Antibodies against Highly Conserved Sites in the Epidermal Growth Factor Receptor Tyrosine Kinase Domain as Probes for Structure and Function†

Nancy A. Brown, Larry A. Compton, and Gail M. Clinton*

Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201-3098

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ABSTRACT: We generated anti-peptide antibodies against four highly conserved sequences in the kinase domain and against two nonconserved sequences surrounding autophosphorylation sites in the carboxyl-terminal domain of the epidermal growth factor receptor (EGFR). These antibodies were used to examine topology and function in catalysis of specific sequences. Two of the highly conserved sites, HRD (residues 811–818) and DFG (residues 827–838), appeared to participate in catalysis since αHRD and αDFG but not the other anti-peptide antibodies inhibited EGFR kinase activity. Examination of the topology of the six sites revealed that epitopes in all except the HRD site appeared to be exposed to antibody binding in the EGFR. The conditions that caused increased exposure of the HRD site to interaction with antibody included autophosphorylation, addition of the ionic detergent sodium dodecyl sulfate (SDS), and elevation in temperature from 4 to 34 °C.

Protein kinases are a large group of regulatory enzymes that play pivotal roles in signal transduction (Cohen, 1986; Hanks & Quinn, 1991). These enzymes are usually inactive until stimulated by their effectors. One mechanism by which protein kinases may be maintained in an inactive state is by blockage of the active site by regulatory regions of the protein which may mimic the substrate (Corbin et al., 1978; Pearson et al., 1988; Hardie, 1988).

The epidermal growth factor receptor (EGFR) is one of the best characterized receptor tyrosine kinases. The receptor transcends the plasma membrane once; the ligand binding domain is outside the cell while the catalytic domain and the C-terminal autophosphorylation domain are cytoplasmic (Ullrich et al., 1984; Gill et al., 1987; Hsuan et al., 1989). The initial events that occur in the process of activation of the EGFR kinase in the intact cell include ligand binding to the extracellular domain followed by autophosphorylation of the C-terminal domain (Gill et al., 1987; Yarden & Ullrich, 1988). Receptor dimerization, induced by ligand, is thought to provide an allosteric regulatory signal that results in intermolecular autophosphorylation and kinase activation (Yarden & Schlessinger, 1987; Yarden, 1988; Ullrich & Schlessinger, 1990). However, other mechanisms that do not involve receptor dimerization also appear to activate the kinase in a ligand-independent fashion (Koland & Cerione, 1988; Northwood & Davis, 1988). Although the processes that lead to receptor activation have been described, the mechanism by which the receptor is maintained in an inactive site and the role of the different domains in this process have not been determined.

If EGFR is maintained in the inactive state because access of protein substrates to the catalytic site is blocked, then sequences that function in catalysis may be inaccessible to interaction with specific antibodies. With this model in mind, we have generated anti-peptide antibodies against highly conserved sites in the catalytic domain to probe the availability of catalytic sequences. These site-directed antibodies were tested for neutralization of kinase activity and for binding to EGFR under conditions that affect EGFR conformation. While anti-peptide antibodies have previously been found to inhibit kinase activity [see Clinton and Brown (1991)] and have been used to show autophosphorylation-dependent exposure of sites in the insulin (Perlman et al., 1989) and PDGF (Bishayee et al., 1988; Keating et al., 1988) receptors, they have not yet been used as probes to measure the exposure of sequences that function in catalysis.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Immunization of Rabbits. Peptides were synthesized by the solid-phase method (Dryland & Sheppard, 1986; Eberle, 1986) using PepSyn KA resin and Fmoc amino acid esters (MilliGen). After cleavage from the resin, the peptides were analyzed by HPLC and by amino acid analysis of trypsin cleavage products. Peptides were conjugated to hemocyanin, and the conjugates were used to immunize rabbits as described (Clinton & Brown, 1991).

Cell Culture, Metabolic Labeling, and Membrane Vesicle Preparation. A431 cells (ATCC CRL 1555) were cultured in modified Eagle's medium containing 5% fetal bovine serum (GIBCO) and 0.5% gentamicin (GIBCO). For metabolic labeling, cultures were washed and then incubated in methionine- and cysteine-free MEM for 90 min, and then 100 μCi/mL [^{35}S]Met/[^{35}S]Cys (New England Nuclear) was added for an additional 4 h. A431 membrane vesicles were prepared by incubation of the cells in roller bottles with a hypotonic vesiculation buffer described by Cohen et al. (1982) and modified by Lin and Clinton (1988). This preparation was stored at −70 °C in 10 mM HEPES, pH 7.4, at 2–4 mg/mL protein. Protein concentrations were determined spectrophotometrically using the Bio-Rad protein assay (Bio-Rad Laboratories).

In Vitro Autophosphorylation and Angiotensin Phosphorylation. For autophosphorylation of EGFR, 2.5 μg of A431 membrane vesicle protein was solubilized in 25 μL of 20 mM HEPES, pH 7.6, 1% nonidet-P40 (NP-40), 10% glycerol, 1% aprotinin, and 1 mM phenylmethanesulfonyl fluoride (PMSF) (solution A). (All chemicals were from Sigma unless

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* To whom correspondence should be addressed.

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specified.) The solubilized membrane vesicles were then incubated for 10 min at room tempeature with 1.0 μM EGF (Collaborative Research) and then for 10 min on ice in a 100-μL kinase reaction mixture [20 mM HEPES, pH 7.6, 2 mM dithiothreitol, 0.5% nonidet-P40 (NP-40), 25 μM Na_3VO_4, and 10 mM $MnCl_2$] containing 10 μM ATP and 2.5 μCi of (γ-^{32}P)ATP [6000 Ci/mmol; New England Nuclear (NEN)]. For autophosphorylation of EGFR following immunoprecipitation, the immune complex was washed twice in the immunoprecipitation buffer and then incubated for 10 min on ice in 10 μL of a kinase reaction mixture containing 10 μM ATP and 5.0 μCi of (γ-^{32}P)ATP. The amount of radiolabeled ATP was higher in the immune complex kinase reaction to compensate for the kinase activity that was lost during immunoprecipitation. This adjustment resulted in nearly equal amounts of ^{32}P incorporated into EGFR which was autophosphorylated first and then immunoprecipitated or autophosphorylated in the immune complex when standard antibody against the ligand binding domain (Lin & Clinton, 1988) was used.

For phosphorylation of EGFR in A431 cell extracts, cells were solubilized in solution A and centrifuged to remove nuclei and insoluble material; 100 μg of the cell extract protein was treated with 1.0 μM EGF at room temperature for 10 min and was then incubated on ice for 10 min in a kinase reaction mixture which contained 100 μM unlabeled ATP.

For phosphorylation of the exogenous substrate angiotensin, 2 mg/mL angiotensin II (Sigma) was added to 100 μL of the kinase reaction mixture described above for the immune complex kinase reaction. Twenty microliters of the kinase reaction mixture was removed after incubation at 34 °C for 5 min, and phosphorylated angiotensin was resolved by high-voltage paper electrophoresis at pH 4.4 as previously described (Lin & Clinton, 1986). The phosphorylated angiotensin was localized by autoradiography and quantified by scintillation counting of excised spots.

Immunoprecipitation. A431 membrane vesicles (2.5 μg of protein), or 200 μg of [^{35}S]Met/[^{35}S]Cys-labeled cell extract protein, were solubilized in solution A. [Greater than 95% of EGFR solubilized in this buffer was accessible to immunoprecipitation by a previously characterized antiserum against the N-terminal ligand binding domain (Lin & Clinton, 1988) or by antiserum against the C-terminal cytoplasmic domain, αP3.] The solubilized material was incubated with antiserum for 1 h with shaking at 4 °C. Immunoprecipitation was conducted in one of three solutions: (A) 20 mM HEPES, pH 7.6, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 1% aprotinin, and 1 mM PMSF; (B) solution A plus 0.05% sodium dodecyl sulfate (SDS); (C) solution A plus 150 mM NaCl, 1% sodium deoxycholate, and 0.1% SDS.

The antibody–EGFR complex was adsorbed at a ratio of 1 part antisera to 3 parts of a 50% suspension of protein A–Sepharose (Sigma) for 45 min at 4 °C on a rotating platform. The immune complex was washed 4 times in solution C, and the proteins were eluted by incubation in a boiling water bath for 2 min in sample buffer containing 0.125 M Tris, pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.1% bromophenol blue. The eluted proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using 7.5% acrylamide. ^{32}P-Labeled proteins were identified by autoradiography of the dried gel using Kodak X-Omat film. For autoradiography of ^{35}S-labeled proteins, gels were soaked in water for 30 min and then in Fluoro-Hance (RPI Corp., Mt. Prospect, IL) for 30 min.

Antibody Characterization. Production of anti-peptide antibodies in the immunized rabbits was initially monitored by Western blotting A431 membrane vesicles (3 μg of protein per lane) resolved by SDS–PAGE. The resolved proteins were electroblotted to nitrocellulose filters as previously described (Lin et al., 1991). The filters, blocked with 5% nonfat evaporated milk, were incubated with immune serum diluted 1:5 with saline or with immune serum plus 100 μg/mL immunizing peptide, or with preimmune serum diluted 1:5. The second antibody employed was Cappel goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad). The immunoblots were developed with diaminobenzidine as previously described (Lin et al., 1990). Sequence-specific reactivity with the EGFR was assessed by the presence of a 170-kD protein that reacted with antisera but not with peptide-blocked antisera or with preimmune sera. The antisera were further characterized by immunoprecipitation of A431 membrane vesicles that were autophosphorylated with [γ-^{32}P]ATP and then suspended into denaturing buffer C. Although the titer varied, each of the six immune sera but not the preimmune sera immunoprecipitated a ^{32}P-labeled 170-kDa protein which comigrated during SDS–PAGE with the ^{32}P-labeled EGFR immunoprecipitated by a standard antibody against the ligand binding domain (Lin & Clinton, 1988). Immunoprecipitation of ^{32}P-EGFR by each of the anti-peptide antibodies was tested for specificity by preincubating the antibody with the synthetic peptide at 15–100 μg/mL peptide at 4 °C for 1 h with shaking.

Anti-peptide antisera against each of the conserved sequences including αHRD, -DFG, -ALE, and -SDV had different properties than antisera against the nonconserved sequences αP3 and αP1 (see Table I for the entire sequence of each peptide). Antisera against the conserved sequences were transiently produced in the immunized rabbits. The production of the antibody that reacted with conserved sequences in EGFR did not correspond to the production of antisera that reacted with the immunizing peptide. Moreover, the antisera against conserved sequences were unstable. For additional discussion of generation and properties of anti-peptide antisera against conserved sequences from the catalytic domain of EGFR, see Clinton and Brown (1991).

Peptide Affinity Chromatography. To purify αHRD IgG, 15 mL of immune serum was precipitated with 40% ammonium sulfate and dialyzed against water. The IgG fraction was then incubated at 4 °C for 1 h with 5-mL bed volume of Affigel 15 (Bio-Rad) cross-linked with the HRD peptide. The resin was washed with 50 mL of 0.1 M sodium acetate, pH 4.0, containing 0.5 M NaCl. Specific antibody was then eluted with the same buffer at pH 2.0; 0.5-mL fractions of the pH 2.0 buffer were collected, neutralized, and monitored by the absorbance at 280 nm and by the concentration of IgG determined by SDS–PAGE and densitometric scanning of Coomassie Blue-stained gel using purified rabbit IgG as the standard.

RESULTS

Sequences from EGFR Used for Production of Synthetic Peptides and Generation of Anti-Peptide Antibodies. To develop anti-peptide antibodies that may react with the active site of the EGFR, we synthesized peptides corresponding to four evolutionarily conserved sequences from the catalytic domain and named them HRD, DFG, ALE, and SDV (Table I). The amino acid residues that are underlined in Table I are almost invariant (greater than 95% conservation) in the catalytic domains of 42 tyrosine kinases (Hanks et al., 1988; Hanks & Quinn, 1991). For comparisons, we synthesized

Table I: Amino Acid Sequence of Synthetic Peptides[a]

	peptide sequence	position in human EGFR
HRD	HRDLAARN	811–818
DFG	VKITDFGLA	827–835
ALE	VPIKWMALES	852–861
SDV	SDVWSYGV	871–878
P3	DDTFLPVPEYINQS	1059–1072
P1	TAENAEYLRVAPQS	1167–1180

[a] The peptide sequences were taken from the human EGFR (Ullrich et al., 1984). Amino acid residues are numbered starting from the amino terminus of the protein. The sequences selected from the catalytic domain were chosen on the basis of the degree of conservation among tyrosine protein kinases (Hanks et al., 1988). The underlined residues are those that are almost invariant in the aligned catalytic domains of 42 tyrosine kinases (Hanks & Quinn, 1991).

two additional peptides with sequences that include the P1 and P3 autophosphorylation sites in the C-terminal autophosphorylation domain of the EGFR (Table I). The C-terminal domain is not required for catalysis (Ullrich et al., 1984; Gill et al., 1987; Hsuan et al., 1989) and is evolutionarily divergent in its amino acid sequence. Production of anti-peptide antibodies and sequence-specific reactivity with EGFR were monitored as described under Experimental Procedures and as previously described (Clinton & Brown, 1991).

Phosphorylation in Immune Complexes Containing Anti-Peptide Antibodies. We initially investigated whether the anti-peptide antibodies interacted with sequences that are critical for catalytic activity by testing whether autophosphorylation of EGFR occurred in the immune complex. A431 membrane vesicles, complexed with EGF, either were autophosphorylated with [γ-^{32}P]ATP as a control autophosphorylation reaction and then immunoprecipitated or were immunoprecipitated first and then autophosphorylated in the immune complex. Antibodies against two conserved sites in the catalytic domain (αALE and αSDV) as well as against the autophosphorylation sites (αP1 and αP3) did not significantly affect autophosphorylation since nearly equivalent amounts of ^{32}P-labeled EGFR were recovered whether the samples were autophosphorylated before or after immunoprecipitation (Figure 1). In contrast, there was significantly less ^{32}P-labeled EGFR recovered from the immune complex kinase reactions with the αHRD and αDFG antibodies than from their controls that were autophosphorylated prior to immunoprecipitation (Figure 1). Similar results were obtained with antisera from multiple rabbits immunized with the HRD and DFG peptides (N.A. Brown and G. M. Clinton, unpublished observations).

Reduced amounts of ^{32}P-labeled EGFR from the immune complex autophosphorylation reaction could be explained if αHRD and αDGF inhibited autophosphorylation, or if unphosphorylated EGFR was not immunoprecipitated by these antibodies. One approach we used to examine whether these two anti-peptide antibodies inhibit kinase activity was to autophosphorylate EGFR first and then immunoprecipitate with the standard antibody against the ligand binding domain as well as with αDFG and αHRD since autophosphorylated EGFR reacts with these anti-peptide antibodies (Figure 1). The immunoprecipitated EGFR was then dephosphorylated with purified prostatic acid phosphatase (Lin & Clinton, 1986), and the phosphatase was washed from the immune complexes which were then incubated in a kinase reaction. While autophosphorylation of EGFR occurred in the phosphatase-treated immune complex containing the control antibody against EGFR, there was no detectable incorporation of ^{32}P

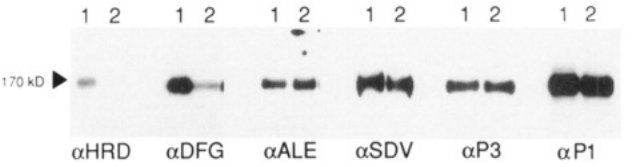

FIGURE 1: Phosphorylation in immune complexes containing anti-peptide antibodies. Solubilized A431 membrane vesicles were suspended in buffer A, treated with EGF, and (lane 1) autophosphorylated by incubation in a kinase reaction mixture containing [γ-^{32}P]ATP prior to immunoprecipitation or (lane 2) first immunoprecipitated and then autophosphorylated in an immune complex kinase reaction mixture containing [γ-^{32}P]ATP (see Experimental Procedures). Five microliters of αP1 and αP3 and 25 μL of αHRD, αDFG, αALE, and αSDV were used for immunoprecipitations. The ^{32}P-labeled proteins were resolved by SDS–PAGE and detected by autoradiography of dried gels.

into EGFR which was complexed with αDFG or with αHRD (data not shown). Thus, αDFG and αHRD, while bound to their cognate sequences in EGFR, neutralized autophosphorylation activity.

Conditions That Affect Immunoprecipitation of EGFR by Anti-Peptide Antibodies. To assess effects of phosphorylation on binding of anti-peptide antibodies, A431 cells were labeled with ^{35}S (Met/Cys), and cell lysates were incubated in a kinase reaction to phosphorylate the ^{35}S-labeled EGFR. To ensure that EGFR was optimally autophosphorylated in the cell extracts, we conducted pilot experiments using a well-characterized standard antibody against the ligand binding domain to immunoprecipitate [γ-^{32}P]ATP-labeled EGFR. Optimal levels of autophosphorylation were achieved in A431 cell extracts by increasing the ATP concentrations to 100 μM in the kinase reaction mixture (data not shown). There appeared to be slightly more EGFR immunoprecipitated with αP1 in samples treated with EGF and ATP, while with αP3, there was a small increase in the amount of EGFR immunoprecipitated from samples treated with EGF alone compared to those treated with ATP (Figures 2 and 3). Regardless of whether the cell extract was incubated in a kinase reaction with or without ATP or EGF, similar amounts of ^{35}S-labeled EGFR and its incompletely glycosylated precursor were immunoprecipitated by αDFG. From these results, we concluded that ligand-activated phosphorylation of EGFR did not affect binding of antibodies to the DGF site. Thus, the decreased amounts of ^{32}P-labeled EGFR observed in Figure 1 for the immune complex phosphorylation reaction containing αDFG must have been caused by inhibition of autophosphorylation.

In contrast, when EGF-treated ^{35}S-labeled cell extracts were incubated with and without ATP, more ^{35}S-labeled EGFR was immunoprecipitated with αHRD from the extract that was incubated with ATP. To ensure that equal amounts of cell extract were immunoprecipitated in each case, we used identical aliquots of ^{35}S-labeled cell extract (200 μg of protein), and the gels were stained to verify that equivalent amounts of immune IgG were recovered from the immunoprecipitations. Differential immunoprecipitation of phosphorylated EGFR was also observed with αHRD that was generated in a different rabbit. In comparison, the antisera against the C-terminus (αP3) immunoprecipitated slightly more EGFR when ATP was not included (Figures 2 and 3). This result suggested that αHRD bound poorly to unphosphorylated EGFR. Thus, the decreased amount of ^{32}P-labeled EGFR from the immune complex kinase reaction containing αHRD (Figure 1) is likely the result of inefficient immunoprecipitation of the unphosphorylated EGFR.

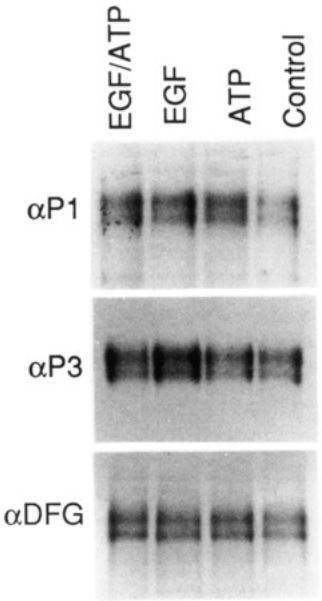

FIGURE 2: Effects of EGF and ATP on immunoprecipitation of EGFR by anti-peptide antibodies. Aliquots (200 μg of protein) of ^{35}S-labeled A431 cell extracts were incubated 5 min at room temperature with EGF and then were incubated 10 min on ice in a kinase reaction mixture containing 100 μM ATP (EGF/ATP), or were incubated with EGF and then in a kinase reaction without ATP (EGF), or were incubated without EGF and then in a kinase reaction mixture with ATP (ATP), or the control was incubated without EGF and without ATP. The aliquots were then immunoprecipitated with 2.5 μL of αP1, 5.0 μL of αP3, or 25 μL of αDFG, and the immunoprecipitated proteins were resolved by SDS–PAGE.

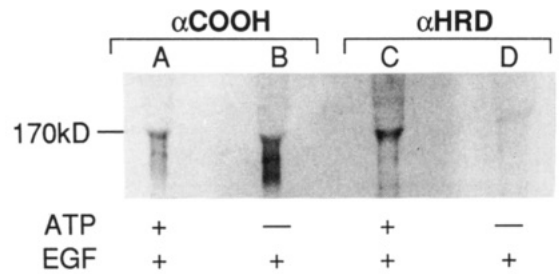

FIGURE 3: Effects of ATP on immunoprecipitation of EGFR by αHRD. Aliquots of ^{35}S-labeled A431 cell extracts were incubated with EGF and with or without ATP as described in Figure 2. The aliquots were then immunoprecipitated with 25 μL of αHRD or with 2.5 μL of αP3 which is designated as αCOOH in this figure. The immunoprecipitated proteins were processed and analyzed as in Figure 2.

While binding of αHRD to EGFR appeared to be increased by receptor autophosphorylation, the efficiency of immunoprecipitation, even of the autophosphorylated receptor, was lower than that of the other anti-peptide antibodies (see Figure 1). We therefore investigated whether conditions that alter protein conformation may affect binding of anti-peptide antibody as indicated by immunoprecipitation. The amount of autophosphorylated EGFR that was immunoprecipitated by αHRD was increased about 5-fold by the addition of 0.05% SDS (Figure 4). This low concentration of SDS in buffer B was not denaturing since kinase activity was only mildly inhibited (10–20%). When the autophosphorylated receptor was briefly incubated with antibody at elevated temperature (5 min at 34 °C), there was about a 5-fold increase in the amount of receptor that was immunoprecipitated in the absence of SDS and about a 2-fold increase when 0.5% SDS was included (Figure 4). Interestingly, increasing the temperature has been shown to enhance the kinase activity of the EGFR (Weber et al., 1984) presumbaly by altering its conformation.

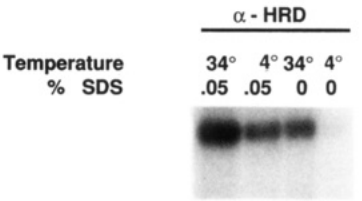

FIGURE 4: Effects of detergent and temperature on immunoprecipitation of ^{32}P-labeled EGFR. αHRD (25 μL) was incubated with EGF-treated, autophosphorylated A431 membrane vesicles. The conditions of incubation with the antibody included: lane 1, 55 min at 4 °C and 5 min at 34 °C; lane 2, 1 h at 4 °C. The buffers used for immunoprecipitation contained either 0.05% SDS (buffer B) or 0% SDS (buffer A) as described under Experimental Procedures. The immune complexes were washed in buffer C, and the ^{32}P-labeled proteins were resolved by SDS–PAGE and detected by autoradiography of the gel.

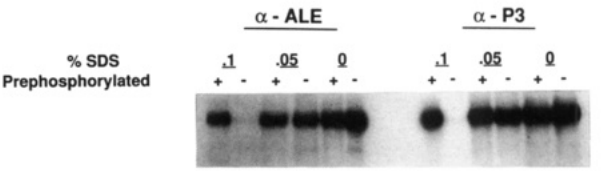

FIGURE 5: Effects of SDS on immunoprecipitation of ^{32}P-labeled EGFR with αALE and αP3. EGF-treated A431 membrane vesicles that were autophosphorylated with [γ-^{32}P]ATP were then incubated with αALE (25 μL) or αP3 (5 μL) on ice for 60 min in buffers containing different amounts of SDS, or were immunoprecipitated in buffer with different amounts of SDS and then autophosphorylated with [γ-^{32}P]ATP in the immune complex.

In contrast, the exposure of epitopes in the DFG, ALE, SDV, P1, and P3 sites was not increased by the addition of up to 0.1% SDS, which eliminated kinase activity [data are shown for αALE and αP3 (Figure 5)]. These findings indicate that exposure of epitopes in the HRD site but not the other five sites was limited and could be increased by conditions that affect protein structure.

Effects of Affinity-Purified αHRD on EGFR Kinase Activity. Because αHRD did not appear to efficiently immunoprecipitate unphosphorylated EGFR, it was difficult to assess the effects of this antibody on EGFR autophosphorylation activity in an immune complex kinase reaction. To further assess whether αHRD inhibits the kinase activity of EGFR, purified αHRD IgG was tested in a soluble kinase assay. EGFR from membrane vesicles was first complexed with EGF and autophosphorylated because αHRD appears to bind more effectively to the phosphorylated receptor. Phosphorylated EGFR was then incubated with different amounts of purified αHRD IgG on ice for 55 min and at 34 °C for 5 min to increase interaction with antibody (see Figure 4). Kinase activity was measured in a reaction containing the exogenous substrate, angiotensin, and [γ-^{32}P]ATP. Figure 6 indicates that greater than 90% of the EGFR kinase activity was inhibited by 3 nM αHRD IgG while less than 5% of the activity was inhibited by αHRD that was preincubated with its cognate peptide. This result strongly suggests that αHRD inhibits the kinase activity of the autophosphorylated EGFR. When the unphosphorylated EGFR was treated with ligand and then incubated with purified αHRD, the kinase activity was not significantly affected, providing further evidence that αHRD interacts poorly with the unphosphorylated EGFR, even in the presence of ligand (Figure 6). While EGFR autophosphorylation should normally occur during the kinase reaction and thus provide binding sites for antibody, the amount of angiotensin used in this assay was sufficient to inhibit autophosphorylation of the EGFR (Bertics & Gill, 1985; L. A. Compton and G. M. Clinton, unpublished observations).

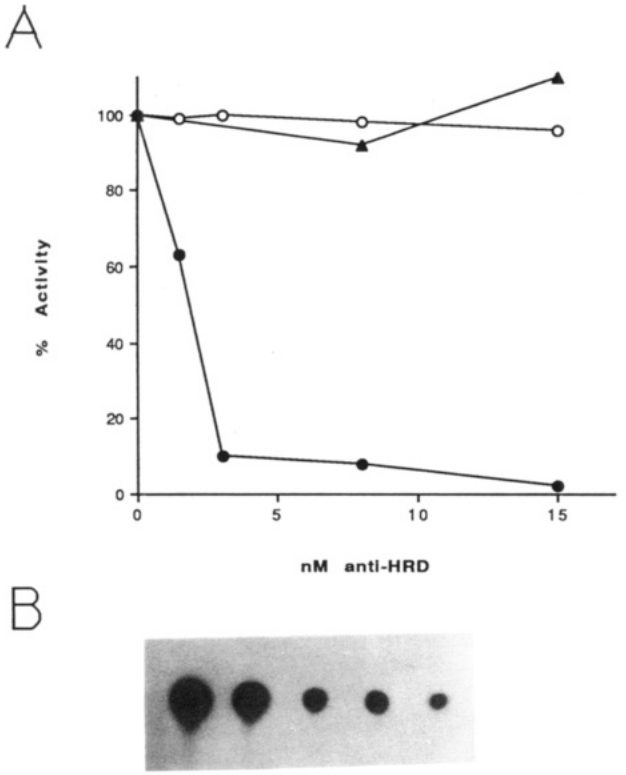

FIGURE 6: Effects of affinity-purified αHRD on EGFR kinase activity. (A) Partially purified EGFR, bound to EGF, was incubated in a kinase mixture with 10 μM ATP to prepare autophosphorylated receptor or without ATP to prepare unphosphorylated receptor. The EGFR samples were dialyzed briefly to remove ATP and then incubated with different concentrations of peptide-affinity-purified αHRD at 4 °C for 55 min and at 34 °C for 5 min. The kinase activity of the different EGFR samples was then measured in a 5-min kinase reaction containing [γ-^{32}P]ATP and 2 mg/mL angiotensin II as substrate. The 100% kinase activity was determined using autophosphorylated EGFR that was incubated without antibody and then incubated in a kinase reaction with angiotensin. (●) Angiotensin phosphorylating activity of the autophosphorylated EGFR. (▲) Angiotensin phosphorylating activity of the autophosphorylated EGFR reacted with αHRD that was preincubated with 15 μg/mL peptide for 30 min at 4 °C. (○) Angiotensin phosphorylating activity of the unphosphorylated EGFR. (B) Autoradiogram of the paper containing the electrophoretically purified ^{32}P-labeled angiotensin from the kinase reaction with autophosphorylated EGFR incubated with different concentrations of αHRD.

DISCUSSION

Anti-peptide antisera against six sequences were used to probe function and topology of specific sites in EGFR. Although four of these sequences are highly conserved and from the catalytic domain, antibodies against only two, αHRD and αDFG, interfered with catalysis. The HRD site, which functions in catalysis, showed increased accessibility to antibody binding following receptor autophosphorylation, by the addition of SDS or by elevation in temperature to 34 °C.

Blocking of phosphotransferase activity by binding of antibodies to the HRD and DFG sites is consistent with several observations suggesting the functional importance of these two highly conserved sequences in catalysis. A computer template developed from analyses of several proteins with phosphotransferase activity contained four critical amino acids in the following positions where X is any amino acid: His-X-Asp-X_4-Asn-X_{13}-Asp (Brenner, 1987). The conserved HRD and DGF sequences in the EGFR fit this pattern exactly (see Table I) and have been proposed to directly function in the phosphotransferase reaction (Taylor et al., 1988; Taylor, 1989). Further evidence for the importance in catalysis of highly conserved residues in these sequences was obtained from in vitro mutagenesis in the catalytic subunit of *Saccharomyces cerevisiae* cAMP-dependent protein kinase (Gibbs & Zoller, 1991). When the highly conserved charged amino acids contained in the equivalent of the HRD and DFG sequences were mutated to Ala, the specific activities of the enzymes were greatly reduced, or the mutants were inviable, indicating complete loss of catalytic activity.

Antibodies against two other highly conserved sites in the catalytic domain, ALE and SDV, did not neutralize catalytic activity. This was surprising since the ALE site is within 17 residues from the DFG site and there are highly conserved amino acid residues within these sequences. However, the results of in vitro mutagenesis studies of Gibbs and Zoller (1991) as well as information obtained from the crystal structure of the cAMP-dependent kinase bound to the peptide inhibitor (Knighton et al., 1991a) indicate that conserved amino acids in these sequences function in stabilization of the catalytic loop whereas nonconserved amino acids appear to function in peptide substrate binding. While it may be expected that attachment of antibody to the ALE and SDV sequences would interfere with peptide substrate binding and consequently inhibit kinase activity, our experiments assessed kinase activity by autophosphorylation rather than by phosphorylation of an exogenous peptide. Thus, autophosphorylation assays may not detect antibody inhibition of kinase activity caused by interference with binding of exogenous peptide substrate. It is also possible that residues within the ALE or SDV site may participate normally in catalysis while bound to the anti-peptide antibody.

Blocked access of substrates to the catalytic site is a proposed mechanism for maintaining kinases in the inactive state (Corbin et al., 1978; Pearson et al., 1988; Hardie, 1988; Gill et al., 1987). Our approach to identify catalytic sites that may be blocked was to test for binding of specific sequences to site-directed antibodies. The only site we found to have regulated exposure to antibody binding was the HRD site. The conditions that resulted in increased exposure of the HRD site included the following: brief incubation at 34 °C, which stimulates kinase activity; the addition of 0.05% SDS where kinase activity is largely retained; autophosphorylation, which has been proposed to stimulate kinase activity (Bertics & Gill, 1985; Lin & Clinton, 1988). The equivalent site in the crystallized cAMP-dependent protein kinase has been localized to the catalytic loop where conserved residues participate in catalysis and nonconserved residues are involved with binding and orientation of the peptide substrate (Knighton et al., 1991a,b). Interestingly, an amino acid residue in the catalytic loop of the cAMP-dependent kinase appears to be accessible to solvent until the addition of peptide substrate, which results in protection from interaction with solvent (Buechler & Taylor, 1990; Knighton et al., 1991a,b). Since we found the HRD site to be inaccessible to antibody binding, even in the absence of exogenous substrate, we speculate that the autophosphorylation domain may be blocking the catalytic loop in the EGFR. The finding that mutant receptors lacking the C-terminal autophosphorylation domain show enhanced EGF-dependent kinase activity (Walton et al., 1990) supports the concept that the C-terminal domain is inhibitory. However, the fact that the C-terminal deletion mutants exhibit ligand-dependent kinase activity suggests that regions outside of the C-terminal domain may function to maintain EGFR in the inactive state prior to ligand binding.

If the catalytic loop of EGFR is blocked, it might be possible that autophosphorylation sites on the receptor may serve this

function since the major autophosphorylation site appears to be a competitive inhibitor of exogenous substrate phosphorylation (Gill et al., 1987; Bertics et al., 1988). However, binding of antibody to the C-terminal autophosphorylation domain at the P1 or the P3 site was not significantly increased by autophosphorylation or by addition of SDS. Therefore, if sequences surrounding the P1 and P3 sites are blocking the HRD site, the epitopes in these sequences are nevertheless exposed to antibody binding. Anti-P1 and -P3 may not directly contact the substrate tyrosines, however, since autophosphorylation was not blocked when these sites were complexed with antibody. Izumi et al. (1988) have also reported that binding of antibody to the sequence surrounding the P1 autophosphorylation site does not inhibit autophosphorylation even at the P1 site.

Because ligand-induced dimerization leads to intermolecular autophosphorylation and kinase activation (Yarden & Schlessinger 1987; Honegger et al., 1989), it is reasonable to consider whether dimerization regulates exposure of the HRD site. Dimerization may result in increased exposure so that autophosphorylation may occur. On the other hand, dimerization may cause blockage of the HRD site with the autophosphorylation domain of the partner receptor. Our data on kinase inhibition by purified αHRD indicate that EGF, which increases dimerization of solubilized receptors (Honegger et al., 1989), is not sufficient to expose the HRD site in the absence of autophosphorylation. Future experiments will need to be conducted to determine whether monomers and dimers differ in the extent of exposure at the HRD site and whether EGFR-associated proteins may be blocking the HRD site.

REFERENCES

Bertics, P. J., & Gill, G. N. (1985) *J. Biol. Chem. 260*, 14642.
Bertics, P. J., Chen, W. S., Hubler, L., Lazar, C. S., Rosenfeld, M. G., & Gill, G. N. (1988) *J. Biol. Chem. 263*, 3610.
Bishayee, S., Majumdar, S., Scher, C. D., & Khan, S. (1988) *Mol. Cell. Biol. 8*, 3696.
Brenner, S. (1987) *Nature 329*, 21.
Bryant, D. L., & Parsons, J. T. (1984) *Mol. Cell. Biol. 4*, 862.
Buechler, J. A., & Taylor, S. S. (1990) *Biochemistry 29*, 1937.
Clinton, G. M., & Brown, N. A. (1991) *Methods Enzymol. 200*, 463.
Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., & McCarthy, D. (1978) *J. Biol. Chem. 253*, 3997.
Dryland, A., & Sheppard, R. C. (1986) *J. Chem. Soc., Perkin. Trans. 1*, 125.
Eberle, A. N. (1986) *J. Chem. Soc., Perkin Trans. 1*, 361.
Gibbs, C. S., & Zoller, M. J. (1991) *J. Biol. Chem. 266*, 8923.
Gill, G. N., Bertics, P. J., & Santon, J. B. (1987) *Mol. Cell. Endocrinol. 51*, 169.
Greengard, P. (1978) *Science 199*, 146.
Hanks, S. K., & Quinn, A. M. (1991) *Methods Enzymol. 200*, 38.
Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42.
Hardie, G. (1988) *Nature 335*, 592.
Honegger, A. M., Kris, R. M., Ullrich, A., & Schlessinger, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 925.
Hsuan, J. J., Panayotou, G., & Waterfield, M. D. (1989) *Prog. Growth Factor Res. 1*, 23.
Izumi, T., Saeki, Y., Akanuma, Y., Takaku, F., & Kasuga, M. (1988) *J. Biol. Chem. 263*, 10386.
Keating, M. T., Escobedo, J. A., & Williams, L. T. (1988) *J. Biol. Chem. 263*, 12805.
Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991a) *Science 253*, 407.
Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991b) *Science 253*, 414.
Koland, J. G., & Cerone, R. A. (1988) *J. Biol. Chem. 263*, 2230.
Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923.
Lin, M.-F., & Clinton, G. M. (1986) *Biochem. J. 235*, 351.
Lin, M.-F., & Clinton, G. M. (1988) *Mol. Cell. Biol. 8*, 5477.
Lin, Y. J., & Clinton, G. M. (1990) *Mol. Cell. Endocrinol. 69*, 111.
Lin, Y. J., & Clinton, G. M. (1991) *Oncogene 6*, 639.
Northwood, I. C., & Davis, R. J. (1988) *J. Biol. Chem. 263*, 7450.
Pearson, R. B. (1988) *Science 241*, 970.
Perlman, R., Bottaro, D. P., White, M. F., & Kahn, C. R. (1989) *J. Biol. Chem. 264*, 8946.
Taylor, S. S. (1989) *J. Biol. Chem. 264*, 8443.
Taylor, S. S., Bubis, J., Toner-Webb, J., Saraswat, L. D., First, E. A., Buechler, J. A., Knighton, D. R., & Sowadski, J. (1988) *FASEB J. 2*, 2677.
Ullrich, A., & Schlessinger, J. (1990) *Cell 61*, 203.
Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., & Seeburg, P. H. (1984) *Nature (London) 309*, 418.
Walton, G. M., Chen, W. S., Rosenfeld, M. G., & Gill, G. N. (1990) *J. Biol. Chem. 265*, 1750.
Yarden, Y., & Schlessinger, J. (1987a) *Biochem. J. 26*, 1443.
Yarden, Y., & Schlessinger, J. (1987b) *Biochem. J. 26*, 1434.
Yarden, Y., & Ullrich, A. (1988) *Annu. Rev. Biochem. 57*, 443.