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A CHIMERIC EGFR/*neu* RECEPTOR IN FUNCTIONAL ANALYSIS OF THE *neu* ONCOPROTEIN

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As the factor binding to the *neu* protein has been unknown, it has not been possible to confirm experimentally the proposed growth-factor receptor like functions of the *neu* protein. To approach this problem we constructed a recombinant receptor which enabled ligand regulation of the *neu* tyrosine kinase. The hybrid receptor consisted of the extracellular ligand binding, transmembrane and protein kinase C-substrate domains joined to the intracellular tyrosine kinase and carboxyl-terminal domains of the *neu* protein. Several properties of NIH3T3 cells carrying this construct were tested. We obtained the first experimental evidence that the *neu* proto-oncogene has mitogenic and transforming activities only in the presence of a ligand stimulating its tyrosine kinase activity. Various cellular and molecular biological parameters indicated that the chimeric receptor behaved very similarly to the EGFR. Also, this chimeric receptor has allowed us to compare the constitutive oncogenic and the ligand-activated non-oncogenic activities of the *neu* tyrosine kinase. In the future we plan to focus on characterization of possible differences between EGFR and *neu* signalling in more differentiated cellular backgrounds.

Key words: *neu* oncogene, EGF receptor, signal transduction.

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The *neu* oncogene

The *neu* proto-oncogene encodes a 185 kD cell surface growth factor receptor-like protein (1–3). p185^{neu} is closely homologous to the epidermal growth factor receptor (EGFR) and has an intrinsic tyrosine kinase activity (1–6). *neu* has been found to be oncogenically activated by a point mutation in its transmembrane region where a glutamic acid residue replaces a valine residue at amino acid 664 (3, 7, 8). The activated form of p185^{neu} shows increased autophosphorylation and induces increased tyrosine phosphorylation of other cellular proteins (9, 10). Unlike the proto-oncogene, the *neu* oncogene transforms cells in vitro (11) and leads to the development of mammary carcinomas in transgenic mice when driven by a mouse mammary tumor virus long terminal repeat promoter (12, 13). The proto-oncogene transforms fibroblasts only when vastly overexpressed (6). A significant correlation has been found between amplification of *neu* (the human counterpart is also called HER-2/*erb*B-2) and poor prognosis of human breast cancer (14–17).

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Structure and expression of the chimeric EGFR/*neu* receptor

The approach used to characterize in our studies was to produce a recombinant receptor which would enable ligand regulation of the *neu* tyrosine kinase. We fused the extracellular, ligand-binding portion of the EGFR to intracellular domains of the *neu* tyrosine kinase with the prediction that EGF would regulate the tyrosine kinase activity

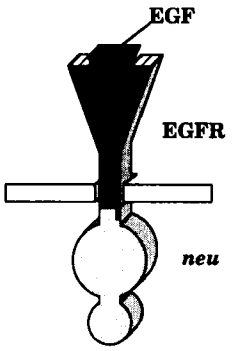

Figure. Structure of the EGFR/*neu* chimeric receptor. EGFR extracellular, transmembrane and protein kinase C domains are fused to the intracellular tyrosine kinase and carboxyl terminal domains of rat *neu* protein.

of the recombinant receptor and induce signal transduction typical for activated *neu*.

The chimeric EGFR/*neu* construct was made utilizing a conserved restriction site in the juxtamembrane domain adjacent to the transmembrane domain of the EGFR and *neu* cDNAs (18). This region is highly conserved between the EGFR and *neu* proteins. The resulting expression vector, pSV2EGFR/*neu*, was used to transfect mouse fibroblasts (NIH3T3 cells). With the help of *neu*-specific antibodies and [^{125}I]-EGF binding, a number of cell clones expressing the EGFR/*neu* hybrid protein were isolated (Figure). Radioactive EGF binding analysis indicated that cells expressing the hybrid protein have specific high-affinity receptors for EGF on their surface. Based on these experiments the approximate number of receptors per cell in characterized clones ranged from 4×10^5 to 2×10^6. This is well above the estimated number of EGFR in these cells ($3-6 \times 10^3$). As expected, the apparent molecular weight of the hybrid protein was slightly higher than that of *neu* protein, approximately 190 000. By immunofluorescence we showed that the protein is located on the plasma membrane in a correct orientation. In all subsequent experiments where NIH3T3 or its neomycin-resistant derivative NN (for NIH *neo*) cells were used as controls, their EGF-dependent responses were found to be absent or barely detectable when compared to the receptor-transfected cells.

Receptor characteristics and autophosphorylation

As is well established for EGFR, also EGFR/*neu* formed two classes of receptors with different affinities for EGF. In cells expressing high amounts of both *neu* and EGF receptors, heterodimers with approximately a 100-fold higher affinity to EGF than the normal high affinity state of the EGFR have been reported (19). No evidence of such extremely high affinity binding was obtained in our experiments, apparently due to minimal EGFR expression in the cells. The formation of heterodimers has also been suggested to be responsible for *neu* transphosphorylation by ligand-activated EGFR (20–22). Wast *neu* overexpression may also lead to cell transformation in the absence of added ligand (6, 19, 23, 24).

Ligand activation of the EGFR/*neu* receptor tyrosine kinase in the transfected cells was monitored using anti-phosphotyrosine antibodies, which specifically recognize tyrosyl side chains with a covalently attached phosphate group. With this method, it was shown that autophosphorylation of the hybrid protein occurs only in the presence of EGF or TGFα.

Growth regulation

In all assays tested, the EGFR/*neu* expressing cells were strongly influenced by EGF. Cellular morphology changed from flat to highly elongated and DNA synthesis of serum starved cells increased after the addition of EGF. The responses were dependent on the concentration of EGF in the assay (25), and the threshold concentration of EGF for maximum induction was dependent on the number of EGFR/*neu* receptors (26). Growth in soft agar was also dependent on EGF and correlated with receptor number/cell. Furthermore, soft agar growth was similar when TGFα was used instead of EGF (18). Our studies indicated that in the presence of EGF, the EGFR/*neu* receptor gave 2- to 5-fold less soft agar colonies than the *neu*NT oncoprotein and about 10-fold less than the c-Ha-*ras* oncogene (18, 27).

By contrast, the *neu* proto-oncogene did not show kinase activity or transforming properties when expressed at similar levels in NIH3T3 cells. Other studies have suggested that the normal *neu* proto-oncogene can transform cells, if it is sufficiently overexpressed (6). However, the level of expression obtained in these studies is apparently higher than in our experiments and in experiments where no transformation was seen even when the amplification level of *neu* was increased with selection (7, 11, 28). Parallel with our study (18), Lee et al. (29) reported a construct where instead of *neu*, c-*erb*B-2 cytoplasmic domain was used. In this vector, the EGFR/c-*erb*B2 joining site was constructed using synthetic oligonucleotides introduced in the proximal portion of the extracellular domain. The results obtained with this construct were similar to ours in all relevant aspects.

These results suggested that the *neu* proto-oncogene possesses mitogenic and transforming properties in the presence of a ligand which stimulates its tyrosine kinase activity and they provided the first model for studies of the function of the *neu* tyrosine kinase. These experiments also provide evidence that the growth promoting properties of the *neu* tyrosine kinase, and therefore apparently of the intact *neu* protein, are similar to those of EGFR. Also, they confirm the hypothesis that heterologous domains of *neu* and EGFR can be combined to form a functional receptor.

Cellular responses to *neu* kinase

When the growth promoting effects of the *neu* tyrosine kinase were found identical with those of EGFR, we went on to test other effects of the ligand activated *neu* tyrosine kinase. These studies had a dual purpose: to further analyse the extent of functional similarities between *neu* and EGFR protein and to find potential differences in cellular responses to ligand binding to the two receptors. It should be noted that coexpression of these two related receptors has been reported in various cell types (21, 30).

Several morphological effects of EGF addition were observed in cells expressing the EGFR/*neu* hybrid protein. Time-lapse videography recorded extensive membrane ruffling, increased pinocytosis and extension of lamellar footpads at the cell periphery. Using immunofluorescence microscopy, the extension of footpads was related to partial reorganization of cellular actin stress fibers (25). More delayed changes were acquisition of an elongated shape, unordered orientation of the cells and formation of multilayered foci of cells (20, 25, 26). Somewhat similar changes have been reported in mouse epithelial cells exposed to EGF (31).

In collaboration with A. Pandiella and J. Meldolesi, we showed that other cellular proteins and pathways are involved in the *neu* tyrosine kinase-induced signal transduction. As is well established for the EGFR, the *neu* tyrosine kinase was shown to induce phospholipid hydrolysis, an increase of intracellular calcium and plasma membrane hyperpolarization (26). These results show that upon ligand binding the chimeric EGFR/*neu* protein undergoes a typical receptor downregulation and transduces cellular signals with characteristics similar to the EGF receptor (32–34).

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