MOLECULAR AND CELLULAR BIOLOGY, Nov. 1990, p. 6064–6068
0270-7306/90/116064-05$02.00/0

Vol. 10, No. 11

Receptor Functions and Ligand-Dependent Transforming Potential of a Chimeric *kit* Proto-Oncogene

SIMA LEV, YOSEF YARDEN,* AND DAVID GIVOL

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 22 May 1990/Accepted 13 August 1990

The c-*kit* proto-oncogene, the cellular homolog of the transforming gene of a feline retrovirus, encodes a transmembrane tyrosine kinase homologous to receptors for growth factors. To study the cellular function of c-*kit*, we constructed a chimeric molecule composed of the extracellular portion of the receptor for epidermal growth factor (EGF) and the transmembrane and cytoplasmic domains of p145^{kit}. The hybrid molecule was properly expressed in murine fibroblasts and displayed specific binding of EGF (K_d, 3×10^{-8} M). Activation of the chimeric receptor by EGF stimulated the tyrosine kinase activity of *kit* and led to the generation of a potent mitogenic signal. Moreover, cells expressing the chimeric receptor acquired a transformed phenotype once they were stimulated with the heterologous ligand.

Cellular interactions that are mediated by soluble factors play a pivotal role in the control of cell growth and differentiation. The receptors for some mitogens display tyrosine kinase activity in their cytoplasmic portions and constitute a family of related molecules (reviewed in reference 27). Several proto-oncogenes, including c-*kit*, were assigned to this family on the basis of structural criteria. Expression studies and immunological analyses demonstrated that p145^{kit} is exposed on the cell surface and carries an intrinsic tyrosine kinase activity (15, 26). These observations led to the hypothesis that p145^{kit} functions as a cell surface receptor for a still unknown growth and/or differentiation factor. This possibility is supported by a genetic linkage analysis that mapped the *kit* gene to the dominant white spotting locus (W) on mouse chromosome 5 (2), and the gene was found to be rearranged in two different W mutants (4). Recent analyses demonstrated an impaired kinase activity in various W mutant mice which was correlated with point mutations at the kinase domain of c-*kit* (16–18, 23).

In an effort to understand the putative receptor function of the c-*kit* product, we designed an experimental model system that permits the functional analysis of c-*kit*. To circumvent the absence of a known ligand of p145^{kit}, we replaced the extracellular portion of the presumed receptor with the epidermal growth factor (EGF)-binding domain of the EGF receptor (EGF-R). The chimeric molecule, when expressed in murine fibroblasts, transmitted growth signals in response to ligand (EGF) binding. Moreover, the transfected cells also displayed characteristics of a transformed phenotype, but only in the presence of the ligand.

Construction and expression of the EKR chimera in transfected cells. To generate the EGF-R–*kit* (EKR) chimera, we introduced a unique *Eco*RV restriction site into the cDNA of EGF-R at position 2090, 11 codons upstream of the transmembrane domain (24). The *Eco*RV site was generated by site-directed mutagenesis with a synthetic oligonucleotide of the sequence (mutated bases are underlined) 5′-GCCA GGTCTTGATATCTGTCCAACG-3′ and a template consisting of a single-stranded phage, M13-um20, which contains the human EGF-R cDNA as an *Xho*I-*Xho*I fragment. The unique *Eco*RV site enabled the exchange of an *Eco*RV-*Sal*I DNA fragment which encodes the cytoplasmic and transmembrane portions of EGF-R with a *Dra*I-*Dra*I DNA fragment which encodes the analogous portions of p145^{kit} (Fig. 1).

The hybrid gene was cloned into an expression vector (pLSV) downstream of the simian virus 40 early promoter (14) and cotransfected with pSVNeo into NIH 3T3 fibroblasts (clone 2.2), which are practically devoid of EGF-R. Antibiotic-resistant clones were obtained after 2 weeks of culturing and assayed for binding of radiolabeled EGF. Two clones, designated EKR-6 and EKR-22, overexpressed the chimeric receptor and were therefore chosen for further analysis. To analyze the biosynthesis of the chimeric receptor and also to confirm its hybrid nature, we labeled it biosynthetically with [^{35}S]methionine. The internally labeled molecule could be specifically immunoprecipitated (Fig. 2) with either an antibody to EGF-R (108, directed against the extracellular portion of EGF-R; 10), or an antibody to p145^{kit} (serum 212, directed against the carboxy-terminal synthetic peptide of the protein; 26). The apparent molecular mass of the chimeric receptor was 153 kDa, as compared with 145 kDa for the *kit* product and 170 kDa for EGF-R (Fig. 2). The unglycosylated form of EKR had an apparent molecular mass of 118 kDa, as shown by an experiment in which glycosylation was inhibited by tunicamycin treatment (Fig. 2B).

Ligand binding and activation of the chimeric receptor. The ligand-binding affinity of the EKR chimera was measured by displacement of ^{125}I-EGF with unlabeled EGF under conditions in which only 5 to 10% of the sites were occupied by the radiolabeled ligand (11). This analysis indicated that the K_d of EGF for EKR was $(2.4 \pm 0.9) \times 10^{-8}$ M, as compared with a K_d of EGF for EGF-R of $(1.6 \pm 0.5) \times 10^{-9}$ M (Fig. 3; the values are averages ± standard deviations for four experiments). The decrease in the EGF-binding affinity of the EKR chimera as compared with EGF-R (approximately 15-fold) may have been due either to some subtle changes in the membrane anchoring or to an effect of the cytoplasmic domain on binding affinity (reviewed in reference 22). It was reported that a secreted recombinant extracellular domain of EGF-R bound EGF with a reduced affinity (5). We next analyzed the ligand stimulation of the chimeric receptor. Cells expressing the chimeric molecule were first starved in 0.1% serum for 12 h and then stimulated with various

* Corresponding author.

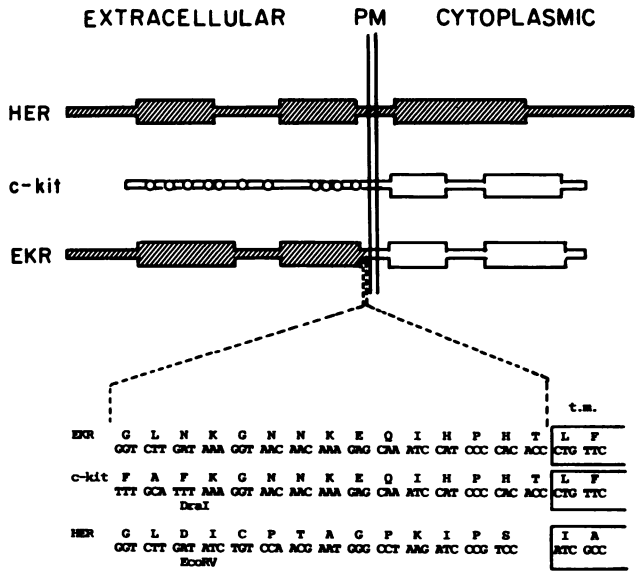

FIG. 1. Schematic structure of the EKR chimera. The structures of human EGF-R (HER) and the *kit* gene product are shown schematically. The locations of the transmembrane domains are indicated by the plasma membrane (PM). Boxes in the extracellular domain of EGF-R represent cysteine-rich domains, whereas the boxes in the cytoplasmic domains represent tyrosine kinase regions. Individual cysteine residues in the extracellular portion of $p145^{kit}$ are represented by open circles. Amino acid and nucleotide sequences at the junction region are shown below the diagram. The amino-terminal parts of the transmembrane domains (t.m.) are boxed, and the locations of restriction enzyme sites used for ligation are indicated (*Dra*I and *Eco*RV).

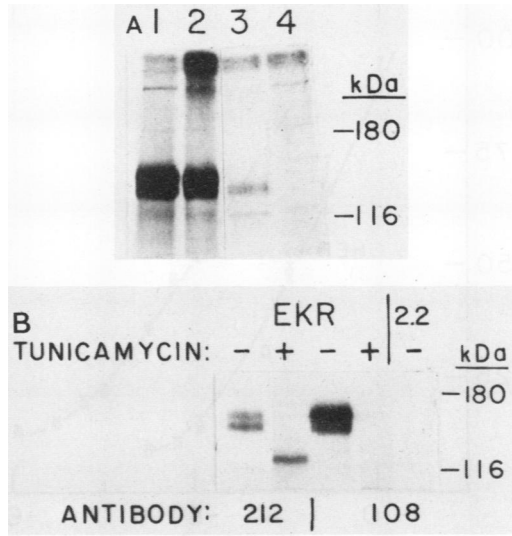

FIG. 2. Identification of the hybrid EKR in transfected cells by immunoprecipitation. (A) Neomycin-resistant clones of cells transfected with different receptor genes were labeled biosynthetically with [^{35}S]methionine and subjected to immunoprecipitation with a monoclonal antibody to EGF-R. An autoradiogram of the following transfected clones is shown: cells expressing the EKR chimera (clone 6 [lane 1] and clone 22 [lane 2]), cells overexpressing the c-*kit* gene (TD cells [lane 3]), and untransfected NIH 3T3 murine fibroblasts (clone 2.2 [lane 4]). Apparent molecular masses of marker proteins are indicated in kilodaltons. (B) Effect of inhibition of protein glycosylation on the chimeric receptor. Cells expressing EKR or untransfected murine fibroblasts (clone 2.2) were biosynthetically labeled with [^{35}S]methionine and either treated (+) or not treated (−) with tunicamycin (for 12 h at 37°C) to inhibit glycosylation. The receptors were immunoprecipitated with either a monoclonal antibody to EGF-R (108), which recognizes the extracellular portion of EGF-R, or a rabbit antibody directed to the carboxy terminus of the *kit* gene product (serum 212). An autoradiogram of the immunoprecipitated and gel electrophoresed samples is shown. Apparent molecular masses of marker proteins are indicated in kilodaltons.

concentrations of EGF. Western blot (immunoblot) analysis (11) of the immunoprecipitated EKR protein with antiphosphotyrosine antibodies (Fig. 4) revealed that the kinase of the chimeric receptor was ligand stimulated. However, relatively high ligand concentrations were required to activate the kinase. In conclusion, the chimeric receptor retained the capability to properly transmit the molecular signal from the ligand-binding domain to the catalytic tyrosine kinase portion.

Transmission of a mitogenic signal by the chimeric receptor. The elevation of tyrosine phosphorylation as a result of receptor activation leads to a proliferative cellular response in most receptor tyrosine kinases. To examine the possibility that the functionally active kinase of the EKR protein was capable of generating a mitogenic signal, we stimulated serum-starved EKR-6 cells with EGF and measured the incorporation of [^{3}H]thymidine into DNA 18 h later (11). EGF induced a strong mitogenic signal in cells expressing the chimeric receptor but not in untransfected murine fibroblasts (Fig. 5). Once again, relatively high concentrations of EGF were required to elicit a half-maximal mitogenic response: 25 ng/ml, as compared with 1 to 5 ng/ml in cells expressing EGF-R (Fig. 5; data not shown). However, when compared with the maximal response to 10% serum (~10-fold), the chimeric receptor yielded a higher signal (~6-fold) than did EGF-R (~3.5-fold). Thus, the *kit*-encoded kinase may be at least as efficient in mitogenesis as is EGF-R.

In vitro transforming potential of the EKR chimera. The strong mitogenic effect displayed by the chimeric receptor led us to test its transforming potential in vitro. Continuous culturing of EKR cells for 10 days with 100 ng of EGF per ml resulted in the appearance of refractile cells with long thin cellular processes, whereas EKR cells that were not treated with EGF and the parental untransfected cell line were flat and grew as single-layered confluent cultures (data not shown). To further analyze the EGF-dependent transformation, we cultured EKR cells in a semisolid medium to measure their anchorage-independent growth properties (13). Essentially no colonies were seen when no EGF was added to the growth medium of the chimera-expressing cells (Fig. 6). Treatment with EGF, however, gave rise to the appearance (after 2 weeks) of large (>100 cells) colonies. Colony sizes and numbers were comparable to those exhibited by B104-1-1 cells (Fig. 6), which are *neu*-transformed murine fibroblasts (1), or Rat1-EJ1 cells, which are *ras*-transformed rat fibroblasts (9; data not shown).

In conclusion, the binding of EGF to the chimeric receptor (EKR) not only elicited a strong growth response but also led to the appearance of a transformed phenotype comparable to that of established oncogenes. It is worth mentioning that a ligand-dependent transforming potential was demonstrated with the platelet-derived growth factor receptor (6), the CSF-1 receptor (21), and EGF-R (3, 20, 25).

The activation of chimeric tyrosine kinase receptors by heterologous ligands was demonstrated with chimeras among EGF-R, $p185^{neu}$, the insulin receptor, and the receptor for IGF-1 (8, 12, 13, 19). This approach is particularly

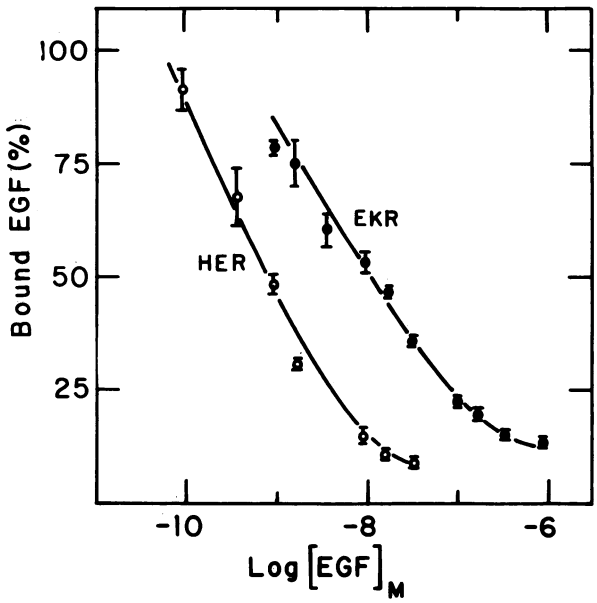

FIG. 3. Ligand binding to the chimeric receptor. Shown are displacement curves of radiolabeled EGF bound to cells that overexpressed either human EGF-R (HER) or the EKR chimera (EKR). ^{125}I-EGF (20 ng/ml) was displaced (for 90 min at 22°C) with increasing concentrations of unlabeled EGF. Cell-associated radioactivity was determined after extensive washing and expressed as a percentage of maximal binding of the radiolabeled ligand when present alone. Averages and ranges of duplicate determinations are given. This experiment was repeated four times, and essentially similar results were obtained (see the text).

useful in the study of receptorlike oncogenic proteins for which cognate ligands have not yet been identified. In the present study, we applied this approach to investigating the cellular function of the c-*kit* gene. Our study established a cellular system in which signal transduction by the c-*kit* product could be analyzed and provided functional evidence in support of the possibility that the c-*kit* proto-oncogene encodes a receptor for a mitogenic factor. The following conclusions can be drawn on the basis of our results. (i) The cytoplasmic tyrosine kinase portion of p145^{kit} can be allosterically regulated by ligand binding to a heterologous extracellular domain. It is therefore conceivable that the ectodomain of p145^{kit} can similarly regulate the *kit* enzymatic function once it binds to its cognate ligand. (ii) Upon stimulation, the active cytoplasmic portion of p145^{kit} generates a strong mitogenic signal which conveys, at least in some aspects, an oncogenic potential.

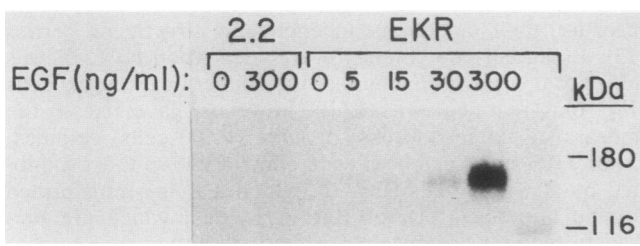

FIG. 4. Ligand-induced tyrosine phosphorylation of the chimeric receptor in living cells. Murine fibroblasts that expressed the EKR chimera (EKR) or untransfected cells (clone 2.2) were incubated with EGF at the indicated concentrations. Following a 15-min incubation at 22°C, cells were lysed and the receptor was immunoprecipitated with anti-EGF-R antibodies. The immunoprecipitates were subjected to get electrophoresis and then blotted to a nitrocellulose filter, which was later reacted with rabbit antibodies directed to phosphotyrosine. Finally, the washed filter was incubated with radiolabeled goat anti-rabbit immunoglobulin G (400,000 cpm/ml), washed, and exposed to X-ray film. The autoradiogram is shown together with the apparent molecular masses of marker proteins.

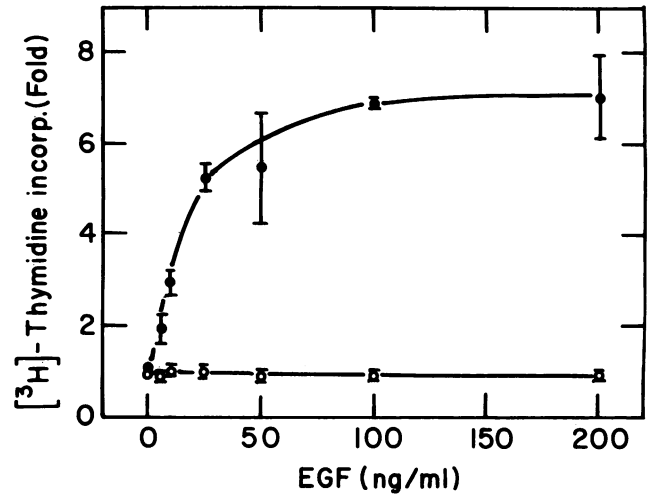

FIG. 5. Ligand-induced DNA synthesis of cells expressing the EKR chimera. Murine fibroblasts that expressed high levels of the EKR chimera (clone EKR-6 [●]) or untransfected murine fibroblasts (clone 2.2 [○]) were incubated with EGF at various concentrations in medium containing 0.5% calf serum. After 18 h at 37°C, [^{3}H] thymidine was added (2 μCi/ml), and the incorporation (incorp.) into DNA was determined 4 h later as described in the text. Duplicate determinations (averages and ranges) of a typical experiment (repeated three times) are expressed as stimulation over basal DNA synthesis.

The observed transforming potential of the chimeric receptor implies that the putative *kit* ligand may also convey oncogenic transformation. In addition, the observed ligand inducibility of the *kit*-encoded kinase (Fig. 4) provides a mechanistic basis for the functional defect in severe W mutants. Thus, mutations that abolish the catalytic function (17, 18, 23) probably render the cell unresponsive to the *kit* ligand.

Our present findings do not provide any hint as to the identification of the elusive ligand of p145^{kit}. However, our results suggest that a soluble factor can properly activate the receptor. This conclusion is important in light of the possibility raised by Kitamura and Fujita (7) that the *kit* ligand is membrane associated. In combination, the structural, genetic, and functional lines of evidence strongly support the possibility that p145^{kit} functions as a receptor for a still-unknown growth factor. In the absence of a ligand molecule, chimeric receptors like the one we constructed are probably the only means available today for comprehensive study of the biochemical pathways involved in the mitogenic and oncogenic actions of the *kit* products.

We thank Joseph Schlessinger for providing antibody 108 and Axel Ullrich for helpful discussions. We thank Esther Majerowich for typing the manuscript and Elior Peles for antiphosphotyrosine antibodies.

This work was supported by research grants form The Leukemia Foundation Inc. (to D.G. and Y.Y.) and The Israel Cancer Research Fund (to D.G.).

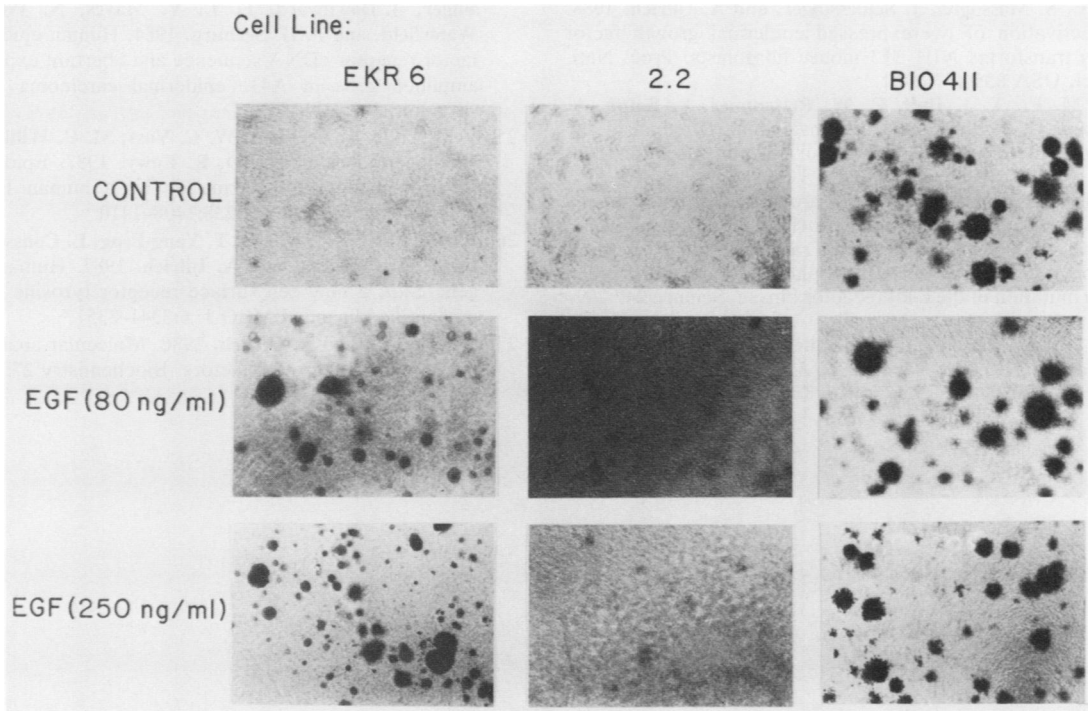

FIG. 6. Ligand-induced growth in soft agar of cells expressing the EKR chimera. Murine fibroblasts that expressed high levels of the chimeric receptor (EKR-6) or untransfected cells (clone 2.2) were suspended with trypsin and plated at 10^4 cells per 35-mm dish in the presence or absence of EGF (at the indicated concentrations) in medium containing agar. As a control, a *neu* oncogene-transformed cell line (B104-1-1) was included. Photographs shown were taken after 14 days of culturing.

LITERATURE CITED

1. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. Multiple independent activation of the *neu* oncogene by a point mutation altering the transmembrane domain of p. 185. Cell **45**:649–657.
2. **Chabot, B., D. A. Stephanson, P. Chapman, P. Besmer, and A. Bernstein.** 1988. The proto-oncogene c-*kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature (London) **335**:88–89.
3. **DiFiore, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, J. Schlessinger, and S. A. Aaronson.** 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Cell **51**:1063–1070.
4. **Geissler, E. D., M. A. Ryan, and D. E. Housman.** 1988. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell **55**:185–192.
5. **Greenfield, C., I. Hiles, M. D. Waterfield, M. Federwisch, A. Wollmer, T. L. Blundell, and N. McDonald.** 1989. Epidermal growth factor binding induces a conformational change in the external domain of its receptor. EMBO J. **8**:4115–4123.
6. **Huang, J. S., S. S. Huang, and T. F. Deuel.** 1984. Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF-cell-surface receptors. Cell **39**:79–87.
7. **Kitamura, Y., and J. Fujita.** 1989. Regulation of mast cell differentiation. Bioassays **10**:193–196.
8. **Lammers, R., A. Gray, J. Schlessinger, and A. Ullrich.** 1989. Differential signalling potential of insulin and IGF-1 receptor cytoplasmic domains. EMBO J. **8**:1369–1375.
9. **Land, H., L. F. Parada, and R. A. Weinberg.** 1984. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
10. **Lax, I., F. Bellot, R. Howk, A. Ullrich, D. Givol, and J. Schlessinger.** 1989. Functional analysis of the ligand binding site of EGF receptor utilizing chimeric chicken/human receptor molecules. EMBO J. **8**:421–427.
11. **Lax, I., A. Johnson, R. Howk, J. Sap, F. Bellot, M. Winkler, A. Ullrich, B. Vennstrom, J. Schlessinger, and D. Givol.** 1988. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol. Cell. Biol. **8**:1970–1978.
12. **Lee, J., T. J. Dull, I. Lax, J. Schlessinger, and A. Ullrich.** 1989. HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. EMBO J. **8**:167–173.
13. **Lehvaslaiho, H., L. Lehtola, L. Sistonsen, and K. Alitalo.** 1989. A chimeric EGF-R-*neu* proto-oncogene allows EGF to regulate *neu* tyrosine kinase and cell transformation. EMBO J. **8**:159–166.
14. **Livneh, E., R. Prywes, O. Kashles, N. Reiss, I. Sasson, Y. Mory, A. Ullrich, and J. Schlessinger.** 1986. Reconstitution of human epidermal growth factor receptor and its deletion mutants in cultured hamster cells. J. Biol. Chem. **261**:12490–12497.
15. **Majumder, S., K. Brown, F.-H. Qiu, and P. Besmer.** 1988. c-*kit* protein, a transmembrane kinase: identification in tissues and characterization. Mol. Cell. Biol. **8**:4896–4903.
16. **Nocka, K., S. Majumder, B. Chabot, P. Ray, M. Cervone, A. Bernstein, and P. Besmer.** 1989. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice—evidence for an impaired c-kit kinase in mutant mice. Genes Dev. **3**:816–826.
17. **Nocka, K., J. C. Tan, E. Chiu, T. Y. Chu, P. Ray, P. Traktman, and P. Besmer.** 1990. Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W^{37}, W^{v}, W^{41} and W. EMBO J. **9**:1805–1813.
18. **Reith, A. D., R. Rottapel, E. Giddens, C. Brady, L. Forrester, and A. Bernstein.** 1990. W mutant mice with mild or severe development defects contain point mutations in the kinase domain of the c-kit receptor. Genes Dev. **4**:390–400.
19. **Riedel, H., T. J. Dull, J. Schlessinger, and A. Ullrich.** 1986. A chimeric receptor allows insulin to stimulate tyrosine kinase activity of epidermal growth factor receptor. Nature (London) **324**:68–70.

20. **Riedel, H., S. Massoglia, J. Schlessinger, and A. Ullrich.** 1988. Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. Proc. Natl. Acad. Sci. USA **85**:1477–1481.
21. **Roussel, M. F., T. J. Dull, C. W. Rettenmier, P. Ralph, A. Ullrich, and C. J. Sherr.** 1987. Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature (London) **325**: 549–552.
22. **Schlessinger, J.** 1988. The epidermal growth factor receptor as a multifunctional allosteric protein. Biochemistry **27**:3119–3123.
23. **Tan, J. C., K. Nocka, P. Ray, P. Traktman, and P. Besmer.** 1990. The dominant W^{42} spotting phenotype results from a missense mutation in the c-kit receptor kinase. Science **247**:209–212.
24. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg.** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells. Nature (London) **309**:418–425.
25. **Velu, T. J., L. Begninot, W. C. Vass, M. C. Willingham, G. T. Merlino, I. Pastan, and D. R. Lowy.** 1987. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science **238**:1408–1410.
26. **Yarden, Y., W.-J. Kuang, T. Yang-Feng, L. Coussens, J. Schlessinger, U. Francke, and A. Ullrich.** 1987. Human proto-oncogene c-*kit*: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. **6**:3341–3351.
27. **Yarden, Y., and A. Ullrich.** 1986. Molecular analysis of signal transduction by growth factors. Biochemistry **27**:3113–3119.