we evaluated the effect of NEM on the insulin-stimulated kinase activity in soluble receptor partially purified from placental membranes using WGA-agarose (Fig. 7). Clearly, although insulin caused a stimulation of phosphorylation in the receptor preparation (Fig. 7, lane D), the presence of NEM abolished the ability of insulin to stimulate the phosphorylation of the 90-kilodalton receptor subunit (Fig. 7, lane B). The instrinsic kinase activity of the membrane glycoprotein fraction, observed in the absence of insulin, was also abolished by NEM (compare lanes A and C, Fig. 7).

DISCUSSION

The main finding of this study is that the receptors for both insulin and EGF possess reactive groups capable of interacting with an organomercurial column. The reaction of both receptors with the mercurial gel was blocked by pretreatment with NEM and was reversed by the addition of DTT. Thus, the data point to the presence of accessible sulfhydryl groups in both receptors. Although the primary goal of our work was focused on the detection of putative receptor sulfhydryls rather than on protein purification, the methods we have explored should prove of use for the isolation of the sulfhydryl-containing receptor domains. Our use of the Affi-Gel 501 column compares favorably with the use of this column for the purification of human red cell membrane glycoproteins [27]. Our previous work [8], in keeping with the observations of others [10–13], has indicated that disulfide-sulfhydryl (SH-SS) exchange reactions may be of importance in terms of the maintenance of insulin receptor structure. Our new data, indicating the presence of sulfhydryl groups in the EGF receptor as well, raise the possibility that such SH-SS exchange reactions may also play a structural role in the regulation of this receptor's properties, even though the EGF receptor appears to be a single-chain species, in contrast with the oligomeric insulin receptor.

The ability of insulin to promote the attachment of its receptor to the organomercurial gel provides independent support for our previous data showing that insulin-binding leads to the exposure of an NEM-reactive group, presumed to be a receptor sulfhydryl [8]. Our observations raise the possibility that cryptic sulfhydryl residues may also be exposed in the course of ligand binding in other receptor systems. In future work, we hope to explore this possibility in depth with the EGF receptor. Experiments demonstrating the presence of a sulfhydryl in the cytoplasmic domain of the α subunit of the T-cell receptor [28] and assessing the influence of sulfhydryl reagents on the muscarinic acetylcholine receptor [29,30], point to a functional role for sulfhydryls in a variety of receptors apart from the two we have examined in the work we describe here.

The ability of NEM to inhibit the kinase activity of the partially purified insulin receptor (Fig. 7) is in agreement with reports that appeared in the course of our studies, indicating that the kinase activity of partially purified insulin and EGF receptor preparations can be inhibited by NEM [25,26]. The inhibition of the EGF kinase activity can also be observed as a consequence of pretreating either placenta membranes [Maturo, Valentine, and Hollenberg, unpublished observations] or A431 cell membranes [31] with NEM. Thus, receptor sulfhydryl groups may be critical not only in terms of regulating receptor structure, but also in terms of controlling the kinase activity of the receptors for both insulin and EGF. Our present study, demon-

strating the attachment of both receptors to an organomercurial gel, presumably via sulfhydryl residues, provides an avenue to identify the receptor domains that participate in the putative SH-SS exchange mechanism that may be involved in the control of receptor function.

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