Identification and Biochemical Characterization of Novel Putative Substrates for the Epidermal Growth Factor Receptor Kinase*

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To gain insight into the mechanisms which control the mitogenic response to epidermal growth factor (EGF), we have partially purified and characterized several intracellular proteins which are phosphorylated on tyrosine residues following activation of the epidermal growth factor receptor (EGFR). Partial purification was achieved by immunoaffinity chromatography using immobilized anti-phosphotyrosine antibodies. Antisera generated against the partially purified proteins were used to identify at least five novel EGF-related putative substrates, designated, on the basis of their apparent molecular weight, p97, p68, p61, p56, and p23. All of these proteins became specifically phosphorylated on tyrosine after EGF treatment of intact cells, as assessed by phosphoamino acid analysis, and none of them represented an EGF degradation product. The phosphorylation of these proteins appeared to be relatively specific for the EGFR. In particular, an EGF-related kinase, erbB-2 was much less efficient than EGFR at phosphorylating p97, p56, and p23 and incapable of phosphorylating p68. The identification of these novel EGFR putative substrates should lead to a better understanding of the mechanisms controlling the specificity of EGF-mediated mitogenic signaling.

Several peptide growth factors regulate cellular growth, metabolism, and differentiation by binding to cell surface receptor-tyrosine kinases (1-4). This interaction starts a cascade of events, most likely involving receptor dimerization/oligomerization (4 and references therein), which culminate in dramatically enhanced receptor catalytic activity (1-4). Active receptor tyrosine kinases are capable of autophosphorylation and the phosphorylation of a number of intracellular substrates (1-4). While receptor autophosphorylation appears to be important in determining the affinity and/or the specificity of the kinase for its cellular substrates (5-8), tyrosine phosphorylation of the latter is thought to represent the initial step of intracellular mitogenic signal transduction.

Several receptor-tyrosine kinase substrates have been identified, including the γ isozyme of phospholipase C (phospholipase C-γ) (9-13), the p21ras GTPase activating protein (14-16), the raf serine-threonine kinase (17, 18), and the p55 subunit of the phosphatidylinositol 3-kinase (19-25). Early attempts to identify receptor-specific signal transduction pathways indicated that a diverse repertoire of effector molecules are used by different growth factor receptors. For instance, the platelet-derived growth factor receptor phospholylates phospholipase C-γ (10, 11) and GTPase activating protein (6, 14, 16) on tyrosine residues; however, other tyrosine kinase receptors do not (14, 26). Therefore, it appears that in addition to receptor expression and/or ligand availability, the specificity of substrate recognition influences the cellular responses to a peptide growth factor.

We are interested in elucidating the mechanisms of mitogenic signal transduction by receptors belonging to the erb subfamily (27). In our previous work, we have demonstrated that two closely related members of this family, the epidermal growth factor receptor (EGFR) and the product of the erbB-2 gene, gp185erbB-2 (13), exert their effects on the cell through different pathway(s) (28-31). In fact, they show qualitative and quantitative differences in their ability to activate mitogenic pathways in different target cells under comparable conditions of expression and enzymatic activation (27, 30). Biochemical analysis of known mitogenic transduction pathways, however, failed to reveal any major difference in the ability of EGF or gp185erbB-2 to induce tyrosine phosphorylation of phospholipase C-γ, GTPase activating protein, or raf (13). In addition, we detected low stoichiometry of tyrosine phosphorylation (≤1% of the total pools) of these substrates by both EGFR and gp185erbB-2 (13). Furthermore, a genetically engineered EGFR mutant which exhibited dramatically reduced mitogenic activity but no alterations of its intrinsic phosphotransferase activity, was indistinguishable from the wild type EGFR as to its ability to tyrosine phosphorylate phospholipase C-γ or GTPase activating protein (32). These results prompted us to hypothesize that other additional signal transduction pathways might exist which are responsible for the different biological effects of members of the erb receptor subfamily. The activation of such alternative signaling pathways should involve early tyrosine phosphorylation of yet unidentified intracellular proteins by active erb receptors. The present studies were undertaken in an attempt to isolate and characterize novel species which are tyrosine-phosphorylated following EGFR kinase activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Genetically engineered NIH-3T3 cells overexpressing EGFR or the chimeric EGFR/erbB-2 receptor (NIH-EGFR and NIH-EGFR and

1 The abbreviations used are: EGF, epidermal growth factor receptor; TK, tyrosine kinase; pTyr, phosphotyrosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
NIH-EGFR/erbB-2, respectively) were described previously (13, 28, 33). These cell lines expressed around 1.5 × 10^6 EGFRs or EGFR/erbB-2 per cell, respectively (13). Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (GIBCO). For EGF triggering experiments, subconfluent cell monolayers were treated with 18 h in the absence (control) or presence of EGF. The cells were stimulated with transferrin (5 μg/ml; Collaborative Research) and selenium (10 μM; Sigma) in the absence of serum and then treated with EGF (Upstate Biotechnology) as described in the text.

Immuneaffinity Chromatography—Cells were lysed on ice with buffer containing 1% Triton X-100 (Pierce), 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin (lysis buffer). EGFR was removed from lysates (50 μg of protein) by anti-EGFR affinity chromatography at 4°C. Anti-EGFR columns were prepared by covalently cross-linking a monoclonal antibody (Ab1, Oncogene Science) directed against the extracellular domain of the EGFR to agarose beads using the Antigen Immobilization kit from Pierce (1 mg of antibody/10 ml of gel). To assess EGFR removal, the columns were eluted with 0.1 M glycine (pH 2.8), and the eluate was neutralized with 50 mM Tris-HCl (pH 9.5) and analyzed by SDS-PAGE and immunostainings as described below.

Proteins that did not bind to the anti-EGFR columns were applied to an anti-phosphotyrosine (anti-pTyr) column (3 ml of agarose-bound monoclonal anti-pTyr; Oncogene Science) at 4°C. Samples were collected over the course several times over a period of 2 h. The column was washed with 50 column volumes of lysis buffer, then eluted with 6 ml of lysis buffer supplemented with 10 mM phenyl phosphate. Fractions were collected and analyzed for pTyr content by dot-immunoblot analysis.

Dot-Immunoblot Analysis—Samples (2 μl) of each fraction eluted from the anti-pTyr column were diluted to 200 μl with a buffer containing 50 mM Tris-HCl (pH 7.6) and 250 mM glycine and boiled for 5 min. Two-fold dilutions of each sample were then spotted onto nitrocellulose filters (pre-equilibrated with buffer) using a microwell filtration apparatus. Wells were washed several times with buffer, and pTyr-containing proteins were detected with an anti-pTyr antibody coupled to 125I-protein A, as described (34). In this and all subsequent experiments, all sera used as immunogens or for immunodetections were absorbed with excess pTyr-containing proteins (which did not inhibit immunorecognition) or phosphotyrosine (which inhibited immunorecognition; data not shown).

Rabbit Immunization—The immuneaffinity-purified pTyr-containing proteins were used to immunize two New Zealand white rabbits as follows. pTyr-containing proteins (25 μg) were mixed with 0.1 ml of incomplete Freund’s adjuvant and injected subcutaneously, near the inguinal lymph nodes. Subsequent injections (10 μg of antigen in incomplete Freund’s adjuvant) were administered subcutaneously 4 weeks after the first injection and were repeated at 3-week intervals. Bleeds were collected 12 days after each boost and screened for the production of antibodies.

Protein Analysis—Antisera were tested for their ability to recognize putative EGFR substrates by immunoprecipitation from cells metabolically labeled with [32P]orthophosphate and treated with EGF in vivo. For labeling in vivo with [32P]orthophosphate, serum-starved NIH/3T3 cells were labeled in [32P]orthophosphate (Du Pont/New England Nuclear Research, 9000 Ci/mmol). Cells were then treated with EGF (100 ng/ml) for 30 min at 4°C. Proteins were immunoprecipitated from cell lysates as described above, separated by SDS-PAGE, and analyzed by autoradiography.

Individual bands were excised from the gels and subjected to phosphoamino acid analysis or V8 protease mapping. Phosphoamino acid analysis was performed as described previously (35). Briefly, peptides were eluted from gel slices by incubation with 200 μg of trypsin (tosylphenylalanyl chloromethyl ketone-treated trypsin; Worthington Biochemicals) in 50 mM ammonium bicarbonate (pH 8.0) for 18 h. Eluted material was dried under vacuum and subjected to acid hydrolysis for 2 h at 110°C using 100 μl of 6 M HCl (Pierce, constant boiling grade). HCl was evaporated under vacuum, and hydrolysates were washed three times with water by repeated vacuum centrifugation, then resuspended in a mixture of phosphoserine, phosphothreonine, and phosphotyrosine (1 mg/ml each phosphorylated aminoacid) and subjected to thin layer electrophoresis. Two-dimensional analysis was performed at pH 1.9 for 60 min at 1 kV (first dimension) and at pH 3.5 for 50 min at 1 kV (second dimension); one-dimensional analysis was performed for 60 min at pH 3.5. Phosphorylated amino acid standards were revealed by ninhydrin staining.

V8 protease peptide mapping was performed by the method of Cleveland et al. (37). Individual gel slices were placed in a single well of a 15% SDS-PAGE gel in the presence of 1.25 mg of Staphylococcus aureus (Boehringer Mannheim), and gels were typically run for 20 h at 15 mA. The gels were dried and subjected to autoradiography at −70°C.

RESULTS

Optimization of Substrate Phosphorylation by the EGFR in Vivo—Our initial efforts were aimed at identifying an optimal system to study tyrosine phosphorylation of cellular substrates by EGFR. Since our final goal was the purification of these proteins, our main concern was to develop an approach yielding high stoichiometry of tyrosine phosphorylation that would allow quantitative recovery of EGFR substrates. In our previous studies, we have shown that overexpression of EGFR in the normally EGF-responsive NIH/3T3 cell line, even at levels of 2–3 × 10^6 receptors/cell (NIH-EGFR cells), did not alter the growth properties of the cells, under standard culture conditions (13, 28, 31). In the presence of EGF, however, NIH-EGFR cells displayed markedly increased DNA synthesis, as compared to mock-transfected NIH/3T3 cells (13, 28, 31). Thus, overexpression of the EGFR amplifies the EGF-triggered mitogenic signal in these cells. These biological findings were paralleled, at the biochemical level, by a dramatic increase in tyrosine phosphorylation of "putative" EGFR substrates, when NIH/3T3 and NIH-EGFR cells were analyzed under conditions of EGF stimulation (13). We therefore elected to use the NIH-EGFR model system for our experiments.

The extent of phosphorylation of the putative EGFR substrates was dependent on the experimental conditions of exposure to EGF in vivo. As shown in Fig. 1, an EGF dose-response analysis indicated that a concentration of 50–100 ng/ml (8–16 nM) induced optimal tyrosine phosphorylation of putative substrates in vivo. Longer exposures of the gel shown in Fig. 1 revealed that differences in substrate phosphorylation over a wide range of EGF concentrations were
more quantitative than qualitative (data not shown). In fact, at doses as low as 1–5 ng/ml (0.16–0.8 nM), at which mostly high affinity binding sites would be occupied at equilibrium, the pattern of phosphotyrosine (pTyr)-containing proteins observed was very similar to that detected by stimulation with higher EGF doses (data not shown). Thus, it appears that the recruitment of a high number of EGFRs does not markedly affect the specificity of its enzymatic activity.

Fig. 2A shows a time course of EGF triggering (at the optimal dose of 100 ng/ml) at 37 °C and 4 °C. We consistently observed higher efficiency of tyrosine phosphorylation of putative substrates at the latter temperature. The signal detected in immunoblot analysis using anti-phosphotyrosine (A) or EGFR antipeptide sera E7 (B) did not show a marked difference. The position of tyrosine-phosphorylated EGFR is indicated by the arrow. Molecular weight markers (×10^3) are indicated on the right. The position of the 170-kDa EGFR protein present in the starting material; this value is in good agreement with previously published estimates of the pTyr protein content of cells stimulated by growth factors or TK oncogenes (38, 39).

Recovery was estimated to be ~0.06% of the total starting material; this value is in good agreement with previously published estimates of the pTyr protein content of cells stimulated by growth factors or TK oncogenes (38, 39).

Blots identical with the one shown in Fig. 2B were stained with AuroDye gold stain (see “Experimental Procedures”) to detect non-pTyr-containing proteins contaminating our preparations.

Proteins which did not bind to the anti-EGFR columns were then loaded on an anti-pTyr column (see “Experimental Procedures”), and the pTyr-containing proteins were finally eluted with 10 mM phenyl phosphate. A dot-blot immunoassay was developed (see “Experimental Procedures”) to analyze eluted fractions for phosphotyrosine content. The fractions with the highest phosphotyrosine content were then pooled and analyzed by SDS-PAGE and conventional anti-pTyr immunoblotting. As shown in Fig. 3A, at least 10 putative substrates were eluted from the anti-pTyr column, which were conspicuously enriched when compared to the starting material (Fig. 3B). We estimated that this preparation was enriched in pTyr-containing proteins at least 500–1000-fold. Recovery was estimated to be ~0.06% of the total starting material; this value is in good agreement with previously published estimates of the pTyr protein content of cells stimulated by growth factors or TK oncogenes (38, 39).

Blots identical with the one shown in Fig. 2B were stained with AuroDye gold stain (see “Experimental Procedures”) to detect non-pTyr-containing proteins contaminating our preparations.
FIG. 3. Purification of tyrosine-phosphorylated proteins using anti-EGFR and anti-phosphotyrosine affinity chromatography. Panel A, total cellular proteins (50 mg for each of four independent preparations) prepared from EGF-stimulated NIH-EGFR cells were loaded on four successive anti-EGFR receptor columns as described under "Experimental Procedures," to remove the EGFR. The anti-pTyr immunoblot of a typical cell lysate (100 µg) before application to the anti-EGFR column is shown in lane 1 of panel A. Proteins bound to the anti-EGFR columns were eluted with 0.1 M glycine (pH 2.8), and fractions were analyzed by immunoblotting with anti-pTyr antibodies (lanes 2, 3, and 4 of panel A) are representative of the entire fractionated eluate. Panel B, proteins that did not bind to the anti-EGFR column were then applied to an anti-pTyr column, and the pTyr-containing proteins were purified as described under "Experimental Procedures." Three different purified preparations (each from 50 mg of starting material) of proteins eluted from the anti-pTyr column are shown in lanes 2–4. An aliquot (500 ng) of each preparation was fractionated on a 3–27% gradient acrylamide gel and then analyzed by anti-pTyr immunoblotting (lanes 2, 3, and 4 of panel B). Lane 1 shows a typical pTyr-containing protein pattern before the purification procedure (100 µg of total cellular proteins). Molecular weight markers (×10^3) are indicated at the left of each panel. The position of tyrosine-phosphorylated EGFR and tyrsoine-phosphorylated putative EGFR substrates are indicated by arrowheads at the left and right sides of panel B, respectively.

Arrows. Furthermore, lysates obtained from cells metabolically labeled with [35S]cysteine and [32P]orthophosphate were subjected to the same purification protocol outlined above and then analyzed by SDS-PAGE. After comparing the anti-pTyr immunoblots with the gold-stained blots and the [32P] labeling experiments, we estimated that co-purified non-pTyr-containing proteins represented only a minor component (<10%) of our preparations (data not shown).

Biochemical Characterization of EGFR Putative Substrates—To further characterize the putative EGFR substrates obtained as described above, we used the purified protein preparations to generate polyclonal antibodies. Two New Zealand rabbits were immunized (see "Experimental Procedures"), and the immune sera (henceforth referred to as sera 450 and 451) were tested for their ability to recognize phosphoproteins. Lysates from cells that had been metabolically labeled with [32P]orthophosphate in the presence of EGFR were immunoprecipitated with either serum 450 or 451 or with the corresponding nonimmune sera (prebleeds from the same animals). As shown in Fig. 4, both sera were able to specifically recognize a number of phosphoproteins. Both sera recognized a Mr = 170,000 species, which was demonstrated by immunodepletion experiments to be EGFR (data not shown). Serum 450 also recognized a major phosphoprotein of an apparent molecular weight of 61,000 (p61), and several other minor phosphoproteins among which was a p23 that comigrated with a similar protein recognized by serum 451 (Fig. 4, A and B).

As shown in Fig. 4, A and B, serum 451 also recognized several major phosphoproteins. Resolution of low (Fig. 4A) and high (Fig. 4B) molecular weight species was obtained by varying the acrylamide percentage and lengths of run in our SDS-PAGE analyses. This allowed us to identify seven major phosphoproteins recognized by serum 451: p109, p97, p72, p68, p56, p50, and p23, respectively. Of note, none of the phosphoproteins recognized by serum 450 or 451 was efficiently detected in parallel immunoprecipitations with the corresponding preimmune serum (Fig. 4, A and B). The anti-EGFR peptide antibody E7 (30) did not recognize any of these phosphoproteins, thus demonstrating that they do not represent EGFR degradation products (Fig. 4, A and B). In addition, Staphylococcus aureus V8 proteolytic patterns of individual proteins did not match that of EGFR, further proving that the identified putative substrates are not EGFR degradation products (data not shown).

To prove that the identified putative substrates were specifically phosphorylated on tyrosine residues following EGFR activation, phosphoamino acid analysis of the proteins recognized by the two sera was performed, before and after EGF treatment of NIH-EGFR cells in vivo. As shown in Fig. 5, in quiescent, unstimulated cells, all of the putative substrates were phosphorylated on serine and/or threonine residues, whereas there was little if any detectable pTyr. EGF treatment led to specific tyrosine phosphorylation of p97, p68, p61, p56, and p23, whereas little, if any, pSer or pThr increase was detectable, even after growth factor stimulation (data not shown), whereas phosphoamino acid analysis of p109 and p50 was not obtained due to the low level of 32P incorporation.

Specificity of Substrate Tyrosine Phosphorylation by EGFR—We compared the ability of the closely related EGFR and erbB-2 kinases to stimulate the phosphorylation of the newly identified substrates. To this end, we utilized two previously described mass populations of NIH-3T3 cells overexpressing either the wild type EGFR or an EGFR/erbB-2 chimera containing the extracellular ligand-binding domain of the EGFR and the intracellular domain of gp185ERBB-2 (13, 33). These two cell lines (NIH-EGFR and NIH-EGFR/erbB-
Phosphoamino acids analysis of putative EGFR substrates. NIH-EGFR cells were metabolically labeled with [32P]orthophosphate and either mock-treated (–) or treated with EGF (+) as described in the text. After immunoprecipitation of radiolabeled cell lysates with immune sera, phosphoproteins were separated by SDS-PAGE, excised from the gels, and digested with trypsin. Eluted tryptic peptides were hydrolyzed and phosphoamino acids were separated by one (upper panels) or two (lower panels)-dimensional thin layer electrophoresis. Phosphoamino acids were detected by autoradiography and identified using standards stained with ninhydrine: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

Fig. 6. Comparison of substrates phosphorylation by EGFR and an EGFR/erbB-2 chimera. Lysates were prepared from 32P-labeled NIH-EGFR and NIH-EGFR/erbB-2 cells treated with (+) or without (−) EGF (100 ng/ml for 30 min at 4 °C). Equal amounts of trichloroacetic acid-precipitable counts (10⁶ cpm, corresponding to about 1.0 mg of total protein) were immunoprecipitated with excess anti-pTyr antibodies. The immunoprecipitates were then eluted with phenyl phosphate (10 mM), immunoprecipitated a second time with serum 451, and analyzed by discontinuous gradient SDS-PAGE (top, 7.5%; bottom, 12.5%). Molecular weight markers (× 10⁶) are indicated at left. The positions of tyrosine-phosphorylated EGFR substrates recognized by sera 451 are indicated by arrowheads at right. Comparable results were obtained in three separate experiments.

Comparable results were also obtained when the EGF triggering was performed at physiological temperature (100 ng/ml EGF for 10 min at 37 °C).

pTyr-containing proteins recognized by serum 451, when compared to NIH-EGFR. As shown in Fig. 6, p97, p56, and p23 seemed to be phosphorylated less efficiently by an active erbB-2 kinase than by EGFR; more importantly, there was no detectable p68 phosphorylation upon EGF stimulation of NIH-EGFR/erbB-2.

DISCUSSION

Second messenger pathways have been implicated as early mitogenic signal transducers for TK growth factor receptors, particularly the pathways activated by phospholipase C-γ tyrosine phosphorylation/L-α-phosphatidylinositol 4,5-di-phosphate breakdown and by p21ras/GTPase activating protein signaling (9–16, 40–42). There is evidence, however, that while these pathways may be necessary, they are not sufficient for receptor-induced mitogenic action. For example, overexpression of phospholipase C-γ and subsequent platelet-derived growth factor receptor activation does not lead to increased mitogenic signaling, despite enhanced L-α-phosphatidylinositol 4,5-di-phosphate turnover (43). In the case of EGFR-activated mitogenic pathway(s), we have previously shown that signal transduction mechanism(s) other than phospholipase C-γ tyrosine phosphorylation/L-α-phosphatidylinositol 4,5-di-phosphate breakdown and the p21ras/GTPase activating protein signaling must contribute to the mitogenic response. A 7-amino acid deletion in the EGFR...
(EGFR Δ660-667), in fact, caused a marked decrease in the mitogenic potency of EGFR despite unaltered kinase activity in vitro and in vivo (32). In particular, the efficiency of tyrosine phosphorylation of phospholipase C-γ in vitro by the EGFR Δ660-667 was indistinguishable from that of wild type EGFR and, under the conditions of overexpression achieved, no GTPase activating protein tyrosine phosphorylation was detectable by either EGFR or EGFR Δ660-667 (32).

The present studies were, therefore, undertaken to identify alternate signaling pathway(s) which contribute to the EGFR-mediated mitogenic response. We approached this question by isolating intracellular proteins phosphorylated on tyrosine upon activation of the EGFR kinase. By taking advantage of polyclonal sera generated against the purified proteins, we were able to identify five proteins, p97, p68, p61, p56, and p23 which were specifically phosphorylated on tyrosine residues immediately upon EGF stimulation. None of these proteins was recognized by anti-peptide sera directed against the carboxyl-terminal domain of the EGFR, and V8 protease mapping confirmed that none were degradation products of the EGFR. These proteins do not appear to correspond to several of the known substrates for tyrosine kinase receptors. By immunoprecipitation, immunodepletion, and immunoblotting techniques, we have determined that p97 is not GTPase activating protein, that none of the newly identified species is phospholipase C-γ, and that p68 is not the ras kinase (data not shown). There have also been reports that members of the src family of tyrosine kinases can be phosphorylated on tyrosine by tyrosine kinase growth factor receptors (44–46). However, the recognition of p97, p61, and p56 by the polyclonal sera 450 and 451 was not abolished in immunodepletion experiments performed with anti-src, anti-fgr, or anti-fyn sera (data not shown). Based on migration in SDS-PAGE, it is also unlikely that any of the proteins we have described are the 85-kDa subunits of phosphatidylinositol 3-kinase (23–25), although this warrants further investigation. Together, these data indicate that we have identified novel proteins which are phosphorylated on tyrosine upon EGFR activation and are likely to be part of the EGF-activated signaling pathway. At present, we do not know whether these proteins directly interact with the EGFR, thereby being phosphorylated or whether they represent “second line” substrates whose phosphorylation is the consequence of secondary tyrosine kinase activation. For this reason, we refer to them as putative substrates. It is to be noted, however, that the kinetics of phosphorylation are consistent with the fact that these proteins represent early direct substrates of the EGFR kinase.

To achieve quantitative tyrosine phosphorylation of substrates for purification purposes, it was necessary to work under conditions of receptor overexpression (approximately 2 × 10^7 EGFR per cell). This raises the question of whether the identified pTyr-containing species are physiologically part of the EGFR-specific mitogenic pathway or are recruited as a result of kinase promiscuity associated with overexpression. At least two lines of evidence indicate that this is not the case. First, we have observed that NIH/3T3 cells overexpressing EGFR at various levels (from 2 × 10^6/cell to 2 × 10^7/cell) display comparable ED50 values for EGF-induced mitogenesis, despite increased maximal response to EGF with increasing receptor numbers (32). This indicates that the abundance of substrates critical for mitogenesis is not limiting, and that at high levels of EGFR expression the mitogenic stimulus is still routed through a physiological pathway. In addition, we have found that increased EGFRs recruitment, obtained by increasing either EGF doses or the time of exposure, resulted in the phosphorylation of a pattern of substrates similar to that detected under conditions in which only a few thousand receptors are activated. Together this evidence indicates that the purified pTyr-containing proteins are physiologic substrates of the EGFR.

We have previously reported biological evidence that the mitogenic signaling pathways activated by EGFR and gp185^erbB-2 must be at least in part different (28, 31, 32). These two highly related kinases, in fact, exhibited markedly different abilities to trigger mitogenic response in different target cells. In particular, in the NIH-3T3 system, the erbB-2 kinase was at least 100-fold more potent than EGFR as a transforming gene (13, 28). In our initial efforts to identify differential coupling of these two kinases to mitogenic pathways, however, we did not detect any difference in their relative ability to phosphorylate known signal transducers, including phospholipase C-γ and GTPase activating protein (13). In contrast, in this study, we found that, under comparable conditions of activation, the erbB-2 kinase is much less active than EGFR at phosphorylating p97, p56, and p23 and cannot efficiently phosphorylate p68. Thus, our results provide a biochemical correlate for the differential mitogenic coupling of EGFR and erbB-2.

Several questions remain as to the functions of p97, p68, p61, p56, and p23. Answers are likely to come from the elucidation of their primary sequences, from gene transfer experiments using eukaryotic expression vectors for cDNAs encoding the novel substrates, and from studies of whether monoclonal antibodies directed against these proteins will block the EGF-activated mitogenic pathway in microinjection experiments. In an effort to address these issues, we have screened an NIH-3T3 cell bacterial expression library with sera 450 and 451, and we are presently characterizing a number of cDNA clones. Initial sequence information has revealed no homology with sequences present in GenBank or in the EMBL data base. While more work will be needed to unequivocally establish that we have cloned the cDNAs for p97, p68, p61, p56, and p23, these studies may lead to a better understanding of the relevance of these proteins in mediating EGF mitogenic actions.

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REFERENCES

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