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The Extracellular Domain of p185/*neu* Is Released from the Surface of Human Breast Carcinoma Cells, SK-BR-3*

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James R. Zabrecky‡, Terence Lam, Sara J. McKenzie, and Walter Carney§

Applied bioTechnology, Inc., Cambridge, Massachusetts 02142 and §E. I. Du Pont deNemours and Co. Inc., North Billerica, Massachusetts 01862

The human breast carcinoma cell line SK-BR-3, expresses the *neu* oncogene product, p185, which is a receptor tyrosine kinase. Using a double monoclonal antibody capture enzyme-linked immunosorbent assay for p185, activity was detected in conditioned media from cultures of SK-BR-3 cells. Two monoclonal antibodies specific for the extracellular domain of p185/*neu* immunoprecipitated a protein with a molecular mass of approximately 105 kDa. p105 was further shown to compete with p185 for binding to monoclonal antibodies and pulse-chase experiments indicate that it was generated by post-translational processing. Peptide maps showed that p105 and p185 are related polypeptides. Since p105 is close to the predicted size for the extracellular domain of p185/*neu*, we propose that SK-BR-3 cells specifically process and release this portion of the receptor into the medium. The release of the extracellular domain may have implications in oncogenesis and its detection could prove useful as a cancer diagnostic.

The *neu* oncogene (HER-2, c-erbB-2) is a member of the tyrosine protein kinase class of oncogenes which possess tumorigenic or transforming activity (1–3). The gene product shows structural and functional homology to growth factor receptors particularly the epidermal growth factor (EGF)[1] receptor. The most extensive homology is in the tyrosine kinase domain and it is this activity that is believed to be responsible for signal transduction and growth regulation.

The *neu* oncogene product (p185) is a 185-kDa transmembrane glycoprotein. From the deduced amino acid sequence it possesses a cysteine-rich extracellular domain believed to function in ligand binding, a single membrane-spanning domain of about 23 amino acids and a cytoplasmic domain that possesses tyrosine protein kinase activity. The putative endogenous ligand has not been identified. p185/*neu* presumably exerts its effects on cell growth through activation of the tyrosine kinase activity; however, the mechanism by which this activity is regulated is not well understood. A single point mutation in the transmembrane region (4, 5) or simply overexpression of the *neu* gene (6) leads to full oncogenic activation. Receptor dimerization has also been discussed as a mechanism for enzymatic, and hence oncogenic, activation (7, 8).

There is mounting evidence that alterations in the structure and/or expression of the *neu* gene product play a role in human malignancies (9–11). Analysis of a series of human mammary tumor cell lines showed overexpression of the *neu* gene as well as elevated levels of the gene product, p185 (12). Gene amplification of *neu* was found in a primary mammary carcinoma (13) as well. The *neu* gene was amplified in 25–30% of human breast and ovarian cancers and this correlated with poor prognosis (10). Overexpression of this gene was found to be the second most predictive independent variable for patient survival after nodal status. The *neu* gene product thus appears to have a significant influence on cellular growth control and its quantitation could have important prognostic value.

We have used a series of *neu*-specific monoclonal antibodies (14) to examine expression of the *neu* gene product in the human mammary adenocarcinoma cell line, SK-BR-3. Using a capture ELISA, we were able to detect soluble p185/*neu* activity in clarified, conditioned media from these cells. Further analysis revealed that this activity is associated with a *neu*-related protein having a molecular mass of about 105 kDa, a value that closely matches the predicted size of the extracellular domain of p185/*neu* (~118 kDa). Several lines of evidence are presented which demonstrate that this is in fact the extracellular domain and that it is released from the surface of SK-BR-3 cells. We will discuss the possible significance of this finding in cell transformation and potential applications in cancer diagnostics.

EXPERIMENTAL PROCEDURES

Materials—SK-BR-3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. PB3, NB3, and TA1 monoclonal antibodies were prepared as described (14). TA1 was biotinylated using NHS-biotin from Sigma. Biotinylated c-*neu* (Ab-1) rabbit polyclonal antibody was purchased from Oncogene Sciences, [^{35}S]Cysteine was from Du Pont-New England Nuclear.

Radioimmunoprecipitation—A subconfluent monolayer of cells in a 10-cm Petri dish was labeled overnight with 500 μCi of [^{35}S]cysteine in 10 ml of cysteine-free Dulbecco's modified Eagle's medium plus 10% fetal calf serum. The supernatant was collected and clarified by centrifugation at 15,000 × *g* for 15 min. The cells were scraped from the plate, washed 2 × with phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4), and lysed with IP (immunoprecipitation) buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 10 mM Tris, 0.65 M NaCl, pH 7.2) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and soybean trypsin inhibitor (100 μg/ml). For immunoprecipitation, samples were incubated overnight at 4 °C with 5 μg of antibody. Each sample was then incubated for 2 h at 4 °C with 50 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.). In the case of TA1, which is an IgG_1, the protein A-Sepharose was first mixed with 20 μg of a capture antibody, rabbit anti-mouse IgG_1 (Organon Teknika). The protein A-Sepharose was pelleted by centrifugation, washed 4 × with

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‡ To whom correspondence should be addressed: Applied bioTechnology, Inc., 80 Rogers St., Cambridge, MA 02142. Tel.: 617-492-7289.

[1] The abbreviations used are: EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

IP buffer and once with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 8.2). The pelleted Sepharose was then boiled for 5 min in SDS sample buffer and run on SDS-polyacrylamide gels (15). The gels were fixed, treated with EN^{3}HANCE (Du Pont-New England Nuclear), dried, and fluorographed.

neu Capture ELISA—96-Well microtiter plates (Nunc) were coated with NB3 (20 μg/ml in 50 mM sodium carbonate, pH 9.6; 100 μl/well) for 2 h at 37 °C. The plate was washed 3 × with wash buffer (10 mM phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween). 100 μl of sample to be assayed, diluted in 50% normal goat serum, was added to each well then incubated overnight at 4 °C. The plate was again washed 3 × with wash buffer, and 100 μl of biotinylated TA1 (0.88 μg/ml in 50% normal goat serum) added to each well and incubated for 2 h at 37 °C. Following another wash, 100 μl of avidin-horseradish peroxidase (1 μg/ml in 10% normal goat serum) was added incubated for 1 h at 37 °C, then the plate was developed for 5 min using tetramethylbenzidine (Sigma) and stopped with H_2SO_4. The absorbance was measured at 450 nm. A whole cell lysate prepared from a *neu*-transfected NIH 3T3 cell line designated 17-3-1 (14) was used to generate a standard curve using an arbitrary definition for units.

Pulse-chase—A nearly confluent 10-cm plate of SK-BR-3 cells was labeled overnight as described above. Aliquots were collected over time and stored at −80 °C. Samples were then thawed, clarified by centrifugation, and immunoprecipitated with the PB3 monoclonal antibody. These were divided and counted for ^{35}S or separated by SDS-PAGE and fluorographed.

Antibody Competition—An ELISA format similar to that described above was used to demonstrate competition for antibody binding between the two antigens p105 and p185. p185 was prepared as a cell lysate from 17-3-1 cells. p105 was prepared from SK-BR-3 culture supernatants that had been clarified then concentrated 10-fold in Amicon concentrator using a YM-10 membrane (Amicon). Units of *neu* activity were determined by capture ELISA. Plates were coated with NB3 as described above. A constant, near-saturating amount of p185 was added to each well. This was followed by the addition of increasing amounts of p105 antigen up to a 10-fold excess over p185. Duplicate samples were developed using one of two biotinylated detection antibodies: TA1 which recognizes the extracellular domain or c-*neu* which recognizes the cytoplasmic domain.

Peptide Map—^{35}S-Labeled SK-BR-3 culture supernatants and 17-3-1 cell lysates were prepared as described, immunoprecipitated with the PB3 antibody, and separated on a 6% SDS-PAGE. Using Rainbow M_r markers (Amersham Corp.) as a guide, bands were cut from the gel at the appropriate molecular weight for p105 and p185. The gel slices were minced with a razor blade, placed in a microcentrifuge tube along with 2.5 μg of Endoproteinase Glu-C (Boehringer Mannheim) in 40 μl of 25 mM ammonium bicarbonate, pH 7.8, and incubated for 1 h at 37 °C. SDS sample buffer was added, the mixture boiled for 5 min then applied to a 15% SDS gel. The gel was fluorographed as described above.

RESULTS

It has been previously demonstrated that SK-BR-3 cells overexpress p185/*neu*. We have confirmed this result by measuring p185 activity in whole cell lysates of SK-BR-3 cells by means of a capture ELISA. Immunoprecipitation using a *neu*-specific monoclonal antibody also revealed the presence of a band at 185 kDa (Fig. 1; *lane 1*). *neu* immunoactivity was also detected in the conditioned media from cultures of SK-BR-3 cells. Table I shows the relative activity from the *neu* capture ELISA which is cell associated compared to that detected in the conditioned media. We found that 10% of the total activity produced in a confluent 10-cm plate of SK-BR-3 cells is present in the media following three days in culture. Prior to analysis, the conditioned media was always clarified by centrifugation at 15,000 × *g* for 15 min. This suggests that the activity is in soluble form and not simply derived from membrane fragments of damaged or dead cells.

To further characterize the activity, 1 ml of clarified conditioned media was immunoprecipitated with two monoclonal antibodies, TA1 and PB3. Fig. 1, *lanes 2* and *3*, shows that using either antibody the predominant immunoprecipitable

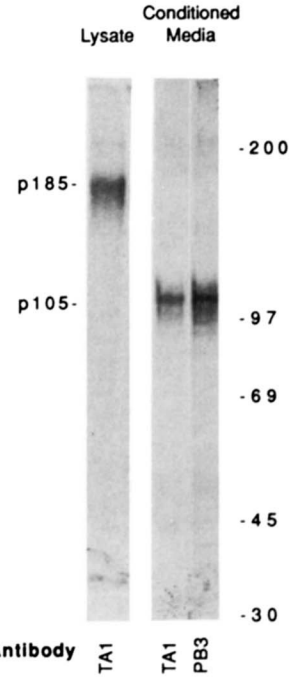

FIG. 1. **Immunoprecipitation of lysate and conditioned media.** [^{35}S]Cysteine-labeled whole cell lysate from *neu*-transfected NIH 3T3 cells and clarified conditioned medium from SK-BR-3 cells were prepared and immunoprecipitated as described under "Experimental Procedures." Two monoclonal antibodies (TA1 and PB3) which are specific for the extracellular domain of p185/*neu* were used. Immunoprecipitated samples were separated by SDS-PAGE and fluorographed.

TABLE I

p185/neu ELISA activity in SK-BR-3 cells

ELISA activity from one 10-cm plate (3 × 10^6 cells) of SK-BR-3 cells after 3 days in culture. Activity is expressed as arbitrary units relative to a whole cell lysate standard.

	Units (× 10^{-6})	% of total
Total activity	4.3	100
Cell-associated	3.9	90
Media	0.42	10

115 kDa and hence will be referred to as p105. This value is close to that predicted for the extracellular domain of p185, about 118 kDa. The three *neu*-specific monoclonals used in these experiments (TA1 and NB3 in the capture ELISA; TA1 and PB3 in immunoprecipitation) have been shown to recognize independent epitopes on the extracellular domain of p185/*neu* (14).

To determine if p105 was generated by *de novo* synthesis or by post-translational processing of p185, a pulse-chase experiment was performed. A confluent 10-cm plate of SK-BR-3 cells was labeled with [^{35}S]Cys for 24 h. The medium was replaced with unlabeled medium, and small aliquots were collected over time. Each sample was clarified by centrifugation, immunoprecipitated with the PB3 monoclonal then either counted for ^{35}S or separated by SDS-PAGE and fluorographed. Fig. 2 shows *neu*-specific immunoprecipitable counts increased over the course of 54 h and this corresponded to an increase in the intensity of the bands at 105 kDa (see *inset*). If this material was synthesized from an alternate message, there should be no increase in incorporation since the labeled amino acid had been removed. On the contrary, p105 that had been labeled during the pulse continued to accumulate in the media over time. Moreover, Northern

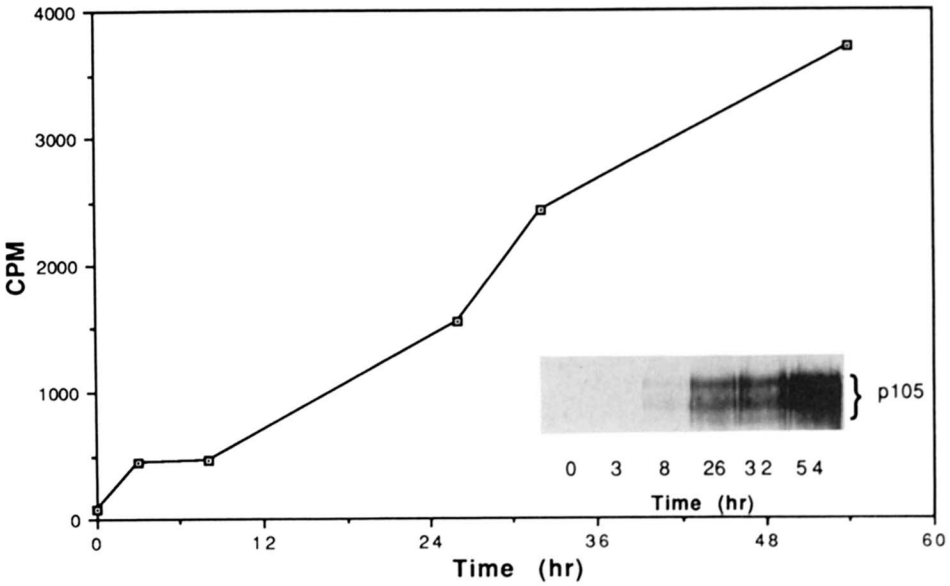

FIG. 2. **Pulse-chase experiment showing release of p105 from SK-BR-3 cells.** A near-confluent culture of SK-BR-3 cells was labeled overnight with [^{35}S]cysteine. The medium was removed and replaced with unlabeled medium (time = 0). Aliquots were collected over time and immunoprecipitated with the monoclonal antibody PB3. Duplicate samples were counted for ^{35}S or separated by SDS-PAGE and fluorographed (*inset*).

experiments (data not shown) detected no aberrantly sized *neu* mRNA. Therefore, p105 appears to be generated as the result of post-translational processing, presumably proteolysis of cell surface p185/*neu*.

Immunoprecipitation of this putative extracellular domain from SK-BR-3 conditioned medium generally yields a rather diffuse band at around 105 kDa (see Figs. 1–3). In fact, this often appears as a broad doublet with the intensity of the lower band varying from one preparation to the next. The same result is sometimes seen with the intact receptor, p185, immunoprecipitated from cell lysates. This heterogeneity could be due either to differences in glycosylation, since the extracellular domain is thought to be heavily glycosylated or to proteolytic processing or degradation.

Immunological analysis was used to confirm the relationship of p105 to the *neu* gene product. If p105 is truly derived from the extracellular domain of p185/*neu* it should be competitive with the intact receptor for binding to the *neu*-specific antibodies. This was tested in two ways. First, immunoprecipitation was used to show that a 10-fold excess of p185 could compete with radiolabeled p105 for binding to the monoclonal antibody, PB3. p185 as part of a whole cell lysate from 17-3-1 cells (see "Experimental Procedures") and ^{35}S-labeled p105 from SK-BR-3 cell conditioned medium were prepared and assayed for activity by ELISA. Antibody was titrated to ensure that its concentration was limiting in the immunoprecipitation. Fig. 3, *lane 1*, shows an immunoprecipitation of the labeled conditioned media. *Lane 2* shows that when the antibody/antigen mixture is co-incubated with a 10-fold excess of unlabeled p185, the p105 band disappears. A comparable amount of nonrecombinant NIH 3T3 cell lysate had no effect (*lane 3*). This experiment indicates that an excess of p185 specifically competes with the p105 antigen for binding to the *neu*-specific monoclonal.

The narrowest interpretation of the above experiment is that the monoclonal antibody has a greater affinity for p185 than for p105. This is not surprising since p185 was the original antigen to which the monoclonal was raised. Therefore, it was important to demonstrate the reverse competition, that is, that an excess of p105 effectively competes off p185. However, it was difficult to prepare sufficient quantities of labeled p105 at high enough concentration to perform this experiment, so a capture ELISA format was used instead.

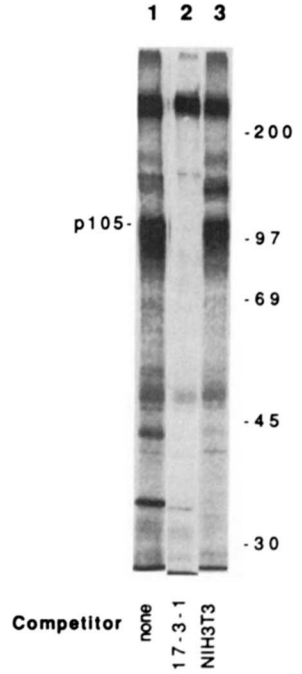

FIG. 3. **Competitive immunoprecipitation of p105.** SK-BR-3 conditioned medium was labeled overnight with [^{35}S]cysteine. Unlabeled p185 was prepared as a whole cell lysate from 17-3-1 cells. Units of activity were determined by capture ELISA. The concentration of monoclonal antibody PB3 was titered so that it would be limiting with respect to antigen. *Lane 1* shows a straight immunoprecipitation of labeled conditioned media. *Lane 2* was immunoprecipitated under the same conditions except that a 10-fold excess of unlabeled p185 was added. To control for nonspecific inhibition, the immunoprecipitation was also done in the presence of a comparable amount of cell lysate from nontransfected NIH 3T3 cells (*lane 3*).

distinguish between captured p105 and p185, a different detector antibody, c-*neu*, which is specific for the C terminus of p185/*neu* was used. The specificity was confirmed by testing 17-3-1 cell lysates (p185) and SK-BR-3 media (p105) samples in this assay. c-*neu* gave a positive signal for p185 but was negative for p105, whereas TA1 produced a positive signal for both antigens (data not shown). For the competition experiment, a constant amount of p185 antigen was added to each well. Increasing levels of p105 antigen up to a 10-fold excess

way using either biotinylated c-*neu* or TA1. Using the signal in the absence of any added p105 as 100%, the results are plotted as the percent of p185 bound *versus* the ratio of p105 to p185 in each sample well (Fig. 4). As the level of competing antigen was increased the signal decreased to as low as 30% of control at a 10-fold excess of competitor (*open squares*). Under ideal conditions where all antigens have equal affinities for the antibodies, one would expect to see about 10% of the p185 remaining bound in the presence of 10-fold excess competitor. To control for nonspecific inhibition by other components in the p105 preparation, an identical series of samples was developed with the TA1 antibody which detects either of the bound antigens. The signal remained near the maximal level (*triangles*). These results indicated that p105 can effectively compete with p185 with near equal affinity.

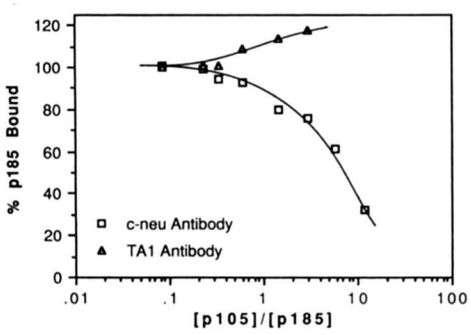

FIG. 4. **Competitive ELISA.** SK-BR-3 cell conditioned media and 17-3-1 cell lysate were prepared and assayed for activity by the standard *neu* ELISA (see "Experimental Procedures"). For the competitive assay, an ELISA plate was coated with monoclonal antibody NB3 and a constant, near saturating level of p185 antigen was added to each well. Each sample was then co-incubated with an increasing concentration of competing antigen, p105, up to a 10-fold excess. The detector antibody was either TA1 (detects p105 and p185) or c-*neu* (only detects p185). The ELISA was developed as described. The signal obtained in the absence of added competing antigen was defined as 100% p185 bound and all values were normalized to this level. Data are plotted as the percent of p185 bound *versus* the ratio of p105 to p185 in the sample well. The signal with the TA1 antibody stays near the saturating level, indicting that there is no nonspecific inhibition from the p105 preparation.

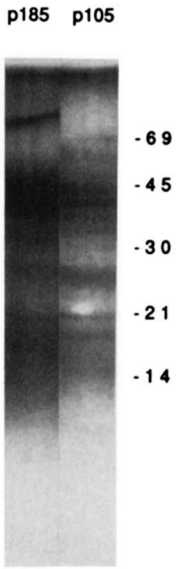

FIG. 5. **Peptide map of p185 and p105.** [^{35}S]Cysteine labeled SK-BR-3 supernatant and 17-3-1 cell lysate were prepared as described. Each was immunoprecipitated with monoclonal antibody PB3 and separated on a 6% SDS-PAGE. Slices of the gel at the appropriate molecular weight for p185 and p105 were cut from the gel, minced with a razor blade, and digested with Endoproteinase Glu-C as described under "Experimental Procedures." Digestion mix-

Finally, both antigens were subjected to peptide mapping analysis. ^{35}S-Labeled SK-BR-3 conditioned medium and 17-3-1 cell lysates were immunoprecipitated with PB3 and separated by SDS-PAGE. The bands corresponding to the appropriate molecular weight were extracted from the gel, minced, digested with Endoproteinase Glu-C (V8 protease) and then separated on a second SDS gel. The results are shown in Fig. 5. Many of the peptide bands line up in the two samples suggesting that p105 and p185 are related proteins. The extra bands seen in the p185 sample were presumably derived from the cytoplasmic domain which is predicted to be absent in p105.

DISCUSSION

We have described a p185/*neu* immunoreactive polypeptide that is found in the media of cultured SK-BR-3 cells. This protein has an apparent molecular mass of 105 kDa and is cross-reactive with three monoclonal antibodies that are specific for independent epitopes on the extracellular domain of p185/*neu*. By two methods, we have demonstrated that this protein is competitive with p185 for binding to *neu*-specific monoclonal antibodies and peptide mapping showed that p105 and p185 are related polypeptides. Pulse-chase results suggest that p105 was generated by post-translational processing. We and others have been unable to detect an alternate transcript that would account for this form as is true for A431 cells which secrete high levels of a truncated EGF receptor (16, 17). On the basis of this evidence, we contend that p105 is the extracellular domain of p185/*neu* and is released from the surface of SK-BR-3 cells probably as the result of proteolysis.

The mechanism by which *neu* and other receptor tyrosine kinases induce transformation is not well understood. However, it is clear that activation of the tyrosine kinase is essential and it now appears that this activity can be induced in several different ways. The role of overexpression of receptor tyrosine kinases has been well documented (18, 19). The binding of ligand is certainly fundamental in the activation and modulation of kinase activity but, in the case of *neu*, the endogenous ligand has not yet been identified. Mutation, as in the replacement of Val with Glu in the transmembrane domain (4), results in constitutive activation of the tyrosine kinase and transforming activity. Finally, many experiments have suggested a role for the extracellular domain in receptor regulation. The oncogenic form of EGF receptor (v-*erb*B) resembles a truncated receptor lacking its extracellular domain (16). This form possesses tyrosine kinase activity that may be constitutively active (20). Furthermore, exposure to EGF was shown to induce an N-terminal truncation in the intact EGF receptor in a protease dependent manner and it was proposed that this form could possess altered intracellular signaling capacity (21). Expression in chicken fibroblasts of a truncated EGF receptor lacking its ligand binding domain resulted in a weak but constitutive oncogenic activation (22). Similar experiments with *neu* have produced more dramatic results. Expression of N-terminally truncated *neu* in NIH 3T3 cells resulted in a 10-fold greater transforming activity compared to the full length gene (6). Cells transfected with this construct were also the most potent in inducing tumors in nude mice.

Much of the evidence discussed above suggests that the remaining cell-associated cleavage product, made up of the transmembrane and cytoplasmic domains, represents an ac-

tion due to overexpression could simply result from a proportional increase in the absolute number of truncated receptors. Ligand binding or mutation in the transmembrane region could induce a conformational change rendering the receptor more susceptible to proteolysis and hence create a transforming molecule. It seems plausible that proteolytic release of the extracellular domain is part of a mechanism by which the tyrosine kinase becomes activated. This process could involve a specific but as yet unidentified membrane-associated protease. Such a protease could be subject to its own set of regulatory constraints and also provide a means for cross-talk between different signal transduction systems. To determine if cleavage of the extracellular domain which we have observed in SK-BR-3 cells is in this way related to their transformed state will require further experimentation.

Proteolysis is likely to be important in the normal process of receptor down-regulation. All known receptor tyrosine kinases are down-regulated in response to the binding of ligand. From studies with receptor mutants, this process has been shown to require an active tyrosine kinase (23–25). However, in the case of the c-*fms* proto-oncogene product, which is the CSF-1 receptor, treatment of cells with phorbol ester induced cleavage and release of the extracellular domain (24). This process, which involves a specific protease activated by protein kinase C, was proposed as an alternate route to ligand induced receptor down-regulation that is independent of tyrosine kinase activity. In preliminary studies in our laboratory, exposure of SK-BR-3 cells to phorbol ester resulted in a 3–4-fold increase in *neu* immuno-reactivity in the cell culture medium (data not shown). The present finding that 10% of the extracellular domain is released in the absence of added mitogen is not well understood. It is possible that this is part of a ligand independent mechanism for normal receptor turnover.

The detection of the extracellular domain of p185/*neu* has potential diagnostic and prognostic value. Amplification and/or overexpression of the gene has been correlated with human malignancies. In a recent study by Hayes *et al.*,[2] the *neu* capture ELISA was used to detect a *neu*-related protein in the plasma of women with breast cancer. Significantly higher circulating levels of this activity were found in patients with metastatic disease as compared to normal control subjects. Biochemical analysis of this circulating activity is underway. It is an interesting possibility that this immuno-reactivity represents the extracellular domain which is shed from tumors overexpressing *neu*.

Much additional work is required in order to establish a role for proteolysis in kinase activation versus normal receptor down-regulation. It will be interesting to identify and explore the effect of specific protease inhibitors on kinase activity and growth control. Identification and purification of the protease(s) and manipulation by recombinant DNA techniques would contribute to a better understanding of the role of proteolysis in carcinogenesis.

[2] D. F. Hayes, W. Carney, C. Tondini, D. Petit, S. J. McKenzie, C. Henderson, D. W. Kufe, manuscript in preparation.

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Note Added in Proof—During the preparation of this manuscript we became aware of a similar observation by Lin and Clinton (Lin, Y. J. and Clinton, G. M. (1991) *Oncogene* **6,** in press) of a soluble form of p185/*neu* released from breast carcinoma cells.

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