Agonistic antibodies stimulate the kinase encoded by the *neu* protooncogene in living cells but the oncogenic mutant is constitutively active

(epidermal growth factor receptor/chemical carcinogenesis)

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Communicated by Michael Sela, January 16, 1990 (received for review October 3, 1989)

ABSTRACT The *neu* protooncogene (also called *c-erbB2* and *HER-2*) undergoes oncogenic activation through a single mutation. The product of the protooncogene, *p185^{neu}*, probably functions as a receptor for a peptide growth factor. To circumvent the absence of a well-characterized ligand, I generated ligand-mimicking monoclonal antibodies directed to the presumed receptor. These antibodies stimulated tyrosine phosphorylation of *p185^{neu}* in living cells and also accelerated the rate of endocytosis and degradation of *p185^{neu}*. A monovalent Fab fragment of such an antibody was ineffective, suggesting a role for receptor dimerization in signal transduction. Unlike the product of the protooncogene, the transforming mutant was not affected by the ligand-like antibodies. However, it undergoes constitutively high phosphorylation on tyrosine residues in living cells, and its turnover rate is remarkably rapid. Nevertheless, the pattern of phosphorylation of the mutant protein is similar to the one exhibited by an antibody-stimulated *p185^{neu}*, suggesting that the mutation mimics activation by the antibody. These results suggest that the kinase of *p185^{neu}* is under allosteric control that may involve ligand-induced dimerization of receptors. This mechanism is deregulated in the oncogenic mutant, which is functionally equivalent to ligand-stimulated receptor.

Receptor-like tyrosine kinases constitute a group of transmembrane glycoproteins having some biochemical and structural attributes shared with established receptors for growth factors (1). The interest in this group of proteins stems from the involvement of most members of the group in malignant transformation (e.g., *met*; ref. 2), whereas others appear to affect early development (e.g., *torso* gene; ref. 3). The protein encoded by the *neu* protooncogene *p185^{neu}* is one of the best characterized members of this group of presumed receptors. (The *neu* protooncogene is also called *c-erbB2* and *HER-2*.) Its overexpression in certain human adenocarcinomas (4) and high homology to the epidermal growth factor (EGF) receptor (5) reinforce the possibility that it functions as a receptor for a yet unknown growth factor.

The rodent *neu* protooncogene presents yet another open question; its oncogenic potential is activated through chemical carcinogenesis that changes a single amino acid. This residue, valine-664, lies within the transmembrane region of *p185^{neu}*, and its replacement by a glutamic acid residue renders the protein highly oncogenic (6). The biochemical mechanism of this activation is currently not understood.

Yarden and Weinberg (7) addressed the presumed receptor function of *p185^{neu}* through biochemical study of a candidate ligand molecule that was detected in medium conditioned by transformed fibroblasts. In the present report I address the cellular function of *p185^{neu}* and its oncogenic mutant through

an alternative approach that involves the generation of stimulatory anti-*p185^{neu}* monoclonal antibodies.

MATERIALS AND METHODS

Materials. Affinity-purified goat anti-mouse IgG was from BioMakor (Rehovot, Israel) and it was radiolabeled with Na^{125}I (Amersham) by the chloramine-T method (8). [^{32}P]Orthophosphate was from the Nuclear Research Center (Negev, Israel). [^{35}S]Methionine was purchased from ICN. Fibronectin was from Boehringer Mannheim.

Generation of Monoclonal Antibodies to *p185^{neu}*. Half-syngeneic NFS mice were injected subcutaneously with 5×10^6 living B104-1-1 cells (9). Tumors appeared after 5–10 days and the mice were bled from the tail 2 weeks after the injection. Antisera were tested by binding to the B104 rat neuroblastoma cells that overexpressed *p185^{neu}* as a result of infection with a *neu*-containing retroviral vector (10). Specific binding to this cell line, designated B104-N12, was indicative for immune reaction with the *p185^{neu}* and was verified by an immunoprecipitation assay. All mice developed a strong response. Spleen cells from two selected mice were fused with NS1 cells by using polyethylene glycol as a fusing agent, by the procedure of Galfre *et al.* (11). Hybrid cells growing in hypoxanthine/aminopterin/thymidine-selective medium were selected for secretion of *p185^{neu}* antibodies. The following assays were employed with the supernatants of cultures: (i) binding to B104-N12 cells that was detected by radiolabeled goat anti-mouse IgG and (ii) immunoprecipitation followed by the addition of MnCl_2 and [$\gamma\text{-}^{32}\text{P}$]ATP to measure precipitation of the *neu* kinase (12). Positive cells were cloned twice in soft agar. Determination of antibody class was done with class-specific second antibodies. Hybridomas were grown as ascites fluid in (NFS $\times$ BALB/c) F_1 mice and were purified by ammonium sulfate precipitation and affinity chromatography on protein A or protein G coupled to agarose (Pharmacia).

Binding Assay to Living Cells. DHFR-G8 cells (13) were plated on fibronectin-coated trays of 24 wells and assayed at confluence. Monolayers were incubated at 22°C with various amounts of the antibodies in binding buffer [Dulbecco's modified Eagle's medium (DMEM) containing 0.1% bovine serum albumin buffered at pH 7.5 with 25 mM Hepes]. After incubation for 2 hr, unbound antibody was washed off, and ^{125}I -labeled goat anti-mouse IgG (1×10^6 cpm) was added. Cell-bound antibody was determined 2 hr later after three 1-ml washes by measuring radioactivity in cell lysates in 0.1 M NaOH/0.1% SDS in a γ counter.

Preparation of the B10 Fab Fragment. The B10 monoclonal antibody (10 mg) (protein G purified) was cleaved with papain essentially as described (14). Separation of the Fab fragment from intact antibody molecules and Fc fragments was

achieved by gel filtration on Sepacryl S-200, ion-exchange chromatography on DEAE-Sephacryl, and finally elution through a column of Fc-specific goat anti-mouse antibody.

Determination of Tyrosine Phosphorylation of p185^{neu} in Living Cells. Confluent monolayers of DHFR-G8 cells or B104-1-1 cells were grown on fibronectin in 1-cm dishes and labeled for 4 hr in DMEM without phosphate but supplemented with 1% dialyzed fetal calf serum and [³²P]orthophosphate (0.6 mCi/ml; 1 Ci = 37 GBq). The radioactive medium was then replaced by fresh medium that contained antibodies at various concentrations and the cells were lysed after a 15-min incubation at 22°C. Tyrosine-phosphorylated p185^{neu} was then immunoprecipitated successively with a monoclonal antibody to phosphotyrosine (15) and a rabbit antiserum to p185^{neu}, exactly as described (7).

Biosynthetic Labeling of Cells with [³⁵S]Methionine. Confluent monolayers of cells growing in 24-well dishes were labeled by incubation (8 hr at 37°C) in DMEM supplemented with 10% dialyzed fetal calf serum and [³⁵S]methionine (ICN; 0.1 mCi/ml). Cells were then chased for various periods of time by incubation in fresh medium in the presence or absence of anti-p185^{neu} antibodies. After a chase, lysates of cells were prepared as described above and p185^{neu} was precipitated with an anti-peptide antibody raised in rabbits. Antigen-antibody complexes were precipitated by protein A-agarose beads, washed with 1-ml washes of high-, medium-, and low-salt buffers (12), and resolved by electrophoresis through SDS gels. The resulting autoradiograms were scanned using an automated densitometer (Bio-Rad) to enable quantitation of the level of p185^{neu}.

Peptide Mapping with V8 Protease. Extensively washed immune complexes resulting from a two-step immunoprecipitation with anti-phosphotyrosine and anti-p185^{neu} antibodies were subjected to proteolysis. Samples were first heated (5 min at 95°C) in gel sample buffer and cooled on ice, and V8 protease (2 µg) was added in a final volume of 0.05 ml. Proteolysis was followed by electrophoresis and the final gel was exposed at -70°C to x-ray film (Kodak XAR-5).

RESULTS

Strategy and Generation of Anti-p185^{neu} Antibodies. To circumvent the absence of a fully characterized ligand of the *neu*-encoded receptor-line protein (7), I sought to generate ligand-mimicking stimulatory monoclonal antibodies. Such antibodies to the EGF receptor (16) and insulin receptor (17) fully or partially mimic the action of the corresponding ligand. To this end mice were injected with various forms of p185^{neu} as antigens, including membrane vesicles derived from *neu*-overexpressing fibroblasts (DHFR-G8 cells, ref. 13), glutaraldehyde-fixed or live DHFR-G8 cells, and living fibroblasts transfected with the oncogenic *neu* gene (B104-1-1 cells, ref. 9). The latter cells gave rise to large tumors, and, when compared to other antigens by immunoprecipitation, they turned out to be the most immunogenic. Furthermore, antisera produced by such tumor-bearing mice stimulated, at a certain dilution, tyrosine phosphorylation of p185^{neu} in living cells (see below). Spleens from these mice were, therefore, used for hybridoma generation.

Four independently obtained hybridomas were selected and cloned, and the antibodies secreted by them were used on living cells. As is depicted in Fig. 1, all monoclonal antibodies bound to normal p185^{neu}. Similar binding curves were obtained with the transforming p185^{neu*} (data not shown). However, these antibodies greatly differed in their apparent affinity to p185^{neu}. Antibodies H4 and C11 had relatively high affinities and bell-shaped curves, whereas antibody D6 had the lowest affinity. An intermediate affinity was displayed by antibody B10, which was more extensively used in this study. All four antibodies immunoprecipitated a single protein band of 185

Fig. 1. Binding of monoclonal anti-p185^{neu} antibodies to transfected murine fibroblasts. Confluent monolayers of DHFR-G8 cells were incubated for 2 hr at 22°C with the indicated concentrations of various monoclonal anti-p185^{neu} antibodies (Ab) and then with radiolabeled goat anti-mouse Fab. Control monolayers were not given any first antibody and their "background" binding was subtracted. Averages of duplicate determinations (<10% variation) are shown.

kDa from metabolically ³⁵S-labeled rat cells but did not react with the human p185^{neu} (data not shown).

Stimulation of Tyrosine Phosphorylation of p185^{neu} in Living Cells and Requirement for Bivalency. The aim of the present study was to generate biologically active anti-p185^{neu} antibodies. A ligand-like anti-p185^{neu} antibody should be capable of stimulating the tyrosine kinase activity of the presumed receptor. To test this possibility DHFR-G8 murine fibroblasts that overexpress the normal *neu* gene were incubated in medium containing [³²P]orthophosphate. The monolayers were then briefly exposed (15 min at 22°C) to the various anti-p185^{neu} antibodies, and the tyrosine-phosphorylated p185^{neu} was immunoprecipitated with anti-phosphotyrosine antibodies, eluted from the antibody with a phosphotyrosine analog, and reprecipitated with an anti-p185^{neu} rabbit antiserum. The results of such an analysis are shown in Fig. 2A. The relatively low basal tyrosine phosphorylation of p185^{neu} was stimulated by short incubation of the living cell with some but not all monoclonal antibodies: Antibodies B10 and H4 significantly elevated tyrosine phosphorylation, whereas a low-affinity antibody (D6) did not affect tyrosine phosphorylation of p185^{neu}. On the other hand antibody C11, which displayed relatively high apparent affinity, and also a polyclonal mouse antiserum to p185^{neu} showed a complex response: stimulatory action at low concentrations and inhibitory activity at high concentrations. This pattern raised the possibility that bivalent interaction is required for activation. This explanation gained support from the use of a monovalent Fab fragment of antibody B10. As is evident from Fig. 2B, the monovalent Fab B10 had very low, if any, stimulatory action, as compared with similar and even lower molar concentrations of the bivalent antibody. This loss of response was not due to lack of antigen binding, as the Fab B10 still bound to p185^{neu}, although with reduced apparent affinity (Fig. 1). In conclusion, tyrosine phosphorylation of p185^{neu} can be stimulated and probably requires close apposition of two receptor molecules.

Constitutively High Tyrosine Phosphorylation of the Transforming p185^{neu*}. If a stimulatory anti-p185^{neu} antibody mimics the action of the putative ligand, such an antibody may provide an insight into the biochemical consequences of the point mutation that activate the transforming potential of

FIG. 2. Antibody-induced stimulation of tyrosine phosphorylation of p185^{neu} in living cells. Monolayers of [³²P]orthophosphate-labeled DHFR-G8 cells were incubated (15 min at 22°C) with the indicated concentrations of the various antibodies. Tyrosine-phosphorylated p185^{neu} was immunoprecipitated with anti-phosphotyrosine antibodies and an anti-peptide antibody. Autoradiograms of the SDS gel-separated immunoprecipitates are shown. Molecular masses of marker proteins are indicated in kDa to the right of the autoradiograms. (A) Various monoclonal anti-p185^{neu} antibodies and a polyclonal mouse antiserum (PC). (B) Increasing concentrations of antibody B10 and its monovalent Fab fragment.

p185^{neu}. Previous analyses of detergent-solubilized p185^{neu} revealed that its kinase activity does not differ dramatically from that of the transforming p185^{neu*}. When analyzed in membrane preparations the oncogenic protein displayed much higher catalytic activity (18). In living cells however, Stern *et al.* (19) did not detect significant differences in the self-phosphorylation of the normal and mutant proteins. It was, therefore, important to resolve this discrepancy by testing the responsiveness of both p185^{neu} and p185^{neu*} to stimulatory antibodies in living cells. Comparison of the basal and antibody-stimulated tyrosine phosphorylation of similar amounts of these proteins surprisingly revealed the following observations (Fig. 3). (i) The basal phosphorylation of p185^{neu*} was dramatically higher than the level displayed by the normal p185^{neu}. (ii) In contrast with the normal p185^{neu}, which was stimulated by some monoclonal antibodies, the level of tyrosine phosphorylation of the oncogenic version of the protein was not affected by antibody binding.

These characteristics are not due to lack of antibody binding to p185^{neu*}, neither do they represent lack of response to a specific antibody or altered concentration-dependence of the induction (Fig. 3B). In addition, the amount of p185^{neu} precipitated could not reflect variation in

the binding by the monoclonal antibody since (i) the first immunoprecipitation step was done with an anti-phosphotyrosine antibody without the inclusion of protein A and (ii) a rabbit antiserum to p185^{neu} was used at over-saturation for final precipitation of p185^{neu}.

Evidently, the data presented here support the long suspected, but unproved, possibility that the transforming mutant of p185^{neu} is a constitutively active tyrosine kinase *in vivo*. The inability of a previous analysis (19) to demonstrate this may reflect methodological differences between the present study and the previously employed Western blot technique. Nevertheless, my results that were obtained with living cells are consistent with observations made with plasma membrane preparations *in vitro* (18).

Antibody-, Mutation- and EGF-Mediated Activation of p185^{neu} Result in Similar Patterns of Tyrosine Phosphorylation. Close inspection of the data in Fig. 3B reveals that maximal stimulation of p185^{neu} with over-saturating concentrations of monoclonal antibody B10 yielded much lower level of tyrosine phosphorylation compared with the transforming p185^{neu*}. This may suggest that the antibody is only a partial agonist of the receptor kinase molecule. Alternatively, the point mutation and the antibody may utilize

FIG. 3. Differential stimulation of p185^{neu} and the transforming mutant (p185^{neu*}). Tyrosine-phosphorylated p185^{neu} was immunoprecipitated from [³²P]orthophosphate-labeled cells that overexpress the normal p185^{neu} (DHFR-G8 cells) or the transforming mutant p185^{neu*} (B104-1-1 cells). Identical numbers of receptors of each type, as determined by radioimmunoassay, were analyzed. Prior to cell lysis, living cells were stimulated for 15 min at 22°C. Molecular masses of marker proteins are indicated in kDa to the right. (A) Effect of increasing concentrations of monoclonal antibody B10 (concentrations given in nM). (B) Effect of various antibodies at the following concentrations: Lanes: B10, 30 nM (left lane) and 300 nM (right lane); D6, 300 nM; C11, 300 nM; H4, 300 nM; Fab-B10, 250 nM; polyclonal antiserum (PC), 1:100 dilution.

different molecular mechanisms to elevate tyrosine phosphorylation. According to the latter explanation, it is possible that each mode of activation will result in phosphorylation of different sites. To test this possibility, I performed peptide map analysis of tyrosine phosphorylated p185^{neu} (after antibody stimulation) and p185^{neu*}. A third route of activation that involves heterologous interaction with the EGF receptor (20, 21) was also analyzed. The results of this analysis are depicted in Fig. 4. p185^{neu} molecules were immunoprecipitated from cells that were stimulated by monoclonal antibody B10 or by EGF and compared with the constitutively phosphorylated p185^{neu*}. Upon treatment of tyrosine-phosphorylated p185 molecules with V8 protease and separation on a polyacrylamide gel, similar patterns of phosphorylated peptides were obtained (Fig. 4B). This result appears to favor, but definitely not prove, the simple model; namely, all three pathways that lead to elevated tyrosine phosphorylation are probably mediated by similar mechanisms. If true, the point mutation within the transmembrane region probably mimics binding of the antibody, and perhaps also binding of the putative ligand to the extracellular domain. What is even more surprising is that heterologous indirect activation by EGF appears to share a similar mode of action that may involve stimulation of the kinase of p185^{neu}, rather than interphosphorylation by the tyrosine kinase of the EGF receptor. However, the possibility that both receptors share the same substrate specificity should also be considered.

Stimulatory Antibodies and Transforming Mutation Accelerate Turnover of p185^{neu}. Ligand-receptor interactions in the family of receptor tyrosine kinases are coupled to rapid endocytosis by coated pits. It follows that a stimulatory antibody should also increase the turnover rate of the receptor. Moreover, for the homologous EGF receptor, phosphorylation on tyrosine residues appears to be essential for ligand-induced endocytosis (22, 23). To test this prediction DHFR-G8 cells were metabolically labeled with [³⁵S]methionine and then chased for 10 hr with fresh medium that contained unlabeled methionine and various antibodies. The residual labeled

FIG. 4. Peptide map analysis of tyrosine-phosphorylated normal and transforming p185^{neu}. Cells expressing either the normal or the mutant (transforming) p185^{neu} were labeled with [³²P]orthophosphate and then stimulated (15 min at 22°C) with antibody B10 (300 nM), EGF (50 ng/ml), or mock-stimulation. Tyrosine-phosphorylated p185^{neu} was isolated by immunoprecipitation with an anti-phosphotyrosine antibody, eluted with phenyl phosphate, and precipitated with an anti-p185^{neu} rabbit antibody. Final immune complexes were heated (5 min at 95°C) in SDS gel sample buffer and treated (B) or untreated (A) with V8 protease (2 µg for 30 min at 37°C). Samples were electrophoresed on a 15% polyacrylamide/SDS gel. Molecular masses in kDa are shown to the right.

FIG. 5. Turnover rates of the normal and the mutant (transforming) p185^{neu}. Monolayers of NIH 3T3 fibroblasts expressing either the normal p185^{neu} (DHFR-G8 cells) or the transforming p185^{neu*} (B104-1-1 cells) were labeled biosynthetically with [³⁵S]methionine (8 hr at 37°C). After a brief wash, cells were chased with unlabeled methionine as indicated, in the presence (closed symbols) or absence (open symbols) of monoclonal anti-p185^{neu} antibodies at 300 nM or a polyclonal mouse antibody (PC) at 1:100 dilution. Residual ³⁵S-labeled p185^{neu} was then subjected to immunoprecipitation with an anti-peptide antiserum and then to SDS gel electrophoresis. (A) Various anti-p185^{neu} antibodies and a monovalent Fab fragment of antibody B10. (B) Quantitative analysis of p185^{neu} turnover as determined by densitometry of autoradiograms.

p185^{neu} present at the end of the chase period was immunoprecipitated and analyzed by autoradiography of polyacrylamide gels. The results of this experiment are given in Fig. 5A. Clearly the stimulatory antibody B10 was able to accelerate the rate of turnover of p185^{neu}, whereas its monovalent fragment was ineffective. Other monoclonal antibodies were less effective, in a way that correlated with their capacity to stimulate tyrosine phosphorylation (Fig. 2A). Yet, even antibody D6, which had no effect on tyrosine phosphorylation, showed significant effect on receptor turnover. The maximal effect obtained with a polyclonal mouse antiserum is probably due to combined effects on receptor degradation and a precipitin reaction after cell lysis (data not shown).

When the same analysis was performed with the transforming p185^{neu*}, a constitutively accelerated turnover was observed (Fig. 5), in line with a previous report (19). To quantitatively compare the normal and the mutated proteins their rates of turnover were followed by densitometry. The data, as summarized in Fig. 5B, indicate that the stimulatory antibody dramatically accelerates degradation of p185^{neu}. However, the antibody was ineffective on the oncogenic mutant that was degraded at a constitutively rapid rate. Interestingly, unlike the effect on tyrosine phosphorylation (Fig. 3), the accelerated turnover obtained with antibody B10 was quantitatively similar to the effect of the point mutation.

Thus, a correlation exists between the induction of tyrosine phosphorylation of p185 and its rate of degradation. Both functions are similarly elicited by anti-receptor antibodies and by the transforming mutation and are expected to be shared also by the authentic ligand of p185^{neu}. This raises the intriguing possibility that the transforming protein is functionally equivalent to a ligand-complexed receptor.

DISCUSSION

Elucidating the functional role of oncogene products is currently a major challenge in many experimental systems. The

present study addressed the putative receptor function of the *neu*-encoded protein and the biochemical mechanism by which chemical carcinogens activate its transforming potential. The experimental approach employed, to circumvent lack of availability of a purified ligand, was to generate monoclonal anti-p185^{neu} antibodies that will be capable of receptor activation. Potentially, this experimental strategy would be useful also in other systems of receptor-like oncogenic proteins.

The main conclusions derived from the experiments presented here are as follows. (i) The p185^{neu}-encoded tyrosine kinase can be activated by the binding of bivalent antibodies to the extracellular domain of the receptor (Fig. 2A). (ii) The oncogenic p185^{neu*}, unlike its normal counterpart, escaped allosteric regulation by the ectodomain (Fig. 3). This kinase, however, displays dramatically elevated and persistent levels of tyrosine phosphorylation in living cells. (iii) Signal transduction by p185^{neu}, at least in response to a stimulatory monoclonal antibody, probably involves receptor dimerization, as suggested by an inactive monovalent antibody fragment (Fig. 2B). (iv) All three known modes by which p185^{neu} is stimulated [namely, antibody, mutation, and EGF (indirectly)] appear to share similar patterns of tyrosine phosphorylation (Fig. 4). (v) Rapid receptor endocytosis and degradation is coupled to elevated tyrosine phosphorylation of p185^{neu} (Fig. 5), which is induced by stimulatory antibodies, and the transforming mutation but not by a monovalent antibody or EGF (7, 20).

Thus these conclusions not only demonstrate the feasibility of an antibody approach to the study of oncogene function but also provide biochemical hints as to the action of the putative ligand. If the presumed hormone is functionally equivalent to the stimulatory antibodies, then its binding to the extracellular portion of p185^{neu} would rapidly activate the catalytic function of the normal receptor but not the transforming mutant. Moreover, this allosteric regulation is probably mediated by receptor dimerization, in analogy to the mechanism proposed for the EGF receptor (24, 25).

Besides the presumed function of p185^{neu} as a growth factor receptor, the aforementioned conclusions relate to the mechanism by which replacement of a single amino acid activates the transforming potential of p185^{neu}. The following model can be envisioned: The transforming p185^{neu*} is functionally equivalent to an antibody-stimulated, and by inference ligand-activated, receptor since it displays a constitutively elevated tyrosine phosphorylation and rapid endocytosis. Thus, a persistent growth stimulus through the active tyrosine kinase probably drives the transformed phenotype. This conclusion is consistent with mutational analysis that indicated that the catalytic function is essential for the transforming potential of *neu* (10). Further, this mechanism is similar to the mode of oncogenic activation of the receptors for EGF and colony-stimulating factor 1, as reflected by the protein products of the *erbB* (26) and the *fms* oncogenes (27). Although the exact biochemical mechanism of activation of p185^{neu*} is not known, the analogy between the point mutation and the stimulatory antibody suggests that the mutation may lead to a strong bias of receptor dimers. Such dimers, according to a model worked out for EGF receptor (24, 25), are functionally activated. This possibility is supported by the rapid turnover of p185^{neu*} as opposed to its normal counterpart (Fig. 5). Dimers of tyrosine phosphorylated receptors (p185^{neu*} or antibody-stimulated p185^{neu}) will certainly be predisposed for endocytosis as compared with monomeric and unphosphorylated receptors. Consistent with this view are the findings that tyrosine phosphorylation of EGF receptor plays a pivotal role in the endocytic destination of occupied receptors (22, 23).

In summary, stimulatory anti-p185^{neu} antibodies appear to be useful in the study of the normal and oncogenic functions of the presumed receptor. It remains to be seen whether these reagents will mimic also late biological effects of the presumed ligand, such as transcriptional activation, mitogenesis, and other cellular effects.

I gratefully acknowledge Dr. Robert Weinberg for encouragement and support in the initial phase of these studies, Dr. R. Frackelton for the 1G2 anti-phosphotyrosine antibodies, and Dr. David Givol for fruitful discussions. This research was supported by The Fund for Basic Research administered by the Israel Academy of Sciences and Humanities and The Israel Cancer Research Fund.

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