Experimental approaches to hypothetical hormones: Detection of a candidate ligand of the neu protooncogene

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Contributed by Robert A. Weinberg, December 22, 1988

ABSTRACT There is a growing list of oncogenes encoding transmembrane tyrosine kinases that have structures reminiscent of growth factor receptors. In most cases, the ligands for these putative receptors are unknown. Using the neu oncogene as a model system, we have developed several experimental approaches for the detection of such hypothetical ligands. The following lines of evidence collectively imply that a candidate ligand of the neu-encoded oncprotein is secreted by ras-transformed fibroblasts: Medium conditioned by ras transformants is able to induce down-modulation of the neu-encoded p185 and to activate its intrinsic tyrosine kinase activity in vitro. In addition, a rapid increase in the phosphorylation in vivo of tyrosine residues of the neu-encoded protein is induced by the conditioned medium. Finally, transfer of the neu gene into hematopoietic cells renders them mitogenically responsive to the conditioned medium. The possibility of indirect activation of the oncprotein through other known receptors, especially the receptor for the epidermal growth factor, was experimentally excluded.

A variety of genetic methods including gene transfer, mutational analysis, and low-stringency hybridization have made it possible to isolate a group of genes that encode transmembrane proteins having the attributes of growth factor receptors. This growing list includes the protooncogenes neu (1–3), c-kit (4), ros (5), met (6), trk (7), ret (8), and other genes such as eph (9), fur (10), and the Drosophila sevenless gene (11). Most of these putative receptors carry a tyrosine-specific protein kinase function in their cytoplasmic portions. Some of these proteins are overexpressed in neoplastic tissues (12–14), suggesting a role in growth regulation. Perhaps the most compelling examples are the neu and kit protooncogenes that display remarkable structural homology with established receptors for the peptide growth factors epidermal growth factor (EGF) and colony-stimulating factor 1 (CSF-1) (15).

We have sought to establish methodological approaches for the detection of ligands to such putative receptors. In practice, systematic screening of potential sources of the ligands calls for assays that are sensitive to low concentrations of ligands and at the same time capable of reliable detection of ligand when it is present in a complex mixture of unfractionated biological macromolecular extracts or body fluids. Using the neu protooncogene as a model system, we present here several independent detection methods that suggest that a candidate ligand of the neu-encoded oncprotein is secreted by fibroblasts upon transformation by ras oncogenes.

MATERIALS AND METHODS

Materials. Affinity-purified goat anti-mouse IgG was from Fischer and it was radiolabeled with Na225I (Amersham) by the chloramine-T method (16). Radiolabeled protein A and 32P were from New England Nuclear (DuPont).

Cell Lines. The DHER-G8 cell line is a derivative of NIH 3T3 fibroblasts transfected with a genomic clone of the normal rat neu gene and selected for overexpression (17). Rat-1-EJ cells are rat fibroblasts transfected with an activated Ha-ras gene (pUC-EJ 6.6; ref. 18). Other cell lines were obtained from the American Type Culture Collection.

Antibodies. Monoclonal anti-p185 neu antibody 7.16.4-IgG (19) was purified by protein A affinity chromatography from ascites fluid. Antiserum to a synthetic peptide corresponding to the C-terminal 15 amino acids of human p185 neu was raised in rabbits injected with the immunogenic peptide coupled to thyroglobulin with maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce). Monoclonal anti-phosphotyrosine antibody 1G2 (20) was purified from ascites fluid.

Plasma Membrane Preparation. The procedure described before (4) was used to obtain purified plasma membrane fraction from DHFR-G8 cells.

Conditioned Media. Cells were grown to confluence in 175-cm² flasks (Falcon), washed with phosphate-buffered saline, and left in serum-free medium for 10–16 hr. The medium was discarded and replaced by fresh serum-free medium that was collected after 3 days in culture. The conditioned medium was cleared from cells and concentrated at 100-fold in an Amicon ultrafiltration cell through a YM2 membrane.

p185 neu Down-Regulation Assay. Confluent monolayers of Rat-1 fibroblasts in 24-well dishes (Costar) were washed with Dulbecco's modified Eagle's medium (DMEM) and then incubated with 0.2 ml of binding buffer [DMEM supplemented with 20 mM Hepes (pH 7.5) and 0.1% bovine serum albumin] with or without conditioned medium and suramin (2 mM). After a 90-min incubation at 37°C, the monolayers were washed with DMEM and incubated at 4°C with 3 μg of the 7.16.4 monoclonal anti-p185 neu antibody per ml of binding buffer. Cell-bound antibody was determined after 2 hr by a 45-min incubation at cold with 125I-labeled protein A.

In Vitro Kinase Assay. Aliquots (50 μg; 20 μl) of purified plasma membrane fraction were incubated with an equal volume of concentrated conditioned medium in a buffer containing 20 mM Hepes (pH 7.5) and bovine serum albumin (1 mg/ml) in the presence or absence of suramin (2 mM). After 30 min of incubation, the membranes were solubilized by the addition of 0.2 ml of solubilization buffer (4), and p185 neu was immunoprecipitated by an anti-C-terminal antibody coupled to Sepharose-protein A. Immune complexes were precipitated after 30 min at cold, washed twice with HNTG solution [20 mM Hepes, pH 7.5/150 mM NaCl/0.1% Triton X-100/10% (vol/vol) glycerol] and resuspended in 30

Abbreviations: EGF, epidermal growth factor; TGF-α, transforming growth factor α; CSF-1, colony stimulating factor 1; PDGF, platelet-derived growth factor.

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Cell Biology: Yarden and Weinberg


µl of HNTG supplemented with 20 mM MnCl₂ and 50 µCi of [α-³²P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq; ICN). The phosphorylation reaction lasted 15 min at 22°C and was followed by three washes in HNTG. Final pellets were mixed with 35 µl of SDS gel sample buffer, heated to 95°C (5 min), and resolved by gel electrophoresis on 7.5% polyacrylamide gel containing SDS.

Metabolic Labeling with ³²P and Immunoprecipitation. Subconfluent monolayers of cells cultured in 24-well dishes were labeled for 4–7 hr in DMEM without phosphate but supplemented with 1% dialyzed fetal calf serum and 0.8 mCi of ³²P per ml (NEN). The monolayers were then incubated at 22°C in fresh medium that contained conditioned medium or growth factors. Fifteen minutes later, the cells were solubilized on ice with 0.4 ml of modified solubilization buffer containing 50 mM sodium pyrophosphate, 100 mM NaF, 5 mM EDTA, and 2 mM sodium orthovanadate. Undissolved material was removed and the cleared supernatant reacted (30 min at 4°C) with anti-phosphotyrosine antibody coupled to agarose. Immune complexes were washed by centrifugation at 4°C with 1 ml of high-, medium-, and low-salt buffers (4). Elution of phosphotyrosine-containing antibodies was achieved by mixing (30 min at 4°C) the final pellets with 1 ml of modified solubilization buffer supplemented with 50 mM p-nitrophenylphosphate. Supernatants were then mixed with rabbit anti-C-terminal peptide antibody coupled to Sepharose-protein A. After a 45-min reaction at 4°C, the beads were washed and immune complexes were subjected to gel electrophoresis as described above.

Infection of Hematopoietic Cells. A retrovirus containing the normal neu gene (21) and the neo selectable marker (22) was harvested from ψ-2 DOL34 cells and used to infect BAC1.2F5 murine macrophages (23) or BAF-3 prepro-B lymphocytes. Selection of neo-expressing clones was started 24 hr later by the addition of G418 ( Gibco) at a concentration of 2 mg/ml. Individual G418-resistant clones were isolated 2 weeks later and tested for expression of p185neo by means of immunoprecipitation and self-phosphorylation within the immune complex.

Western Blot Analysis. Proteins resolved by polyacrylamide gel electrophoresis were transferred onto a nitrocellulose filter. The filter was then saturated by incubation with bovine serum albumin (5% in 50 mM Hepes, pH 7.5/150 mM NaCl) and reacted for 8 hr with the anti-phosphotyrosine antibody at 2 µg/ml. Radiolabeled goat anti-mouse antibody (400,000 cpm/ml) was allowed to react for 3 hr with the washed filter, and the filter was washed again before exposure at -70°C to an x-ray film.

Cell Proliferation Assay. The MTT uptake method was used as described (24) except that the assay was done in medium that contained 0.5% calf serum, and the growth factors on which the cell lines are dependent were omitted.

RESULTS

Strategy for Ligand Detection. The putative receptor p185neo was selected as an experimental system because of its relatively detailed biochemical characterization (25, 26) and its possible involvement in human malignancies (13, 14). Because of the extensive structural homology between p185neo and EGF receptor, we hypothesized that its ligand would be related in structure and physiology to the ligands of EGF receptor: EGF/urogastrone, which is enriched in saliva and urine (27), and transforming growth factor α (TGF-α), which is secreted by various carcinoma cell lines (28). As a model system in which to test potential ligand-detection assays, we studied the EGF receptor displayed by human skin fibroblasts (data not shown). In addition, as a possible modulator of effects due to ligand–receptor interactions, we used the drug suramin. It has been shown that suramin inhibits binding of platelet-derived growth factor (PDGF) (29) and EGF (unpublished observation) to their receptors and therefore may well inhibit the binding of the putative ligand to p185neo.

Down-Regulation of p185neo upon Treatment with Conditioned Medium Derived from ras-Transformed Fibroblasts. Receptor down-regulation in response to exposure to its cognate ligand is a property shared by all known receptor tyrosine kinases. Moreover, the effect is specific to the ligand and in the case of the EGF receptor and p185neo is not achieved through trans-modulation by heterologous receptors (30, 31). The assay used here is based on quantitative immunological determination of surface-exposed receptors after incubation with the potential ligand at 37°C. When tested on human fibroblasts and human A431 cells, EGF at a concentration as low as 1 ng/ml could be readily detected by our down-regulation assay (data not shown). Analogously, when medium conditioned by Ha-ras-transformed fibroblasts (Rat-1-EJ) was incubated at 37°C with rat fibroblasts, we observed partial disappearance of cell surface p185neo (Fig. 1A). This effect was significantly inhibited by suramin at 2 mM, suggesting that it was due to receptor–ligand interactions. Furthermore, incubation at 4°C resulted in no apparent down-regulation of p185neo (Fig. 1B), and medium conditioned by untransformed rat fibroblasts was much less active, as shown in Fig. 1C. Other conditioned media like those

![Fig. 1](image-url)

**Fig. 1.** Down-regulation of p185neo by conditioned medium of ras-transfected Rat-1 fibroblasts. Confluent monolayers of Rat-1 fibroblasts were incubated with the indicated dilutions of concentrated conditioned medium in the presence (•) or absence (○) of suramin (2 mM). After a 90-min incubation period, the relative amount of surface-exposed p185neo was assayed by binding of a monoclonal anti-p185neo antibody. (A and B) Incubation with conditioned medium from Rat-1-EJ fibroblasts at 37°C (A) or 4°C (B). (C) Incubation at 37°C with conditioned medium from untransfected Rat-1 fibroblasts.
derived from the B-50 neuroblastoma line and the B-16 melanoma line were less active than Rat-1-EJ, whereas EGF and TGF-α at concentrations up to 100 ng/ml had no effect. Encouraged by these observations, we used alternative assays to examine the Rat-1-EJ-conditioned medium for the presence of the putative ligand.

**Stimulation of Self-Phosphorylation of p185<sub>neo</sub> in Vitro.** Ligand–receptor interactions should lead to activation of the tyrosine-specific kinase function of p185<sub>neo</sub>. However, less direct mechanisms may also lead to an apparently similar effect. Thus, TGF-α/EGF treatment of intact cells may induce phosphorylation of p185<sub>neo</sub> through the action of EGF receptor (30, 31). Although there is no evidence that such trans-modulation also operates in broken cells, we sought to prevent such effects by the incubation of p185<sub>neo</sub>-rich plasma membranes with Rat-1-EJ-conditioned medium under conditions that do not allow protein phosphorylation. Accordingly, we omitted bivalent ions and nucleotides from the binding mixture. Later, the state of activation of the tyrosine kinase was assayed by the degree of self-phosphorylation within an immune complex.

As depicted in Fig. 2, preincubation of p185<sub>neo</sub>-containing membranes with Rat-1-EJ-conditioned medium leads to selective enhanced self-phosphorylation of p185<sub>neo</sub>. Slightly higher p185<sub>neo</sub> phosphorylation is seen when the preincubation is done at 4°C rather than at 37°C, and it is completely abolished if preincubation with the conditioned medium is performed in the presence of suramin (Fig. 2). EGF and conditioned medium of normal fibroblasts were inactive in this kinase activation assay, suggesting that a component of Rat-1-EJ-conditioned medium interacts directly with p185<sub>neo</sub> to activate its enzymatic function.

This conclusion is underscored by the examination of the group of proteins in the molecular mass range of 80–100 kDa that coprecipitate with p185<sub>neo</sub> in the in vitro kinase assay (Fig. 2). These proteins reacted with two rabbit antiserum directed to different peptide epitopes of the cytoplasmic domain of p185<sub>neo</sub> but not with a series of monoclonal and polyclonal antibodies that are directed against the extracellular portion of p185<sub>neo</sub>. In addition, the p80–p100 phosphoproteins were capable of self-phosphorylation in vitro and their level was proportional to the level of expression of p185<sub>neo</sub> in transfected mouse cells (data not shown). We conclude that p100 phosphoprotein is derived from p185<sub>neo</sub>, perhaps by proteolysis, and they are devoid of most or all the extracellular region. Phosphorylation of these truncated versions of p185<sub>neo</sub> was not significantly enhanced by the incubations analyzed in Fig. 2. Thus, the differential phosphorylation effect of p185 but not p80–p100 is attributable to interaction of the putative ligand with the extracellular domain of p185<sub>neo</sub> that is missing in the p80–p100 fragments.

**Stimulation of Tyrosine Phosphorylation of p185<sub>neo</sub> in Human Mammary Carcinoma Cells.** To test the ability of the conditioned medium of Rat-1-EJ cells to stimulate tyrosine phosphorylation of p185<sub>neo</sub> in vivo, we metabolically labeled human mammary carcinoma cells (SKBR-3) with 32P, challenged these cells with various conditioned media at 22°C, and analyzed tyrosine-phosphorylated p185<sub>neo</sub> by successive immunoprecipitations. In this cellular system, both media conditioned by Rat-1-EJ and sis-NRK cells stimulated tyrosine phosphorylation of p185<sub>neo</sub>, whereas media conditioned by other cells were inactive (data not shown).

Since both ras- and sis-transformed fibroblasts secrete TGF-α (32, 33), we examined the possibility that the observed increased tyrosine phosphorylation of p185<sub>neo</sub> was due to TGF-α acting through the EGF receptor. Accordingly, we used cells from another human mammary carcinoma line (MDA-MB453) that reportedly show undetectable levels of mRNA of the EGF receptor (13) but nevertheless overexpress p185<sub>neo</sub>. We confirmed the absence of EGF receptor on MDA-MB453 cells by immunoprecipitation with two different monoclonal antibodies (R1 and 528; ref. 34) to human EGF receptor and two polyclonal rabbit antibodies directed to synthetic peptides of EGF receptor. In addition, no specific binding of radiolabeled EGF to MDA-MB453 could be detected (data not shown). As expected, EGF and TGF-α were unable to elicit enhanced tyrosine phosphorylation of p185<sub>neo</sub> of the MDA-MB453 cells (ref. 30; Fig. 3A), unlike the SKBR-3 cells. However, media conditioned by either ras- or sis-transformed fibroblasts, but not by untransformed rat fibroblasts, induced a significant increase in tyrosine phosphorylation of p185<sub>neo</sub> (Fig. 3A). Furthermore, this effect was completely abolished by suramin, compatible with its origin in ligand–receptor interactions.

To examine the possibility that the effect of Rat-1-EJ-conditioned medium on p185<sub>neo</sub> phosphorylation is mediated by a receptor other than the EGF receptor and p185<sub>neo</sub> itself, we used a truncation mutant of p185<sub>neo</sub> that lacks virtually all the extracellular putative ligand-binding domain (35). As shown in Fig. 3B, the truncated form of the neu-encoded protein, when expressed in mouse fibroblasts (B-14-21 cell line), lost the ability to respond to Rat-1-EJ-conditioned medium but also to EGF alone. Thus, intact extracellular domain appears to be essential for p185<sub>neo</sub> activation by the presumptive ligand. Lack of effect of EGF may be due to defective heterologous dimerization of EGF receptor and its truncated homologue (36). Nevertheless, this receptor form retained the capacity to undergo phosphorylation in response to the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate, PDGF, and fibroblast growth factor as indicated by a moderate increase in its phosphorylation and a slight decrease in its electrophoretic mobility (Fig. 3B). In conclusion, this data suggest that p185<sub>neo</sub> undergoes at least partial trans-regulation, but unlike the intact molecule is devoid of responsiveness to the candidate ligand.

**Establishment and Responsiveness of Hematopoietic Cell Lines Expressing p185<sub>neo</sub>.** To further exclude the possibility that transregulation through other receptors mediated the observed effects on p185<sub>neo</sub>, we sought a cellular system that expresses a set of membrane receptors that differs from those displayed by mesenchymal cells. Hematopoietic cells offer such a model. Moreover, these cells carry an intracellular signal-transducing network responsive to ectopically expressed receptors since the EGF receptor is biologically active when its gene is transduced into a myeloid cell line.
The mitotic response of the factor-dependent B-lymphocyte and macrophage cell lines was determined by the cell proliferation assay of Mosmann (24). In the absence of lymphokine, both BAF-3 and BAC1.2F5 cells show low if any proliferation. In addition, they both retain responsiveness to their normal mitogens, interleukin 3 and CSF-1, respectively (Table 1). However, the response to conditioned medium of ras-transformed fibroblasts is quite different: whereas the parental lines are inhibited by the conditioned medium, their neu-expressing derivatives are mitogenically stimulated (Table 1). We conclude that ras-transformed fibroblasts secrete both stimulatory and inhibitory factors, but the former are operative only on neu-expressing derivatives of the hematopoietic lines.

**DISCUSSION**

The major question addressed in the present work is whether it is feasible to use a putative receptor for a systematic screening that will lead to detection of its ligand. Answering this question is increasingly important as more and more putative receptors, most of them products of protooncogenes, are being identified. The definitive proof of suspected ligand-receptor relationships must rely on binding or chemical crosslinking of a radiolabeled ligand to its receptor. However, this approach is not practical when dealing with a very limited amount of labeled ligand.

**Table 1. Comparison of the proliferative response of neu-infected hematopoietic cell lines and their uninfected parental lines**

<table>
<thead>
<tr>
<th></th>
<th>BAF-3</th>
<th>BAF-3/neu</th>
<th>BAC1</th>
<th>BAC1/neu</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0 ± 14</td>
<td>0 ± 14</td>
<td>0 ± 11</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>Interleukin 3</td>
<td>100 ± 17</td>
<td>84 ± 15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CSF-1</td>
<td>ND</td>
<td>ND</td>
<td>100 ± 20</td>
<td>173 ± 33</td>
</tr>
<tr>
<td>Rat-1-EJ conditioned medium</td>
<td>70 ± 14</td>
<td>84 ± 14</td>
<td>47 ± 1</td>
<td>76 ± 17</td>
</tr>
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BAF1.2F5 murine macrophages, either infected or uninfected with the neu gene, were grown to 80% confluency in 96-well dishes and then starved for 2 days in 0.5% calf serum in MEM medium without CSF-1. BAF-3 murine prepro-B cells plated into a 96-well dish at 2 \times 10^5 cells per ml in 0.5% calf serum (in RPMI medium). Interleukin 3 and CSF-1 were from media conditioned by Wehi-3 and L929 cells, respectively, and were added at 1:10 dilution. The conditioned medium from Rat-1-EJ cells was used at a final protein concentration of 0.1 mg/ml. The proliferative response was assayed by means of a colorimetric determination of the uptake of the mitochondrial dye MTT (24). Results of triplicate determinations are given as mean ± SD and are expressed as percentage of the response obtained with the corresponding hematopoietic growth factor. Qualitatively identical results were obtained twice with BAF-3 cells and three times with BAC1.2F5 cells. ND, not determined.
still unidentified molecule and with large numbers of potential biological sources of ligand. As a practical alternative, we sought to establish several independent ligand detection assays that are based on later steps of the ligand–receptor interaction rather than on the initial binding. Although indirect, such tests offer the advantage of ligand detection in crude unfractionated biological fluids.

Aiming at the ligand for the neu protooncogene product, we have used the structurally related EGF receptor as a model system to test the reliability, sensitivity, and specificity of each detection assay and then applied each assay to the neu system. The following lines of evidence collectively imply that a candidate ligand for p185new is present in medium conditioned by cultured fibroblasts that are transformed by a ras oncogene. (i) p185new is down-regulated in a temperature-dependent and suramin-sensitive manner by conditioned medium from ras-transformed cells but not by other media. (ii) The catalytic kinase function of p185new is stimulated in vitro in a suramin-sensitive but temperature-independent process by the above-mentioned conditioned medium. A truncated receptor that lacks the ligand-binding domain is not affected. (iii) An increased phosphorylation on tyrosine residues of p185new is induced in vivo by conditioned medium from ras-transformed cells but not by media conditioned by other cells. The increase is seen in mesenchymal, hematopoietic, and epithelial cells, including EGF receptor-deficient cells. (iv) Transduction of the new gene into hematopoietic cells renders them mitotically responsive to conditioned medium from ras-transformed fibroblasts.

We must consider the possibility that the observed effects are due to a factor that acts only indirectly on p185new. Transmodification of EGF receptors through the activation of protein kinase C represents one well-characterized mechanism (39). Thus, tyrosine phosphorylation of p185new is rapidly induced through activation of the kinase of EGF receptor (30, 31). Since both TGF-α (40) and a PDGF-like factor (unpublished observation) are present in medium conditioned by ras-transformed cells, trans-modulation rather than direct activation of p185new may have potentially contributed to the observed effects. However, under the conditions used to obtain the aforementioned lines of evidence, neither PDGF, EGF, nor the EGF analogue TGF-α, induced the down-regulation response or stimulation of the catalytic function of p185new in vitro. Furthermore, detection of elevated tyrosine phosphorylation of p185new in epithelial cells that do not express either the PDGF or EGF receptor, and the selective mitotic responsiveness of neu-expressing hematopoietic cells also exclude their involvement.

It remains possible though unlikely that an as yet unknown factor is responsible for the observed biological effects, acting through a receptor other than p185new. Such a receptor would need to be broadly expressed in mesenchymal (Fig. 3) and hematopoietic cells (Fig. 4). However, the differential activation of intact p185new compared with its truncated ectodomain-deficient forms (Figs. 2 and 3B) strongly suggests that the putative ligand binding domain indeed directly mediates kinase activation by the factor of the conditioned medium. In the end, we cannot rule out absolutely the possibility that our results are due to a factor other than the neu ligand. The ultimate proof, as already discussed, will depend on the availability of a purified factor.

We gratefully acknowledge M. Waterfield for the gift of R1 antibodies, J. Mendelsohn for the 528 IgG, R. Frackelton for the Ig2 antibody, and C. Morgan and R. Stanley for the gift of the BAC1.1FS cells. This work was supported by the National Institutes of Health Grant CA39826.