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p185, a Product of the *neu* Proto-Oncogene, Is a Receptorlike Protein Associated with Tyrosine Kinase Activity

DAVID F. STERN,* PATRICIA A. HEFFERNAN,† AND ROBERT A. WEINBERG

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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The *neu* oncogene was originally identified in cell lines derived from rat neuroectodermal tumors. *neu* is related to but distinct from the c-*erbB* gene, which encodes the epidermal growth factor (EGF) receptor. *neu* encodes a protein, designated p185, that is serologically related to the EGF receptor. Identification of the normal homolog of p185 encoded by the *neu* proto-oncogene enabled us to compare the product of the *neu* proto-oncogene with the mutated version specified by the *neu* oncogene and with the EGF receptor. The normal form of p185 was structurally similar to its transforming counterpart, indicating that activation of the *neu* oncogene did not cause major structural alterations in the gene product. Both normal and transforming forms of p185 were associated with tyrosine kinase activity, supporting the idea that normal p185 functions as a growth factor receptor. p185 differed both structurally and functionally from the EGF receptor. p185 and the EGF receptor had distinct electrophoretic mobilities when synthesized under normal culture conditions or in the presence of tunicamycin. EGF did not stimulate increased turnover of p185 and did not bind quantitatively to p185. A number of other growth factors failed to stimulate degradation of p185 or tyrosine phosphorylation of p185 and are therefore unlikely to be ligands for p185.

More than 20 different oncogenes have been implicated in virus-mediated tumorigenesis, and a number of these are likely to play a direct role in human cancer (reviewed in references 4 and 35). It is now evident that some of these oncogenes operate by disrupting pathways regulated by polypeptide growth factors (reviewed in references 23, 25, and 59). Thus the v-*sis* oncogene has been found to encode a protein which is structurally and functionally homologous to platelet-derived growth factor (PDGF) (13, 15, 30, 48, 64). v-*sis* apparently transforms cells by causing them to constitutively secrete a mitogenic factor to which these same cells respond. Two viral oncogenes, v-*erbB* and v-*fms*, are derived from cellular genes encoding growth factor receptors. v-*erbB* specifies a truncated version of the cellular epidermal growth factor receptor (EGFr) (16, 63), while v-*fms* specifies a protein related to the macrophage colony-stimulating factor 1 receptor (56). The two defective receptor proteins may transform cells by constitutively transmitting growth excitatory signals that are normally produced only in the presence of their respective ligands.

We have been studying another oncogene, designated *neu*, which apparently specifies a mutated growth factor receptor. In contrast to v-*erbB* and v-*fms*, which were discovered as transforming genes of retroviruses, the *neu* oncogene was identified by transfection of DNA from chemically induced tumors (57). DNA from four ethyl-nitrosourea-induced rat neuroectodermal tumor cell lines induced foci when introduced by transfection into NIH 3T3 cell monolayers (57). Subsequent studies revealed that these four neuroectodermal tumor cell lines all contained activated versions of the *neu* oncogene (53).

The initial studies showed that the transfected *neu* oncogene is invariably associated with a specific tumor antigen designated p185 (17, 18, 45). The tight correlation of expression of p185 with the presence of a transfected *neu* oncogene suggested that p185 is the *neu* gene product. p185 is a phosphoprotein that is associated with the plasma membrane (18, 45; A. L. Schechter, unpublished data). These properties suggested that the normal (nontransforming) version of p185 is a growth factor receptor. Strong support for this idea came from the findings that the *neu* gene is homologous to *erbB*, the gene for the EGFr and that p185 is serologically related to the EGFr (53). These experiments demonstrated that *erbB* and *neu* are related but did not indicate whether they are versions of the same gene or represent distinct but related genes.

To understand the mechanism of transformation by the *neu* oncogene, we wish to determine the function of the normal *neu* gene product and the functional consequences of the mutations that activate *neu* oncogenes. We have identified the product of the normal cellular *neu* proto-oncogene. This has permitted us to compare the normal *neu* gene product with transforming forms of p185 and with the EGFr. The results indicate that the *neu* gene product differs both structurally and functionally from the EGFr.

MATERIALS AND METHODS

Cells. Cells were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% calf serum (CS) or inactivated fetal serum under an atmosphere of 5% CO_2 at 37°C. B104 cells were derived from an ethyl-nitrosourea-induced rat neuroectodermal tumor (54); B104-1-1 cells are NIH 3T3 cells transformed by transfection with an *neu* oncogene originally derived from B104 cells (45); DHFR/G-6 and DHFR/G-8 cells are NIH 3T3 cells transfected with a genomic clone of the *neu* proto-oncogene (24).

Growth factors. EGF (receptor grade) and nerve growth factor were obtained from Collaborative Research, Inc. Transforming growth factor-α (TGF-α) was obtained from Rik Derynck (Genentech, Inc.), TGF-β was from Anita Roberts (National Institutes of Health), PDGF was from A. R. Frackelton, Jr. (Roger Williams Hospital), and L. T.

* Corresponding author.
† Present address: Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139.

Williams (University of California at San Francisco), insulin was from Sigma Chemical Co., and pituitary fibroblast growth factor was from D. Gospodarowicz (University of California at San Francisco). A platelet extract, prepared as described previously (2) was a gift from C. D. Stiles (Dana-Farber Cancer Institute).

Antisera. Monoclonal antibody 7.16.4 (18) was obtained from J. A. Drebin and M. I. Greene (Tufts New England Medical Center), polyclonal anti-EGFr serum (11) was from S. Decker (Rockefeller University), polyclonal anti-insulin receptor A410 (27) was from S. Jacobs (Wellcome Research Laboratories), polyclonal anti-insulin-like growth factor 2 (IGF-2) receptor serum (43) was from M. P. Czech (University of Massachusetts Medical Center).

Radiolabeling cells. (i) Overnight labeling. Unless otherwise indicated, cells were seeded the day before immunoprecipitation at a density equivalent to 1×10^6 cells (Rat-1 cells) or 3×10^6 cells (B104, B104-1-1, or A431 cells) per 60-mm culture dish. Cells labeled with [^{35}S]cysteine were incubated in Hanks minimal essential medium containing 1/10 the normal amount of cysteine and [^{35}S]cysteine (980 Ci/mmol; New England Nuclear Corp.) at an activity of 0.2 to 0.5 mCi/ml (1 ml per 60-mm dish). Cells labeled with [^{35}S]methionine were incubated in DMEM containing 1/10 the normal amount of methionine and [^{35}S]methionine (New England Nuclear) at an activity of 0.25 mCi/ml. Cells labeled with $^{32}P_i$ were incubated in phosphate-free DMEM containing $^{32}P_i$ (carrier-free; New England Nuclear) at an activity of 100 μCi/ml (see Fig. 3), 500 μCi/ml (see Fig. 2), or 1,000 μCi/ml (markers, see Fig. 8). Labeling media were supplemented with 2% dialyzed CS (see Fig. 1, 4, and 6) or 4% CS (see Fig. 2, 3, 5, 7, and 10).

(ii) Pulse-chase experiments. For the experiment shown in Fig. 3, cells in 60-mm culture dishes were washed once in cysteine-free or phosphate-free DMEM and incubated for 10 min at 37°C in 1.0 ml of cysteine-free medium containing 0.5 mCi of [^{35}S]cysteine and 0.1% CS or 1.0 ml of phosphate-free DMEM containing 0.5 mCi of $^{32}P_i$ and 0.1% CS. Chases were performed by washing the cultures twice with DMEM–0.1% CS and incubating the cultures in the same medium for 120 min at 37°C. For the experiment shown in Fig. 6, dense cultures of cells in 35-mm dishes were pulse-labeled for 15 min in 0.5 ml of methionine-free DMEM containing 0.5 mCi of [^{35}S]methionine, washed twice, and chased in DMEM–2% CS.

Tunicamycin treatment. Cells were incubated (without radiolabel) for 2 h at 37°C in DMEM containing tunicamycin (final concentration, 1 μg/ml; Calbiochem-Behring). The cells were then labeled in medium containing tunicamycin (1 μg/ml). For the experiments shown in Fig. 4 and 5, 50 μM leupeptin (Sigma Chemical Co.) was included in the labeling medium to inhibit proteolysis (39).

Immunoprecipitation. All operations were performed at 4°C. Cells were washed once with cold phosphate-buffered saline, scraped into 0.5 ml of lysis buffer (phosphate-buffered RIPA [55] containing 1 mM ATP, 2 mM EDTA, 20 mM sodium fluoride, and 100 μM sodium orthovanadate [Sigma] [for experiments shown in Fig. 3, 5, 7, and 8]) per 60-mm dish, and incubated for 25 min. The lysates were cleared for 10 min in a microcentrifuge, and the supernatants were incubated for 10 min with 50 μl of 50% (wt/vol) fixed *Staphylococcus aureus* (IgGSorb; The Enzyme Center, Inc.) with constant rotation and centrifuged again for 10 min. Portions of the cleared lysates were incubated for 1 to 3 h with antiserum (1 to 3 μl of normal mouse and rabbit sera, 1 to 2 μl of 60× concentrated antibody 7.16.4, 3 to 6 μl of anti-EGFr serum, 25 μl of A410 anti-insulin receptor, or 6 μg of anti-IGF-2 receptor per 1×10^6 to 3×10^6 cells). Antibody 7.16.4 was in excess for all experiments. The lysates were then incubated with 2 μl of sheep anti-mouse immunoglobulin (Cooper Biomedical, Inc.) for 20 min and 50 μl of 10% (wt/vol) fixed *S. aureus* for 30 to 60 min with rotation. The *S. aureus* was prepared by preadsorbing with lysates of nonlabeled Rat-1 or NIH 3T3 cells (15 to 30 min) and washing twice with RIPA. Immune complexes were collected by centrifugation in a microcentrifuge, suspended in 0.5 ml of lysis buffer, and centrifuged through 0.5 ml of 10% (wt/vol) sucrose in lysis buffer. The pellets were washed three times with 0.5 ml of lysis buffer and once with phosphate-buffered saline. For testing anti-IGF-2 receptor serum, a different lysis buffer was used. HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (50 mM; pH 7.4)–2% Triton X-100–0.15 M NaCl–1% bovine serum albumin–0.198 TIU of aprotinin per ml (Sigma)–1 mM phenylmethylsulfonyl fluoride–1 mM ATP was used to lyse cells and wash immunoprecipitates. Lysates were incubated at room temperature for 2 h with antibody, and washes were performed at 4°C.

SDS-polyacrylamide gel electrophoresis. Samples were suspended in electrophoresis sample buffer (5 mM sodium phosphate [pH 7.0], 2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue), incubated at 100°C for 1 min, and analyzed by electrophoresis on discontinuous 7.5% acrylamide–0.17% bisacrylamide gels as described (55). Gels were 14-cm long and 1.2-mm thick. Gels were fluorographed (58) and exposed to Kodak X-Omat R film.

Immune complex kinase assays. Lysis and immunoprecipitation were carried out at 4°C. Dense cultures of cells on 100-mm dishes were washed once with cold phosphate-buffered saline, scraped into 1.3 ml of TG (1% [vol/vol] Triton X-100–10% [vol/vol] glycerol–0.198 TIU of aprotinin per ml, prepared in calcium- and magnesium-free phosphate-buffered saline), and incubated for 20 min. The lysates were triturated, incubated for 10 min with 100 μl of 10% (wt/vol) fixed *S. aureus* with rotation, and centrifuged in a microcentrifuge for 5 min. Supernatants were incubated consecutively with primary antibody for 45 min, sheep anti-mouse immunoglobulin for 15 min, and 90 μl of 50% (vol/vol) *S. aureus* protein A-Sepharose (Pharmacia Fine Chemicals) for 30 min with rotation. Immune complexes were collected by centrifugation and washed twice with 750 μl of TG and twice with 20 mM HEPES (pH 7.4) plus 3 mM $MnCl_2$. The pellets were suspended in 50 μl of 20 mM HEPES (pH 7.4)–3 mM $MnCl_2$–40 μM 5'-adenylylimidodiphosphate (Sigma). [γ-^{32}P]ATP (New England Nuclear) was added to yield a final concentration of 10 μM at a specific activity of 30 Ci/mmol (15 μCi per 50-μl reaction). The samples were incubated at 30°C for 8 min, and the reactions were terminated by addition of 2× electrophoresis sample buffer containing 2 mM ATP and 2 mM EDTA and incubation at 100°C for 2 min.

Phosphoamino acid analysis. Gel slices containing ^{32}P-labeled proteins were homogenized in 1.0 ml of 50 mM ammonium bicarbonate–0.1% SDS–1 mM EDTA–10 μg of bovine serum albumin per ml and shaken overnight at 37°C. After centrifugation of the homogenates in a microcentrifuge for 10 min at room temperature, the supernatants were filtered through siliconized glass wool and precipitated by addition of tricholoroacetic acid to a final concentration of 20% and incubation on ice for 2 h. Precipitates were collected by centrifugation for 10 min, washed with cold ethanol and then cold ethanol-diethyl ether (50% [vol/vol]), sus-

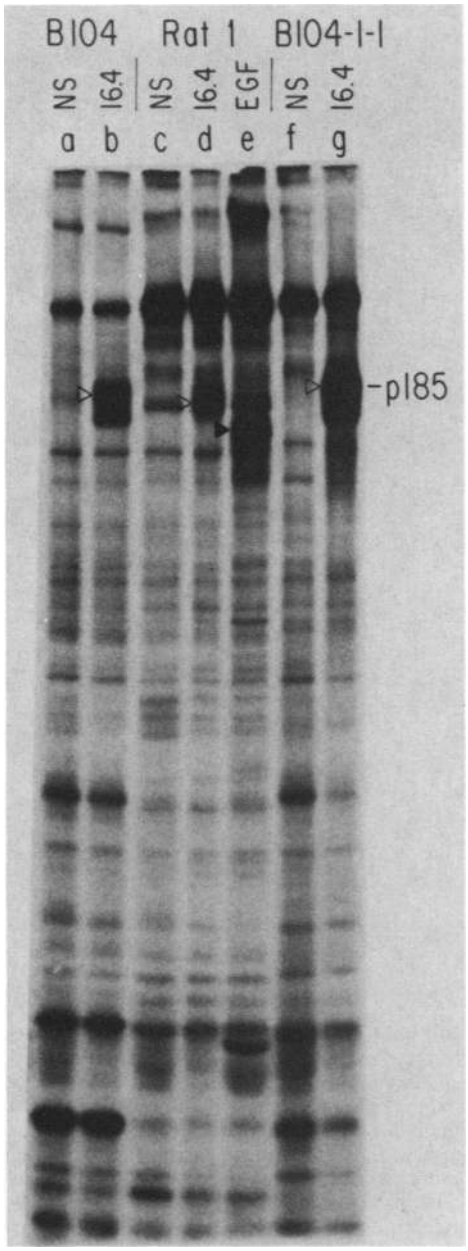

FIG. 1. p185 immunoprecipitated from normal and *neu*-transformed cells. B104, Rat-1, and B104-1-1 cells were labeled overnight with [^{35}S]cysteine and lysed, and portions were immunoprecipitated with a mixture of normal mouse and rabbit sera (lanes NS), with anti-p185 antibody 7.16.4 (lanes 16.4), or with polyclonal anti-EGFr serum (lanes EGF). The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Precipitates were prepared from approximately 5×10^5 cells (B104 and B104-1-1) or 2.4×10^5 cells (Rat-1). The fluorographed gel was exposed to preflashed film for 7 days at −70°C. Lysates of B104 cells were immunoprecipitated with mixed normal mouse and rabbit sera (lane a) and antibody 7.16.4 (lane b). Lysates of Rat-1 cells were immunoprecipitated with mixed normal mouse and rabbit sera (lane c), antibody 7.16.4 (lane d), and anti-EGFr serum (lane e). Lysates of B104-1-1 cells were immunoprecipitated with mixed normal mouse and normal rabbit sera (lane f) and antibody 7.16.4 (lane g). ▷, p185; ▶ EGFr.

pended in 100 μl of constant-boiling hydrochloric acid (Sequenal grade, Pierce Chemical Co.), and incubated at 105°C for 60 min. Samples were lyophilized, suspended in 100 μl of water, lyophilized, and suspended in thin-layer electrophoresis buffer (pH 3.5) (26) for application to thin-layer plates. Phosphoamino acids were separated by two dimensional electrophoresis on plastic-backed 0.1-mm cellulose plates (EM Reagents) as described previously (26) with the modifications described previously (20).

EGF binding to cell monolayers. Cells (10^5 per 35-mm well) were seeded in 6-well cluster dishes in DMEM–5% CS. Binding was measured the following day. Cells were washed twice with 2.0 ml of warm binding medium (DMEM–0.5% bovine serum albumin–50 mM HEPES [pH 7.5]). Cells were incubated with 0.5 ml of binding medium containing [^{125}I]EGF (New England Nuclear; 150 to 200 μCi/μg) at 37°C for 40 min and chilled on ice for 10 min. The wells were washed six times rapidly with 2 ml of binding medium; the washes were complete within 3 min. Cells were solubilized in 0.5 ml of 1 N sodium hydroxide at room temperature, and ^{125}I activity was determined with a scintillation counter. Of the six wells on each cluster dish, two were incubated with binding medium lacking EGF but were otherwise treated like the remaining wells. Cell number was determined in these wells and used to correct for differences in cell number resulting from nonspecific losses of cells during the procedure. Specific binding of EGF was calculated from the difference between the number of counts per minute bound in the absence and presence of 1 μg of nonradiolabeled EGF (receptor grade, Collaborative Research) per ml.

RESULTS

Product of the *neu* proto-oncogene. We previously identified a candidate for the product of the normal *neu* proto-oncogene in nontransformed rat fibroblasts (53). This protein can be immunoprecipitated from Rat-1 cells (a nontransformed rat fibroblast cell line) by use of monoclonal antibody 7.16.4 (18), which recognizes rat p185 (Fig. 1, lane d). The protein migrates slightly more rapidly than the p185 immunoprecipitated from B104-1-1 cells, which are NIH 3T3 cells transfected with the *neu* oncogene (Fig. 1, lane g). To determine whether the protein detected in Rat-1 cells is indeed a normal version of the p185 found in the B104-1-1 transformants, we compared the two proteins by partial proteolytic mapping. The proteins were purified by immunoprecipitation and gel electrophoresis from Rat-1 and B104-1-1 cells that had been metabolically labeled with $^{32}P_i$ and then digested with *S. aureus* V-8 protease or papain (Fig. 2). The near identity of the partial proteolytic fragments yielded by the two proteins confirmed that the protein detected in Rat-1 cells is closely related in structure to p185 and is probably the normal version of the p185 encoded by the *neu* oncogene. These results demonstrated that the mutation that activated the *neu* oncogene did not cause major structural changes in the gene product. We will denote the oncogenic alleles of *neu* and encoded gene products with an asterisk (*neu**, p185*).

p185* was also detected in the B104 cell line (54), the DNA of which originally yielded the B104 allele of the *neu* oncogene (Fig. 1, lane b). This protein was even less abundant in the B104 neuroblastoma cells than in Rat-1 cells (compare lanes b and d), suggesting that activation of the *neu* oncogene was not accompanied by increased expression of its gene product.

Biogenesis of p185. The diffuse profile of p185 on polyacrylamide gels suggested that the core polypeptide of p185

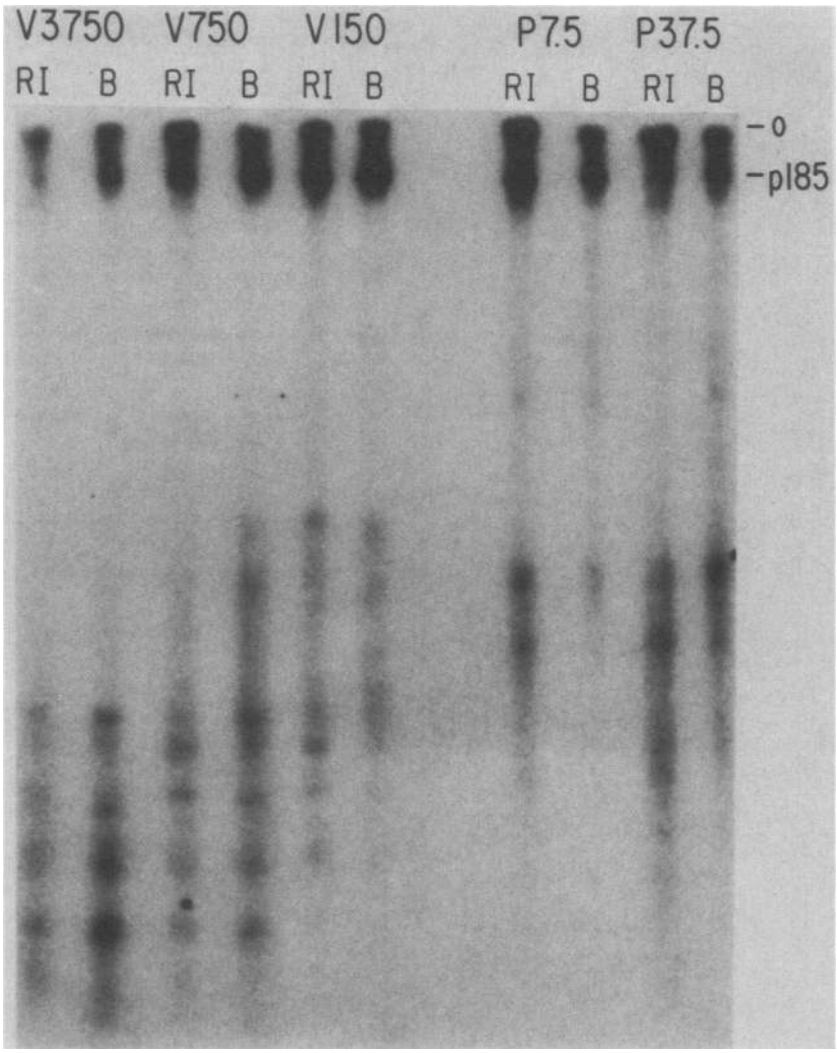

FIG. 2. Partial proteolytic analysis of p185 immunoprecipitated from Rat-1 cells and B104-1-1 cells. Two dense 100-mm cultures of Rat-1 cells and one dense culture of B104-1-1 cells were labeled with $^{32}P_i$ for 24 h. The cell lysates were immunoprecipitated with antibody 7.16.4 and fractionated by SDS-polyacrylamide gel electrophoresis. The gel was dried without fixation. p185 was analyzed by partial proteolysis as described by Cleveland et al. (5), except that EDTA was omitted from all solutions and the buffer for enzyme dilution and rehydration of the gel slices contained 1 mM 2-mercaptoethanol. Gel slices containing p185 were inserted into wells of a 20% acrylamide–0.08% bisacrylamide discontinuous SDS gel with a 20-mm stacking gel and overlaid with *S. aureus* protease (Miles Laboratories, Inc.) or papain (Sigma). The proteins were electrophoresed into the stacking gel and digested for 30 min at room temperature. The gel was exposed to preflashed film with an intensifying screen for 21 days at −70°C. 0, The origin of the resolving gel. Lanes: RI, p185 from Rat-1 cells; B, p185 from B104-1-1 cells; V3750, V750, and V150, p185 digested with 3,750, 750, and 150 ng of *S. aureus* V-8 protease, respectively; P7.5 and P37.5, p185 digested with 7.5 and 37.5 ng of papain, respectively.

is modified. To determine whether p185 undergoes posttranslational modification, we performed a pulse-chase experiment. B104-1-1 cells, which carry a transfected *neu** gene, were labeled with [^{35}S]cysteine for 10 min and lysed either immediately or after a 120-min chase (Fig. 3). The protein labeled during the 10-min pulse migrated at a rate suggesting that it was approximately 15-kilodaltons (kDa) smaller than its mature counterpart (Fig. 3, lane j). This more rapidly migrating protein was also visible as a minor band in lysates labeled to equilibrium (Fig. 3, lane l). The mobility of the protein decreased during the 2-h chase so that it comigrated with the bulk of p185* labeled to steady state (Fig. 3, lanes k and l), indicating that these posttranslational modifications are complete within 2 h of synthesis of the polypeptide.

We had shown previously that p185* is modified by phosphorylation (45). To determine whether addition of phosphate to the polypeptide is an early or late event in maturation of the protein, we performed a pulse-labeling experiment with $^{32}P_i$. In cells labeled for 10 min, most of the phosphate label appeared in the mature form of p185* (Fig. 3, lane g). This showed that the most abundant form of the protein is also the predominant substrate for phosphorylation.

To determine whether p185 bears asparagine-linked oligosaccharides, we examined the effect of tunicamycin on the size of the protein. This drug inhibits addition of N-linked oligosaccharides by blocking synthesis of the dolichol-linked precursor to the oligosaccharides (reviewed in reference 44). Because of the low amount of p185 in Rat-1

cells, we used DHFR/G-8 cells in these experiments. DHFR/G-8 cells were derived by transfection of a genomic clone of the *neu* proto-oncogene into NIH 3T3 cells and express high levels of p185 (24). Monoclonal antibody 7.16.4 specifically immunoprecipitated 170-kDa proteins from tunicamycin-treated B104-1-1 cells (Fig. 4, lane b) and DHFR/G-8 cells (Fig. 5, lane e). These proteins are likely to be p185* and p185 lacking N-linked oligosaccharides. p185* synthesized in the presence of tunicamycin migrated more rapidly than either mature p185* or its precursor (Fig. 6, compare lane c [tunicamycin] with lanes d and e). These results indicated that p185 bears N-linked oligosaccharides and that the oligosaccharides are added to the protein as it is being synthesized. The apparent increase in the size of the protein that occurs after translation is probably due to addition of terminal sugars and to phosphorylation, but other as yet unidentified modifications cannot be ruled out.

p185 is not the EGFr. We had shown previously that the *neu* oncogene is homologous to c-*erbB*, the gene that encodes the EGFr, and that p185 is serologically related to the EGFr (53). This raised the possibility that p185 is a form of the EGFr itself. The EGFr immunoprecipitated from Rat-1 cells and human A431 cells had an apparent molecular weight of 175,000, in agreement with published reports (Fig. 7, lane f and i) (7, 10, 12). This protein was well resolved from p185* immunoprecipitated from B104-1-1 cells (Fig. 7, lane b), and p185 from Rat-1 cells (Fig. 7, lane e) and DHFR/G-8 cells (Fig. 7, lane k). We have noted previously that the polyclonal anti-EGFr serum used cross-reacts with p185 (Fig. 7, lanes c and l) (53). These data demonstrated that p185 and the EGFr are distinct and that both proteins are displayed by normal rat cells. Since normal p185 could be distinguished from the EGFr, we concluded that differences between p185 and the EGFr are not attributable to the mutation(s) which activated *neu**.

It remained possible that the difference between the mobilities of p185 and the EGFr resulted from differential processing of a single-core polypeptide. Consequently, we compared the sizes of the polypeptides synthesized in the presence of tunicamycin. The EGFr synthesized in tunicamycin-treated Rat-1 cells and A431 cells had an apparent molecular weight of 140,000, consistent with previous reports (Fig. 4, lanes f and i) (12, 39). No large protein was specifically detected in tunicamycin-treated Rat-1 cells with 7.16.4 serum (Fig. 4, lanes d and e). This may be due to the instability of proteins synthesized in the presence of tunicamycin (44) and the low amount of p185 in these cells. However, B104-1-1 cells and DHFR/G-8 cells contained the 170-kDa protein described above, which migrated more slowly than the EGFr core polypeptide (compare Fig. 5, lanes a, c, and e). Since p185 and the EGFr exhibited different mobilities when synthesized in the presence of tunicamycin, it appeared unlikely that they are derived from differential modification of a common core polypeptide.

p185 is associated with tyrosine kinase activity. A number of oncogene-encoded proteins as well as the receptors for EGF, PDGF, insulin, and IGF-1 are tightly associated with tyrosine kinase activity (4, 6, 7, 19, 29, 32, 42, 47, 51). To determine whether p185 is also associated with tyrosine kinase activity, we prepared extracts of Rat-1 cells with nonionic detergent and isolated p185 and the EGFr by immunoprecipitation with appropriate antibodies. The immune complexes were then incubated with [γ-^{32}P]ATP and $MnCl_2$ in an attempt to label p185 by autophosphorylation. The Rat-1 EGFr was readily labeled under these conditions (Fig. 8, lane d). The monoclonal anti-p185 serum specifically

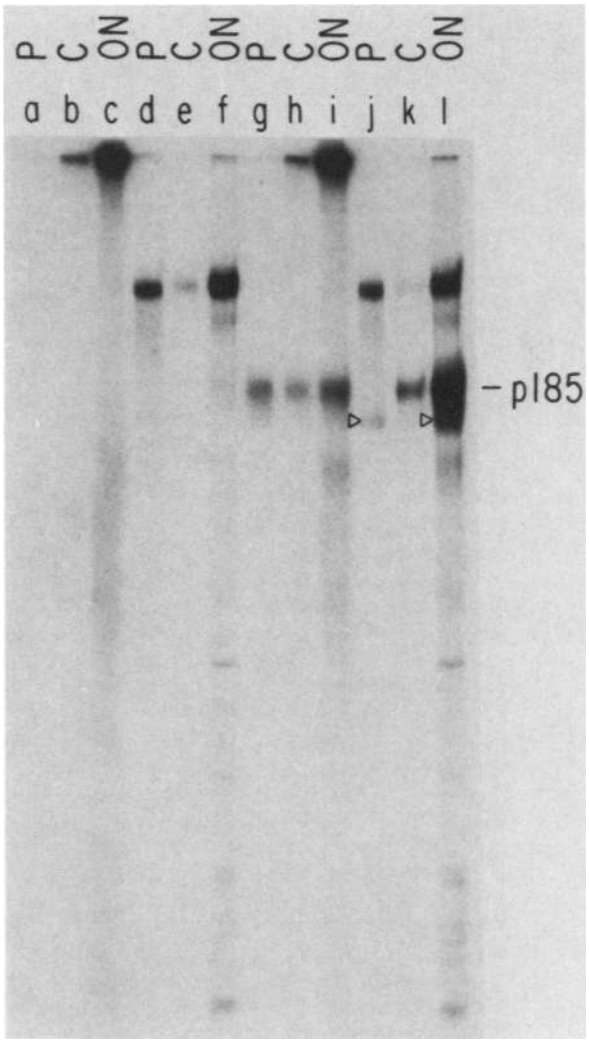

FIG. 3. Pulse-chase analysis of biogenesis of p185. B104-1-1 cells (3×10^6 cells per 60-mm dish) were labeled with $^{32}P_i$ (lanes a, b, c, g, h, and i) or [^{35}S]cysteine (lanes d, e, f, j, k, and l), and lysates were immunoprecipitated with normal mouse serum (lanes a, b, c, d, e, and f) or antibody 7.16.4 (lanes g, h, i, j, k, and l). Cells were labeled for 10 min (P; lanes a, d, g, and j), for 10 min, followed by a 120-min chase (C; lanes b, e, h, and k), or overnight (ON; lanes c, f, i, and l). The labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorographed gel was exposed to film for 7 days at room temperature. ▷, p185 precursor.

precipitated a protein that was labeled in vitro (Fig. 8, lane c) and migrated slightly faster than p185 precipitated from Rat-1 cells labeled metabolically (Fig. 8, lane a). Similarly, a protein that migrated slightly faster than the p185* labeled in vivo was detected in immune complexes prepared from B104-1-1 cells (Fig. 8, lanes f and h). The small mobility differences observed between the proteins labeled in vitro and in vivo are possibly due to differences in phosphorylation of the proteins.

The failure to detect the EGFr in immune complexes derived from B104-1-1 cells is due to the low amount of EGFr in these cells (see below; Table 1). Although the anti-EGFr serum could be used to precipitate p185* from RIPA lysates of cells labeled in vivo (Fig. 7, lane c), it did not precipitate p185* which could be labeled in vitro (Fig. 8, lane e). When immune complexes were prepared with a combi-

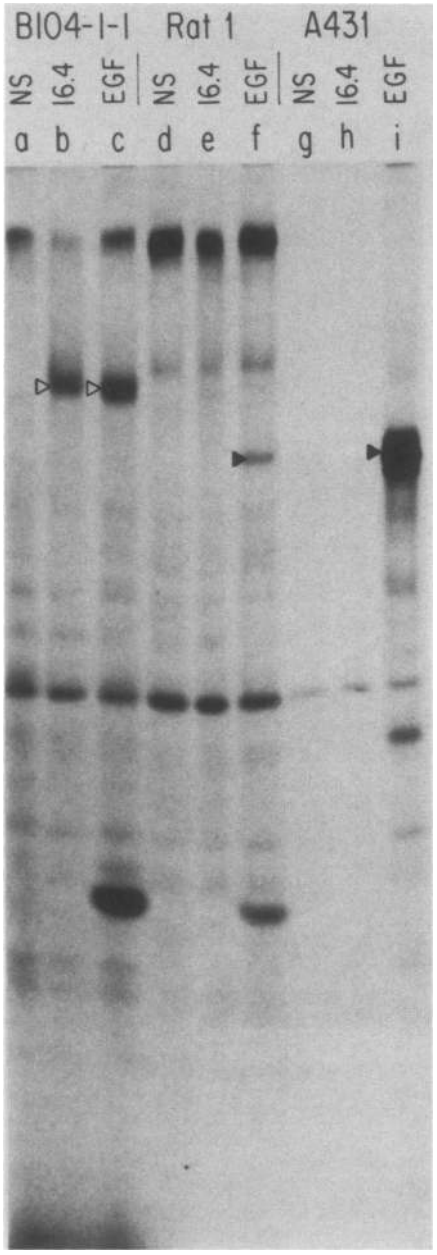

FIG. 4. Effect of tunicamycin on p185* and the EGFr. Cells (B104-1-1, Rat-1, or A431; 3×10^6) were labeled overnight with [^{35}S]cysteine in the presence of tunicamycin. Cell lysates were divided into three portions and immunoprecipitated with mixed normal mouse and rabbit sera (NS; lanes a, d, and g), monoclonal antibody 7.16.4 (16.4; lanes b, e, and h), or polyclonal anti-EGFr serum (EGF; lanes c, f, and i). The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorographed gel was exposed to preflashed film for 2 days at −70°C. ▷, p185; ▶, EGFr.

nation of both anti-EGFr serum and monoclonal 7.16.4 antibody, p185* could be labeled in vitro, indicating that the anti-EGFr serum does not inhibit the kinase activity of p185* (data not shown). This suggested either that the anti-EGFr serum recognizes only inactive (denatured) forms of p185 or that it fails to react at all with p185 solubilized under the mild conditions used for the immune complex kinase assays. The intensity of labeling of p185 and p185* in immune complexes prepared from Rat-1, B104-1-1, and DHFR/G-8 cells was proportional to the abundance of p185 and p185* in these cell lines. Thus p185 and p185* are labeled to the same extent in these assays.

We next determined the identity of the phosphate acceptor amino acids labeled in vitro. As expected, the EGFr contained one major labeled amino acid, phosphotyrosine (Fig. 9A). Similarly, the predominant labeled phosphoamino acid in both p185 and p185* was phosphotyrosine (Fig. 9B and C). We concluded that both normal and transforming forms of p185 are tightly associated with a tyrosine kinase activity.

Functional differences between p185 and the EGFr. Although p185 differs structurally from the EGFr, the homology of *neu* and *erbB* (53) suggested that they might nevertheless have similar or identical functions. To determine whether p185 and the EGFr respond in the same way to EGF, we compared the stability of the two proteins in normal Rat-1 cells after exposure to EGF. Binding of EGF to the EGFr is followed by internalization of the receptor-

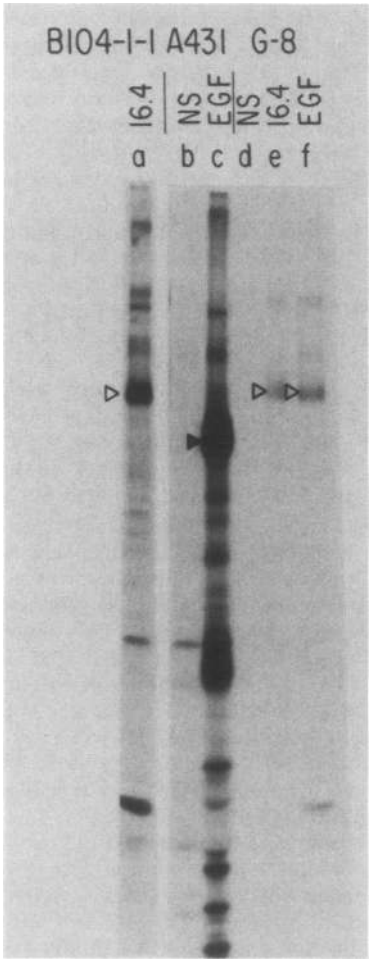

FIG. 5. Effect of tunicamycin on p185. B104-1-1 cells (1×10^6 cells), A431 cells (1×10^6 cells), and DHFR/G-8 cells (G-8; 3×10^5 cells) were labeled overnight with [^{35}S]cysteine in the presence of tunicamycin. Cell lysates were divided into three portions and immunoprecipitated with mixed normal mouse and rabbit sera (NS; lanes b and d), antibody 7.16.4 (16.4; lanes a and e), or polyclonal anti-EGFr serum (EGF; lanes c and f). The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorographed gel was exposed to preflashed film for 2 days at −70°C. ▷, p185; ▶, EGFr.

ligand complex and degradation of the receptor (12, 60). To compare the effects of EGF on the stability of the EGFr and p185, we labeled Rat-1 cells to steady state with [^{35}S]cysteine and chased with cold cysteine in the presence of EGF. After a 2-h chase, the EGFr was detectable in control cells (Fig. 10, lane c) but not in cells incubated with 15 nM EGF (Fig. 10, lane i). In contrast, the stability of p185 was unaffected by the presence of EGF (Fig. 10, compare lanes b and h).

In the same experiment we compared the response of p185 and the EGFr to another growth factor, TGF-α. This factor bears some sequence homology to EGF, binds to the EGFr, and induces the same physiological effects as EGF (A. B. Roberts and M. B. Sporn, Cancer Surv., in press). Incubation of Rat-1 cells with TGF-α resulted in the loss of immunoprecipitable EGF receptors (Fig. 10, lane f), indicating that this factor was binding to the EGFr and causing its turnover, as expected. Like EGF, TGF-α had no effect on the stability of p185 (Fig. 10, lane e). These results demonstrated functional distinctions between p185 and the EGFr and indicated that p185 is unlikely to be a receptor for EGF or TGF-α.

A partly purified preparation of PDGF and a crude platelet extract (which contained activity equivalent to 1 nM PDGF) were also assayed for their effects on the stability of p185 and the EGFr. Neither PDGF nor the platelet extract affected the stability of either p185 or the EGFr (Fig. 10, lanes k, l, n, and o). This suggested that PDGF and the factors present at high concentration in the platelet extract were not ligands for p185.

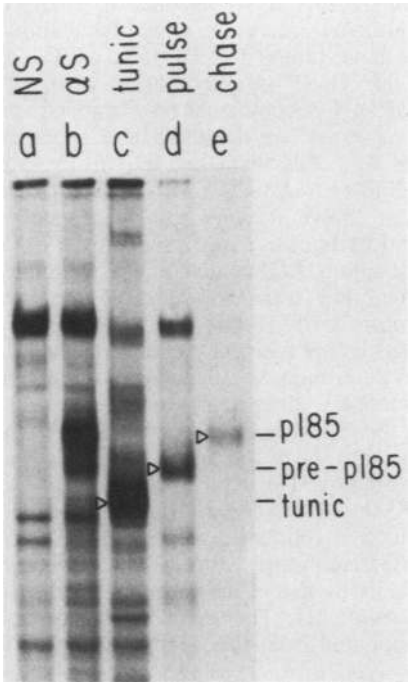

FIG. 6. Comparison of p185* labeled in the presence of tunicamycin with precursor and mature forms. Dense 35-mm cultures of B104-1-1 cells were labeled with [^{35}S]methionine and lysed for immunoprecipitation. Cells shown in lanes a, b, and c were labeled for 20 h in the absence (lanes a and b) or presence (tunic; lane c) of tunicamycin. Cells shown in lane d were labeled for 15 min (pulse), and cells shown in lane e were labeled for 15 min and then chased for 120 min in the absence of radiolabel (chase). Lysates were immunoprecipitated with normal rabbit serum (ns; lane a) or polyclonal anti-p185 serum (lanes b, c, d, and e). The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and the fluorographed gel was exposed to preflashed film for 8 days at −70°C. ▷, p185*.

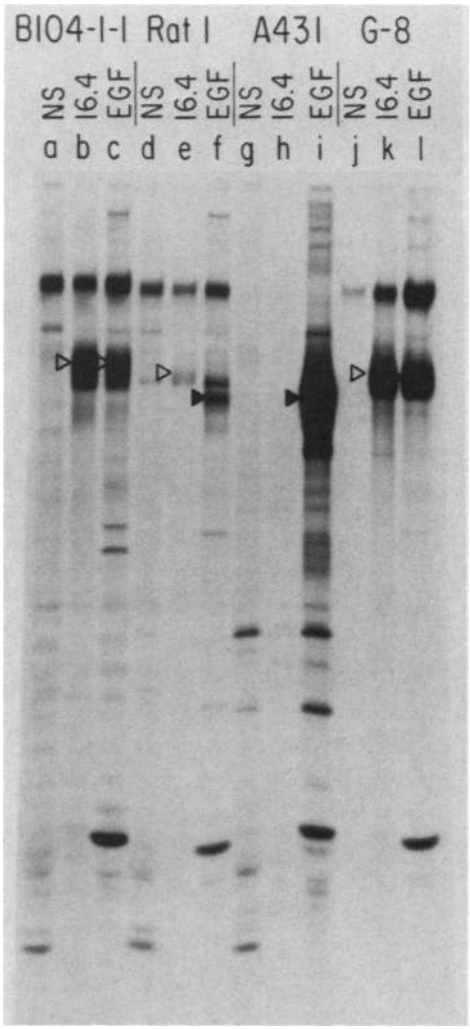

FIG. 7. Comparison of p185 and the EGFr. B104-1-1 cells (1 × 10^6 cells), Rat-1 cells (1 × 10^6 cells), A431 cells (1 × 10^6 cells), and DHFR/G-8 cells (G-8; 3 × 10^5 cells) were labeled for 15 h with [^{35}S]cysteine. The lysates were divided into three portions and immunoprecipitated with mixed normal mouse and rabbit sera (NS; lanes a, d, g, and j), monoclonal antibody 7.16.4 (16.4; lanes b, e, h, and k), or polyclonal anti-EGFr serum (EGF; lanes c, f, i, and l). The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis, and the fluorographed gel was exposed to preflashed film for 2 days at −70°C. ▷, p185; ▶, EGFr.

EGF binding to p185. The absence of p185 downmodulation by EGF did not rule out the possibility that EGF can bind to p185. To assess this, we used NIH 3T3 cells transfected with either a transforming allele of *neu* (B104-1-1) or a genomic clone of the *neu* proto-oncogene (DHFR/G-6 and DHFR/G-8) (24). These transfected cell lines contain high levels of p185* or p185 expressed at the cell surface. The B104-1-1 and DHFR/G-6 cells display approximately 10^5 molecules per cell, while the DHFR/G-8 cells display approximately 10^6 molecules per cell (as estimated by immunofluorescence and immunoprecipitation; [24]; J. Drebin, personal communication; D. F. Stern, un-

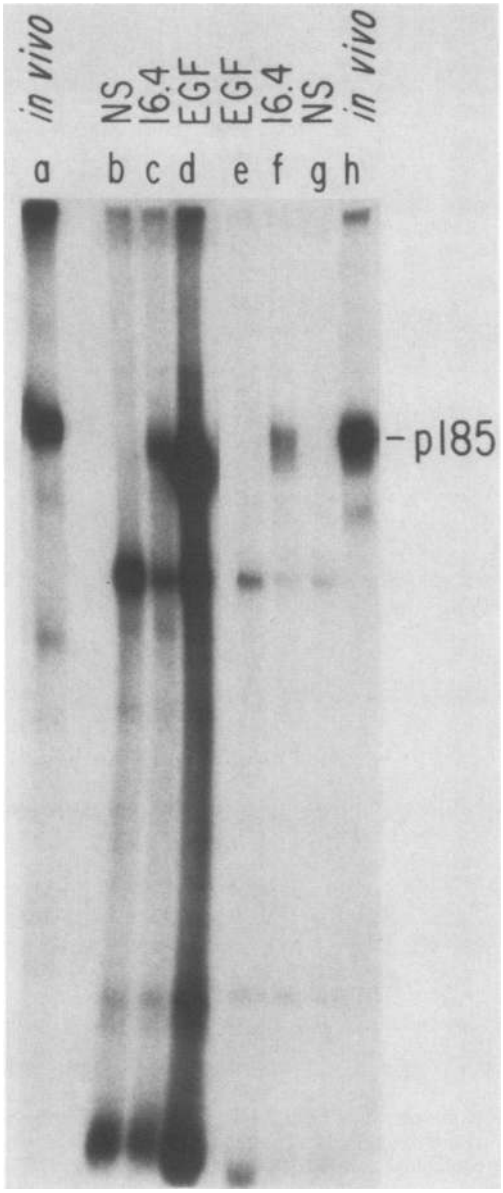

FIG. 8. Immune complex kinase assays. Immune complexes were prepared from Rat-1 cells (lanes b, c, and d) and B104-1-1 cells (lanes e, f, and g) and labeled by incubation with [γ-^{32}P]ATP as described in Materials and Methods. For markers, p185 was immunoprecipitated from Rat-1 cells (lane a) or B104-1-1 cells (lane h) labeled for 4 h in vivo with $^{32}P_i$ and immunoprecipitated with antibody 7.16.4. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes a through d and e through h are different exposures of adjacent sections of a single gel. Lanes a, b, c, and d were exposed to film for 20 h; lanes e, f, g, and h were exposed for 2 h. Lane a, Rat-1 cells labeled in vivo with $^{32}P_i$ and immunoprecipitated with antibody 7.16.4. Immune complexes from Rat-1 cells were prepared by immunoprecipitation with a mixture of normal mouse and rabbit sera (NS, lane b), antibody 7.16.4 (16.4, lane c), and anti-EGFr serum (EGF, lane d) and labeled in vitro. Immune complexes from B104-1-1 cells were prepared by immunoprecipitation with anti-EGFr serum (EGF, lane e), antibody 7.16.4 (16.4, lane f), and a mixture of normal mouse and rabbit sera (NS, lane g) and labeled in vitro. Lane h, B104-1-1 cells labeled with $^{32}P_i$ in vivo and immunoprecipitated with antibody 7.16.4.

TABLE 1. Binding of [^{125}I]EGF to cell monolayers[a]

Expt. no. and cell line	10^4 Molecules of EGF bound/cell at following EGF concn (nM)[b]:			
	1.6	8.5	15	45
1				
Rat-1	0.84 (3,477)		1.7 (7,013)	
B104-1-1	0 (−436)		0 (−13)	
2				
Rat-1	1.2 (3,786)	2 (6,227)		
DHFR/G-6	0 (−26)	0 (28)		
DHFR/G-8	0 (−46)	0 (−159)		
A431	55 (22,487)	190 (78,452)		
3				
Rat-1			0.4 (222)	0.7 (358)
DHFR/G-8			0.2 (242)	0.2 (218)
A431				120 (6,812)

[a] Binding of [^{125}I]EGF to cells was determined as described in Materials and Methods.

[b] The data shown in parentheses are the differences between the number of counts per minute bound in the absence and presence of 1 μg of nonlabeled EGF per ml, and they represent specific counts per minute bound. These values are not corrected for differences in cell number (which were corrected for in the final calculation of EGF bound per cell). In experiments 2 and 3, [^{125}I]EGF was diluted with nonlabeled EGF to a 50-fold lower specific activity for binding to A431 cells than that for binding to other cell lines.

published data). At concentrations of EGF near 10 to 15 nM (the dissociation constant of the EGFr [1]), Rat-1 cells bound up to 1×10^4 to 2×10^4 molecules of [^{125}I]EGF per cell, while A431 human tumor cells, which have amplified copies of the EGFr gene, bound 1×10^6 to 2×10^6 molecules per cell (Table 1). These numbers agree with the reported abundance of EGF receptors in rat fibroblasts and in A431 cells (1). In contrast, we detected little specific binding of EGF to NIH 3T3 cells (data not shown) or to the various NIH 3T3 cell lines transfected with normal or transforming alleles of *neu*. Since the *neu*-transfected cells contained between 5 and 50 times as many molecules of p185 per cell as Rat-1 cells contain EGFr molecules, we concluded that neither normal nor transforming p185 function as high-affinity receptors for EGF.

Role of p185 as the receptor for other growth factors. We attempted to determine whether p185 is identical to several previously identified growth factor receptors (Table 2). In some cases, the reported sizes of receptors for these factors differ substantially from the size of p185 (Table 2). Where possible, we employed structural or serological comparisons between p185 and the candidate receptors. (i) In an experiment performed in collaboration with A. R. Frackelton, we found that p185 comigrates with the PDGF receptor purified from NRK cells by use of an anti-phosphotyrosine column (data not shown; 21). The PDGF receptor could not be immunoprecipitated from this preparation with monoclonal 7.16.4 antibody. However, p185* was immunoprecipitated when the PDGF receptor preparation was mixed with radiolabeled B104-1-1 extract in a control reconstruction experiment. (ii) The rat TGF-β receptor, radiolabeled by cross-linking to [^{125}I]TGF-β, was not immunoprecipitated by 7.16.4 serum (Maureen O'Connor-McCourt, personal communication). (iii) The published nucleotide sequence of the human insulin receptor is only distantly related to the sequence of rat *neu* (3, 8, 62, 66). Polyclonal anti-insulin receptor serum A410 (27) failed to immunoprecipitate p185*. (iv) Anti-IGF-2 receptor serum (43) did not react with p185*.

We also tested whether p185 reacted with several known

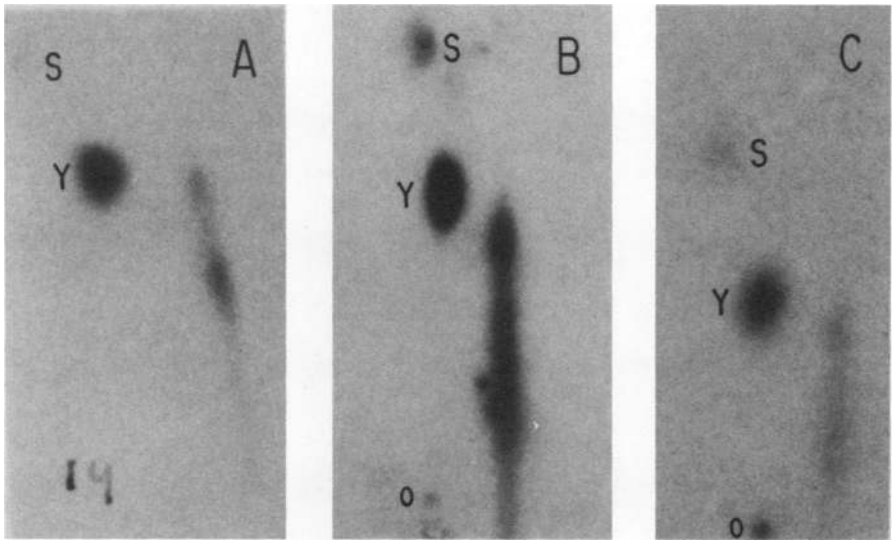

FIG. 9. Phosphoamino acid analysis of proteins labeled in the immune complex kinase assay. Immune complexes were prepared from Rat-1 cells or B104-1-1 cells with antibody 7.16.4 (B and C) or anti-EGFr serum (A) and labeled in vitro with [γ-^{32}P]ATP as a phosphate donor. The labeled proteins were resolved by preparative SDS-polyacrylamide gel electrophoresis, eluted from the gel, and subjected to partial acid hydrolysis. Phosphoamino acids were analyzed by two-dimensional electrophoresis on thin-layer cellulose plates. Electrophoresis in the first dimension was at pH 3.5 (anode at the top), and that in the second dimension was at pH 1.9 (anode at the left). Nonlabeled phosphoamino acid standards mixed in with the samples were located by staining with ninhydrin. S, The position of phosphoserine; Y, the position of phosphotyrosine; 0, the origin. Samples shown in panels A and C were isolated from the gel shown in Fig. 8, lanes d and f. The sample shown in panel B was prepared in a separate experiment. Thin-layer plates were exposed to preflashed film for 18 h (A and C) or 7 days (B) at −70°C with an intensifying screen. Phosphoamino acid analysis of EGFr from Rat-1 cells (A), p185 from Rat-1 cells (B), and p185* from B104-1-1 cells (C) labeled in immune complexes is shown.

growth factors. We used two functional assays to test every candidate ligand. Since binding of many growth factors to their receptors results in down-regulation of the receptor, we first determined whether the candidate factors caused increased turnover of p185 in Rat-1 cells. These experiments were similar to those shown in Fig. 10. Both 2- and 11-h chases were used to test all factors, since the time course of down-regulation can vary with the receptor and perhaps with the cell line. None of the factors tested substantially decreased the stability of p185 (Table 2).

A second assay measured the in vivo incorporation of $^{32}P_i$ into phosphotyrosine in p185. Since autophosphorylation of growth factor receptors associated with tyrosine kinases is stimulated by incubation of cells with the appropriate ligand, we determined whether any of the candidate factors stimulate tyrosine phosphorylation of p185. Rat-1 cells were labeled with $^{32}P_i$, then incubated with the candidate ligand for 5 min, and lysed. p185 was immunoprecipitated and purified by gel electrophoresis, and the radiolabeled phosphoamino acids analyzed. The major phosphoamino acid found in p185 labeled in vivo was phosphoserine, which was severalfold more abundant than phosphothreonine (data not shown). Phosphotyrosine was not detectable. EGF was the only one of the factors listed in Table 2 that stimulated phosphorylation of p185 on tyrosine (IGF-1 and IGF-2 were not tested, but insulin was used at concentrations at which it should bind to the IGF-1 receptor). Since EGF itself does not bind to p185, this result may indicate that p185 can serve as a substrate for the EGFr kinase. The results acquired to date suggest (but do not prove) that none of the factors tested is a bona fide ligand for p185.

DISCUSSION

Recent Southern blotting experiments have shown that *neu* and *erbB* are distinct genes and reside on different human chromosomes (34, 52). Furthermore, determination of the nucleotide sequence of cDNA clones of *neu** (3) and *neu* (8, 66) have permitted direct comparisons of the primary structures of p185 and the EGFr. As expected from the results presented here, the predicted amino acid sequence includes a domain that closely resembles the kinase domain of other tyrosine kinases. This is the region of greatest homology between the *neu* and *erbB* genes. *neu* is more closely related to *erbB* than are any of the other tyrosine kinase-encoding genes. However, as we show here, this structural homology is not paralleled by close functional analogy. If p185 functions as a growth factor receptor it must recognize a ligand other than EGF.

Architecture of p185. p185 and p185* lacking N-linked oligosaccharides have apparent molecular weights of 170,000. The mature form is 15 to 25 kDa larger, so as much as 25 kDa of the estimated molecular weight may be contributed by N-linked oligosaccharides. Assuming a mobility shift of 3 kDa per oligosaccharide chain, this is consistent with the six potential sites for addition of N-linked oligosaccharides predicted from the *neu* nucleotide sequence (3, 8, 66). One issue raised by the present work stems from the discrepancy between the apparent molecular weight of p185* synthesized in the presence of tunicamycin (170,000) and the 140-kDa size of the core polypeptide predicted from the nucleotide sequence (3). This discrepancy could be explained by aberrant mobility of the core polypeptide or other still unidentified modifications, e.g., *O*-glycosylation. Interestingly, the low-density lipoprotein receptor undergoes an unidentified modification which increases its apparent molecular weight by 40,000 (61).

Both the normal and transforming forms of p185 are associated with tyrosine kinase activity, a property that they share with several known growth factor receptors. Still other traits of p185 suggest that it functions as a growth factor

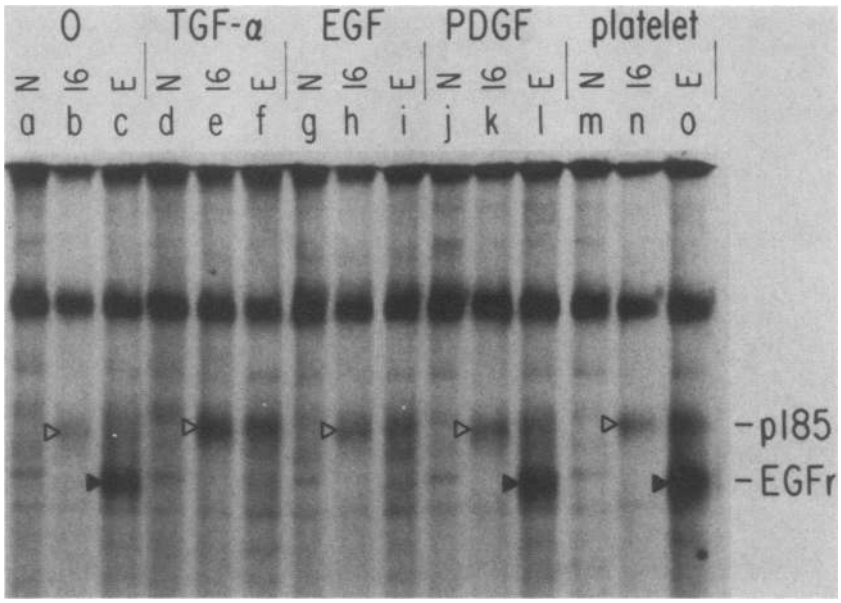

FIG. 10. Stability of p185 and the EGFr in the presence of growth factors. Rat-1 cells (10^6 cells per 6-cm plate) were labeled overnight with [^{35}S]cysteine. The labeling medium was washed out and replaced with 1.0 ml of DMEM containing 2% CS and 15 nM TGF-α (lanes d, e, and f), 15 nM EGF (lanes g, h, and i), 0.4 nM PDGF (lanes j, k, and l), or platelet extract (activity equivalent to 1 nM PDGF; lanes m, n, and o), and the cells were incubated at 37°C for 120 min. Cell lysates were divided into three portions and immunoprecipitated with a mixture of normal mouse and rabbit sera (N, lanes a, d, g, j, and m), antibody 7.16.4 (16, lanes b, e, h, k, and n), or anti-EGFr serum (E, lanes c, f, i, l, and o). The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorographed gel was exposed to preflashed film for 7 days at −70°C. The cells were chased as follows. 0, Rat-1 cells were chased for 2 h without additional growth factors; TGF-α, cells were chased in the presence of TGF-α; EGF, cells were chased in the presence of EGF; PDGF, cells were chased in the presence of PDGF; platelet, cells were chased in the presence of a platelet extract. ▷, p185; ▶, EGFr.

receptor. Like other, well-characterized receptors, it is a membrane-bound protein and is exposed at the cell surface. It is also closely related to a known growth factor receptor. The ability of altered p185 to transform cells is also compatible with a role of normal p185 in regulating cell proliferation.

We have failed to date to identify a ligand for p185. It is possible that the true ligand for p185 has not yet been purified or that it is one of the many partially characterized growth factors that we have not yet tested. The finding that neuropeptides such as bombesin, substance P, and substance K are mitogens (41, 50) indicates that the number of candidate factors remains quite large.

The identification of p185 in nontransformed rat cells has enabled us to compare the structures of p185 and p185*. These proteins had nearly identical electrophoretic mobilities. The forms of p185* encoded by *neu* oncogenes from four rat tumor cell lines, B50, B82, B103, and B104, all have the same mobility (45). Thus the activation of the *neu* oncogene that occurred during the creation of four independent neuroectodermal tumors was not accompanied by gross structural changes in the gene product. This raised the possibility that the mutations that activated the *neu* oncogene affected the abundance rather than the structure of the protein. However, this seems unlikely for two reasons. First, p185 is no more abundant in the B104 tumor cell line than it is in nontransformed Rat-1 cells (Fig. 1). Second, cell lines which overexpress normal p185 by a factor of 50 or 100 are not transformed, indicating that high-level expression of p185 is not sufficient for transformation (24). Taken together, these results imply that activation of the *neu* oncogene in rat neuroectodermal tumors is due to a subtle change in the structure of the encoded protein p185. Because the rat neuroectodermaltumors were induced by ethyl-nitrosourea,

TABLE 2. Assays for the p185 ligand[a]

Factor	Immunoprecipitation of:		Reported receptor subunit size (kDa)[d]
	p185 with anti-receptor serum[b]	Receptor with anti-p185 serum[c]	
EGF	+	−	170
TGF-α			170
TGF-β		−	280
PDGF		−	180
Insulin	−		90 + 135 (210 precursor)
IGF-1			90 + 135 (210 precursor)
IGF-2	−		220
FGF (pituitary)[e]			
NGF[f]			75 or 140

[a] Turnover of p185 was measured exactly as described in the legend to Fig. 10. Both 2- and 11-h chases were used to assay each factor. Increased turnover was not found with any factor used. Factors and concentrations were as follows: EGF, 15 nM; TGF-α, 15 nM; TGF-β, 100 ng/ml; PDGF, 0.4 nM; insulin, 50 μg/ml; pituitary fibroblast growth factor, 210 ng/ml; nerve growth factor, 100 ng/ml.

[b] Immunoprecipitation of p185 with anti-EGFr serum was shown previously (53). Rat-1 and B104-1-1 cells were labeled with [^{35}S]cysteine and immunoprecipitated with anti-EGF receptor serum, A410 anti-insulin receptor serum, or anti-IGF-2 receptor serum.

[c] See Fig. 1, lane d, and Fig. 10 for the EGFr. TGF-β and PDGF receptors are discussed in the text.

[d] References: EGF, 7, 9; TGF-β, 36; PDGF, 9, 22, 65; insulin and IGF-1, 14, 28, 31, 33; IGF-2, 33, 37, 46; NGF, 38, 49.

[e] FGF, Fibroblast growth factor.

[f] NGF, Nerve growth factor.

which causes point mutations, it is possible that p185 and p185* differ in only a single amino acid residue.

This subtle alteration in p185 structure is to be contrasted with the profound changes in structure observed in oncogenic forms of the EGFr (16, 40, 63). Thus studies of p185 may yield clues to receptor function not provided by the EGFr model. Moreover, the determination of functional differences between normal and transforming p185 provides a unique opportunity to identify the important second messenger(s) regulated by this family of receptors.

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