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Different Structural Alterations Upregulate In Vitro Tyrosine Kinase Activity and Transforming Potency of the *erbB*-2 Gene

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Compared with normal *erbB*-2 gp185, mutant *erbB*-2 proteins generated by mutations either in the transmembrane domain or by NH_2-terminal deletion are able to transform NIH 3T3 cells at a 10- to 100-fold greater efficiency. Mutant proteins of both classes show increased tyrosine kinase activity, suggesting that an abnormal level of receptor-associated tyrosine kinase activity is a major determinant of *erbB*-2 oncogenic potential.

The *erbB*-2 gene encodes a glycoprotein of M_r 185,000 (gp185) possessing intrinsic tyrosine kinase activity (1) and displaying structural features typical of growth factor receptor molecules (4, 19). Several lines of evidence suggest that alterations of the *erbB*-2 gene can be involved in the transition from the normal to the malignant phenotype. In fact, *erbB*-2 was first identified as an amplified gene in a human mammary carcinoma (7) and in a salivary gland carcinoma (15). Subsequent studies have shown that this gene is amplified and overexpressed in as many as 40% of human breast carcinomas (8, 17, 18). Further evidence that alterations in *erbB*-2 gene expression can be involved in the transition from the normal to the malignant state comes from the observation that overexpression of *erbB*-2 gp185 at levels comparable with those of human mammary carcinoma cell lines containing an amplified *erbB*-2 gene is sufficient to transform NIH 3T3 cells (5, 6).

A different mechanism seems to be involved in the activation of *neu*, the rat homolog of *erbB*-2 (2), in ethylnitrosourea-induced rat neuroblastomas (16). *neu* has been shown to be converted into a transforming gene by a point mutation in the region coding for the transmembrane domain of this putative receptor protein (3). Another structural alteration able to activate the transforming potential of the *erbB*-2 protein is the deletion of its NH_2-terminal extracellular domain (5). This protein, termed *erbB*-2ΔN, can transform NIH 3T3 cells at levels of expression 5- to 10-fold lower than those necessary to achieve transformation with the normal protein (5). Thus, it appears that different molecular alterations, as well as overexpression of the normal gene, can activate *erbB*-2-transforming properties.

In this study, we sought to examine whether common or distinct biochemical mechanisms account for the increased transforming capability of different structurally altered *erbB*-2 proteins. By site-directed mutagenesis (9) on an *Eco*RI subclone of the *erbB*-2 cDNA (8), we engineered a mutant bearing at position 659 the Val-to-Glu substitution, i.e., the lesion responsible for activation of the *neu* oncogene (3). Since this mutation requires two base changes in the human sequence (from GTT to either GAA or GAG) and is therefore unlikely to occur in spontaneous human tumors, we also engineered a Val-to-Asp mutation, which is structurally similar and requires a single-base substitution from GTT to GAT. *erbB*-2 cDNAs harboring these mutations were expressed under the transcriptional control of either the Moloney-murine leukemia virus long terminal repeat (LTR) (LTR/*erbB*-2 Glu and LTR/*erbB*-2 Asp) or the simian virus 40 (SV40) early region promoter (SV40 *erbB*-2 Glu and SV40 *erbB*-2 Asp) in order to compare their transforming activities at different levels of expression with that of normal *erbB*-2 cDNA as well as that of the previously described mutant *erbB*-2ΔN (5).

Different classes of structural alterations upregulate *erbB*-2-transforming activity to a similar extent. Under SV40 promoter influence, both *erbB*-2 Glu and *erbB*-2 Asp cDNAs were able to transform NIH 3T3 cells with efficiencies of around 2×10^2 focus-forming units per pmol, comparable with that of the truncated *erbB*-2 gene (SV40/*erbB*-2ΔN) (Table 1). The normal full-length cDNA (SV40/*erbB*-2) failed to induce transformed foci under the same assay conditions. When expressed under LTR influence, the mutant cDNAs displayed a 10-fold-higher transforming efficiency than the full-length normal *erbB*-2 (Table 1). Thus, the Glu-659 and Asp-659 mutations both upregulate *erbB*-2-transforming efficiency to an extent similar to that of the previously described NH_2-terminal truncation.

Rate of synthesis and subcellular distribution are similar for normal and mutant *erbB*-2 proteins. Since we have previously reported that the transforming activity of *erbB*-2 gp185 is strictly dependent upon its level of expression, it was possible that the greater oncogenic activities of these mutant *erbB*-2 proteins were determined by increases in their steady-state levels of expression. Therefore, we subjected lysates prepared from mass cell populations transfected with different *erbB*-2 constructs to Western blot (immunoblot) analysis with anti-*erbB*-2 peptide sera, as previously described (8). LTR/*erbB*-2 Glu and LTR/*erbB*-2 Asp transformants (Fig. 1, lanes 3 and 4, respectively) expressed 185-kilodalton (kDa) proteins, which comigrated with the *erbB*-2 gp185 detected in LTR/*erbB*-2 transfectants (lane 2). In contrast, we detected an 86- to 98-kDa complex in LTR/*erbB*-2ΔN-transformed cells (Fig. 1, lane 5). In each case, the level of expression of the mutant protein was no greater than that of normal *erbB*-2 gp185. These results suggested

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TABLE 1. Comparison of transforming activities of different *erbB*-2 mutants[a]

erbB-2 cDNA clone	Transforming efficiency[b] with:	
	SV40 vector	LTR vector
erbB-2	$<5 \times 10^0$	2×10^3
erbB-2ΔN	1.5×10^2	4×10^4
erbB-2 Glu	2×10^2	3×10^4
erbB-2 Asp	2×10^2	3.5×10^4

[a] Transfection was performed by the calcium phosphate precipitation technique (5) with 40 μg of calf thymus DNA as a carrier and linear dilutions of circular plasmid DNA. Assays were scored at 14 to 21 days as previously described (5).

[b] Expressed as focus-forming units per picomole.

that differences in transforming activity could not be accounted for by alterations in the steady-state levels of the mutant proteins. Similar results were obtained with SV40 transfectants in which the respective levels of *erbB*-2 protein expression were 5- to 10-fold lower than in the LTR transfectants (data not shown).

Alteration in the subcellular localization of an oncogene product might be involved in the activation of its transforming potential. In fact, the structural alteration responsible for oncogenic activation of the membrane-spanning protein encoded by the *trk* proto-oncogene (11) is associated with an abnormal compartmentalization of the transforming gene product (12). Thus, we compared the subcellular distribution of normal *erbB*-2 gp185 with that of mutant proteins. A membrane fraction and a soluble cytoplasmic fraction were prepared from LTR/*erbB*-2, LTR/*erbB*-2ΔN, and LTR/*erbB*-2 Glu transfectants by the procedure of Robbins et al. (14). For each cell line, 5 μg of membrane proteins and 50 μg of cytosolic proteins were subjected to Western blot analysis. As expected for proteins with the structural features of membrane-spanning receptors, normal *erbB*-2 and *erbB*-2 Glu gp185 were localized to the membrane fraction (Fig. 2, lanes 1 and 3). In LTR/*erbB*-2ΔN transfectants, the majority of the *erbB*-2 protein was detected in the membrane fraction as an 86- to 98-kDa complex (Fig. 2, lane 5). An 86-kDa species accounting for about 20% of the total *erbB*-2ΔN protein was detected in the cytosolic fraction (Fig. 2, lane 6). In other studies, we found that this polypeptide comigrated with the single 86-kDa form specifically detected in tunicamycin-treated cells (data not shown). These data argue that some 86-kDa nonglycosylated *erbB*-2ΔN protein accumulates in the cytoplasm. This is probably due to inefficient translocation across the endoplasmic reticulum, since this molecule lacks the *erbB*-2 signal peptide sequence (5). The transferrin receptor also lacks a typical signal peptide and uses its transmembrane domain for translocation across the cellular membranes (22). It seems likely that the *erbB*-2 transmembrane domain serves this purpose in the *erbB*-2ΔN molecule but does so at a somewhat reduced efficiency.

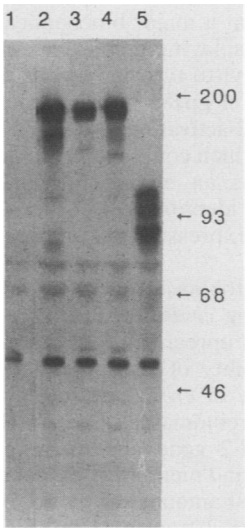

FIG. 1. Comparison by immunoblot analysis of steady-state expression of normal and mutant *erbB*-2 proteins in LTR transfectants. Total cellular protein (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blot analysis. Lanes: 1, NIH 3T3; 2, LTR/*erbB*-2; 3, LTR/*erbB*-2 Glu; 4, LTR/*erbB*-2 Asp; 5, LTR/*erbB*-2ΔN. Protein sizes are shown in kilodaltons.

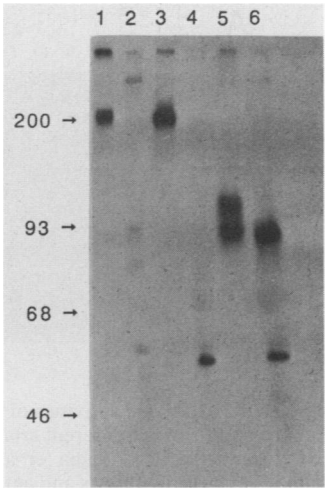

FIG. 2. Subcellular distribution of normal and mutant *erbB*-2 proteins in LTR transfectants. Membrane protein (5 μg; P100 fraction) and cytosolic protein (50 μg; S100 fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters for immunoblot analysis. Lanes: 1, 3, and 5, P100 fractions from LTR/*erbB*-2, LTR/*erbB*-2 Glu, and LTR/*erbB*-2ΔN, respectively; 2, 4, and 6, S100 fractions from LTR/*erbB*-2, LTR/*erbB*-2 Glu, and LTR/*erbB*-2ΔN, respectively.

Previous studies have shown that recombinant insulin receptor molecules which lack an extracellular domain are able to promote constitutive glucose uptake only if localized to the membrane compartment (10). In contrast, the *trk* oncogene product (11) represents a cytosolic transforming variant of its normal counterpart, a membrane-spanning receptor-like protein with tyrosine kinase activity (12, 13). It will be of interest to generate and express cytosolic *erbB*-2 variants in order to assess the possible contribution of p86 to the transforming activity of the NH_2-terminal truncated *erbB*-2 product.

Mutant *erbB*-2 proteins with increased transforming activity display increased in vitro tyrosine kinase activity. Since the above studies did not show major differences between the rates of synthesis and processing of our *erbB*-2 mutant proteins and that of normal *erbB*-2 gp185, we speculated that a critical difference might reside in the level of their tyrosine kinase activity. To address this issue, we used an immune complex kinase assay, essentially as described by Akiyama et al. (1). Equal amounts of *erbB*-2 proteins were immunoprecipitated from the individual lysates, as confirmed by

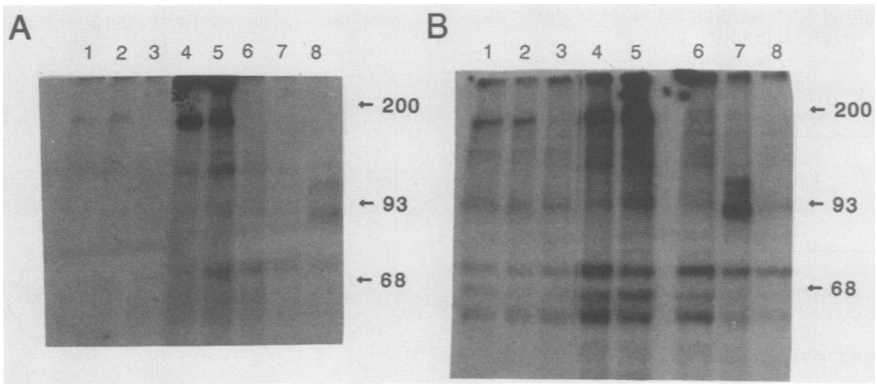

FIG. 3. Comparison of autokinase activities of normal and mutant proteins. Subconfluent cultures of LTR transfectants were subjected to detergent lysis. Equal amounts of the different *erbB*-2 proteins were immunoprecipitated from each lysate. The immune complex kinase assay was performed as described by Akiyama et al. (1) for 2 min (A) and 10 min (B). Reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. (A) Lanes: 1 and 2, LTR/*erbB*-2; 4, LTR/*erbB*-2 Asp; 5, LTR/*erbB*-2 Glu; 8, LTR/*erbB*-2ΔN; 3, 6, and 7, same as lanes 1, 4, and 8, respectively, except that immunoprecipitation was carried out with peptide-absorbed antiserum. (B) Lanes: 1 and 2, LTR/*erbB*-2; 4, LTR/*erbB*-2 Asp; 5, LTR/*erbB*-2 Glu; 7, LTR/*erbB*-2ΔN; 3, 6, and 8, same as lanes 1, 4, and 7, respectively, except that immunoprecipitation was carried out with peptide-absorbed antiserum. Both autoradiograms were obtained after exposure for 30 min.

Western blot analysis of cold immunoprecipitates (not shown). Preliminary kinetic experiments showed that under the assay conditions used, the gp185 autokinase reaction was linear in the first 5 min and reached a plateau by 10 to 15 min (data not shown). Therefore, we compared the autokinase activities of normal and *erbB*-2 mutant proteins in 2- and 10-min reactions, i.e., in both the linear and plateau phases of the reaction.

The proteins encoded by *erbB*-2 Glu, *erbB*-2 Asp, and *erbB*-2ΔN displayed four- to fivefold increases in autokinase activity compared with the normal *erbB*-2 gp185 (Fig. 3). This difference was detectable in both the early (2-min) and late (10-min) stages of the autokinase reaction (Fig. 3A and B, respectively). These results were confirmed in a series of four experiments using cell lines derived from two independent transfections (Table 2). The average increases in mutant protein autokinase activity over *erbB*-2 gp185 autokinase activity were between four- and sevenfold at 2 min and between 3.7- and 5.3-fold at 10 min. A formal possibility was that these differences were due to a different extent of tyrosine phosphorylation of the immunoprecipitated *erbB*-2 proteins before in vitro ATP triggering. However, under the lysing conditions used (i.e., a relatively low detergent concentration and no phosphatase inhibitors), the different *erbB*-2 proteins did not show detectable phosphorylation on tyrosine residues before ATP triggering. Therefore, the observed differences represent entirely de novo tyrosine phosphorylation.

TABLE 2. Comparison of tyrosine kinase activities of *erbB*-2 mutant proteins and normal *erbB*-2 gp185[a]

Mutant protein	Increase (fold) over normal *erbB*-2 gp185 activity			
	Autokinase		Polyglutamic acid-tyrosine kinase	
	2 min	10 min	5 min	15 min
erbB-2ΔN gp86-98	7.2	5.3	3.2	3.5
erbB-2 Glu gp185	5.6	4.1	5.5	2.6
erbB-2 Asp gp185	4.1	3.7	3.1	2.5

[a] For each reaction, equal amounts of *erbB*-2 protein were immunoprecipitated from lysates of mass cell populations transfected with different LTR vectors. Reactions were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and specific radioactivity was counted after excision of the specific bands from the gel. As a control, reactions were performed on immunoprecipitates obtained with peptide-absorbed antiserum. Data represent the mean values of four separate experiments.

We also compared the catalytic function of the two classes of *erbB*-2 mutant proteins with that of the normal *erbB*-2 product by assaying their abilities to phosphorylate in vitro the exogenous substrate polyglutamic acid-tyrosine. This assay was performed essentially as the immune complex kinase assay, with the addition of 2 μg of polyglutamic acid-tyrosine and 20 μM cold ATP in each reaction. The mutant proteins encoded by *erbB*-2 Glu, *erbB*-2 Asp, and *erbB*-2ΔN were three- to fourfold more efficient than normal gp185 in the polyglutamic acid-tyrosine kinase assay in both 5- and 15-min reactions (Table 2). Taken together, these results indicate that a major biochemical marker of mutant *erbB*-2 proteins displaying increased transforming potential is an enhanced in vitro tyrosine kinase activity.

Specific activity of gp185 tyrosine kinase as a marker for detection of *erbB*-2-activating lesions in human tumors. Although cell lines which contain an amplified *erbB*-2 gene and which overexpress an electrophoretically normal *erbB*-2 protein have been identified (8), it has not yet been proven that the protein expressed has a normal, unmutated sequence.

The in vitro autokinase assay provided us with an approach to analyzing *erbB*-2 proteins for genetic alterations associated with an upregulation of their catalytic functions. To test the feasibility of this approach, we measured the autokinase activity of *erbB*-2 protein synthesized by a human mammary carcinoma cell line, SKBr3, which contains an amplified *erbB*-2 gene and overexpresses normal-size *erbB*-2 mRNA (8) and membrane-associated 185-kDa *erbB*-2 glycoprotein. Equal amounts of gp185 expressed by SKBr3 cells and LTR/*erbB*-2 transfectants (Fig. 4, lanes 1 and 2, respectively) displayed very similar autokinase activity, which was fivefold lower than those of LTR/*erbB*-2ΔN and LTR/*erbB*-2 Glu gp185 (lanes 3 and 4, respectively). These findings provide evidence that amplification of a normal rather than a mutated *erbB*-2-coding sequence was selected for in the SKBr3 tumor cell line.

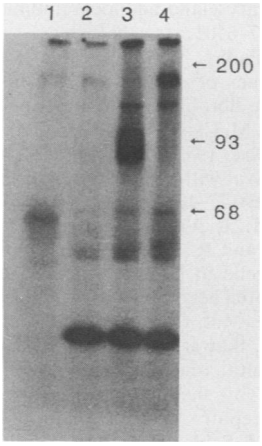

FIG. 4. Specific tyrosine kinase activity of *erbB*-2 gp185 expressed by human tumor cells. Subconfluent cultures of SKBr3 cells and LTR/*erbB*-2, LTR/*erbB*-2 Glu, and LTR/*erbB*-2ΔN transfectants were subjected to detergent lysis. Equal amounts of the different *erbB*-2 proteins were immunoprecipitated from each lysate. The immune complex kinase assay was performed for 2 min. Reactions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, SKBr3; 2, LTR/*erbB*-2; 3, LTR/*erbB*-2ΔN; 4, LTR/*erbB*-2 Glu.

The oncogenic activity of the *erbB*-2 gene product appears to be a function of its overall kinase function. Different classes of structural alterations were shown to upregulate the transforming activity of the *erbB*-2 gene product to a similar extent. This effect was particularly striking when the mutant cDNAs were expressed under the SV40 early region promoter. Under these experimental conditions, the normal *erbB*-2 cDNA did not induce cell transformation, whereas the three mutant cDNAs were able to transform NIH 3T3 cells with an efficiency of around 2×10^2 focus-forming units per pmol of DNA. This difference in transforming activity could not be accounted for by alterations in the rate of synthesis or in the subcellular compartmentalization of the mutant proteins compared with normal *erbB*-2 gp185. However, a good correlation emerged between the increased transforming ability of different classes of *erbB*-2 mutant proteins and their in vitro tyrosine kinase activities. Thus, it appears that a crucial event in *erbB*-2-induced cell transformation is the achievement of a critical threshold of receptor-associated tyrosine kinase activity. In a given cell this can be caused by low levels of expression of structurally altered *erbB*-2 proteins possessing increased tyrosine kinase activity or by overexpression of normal gp185. The concept that abnormally high levels of *erbB*-2 gp185 can be sufficient to confer a growth advantage in tumorigenesis is supported by our demonstration that the *erbB*-2 sequences amplified in the tumor cell line SKBr3 encode a protein displaying normal tyrosine kinase activity.

In view of our demonstration that the Asp-659 mutation (GTT→GAT) was as effective as the Glu-659 mutation (GTT→GAA or GAG) in upregulating *erbB*-2-transforming activity as well as its tyrosine kinase activity, it is possible that point mutations at codon 659 of the *erbB*-2 gene might be selected for in human tumors. The immune complex kinase assay outlined in this study represents a relatively simple assay for screening gp185 produced by human tumors for abnormalities in their levels of tyrosine kinase activity and should therefore help in assessing the frequency of such activating lesions in human malignancies.

The question arises as to how two very different structural alterations of the *erbB*-2 protein induce similar biochemical effects. There is some evidence that ligand-induced activation of epidermal growth factor receptor tyrosine kinase activity is associated with the formation of highly active dimeric or oligomeric structures (20, 21). Thus, it is possible that the two classes of *erbB*-2 mutants display an increased ability to form catalytically active oligomers in vivo. Alternatively, it is possible that ligand binding induces an allosteric conformational change which activates the receptor catalytic domain by an intramolecular mechanism. The NH_2-terminal truncation may deprive the *erbB*-2 molecule of its ligand-binding domain and may thus mimic the allosteric activation of the tyrosine kinase domain. Similarly, substitution of Val-659 in the highly hydrophobic transmembrane domain with a negatively charged amino acid residue may induce a structural alteration which mimics the ligand-induced conformational change. Whether or not this increased in vitro tyrosine kinase activity of *erbB*-2 mutants is reflected in vivo by an increased rate of phosphorylation of cellular substrates awaits further studies. The availability of cell lines expressing different levels of *erbB*-2 mutant proteins with increased tyrosine kinase activity will probably facilitate the characterization of cellular substrates for the *erbB*-2 receptor kinase.

ADDENDUM

After the submission of our manuscript, Bargmann and Weinberg have reported similar studies on the rat *neu* gene product. While their data differ somewhat from our own as to the extent of activation of the *neu* gp185 by NH_2-terminal truncation or Asp mutation (EMBO J., 7:2043–2052, 1988), they similarly observe a good correlation between upregulation of *neu* gp185-transforming ability and increase in its tyrosine kinase activity (Proc. Natl. Acad. Sci. USA **85:** 5394–5398, 1988).

LITERATURE CITED

1. **Akiyama, T., C. Sudo, H. Ogawara, K. Toyoshima, and T. Yamamoto.** 1986. The product of the human c-*erb*B-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science **232:**1644–1646.
2. **Bargmann, C. I., M. C. Hung, and R. A. Weinberg.** 1986. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature (London) **319:**226–230.
3. **Bargmann, C. I., M. C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell **45:** 649–657.
4. **Coussens, L., T. L. Yang-Feng, Y.C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science **230:**1132–1139.
5. **Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson.** 1987. *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science **237:**178–182.
6. **Hudziak, R. M., J. Schlessinger, and A. Ullrich.** 1987. Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH/3T3 cells. Proc. Natl. Acad. Sci. USA **84:**7159–7163.
7. **King, C. R., M. H. Kraus, and S. A. Aaronson.** 1985. Amplification of a novel v-*erb*B-2 related gene in a human mammary carcinoma. Science **229:**974–976.
8. **Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King.** 1987. Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different

molecular mechanisms. EMBO J. **6**:605–610.
9. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**: 488–492.
10. **Leland, E., D. O. Morgan, E. Clauser, R. A. Roth, and W. Rutter.** 1987. A membrane-anchored cytoplasmic domain of the human insulin receptor mediates a constitutively elevated insulin-independent uptake of 2-deoxyglucose. Mol. Endocrinol. **1**: 15–24.
11. **Martin-Zanca, D., S. H. Hughes, and M. Barbacid.** 1986. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature (London) **319**: 743–748.
12. **Mitra, G., D. Martin-Zanca, and M. Barbacid.** 1987. Identification and biochemical characterization of $p70^{TRK}$, product of the human TRK oncogene. Proc. Natl. Acad. Sci. USA **84**:6707–6711.
13. **Oskam, R., R. Coulier, M. Ernst, D. Martin-Zanca, and M. Barbacid.** 1988. Frequent generation of oncogenes by *in vitro* recombination of TRK protooncogene sequences. Proc. Natl. Acad. Sci. USA **85**:2964–2968.
14. **Robbins, K. C., R. Leal, J. H. Pierce, and S. A. Aaronson.** 1985. The v-*sis*/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. **4**:1783–1792.
15. **Semba, K., N. Kamata, K. Toyoshima, and T. Yamamoto.** 1985. A v-*erb*B-related protooncogene, c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA **82**:6497–6501.
16. **Shih, C., L. C. Padhy, M. Muray, and R. A. Weinberg.** 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature (London) **290**:261–264.
17. **Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire.** 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science **235**:177–182.
18. **Van de Vijver, M., R. Van de Bersselaar, P. Devilee, C. Cornelisse, J. Peterse, and R. Nusse.** 1987. Amplification of the *neu* (c-*erbB*-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erbA* oncogene. Mol. Cell. Biol. **7**:2019–2023.
19. **Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito, and K. Toyoshima.** 1986. Similarity of protein encoded by the human c-*erb*B-2 gene to epidermal growth factor receptor. Nature (London) **319**:230–234.
20. **Yarden, Y., and J. Schlessinger.** 1987. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry **26**:1443–1451.
21. **Yarden, Y., and J. Schlessinger.** 1987. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. Biochemistry **26**:1434–1442.
22. **Zerial, M., P. Melancon, C. Schneider, and H. Garoff.** 1986. The transmembrane segment of the human transferrin receptor functions as a signal peptide. EMBO J. **5**:1543–1550.