The EMBO Journal vol.15 no.2 pp.254–264, 1996

ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer

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Overexpression of the *erbB-2* gene contributes to aggressive behavior of various human adenocarcinomas, including breast cancer, through an unknown molecular mechanism. The *erbB-2*-encoded protein is a member of the ErbB family of growth factor receptors, but no direct ligand of ErbB-2 has been reported. We show that in various cells ErbB-2 can form heterodimers with both EGF receptor (ErbB-1) and NDF receptors (ErbB-3 and ErbB-4), suggesting that it may affect the action of heterologous ligands without the involvement of a direct ErbB-2 ligand. This possibility was addressed in breast cancer cells through either overexpression of ErbB-2 or by blocking its delivery to the cell surface by means of an endoplasmic reticulum-trapped antibody. We report that ErbB-2 overexpression enhanced binding affinities to both EGF and NDF, through deceleration of ligand dissociation rates. Likewise, removal of ErbB-2 from the cell surface almost completely abolished ligand binding by accelerating dissociation of both growth factors. The kinetic effects resulted in enhancement and prolongation of the stimulation of two major cytoplasmic signaling pathways, namely: MAP kinase (ERK) and c-Jun kinase (SAPK), by either ligand. Our results imply that ErbB-2 is a pan-ErbB subunit of the high affinity heterodimeric receptors for NDF and EGF. Therefore, the oncogenic action of ErbB-2 in human cancers may be due to its ability to potentiate *in trans* growth factor signaling.

Keywords: growth factors/oncogene/receptor dimerization/signal transduction/tyrosine kinase

Introduction

Many growth and differentiation signals are transmitted into the cell's interior by means of receptors whose cytoplasmic domains share an intrinsic tyrosine kinase catalytic activity (Fantl *et al.*, 1993). On the basis of structural determinants it is possible to classify the known receptors into subgroups that contain two to nine highly related receptors. This multiplicity does not exist in insect cells, suggesting that it has been evolved in order to accommodate increasing physiological requirements. While the mechanism of signal transduction by a single receptor is relatively well characterized and involves sequential steps of receptor activation and association with cytoplasmic effector proteins, the mechanisms that propagate signals from one receptor to other signaling systems remain poorly understood. One example is the ErbB subfamily of receptor tyrosine kinases. All of the four members of the group share a similar primary structure but they differ remarkably in their ligand specificity and kinase function (Peles and Yarden, 1993; Carraway and Cantley, 1994): ErbB-1 binds to several distinct ligands whose prototype is the epidermal growth factor (EGF), while both ErbB-3 and ErbB-4 bind all of the isoforms of a related factor called Neu differentiation factor (NDF, or heregulin). ErbB-2 binds no known ligand (Dougall *et al.*, 1994), but unlike ErbB-1 whose kinase activity is strictly ligand dependent, and ErbB-3, which is characterized by very low kinase activity (Kraus *et al.*, 1993; Guy *et al.*, 1994; Prigent and Gullick, 1994), the catalytic action of ErbB-2 is relatively high, even in the absence of a ligand (Lonardo *et al.*, 1990).

Although several ErbB ligands and receptors were implicated in cancer, most of the interest has been focused on ErbB-2 (Hynes and Stern, 1994). Whereas the rodent homolog of the gene can undergo oncogenic activation, as a result of a point mutation (Bargmann *et al.*, 1986), the unmutated human gene is frequently amplified or otherwise overexpressed in several types of human adenocarcinomas, including breast tumors (Slamon *et al.*, 1987; Varley *et al.*, 1987). Importantly, retrospective analyses of several types of human tumor correlated *erbB-2* overexpression with poor prognosis of breast, ovarian and lung cancer (Slamon *et al.*, 1989; Kern *et al.*, 1990). The gene is amplified in mammary carcinomas of all stages but not in benign breast tumors, and overexpression is maintained in the metastatic lesions (Igelhart *et al.*, 1990). The mechanism by which an overexpressed ErbB-2 contributes to the development of a more aggressive phenotype of cancer cells is currently unknown. The answer to this question may be related to another open issue, namely whether or not ErbB-2 has a ligand of its own. Several putative ligands that presumably interact directly with ErbB-2 were purified from breast cancer cells (Lupu *et al.*, 1992), bovine kidney (Huang and Huang, 1992), activated macrophages (Tarakhovsky *et al.*, 1991) and human T cells (Dobashi *et al.*, 1991). However, in no case was it proven by molecular cloning that the activity corresponds to a direct ErbB-2 ligand.

Even without a ligand of its own, ErbB-2 can undergo activation by heterologous ligands. EGF has been shown

to elevate tyrosine phosphorylation of ErbB-2 in living cells through the formation of heterodimers between ErbB-1 (EGF receptor) and ErbB-2 (Goldman *et al.*, 1990; Wada *et al.*, 1990). Moreover, simultaneous overexpression of the two proteins synergistically affected malignant transformation of murine fibroblasts (Kokai *et al.*, 1989). This was accompanied by an increased binding affinity and mitogenic responsiveness to EGF (Wada *et al.*, 1990). Likewise, NDF elevates tyrosine phosphorylation of ErbB-2 through binding to its own receptors (Peles *et al.*, 1993; Plowman *et al.*, 1993; Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1994), and heterodimerization of ErbB-3 with ErbB-2 has been implicated in the elevation of binding affinity to NDF (Sliwkowski *et al.*, 1994). On the other hand, ErbB-1–ErbB-3 heterodimers presumably enable EGF to recruit phosphatidylinositol 3′-kinase through ErbB-3 (Soltoff *et al.*, 1994). It thus appears that ErbB-2 may participate in combinatorial receptor–receptor interactions, and thereby it may play an important role in signal transduction by both NDF and EGF, even without the involvement of an endogenous ligand. This possibility has been recently supported by the observation that blocking ErbB-2 expression at the cell surface impaired various aspects of cytoplasmic signaling by both NDF and EGF (Graus-Porta *et al.*, 1995). To further examine the role of ErbB-2 in signal transduction and oncogenesis by these two families of growth factors, we analyzed kinetic and signaling effects of either overexpression or complete removal of the protein from the surface of mammary tumor cells. Our results imply that ErbB-2 is an essential component of the high affinity receptors of NDF and EGF, and thereby it significantly affects signaling by the two growth factors.

Results

ErbB-2 heterodimerizes with ErbB-1, ErbB-3 and ErbB-4

To examine the effect of ErbB-2 on binding and responsiveness of mammary tumor cells to heterologous ErbB ligands, we used two human breast cancer cell lines, MCF-7 and T47D. Two derivatives of these cell lines were employed as model cellular systems. In the first one, the endogenous low level of *erbB-2* expression in MCF-7 cells was elevated through gene transfer, resulting in MCF-7/ErbB-2 cells (Peles *et al.*, 1993). The second cellular system comprised two derivatives of the T47D cell line, that originally has moderate *erbB-2* expression: T47D-puro; a control infected cell line, and T47D-5R cells that display no ErbB-2 at the cell surface as a result of ectopic expression, in the endoplasmic reticulum, of an engineered single chain monoclonal antibody to ErbB-2 (Beerli *et al.*, 1994). Immunoprecipitation of biosynthetically-labeled ErbB-2 from extracts of MCF-7/ErbB-2 cells showed that expression of the protein was elevated by 8-10-fold relative to the parental cell line (Figure 1A). Conversely, the level of ErbB-2 expression is the same in both derivatives of the T47D cell line. However, the corresponding protein band in the 5R cells displayed faster electrophoretic migration (Figure 1A) because of its retention in the endoplasmic reticulum (Graus-Porta *et al.*, 1995). In order to determine if the changes in ErbB-2 expression were accompanied by alterations in the

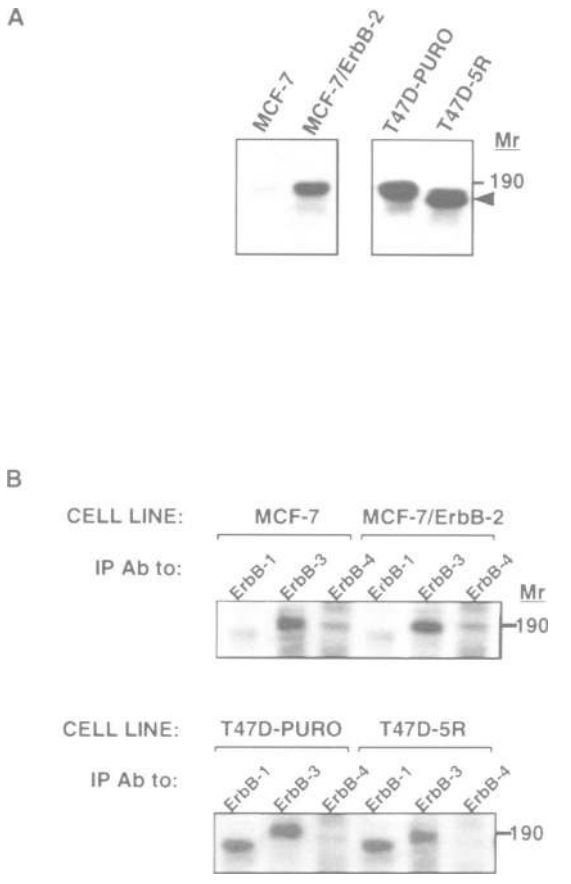

Fig. 1. Immunoprecipitation of biosynthetically-labeled ErbB proteins from MCF-7 and T47D cells. (**A**) The indicated cell lines were incubated for 16 h in [^{35}S]methionine-containing medium and their ErbB-2 proteins were subjected to immunoprecipitation by using rabbit antibodies to the carboxy-terminal peptide of ErbB-2. Note overexpression of ErbB-2 in MCF-7/ErbB-2 cells and the increased electrophoretic mobility of the endoplasmic reticulum-trapped ErbB-2 in T47D-5R cells (arrowhead). (**B**) Immunoprecipitates of the indicated ErbB-1, ErbB-3 and ErbB-4 proteins were prepared from the indicated pairs of biosynthetically-labeled cells by using monoclonal antibodies to ErbB-1 (Kawamoto *et al.*, 1983), ErbB-2 (Stancovski *et al.*, 1991) or ErbB-4, or polyclonal rabbit antibodies to a recombinant ectodomain of ErbB-3. This was followed by electrophoresis and autoradiography as described above. Equal amounts of acid-precipitable radioactivity in cell lysates were taken for each lane. The extensively washed immunoprecipitates were resolved by gel electrophoresis and autoradiography.

expression of other ErbB proteins, we performed additional immunoprecipitation analyses with antibodies to ErbB-1, ErbB-3 and ErbB-4. These experiments indicated that both MCF-7 cells and T47D cells express the three other members of the ErbB family, but their expression levels displayed no significant change in the derivative cell lines (Figure 1B). This conclusion was supported also by determination of cell surface expression of the three ErbB proteins by using specific monoclonal antibodies and a radiolabeled secondary antibody (data not shown). In both parental cell lines, ErbB-4 was the least abundant member and ErbB-3 displayed relatively high levels of expression.

Consistent with the presence of all ErbB proteins, radiolabeled NDF and EGF underwent covalent crosslinking to monomers and dimers of their receptors in the parental cell lines and in their derivatives (Figure 2). Significantly, immunoprecipitation of ErbB-2 from lysates

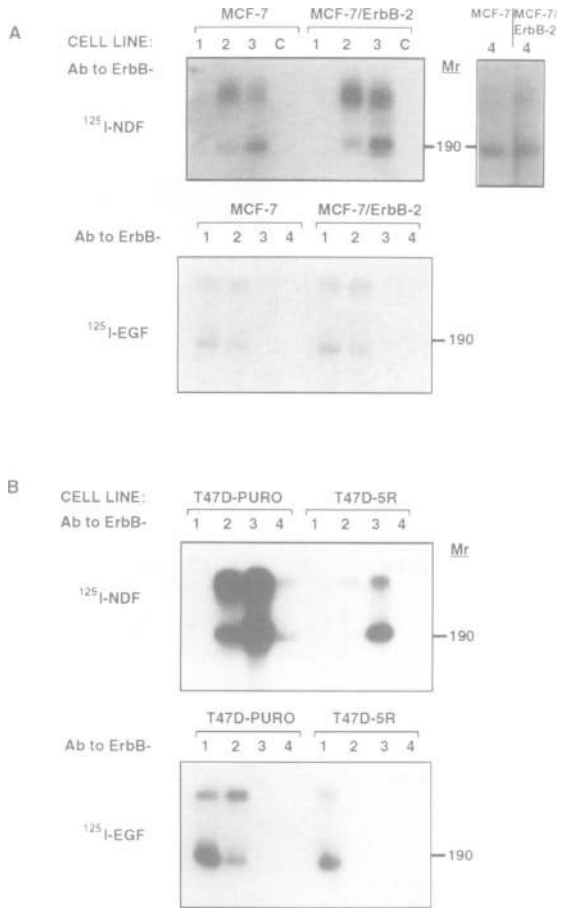

Fig. 2. Affinity labeling of NDF and EGF receptors and their co-immunoprecipitation with the ErbB-2 protein. Monolayers of either MCF-7 cells and their derivative line MCF-7/ErbB-2 (**A**) or T47D-puro cells and their 5R derivatives (**B**) were incubated with the indicated radiolabeled ligands and then subjected to covalent crosslinking analysis at 4°C as described in Materials and methods. Cell lysates were prepared and antibodies to the indicated ErbB proteins or control antibodies (labeled C) were used in immunoprecipitation. Autoradiograms of the gel-resolved immunoprecipitates are shown.

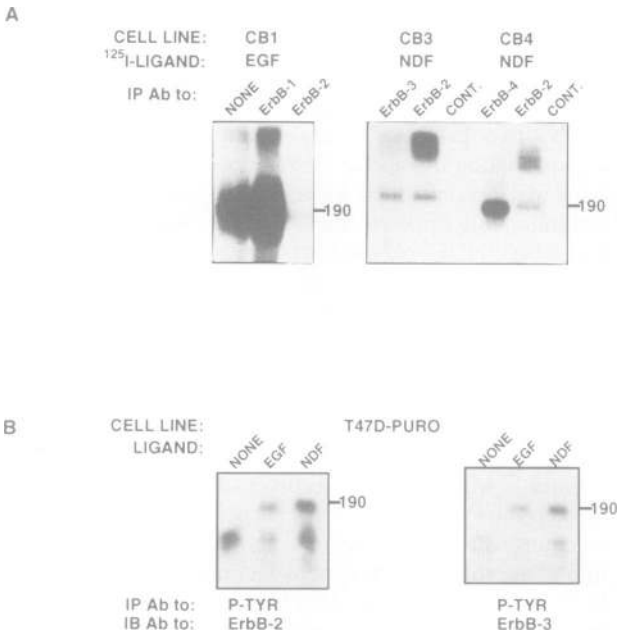

Fig. 3. Inter-receptor interactions and transphosphorylation. (**A**) CHO cell lines that overexpress either EGF receptor/ErbB-1 (CB1 cells), ErbB-3 (CB3 cells) or ErbB-4 (CB4 cells) were subjected to covalent crosslinking of radiolabeled NDF or EGF as described in the legend to Figure 2. The indicated receptors were immunoprecipitated (IP) by using specific antibodies. For control, we directly subjected whole cell lysates to gel electrophoresis (lane labeled NONE) or used a monoclonal antibody to the fibroblast growth factor receptor (lanes labeled CONT.). Immunoprecipitates were washed extensively and resolved by gel electrophoresis (6.5% acrylamide), which was followed by autoradiography. (**B**) Monolayers of T47D-puro cells were treated for 10 min at 37°C with NDF or EGF (each at 50 ng/ml), or left untreated (NONE). The cells were transferred to 4°C, washed in PBS and solubilized in solubilization buffer that contained phosphatase inhibitors. Phosphotyrosine-containing proteins were immunoprecipitated (IP) by using Sepharose–protein A-immobilized antibody to phosphotyrosine and the complexes resolved by gel electrophoresis. After electrophoretic transfer to nitrocellulose, the filters were immunoblotted (IB) with rabbit antibodies that are directed to the carboxy-terminal peptide of either ErbB-2 or ErbB-3.

of the four cell lines with a specific antiserum led to coprecipitation of monomeric and dimeric forms of affinity-labeled NDF and EGF receptors. This observation implied that in both MCF-7 and T47D cells, ErbB-2 physically interacts with the three other ErbB proteins. However, although ErbB-2 does not bind to any of the two ligands (Peles *et al.*, 1993), its presence enhanced affinity labeling of the other ErbB proteins. For example, overexpression of ErbB-2 in MCF-7 cells resulted in an increased covalent crosslinking of NDF to its receptors, namely ErbB-3 and ErbB-4 (Figure 2A). Similarly, the absence of ErbB-2 at the surface of T47D-5R cells caused reduced labeling of the two NDF receptors (Figure 2B). The effect of ErbB-2 on affinity labeling of the EGF receptor was similar but less prominent (compare for example the signals observed with T47D-puro cells to those of T47D-5R cells in the lower panel of Figure 2B). In contrast to the strong signals of [^{125}I]EGF and [^{125}I]NDF, that were precipitated with antibodies to ErbB-2, we observed only faint NDF signals in immunoprecipitates of ErbB-1/EGF receptor (compare for example the first two lanes in the upper panels of Figure 2A and B). Likewise, [^{125}I]EGF signals were hardly precipitable by ErbB-3- and ErbB-4-specific antibodies, suggesting that ErbB-2-containing heterodimers are preferred over heterodimeric complexes of ErbB-1 with ErbB-3, and probably also with the least abundant receptor, ErbB-4.

In order to further analyze the interactions of NDF and EGF receptors with ErbB-2, and also compare the relative stability of the various ErbB-2-containing heterodimers, we established another model cellular system, namely transfected Chinese hamster ovary (CHO) cells. These cells express a moderate amount of endogenous ErbB-2, but no other member of the family. Therefore, introduction of the three other ErbB proteins, by means of transfection of the corresponding expression vectors, should enable examination of individual heterodimers. To this end we established three stable CHO cell lines that express either ErbB-1 (denoted CB1), ErbB-3 (CB3 cell line) or ErbB-4 (CB4 cell line). Despite overexpression, we were unable to detect ligand-induced ErbB-2 heterodimers in these cell lines, by blotting gel-separated immunoprecipitates with antibodies to ErbB-2 or to the transfected receptor. How-

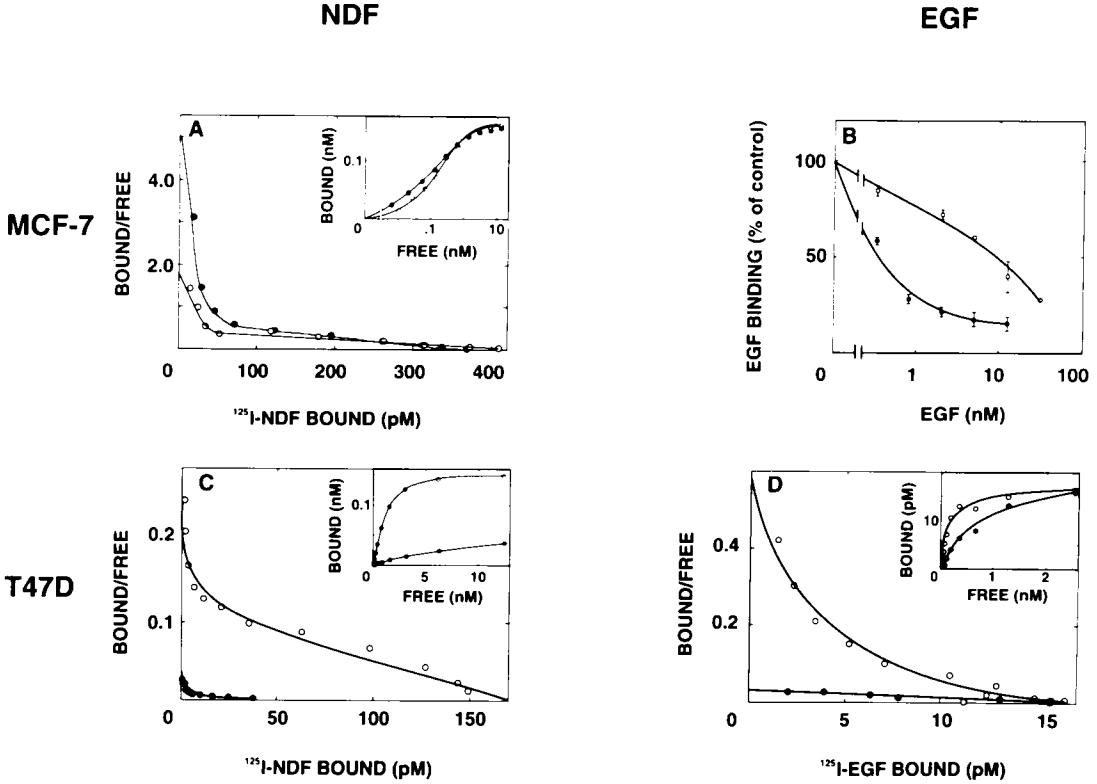

Fig. 4. Demonstration of ErbB-2-dependent changes in the cell surface binding of NDF and EGF. Monolayers ($1–2\times10^5$ cells/well in 48-well dishes) of the indicated cell lines were incubated for 2 h at 4°C with different concentrations of ^{125}I-labeled NDF-$\beta1_{177-246}$ or [^{125}I]EGF, as indicated (**A**, **C** and **D**), or with radiolabeled EGF (5ng/ml) in the presence of increasing concentrations of the unlabeled ligand (**B**). Cells were then washed three times with ice-cold binding buffer, lysed in 0.5 ml of 0.1N NaOH containing 0.1% SDS and the radioactivity was determined. Binding results are presented as Scatchard plots, saturation curves (insets) or displacement curves (B). Open circles refer to the control parental cell lines and closed circles indicate results obtained with the respective modified cell lines MCF-7/ErbB-2 and T47D-5R. Each data point represents the average and standard deviation (bars) of triplicate determinations, after substraction of non-specific ligand binding. The assays were performed at least twice.

ever, the more sensitive assay that uses co-immunoprecipitation of affinity-labeled NDF or EGF receptors together with the endogenous ErbB-2 protein of CHO cells, provided direct support for the conclusion that ErbB-2 can form heterodimers with ErbB-1, ErbB-3 and ErbB-4. Thus, by using CB1 cells, we detected the affinity-labeled EGF receptor in ErbB-2 immunoprecipitates (Figure 3A). In contrast to this relatively weak interaction, affinity labeling of the two NDF receptors in CB3 and CB4 cells revealed extensive interactions of these receptors with ErbB-2. Similar to the breast cancer cells (Figure 2), anti-ErbB-2 antibodies precipitated from CB3 and CB4 cell extracts, primarily a dimeric form of NDF receptors, most likely a heterodimer that contains ErbB-2 (Figure 3A). The reason why a larger amount of the presumed heterodimer is precipitated by antibodies to ErbB-2, as compared with anti-ErbB-3 and anti-ErbB-4 precipitates, may be related to better accessibility of ErbB-2 epitopes in the heterodimer. The interaction between ErbB-4 and ErbB-2 is especially important, because we could not rigorously establish it in MCF-7 and in T47D cells due to the very low expression level of ErbB-4 in these cells. In conclusion, ErbB-2 interacts with the three other members of the family in a manner that is independent of the specific cellular environment. In addition, ErbB-2 heterodimers that contain NDF receptors are apparently more stable than ErbB-1-ErbB-2 heterodimers.

Another aspect of heterodimer formation between ErbB-2 and either NDF or EGF receptors is the ability of these receptors to increase tyrosine phosphorylation of ErbB-2, possibly via an *in trans* mechanism. We chose to examine this aspect in T47D-puro cells, because they express relatively high levels of all four members of the ErbB family, unlike MCF-7 cells in which ErbB-1 is expressed at very low levels (Figure 1B). The results shown in Figure 3B indicate that in T47D cells, ErbB-2 underwent increased tyrosine phosphorylation after binding of either EGF or NDF. It is important to note that the latter ligand induced a stronger signal, in accordance with the superior ability of NDF receptors to heterodimerize with ErbB-2 (Figure 3A). Consistent with previous reports that documented interactions between EGF receptor and ErbB-3 (Soltoff *et al.*, 1994), we found that EGF, as well as NDF, increased phosphorylation of ErbB-3 in T47D cells (Figure 3B). Because ErbB-3 has very low, if any, tyrosine kinase activity (Prigent and Gullick, 1994), the observed phosphorylation should be mediated *in trans*, by either ErbB-2 or ErbB-1.

Binding of NDF and EGF to breast cancer cells depends on the presence of ErbB-2 at the cell surface and is enhanced by ErbB-2 overexpression

We next analyzed direct binding of radiolabeled NDF and EGF to the T47D-5R and MCF-7/ErbB-2 cell lines (Figure 4). Scatchard analyses of the binding data revealed that in the absence of ErbB-2 at the cell surface, binding of

Table I. Summary of ligand binding parameters of *erbB-2*-expressing cells

	NDF			EGF		
	K_d (nM)		k_{off} ($s^{-1} \times 10^{-4}$)	K_d (nM)		k_{off} ($s^{-1} \times 10^{-4}$)
	High	Low		High	Low	
MCF-7	0.04	0.8	0.91	(9)		6.7
MCF-7/ErbB-2	0.02	0.6	0.26	(0.8)		3.9
T47D	0.05	1.2	0.5	0.01	0.08	2.6
T47D-5R	0.2	8.5	2.3	None	0.4	6.1

Listed are the parameters obtained from the data presented in Figure 4 (K_d equilibrium) and Figure 5 (k_{off}). The data in parentheses refer to displacement curves (Figure 4B). Ligand dissociation constants were derived from the initial (fast) components of the corresponding dissociation curves.

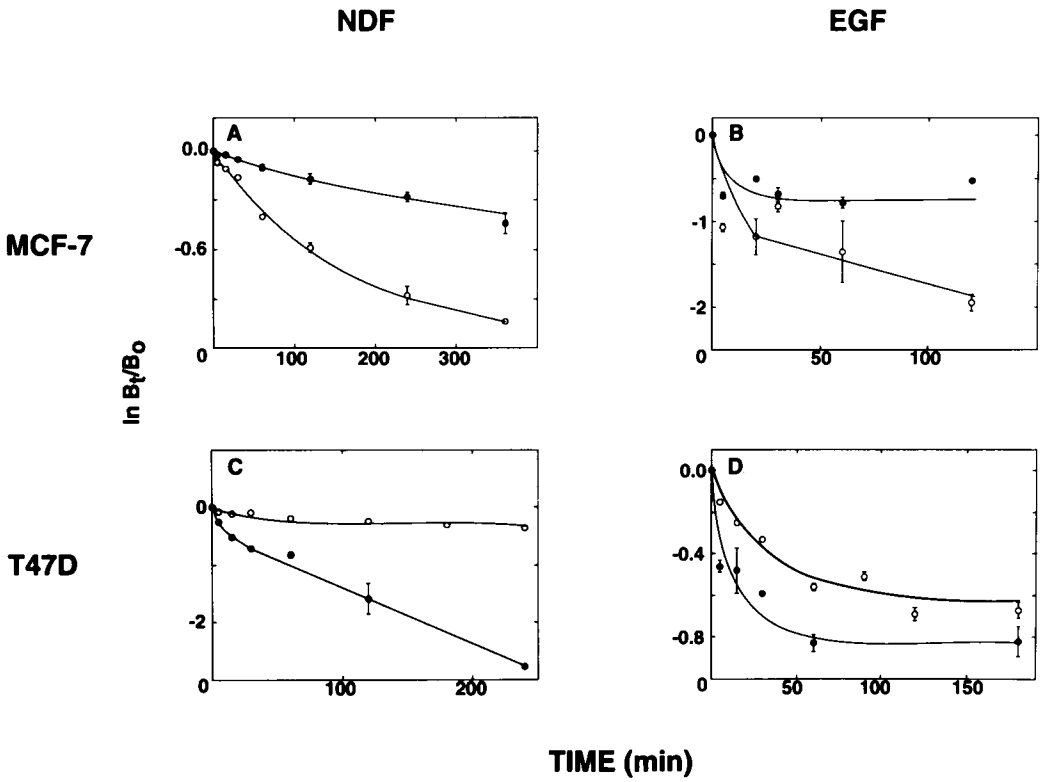

Fig. 5. ErbB-2 delays dissociation of NDF and EGF from their receptors. Dissociation kinetics of the indicated radiolabeled ligands were analyzed on confluent monolayers of MCF-7 cells and T47D cells, that were preincubated at 4°C with the corresponding ligand at saturating concentrations. After removal of unbound ligand, the cells were incubated with the unlabeled ligand and radioactivity that was released from cells, as well as the residual cell-bound ligand, was determined as a function of the time of incubation at 4°C. The results were expressed in terms of the ratio between the amount of ligand that remained bound at time t (B_t) and the initially bound radioactivity (B_o). Nonspecifically bound and released radioactivity was subtracted. Open circles refer to the parental cell lines and closed circles refer to the derivative lines MCF-7/ErbB-2 and T47D-5R. The experiments were repeated twice.

both ligands was dramatically reduced. The curvilinear Scatchard curve of NDF binding to the parental T47D-puro cells indicated the existence of two populations of NDF binding sites: a major population of low affinity receptors (~87% of the total number of sites), and a minor high affinity class of ligand binding sites, whose affinity was higher by 24-fold (Figure 4C). Both sites almost completely disappeared in T47D-5R cells, and their affinities were remarkably reduced (Table I). Likewise, in the absence of cell surface ErbB-2, all of the high affinity binding sites of EGF disappeared and the affinity of the major population of sites was reduced (Figure 4D and Table I). However, in contrast to the case of NDF receptors, the number of the low affinity EGF binding sites remained unchanged.

Consistent with the *trans*-regulatory effect of ErbB-2 in T47D cells, ErbB-2 overexpression in MCF-7 cells was associated with increased binding affinities to both NDF (Figure 4A) and EGF (Figure 4B), in agreement with previous observations that were made in rodent and insect cells (Wada *et al.*, 1990; Sliwkowski *et al.*, 1994). Due to the relatively low number of EGF receptors in MCF-7 cells, we were unable to perform Scatchard analysis. However, ligand displacement analysis indicated an 11-fold increase in EGF binding affinity (Figure 4B) as a result of the ~8-fold enhanced expression of ErbB-2 in

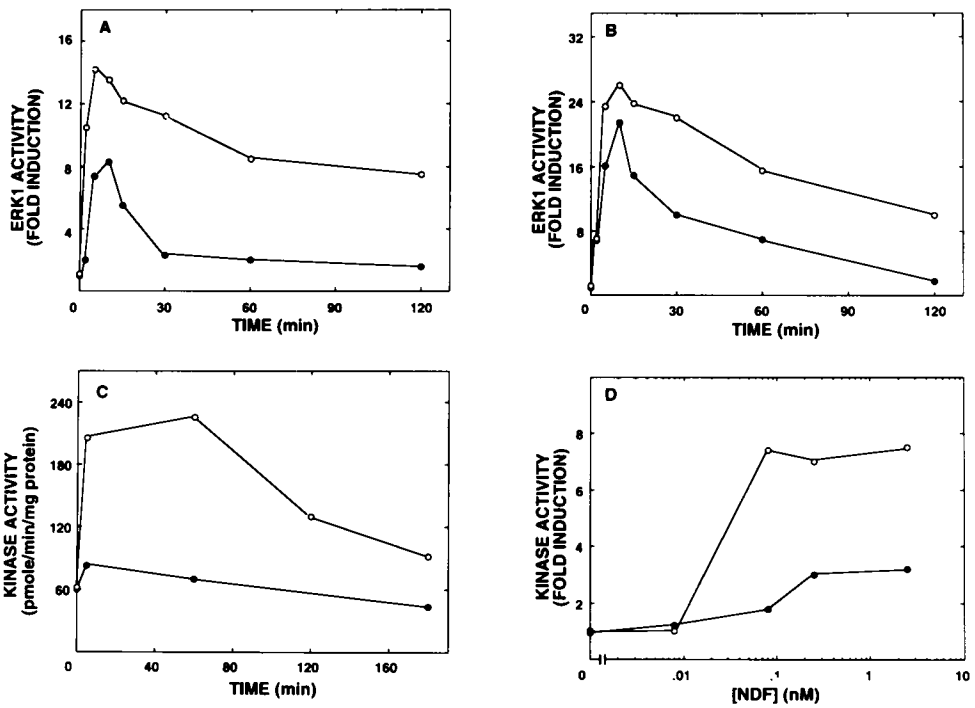

Fig. 6. Cell surface expression of ErbB-2 potentiates and prolongs the activation of MAP kinase by NDF and EGF. Monolayers of T47D-puro cells (open circles) or their 5R derivatives (closed circles) were serum-starved for 12 h and then treated with either NDF or EGF. Cell lysates were prepared and assayed for MAP kinase activity. (**A** and **B**) Cells were stimulated for the indicated time intervals with 0.5 nM NDF (A) or EGF (B) and the ERK1 isoform of MAP kinase was immunoprecipitated from cell lysates by using a specific antibody. Kinase activity was determined by using myelin basic protein (MBP) as a substrate, gel electrophoresis, and quantitation of MBP phosphorylation by image analysis. (**C**) Cells were stimulated with 0.5 nM NDF for the indicated time intervals. A cellular fraction that contains >85% of both ERK isoforms was isolated by ion-exchange chromatography on mini-columns (Seger *et al.*, 1994). MAP kinase activity was determined by using MBP as a substrate. (**D**) Cells were stimulated for 10 min with the indicated concentrations of NDF. Cell lysates were prepared and their MAP kinase activity was determined in MBP-containing polyacrylamide gels as described (Tobe *et al.*, 1991). Briefly, cell lysates were electrophoresed on an SDS–polyacrylamide gel that was polymerized with MBP (0.5 mg/ml). After electrophoresis, denaturation and renaturation, the gel was incubated for 1 h in phosphorylation buffer containing [γ-^{32}P]ATP. The amount of radioactivity that was incorporated into MBP in the gel region corresponding to 42–45 kDa bands, was quantitated after extensive washing.

MCF-7/ErbB-2 cells. Although the effect on NDF binding to MCF-7 cells was lower than that observed in T47D cells, in both cases ErbB-2 affected mostly the high affinity population of binding sites (Figure 4A), which has previously been attributed to receptor dimers and heterodimers (Wada *et al.*, 1990; Ben-Levy *et al.*, 1992).

ErbB-2 decelerates dissociation rates of its heterologous ligands

Because ligand binding is determined by both the rate of association with the receptor and by the rate of dissociation of the ligand–receptor complex, we examined the question concerning which of these processes is affected by ErbB-2. While analyses of the association rates of NDF and EGF revealed relatively small effects upon changes in ErbB-2 expression (data not shown), determination of the rates of dissociation of both ligands indicated that ErbB-2 significantly reduced the release of each ligand from its own receptor (Figure 5). The rates of dissociation of NDF and EGF from the parental MCF-7 cells were rapid and displayed biphasic kinetics (Figure 5A and B). Conversely, in the presence of an overexpressed ErbB-2 the rate of release of either ligand was slower, and in the case of NDF it displayed linear kinetics (Figure 5A and Table I). Similarly, blocking ErbB-2 delivery to the surface of T47D cells accelerated NDF dissociation and replaced the linear kinetics of release with a biphasic behavior (Figure 5C and Table I). It is important to note that the effect of ErbB-2 on the release of NDF from both MCF-7 and T47D cells was significantly more extensive than that observed with EGF (Figure 5D), consistent with our conclusion that the interaction of ErbB-2 with NDF receptor(s) is stronger than with the EGF receptor.

ErbB-2 potentiates and prolongs NDF- and EGF-induced activation of MAP kinase and c-Jun kinase

To examine the intracellular effect of the ErbB-2-mediated lengthening of the half lives of ligand–receptor complexes, we concentrated on the activation of two protein kinase cascades that funnel many extracellular signals to the nucleus (Marshall, 1994; Seger and Krebs, 1995): c-Jun kinase (JNK, also known as stress-activated protein kinase, SAPK) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994), and mitogen-activated protein kinase (MAP kinase/ERK). Importantly, the latter mediates the oncogenic action of ErbB-2 (Ben-Levy *et al.*, 1994), and is included in a group of other signaling molecules (e.g. Shc and p70/p85^{S6K}), whose activation in T47D cells is potentiated by ErbB-2 (Graus-Porta *et al.*, 1995). We first concentrated on T47D cells and their 5R derivatives, in which ErbB-2 has been shown to enhance activation of the ERK2 isoform of MAP kinase (Graus-Porta *et al.*, 1995). Determination of kinase activity in immunoprecipitates of the ERK1 isoform, that were prepared from NDF- (Figure 6A) or EGF-

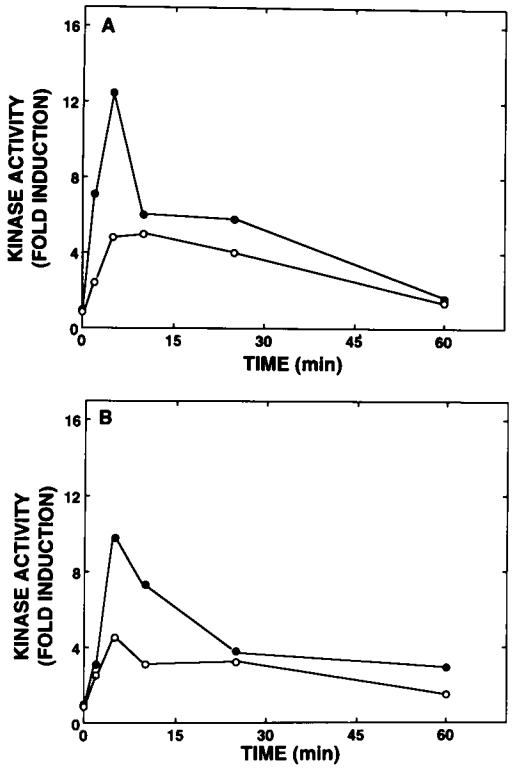

Fig. 7. Overexpression of ErbB-2 potentiates activation of MAP kinase in MCF-7 cells. Monolayers of MCF-7 cells (open circles) or MCF-7/ErbB-2 cells (closed circles) were incubated for different time intervals with 0.5 nM of either NDF (**A**) or EGF (**B**). MAP kinase activity in cell extracts was determined by using an in-gel kinase assay with MBP as a substrate, as described in the legend to Figure 6D.

treated cells (Figure 6B), revealed that the absence of ErbB-2 at the cell surface caused significant reduction in the extent and duration of kinase activation by either growth factor. The effect of ErbB-2 on the NDF-induced signal was larger than on the EGF-induced activation, in accordance with the results of ligand binding and dissociation analyses. We therefore concentrated on the effect of NDF and attempted to confirm it by using two alternative MAP kinase assays, that do not require the use of MAP kinase-specific antibodies, which may interfere with kinase activity *in vitro*. The assays involved fractionation of whole lysates from NDF-treated cells by using either column chromatography or gel electrophoresis, and was followed by phosphorylation of myelin basic protein (MBP) *in vitro*. Isolation of a fraction that contains >85% of ERK activity confirmed that T47D cells that express no ErbB-2 at the cell surface lost most of the effect of NDF on MAP kinase activation (Figure 6C). By using the in-gel assay of MAP kinase, we found that in the absence of ErbB-2 the concentration-dependency of MAP kinase activation by NDF was dramatically shifted to the right (Figure 6D). This means that the presence of ErbB-2 allowed T47D cells to respond to lower concentrations of NDF than cells that do not express this co-receptor, which is in accordance with observations made by using the two other assays of MAP kinase.

Comparison of the response of MCF-7 cells with that of their *erbB-2*-overexpressing derivatives, by using the in-gel assay of MAP kinase, indicated that ErbB-2 potentiated MAP kinase activation by either NDF (Figure 7A) or EGF (Figure 7B) and also prolonged the ligand-induced active state of the enzyme. Qualitatively similar results were obtained when we used the two other methods of MAP kinase assays (data not shown).

In order to examine JNK activation we prepared whole cell extracts from growth factor-stimulated cells and determined their ability to phosphorylate a recombinant c-Jun protein (Hibi *et al.*, 1993). The extent of c-Jun phosphorylation *in vitro* was determined after gel electrophoresis and autoradiography (insets in Figure 8) that was complemented by densitometry. Analysis of the T47D cell lines revealed that in the absence of a cell surface-localized ErbB-2, JNK activation by NDF was significantly reduced at all concentrations of the ligand (Figure 8A). In addition, in the presence of ErbB-2 at the cell surface, the kinetic of JNK activation was longer than in its absence and it returned to the base line only after 2 h (Figure 8B and data not shown). Kinetic analyses of the activation of c-Jun kinase in MCF-7 cells by either NDF (Figure 8C) or EGF (Figure 8D) similarly indicated that ErbB-2 overexpression augmented and extended the stimulatory action of both ligands. For example, whereas in the parental MCF-7 cells maximal JNK activation by NDF was 3-fold and it returned to base line after 1.5 h, in ErbB-2-overexpressing cells maximal activation reached 8-fold and it returned to the initial level after 3 h. The response of MCF-7 cells to EGF was relatively small (Figure 8D), probably because these cells express very low levels of EGF receptor. Nevertheless, overexpression of ErbB-2 augmented the JNK signals. Taken together, these results imply that ErbB-2 presence at the cell surface, and the extent of its overexpression, significantly affect the potency and duration of MAP kinase and JNK activation by either NDF or EGF, probably because ErbB-2 decelerates the rates of dissociation of the heterologous ligands.

Discussion

Despite extensive knowledge on the phenomenology of *erbB-2* overexpression in human cancer and detailed structure–function analyses of the putative receptor, the exact molecular mechanism that underlies transformability by the apparently normal gene product remained obscure (Hynes and Stern, 1994). This is due in part to the failure of many attempts to identify a direct ligand of ErbB-2 (Peles and Yarden, 1993). The results of our present study indicate that ErbB-2 may be considered an integral subunit of NDF and EGF receptors, and as such it may have no direct ligand. Consequently, we raise the possibility that at least part of the transforming ability of an overexpressed ErbB-2 is due to transactivation of growth factor signaling.

ErbB-2 is a pan-ErbB receptor subunit

The major support to our contention that ErbB-2 should be considered an essential common subunit of NDF and EGF receptors is based on the remarkable reduction in binding of the two ligands in the absence of ErbB-2 at the cell surface (Figure 4). The results of affinity labeling experiments are also supportive, but the effect of ErbB-2 expression is less extensive (Figure 2). We attribute this difference to the observation that ErbB-2 primarily affects the process of ligand dissociation (Figure 5), which is blocked by the procedure of covalent crosslinking. Because

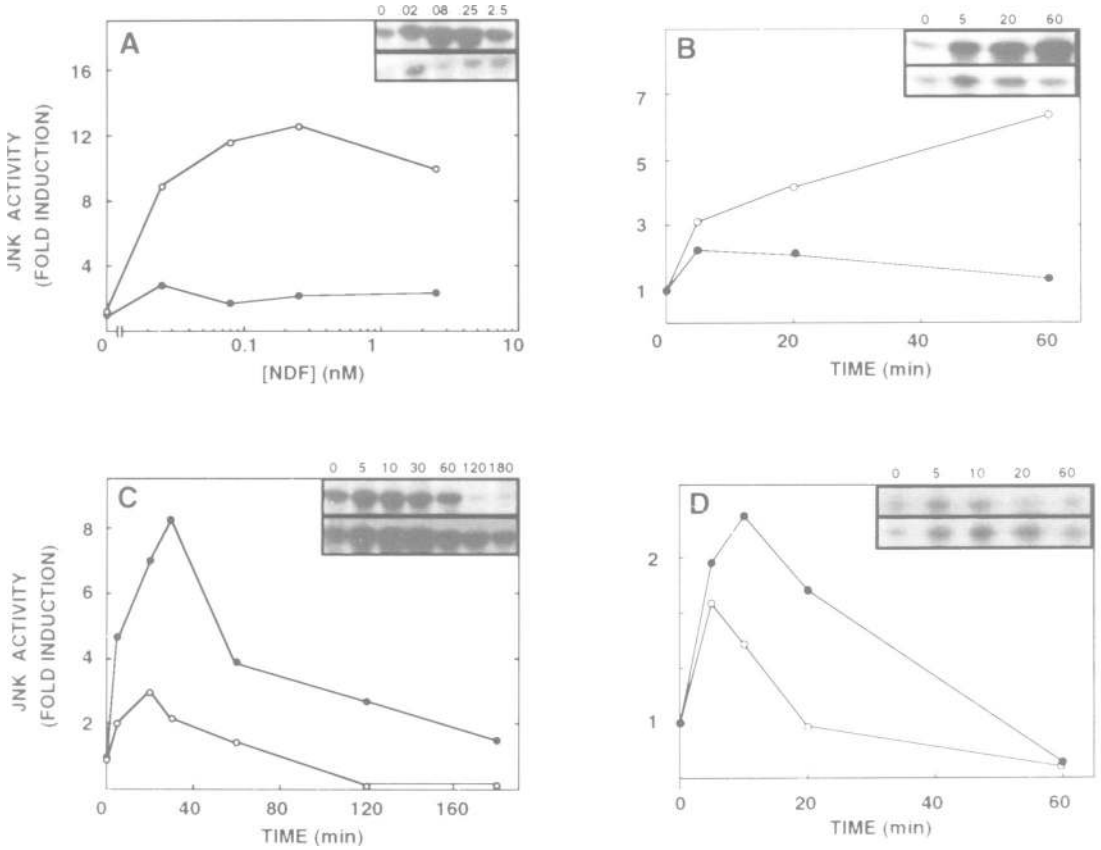

Fig. 8. Potentiation of ligand-induced activation of c-Jun kinase (JNK) by an overexpressed ErbB-2. (**A** and **B**) NDF-induced activation of c-Jun kinase was determined with either T47D-puro cells (open circles) or with their 5R derivative cells (closed circles). Cells were serum-starved for 12 h and then incubated for 10 min with different concentrations of NDF (A), or with a fixed concentration of NDF (1 nM) for the indicated time intervals (B). c-Jun kinase activity was determined in cell lysates by using recombinant GST–Jun fusion protein as a substrate and [γ-^{32}P]ATP, as described (Hibi *et al.*, 1993). Phosphorylation *in vitro* was followed by gel electrophoresis and quantitation of the amount of radioactivity that was incorporated into c-Jun. Insets show the corresponding autoradiograms of phosphorylated recombinant c-Jun. The upper parts of the insets refer to T47D-puro cells and the lower parts to T47D-5R cells. Each experiment was repeated twice. (**C** and **D**) Monolayers of MCF-7 cells (open circles) or MCF-7/ErbB-2 cells (closed circles) were incubated for the indicated time intervals with 0.5 nM of either NDF (C) or EGF (D). c-Jun kinase activity was quantitated as described above. The upper parts of the insets show the phosphorylated c-Jun band in MCF-7 cells and the lower parts refer to MCF-7/ErbB-2 cells. Each experiment was repeated thrice.

ErbB-2 can form heterodimers with the three other ErbB proteins (Figures 2 and 3), we favor the possibility that it affects binding of NDF and EGF through heterodimer formation with the respective receptors. Conceivably, both ligands bind with higher affinities to heterodimers that include ErbB-2 than they bind to the corresponding homodimers. According to this model, in a heterodimeric configuration ErbB-2 is able to inhibit ligand release from the single binding site of the heterodimer. Previous results have already indicated that homodimeric receptors that were stabilized by either a mutation or by a bivalent reagent, displayed higher ligand binding affinity as compared with their monomeric species, and this was due to slower rates of ligand dissociation (Ben-Levy *et al.*, 1992; Zhou *et al.*, 1993). In addition, the apparent forcing of heterodimer formation between ErbB-2 and either ErbB-1 (Wada *et al.*, 1990) or ErbB-3 (Sliwkowski *et al.*, 1994), by means of overexpression of ErbB-2, has been shown to augment binding of EGF and NDF respectively. We note that relatively moderate overexpression of ErbB-2 (<10-fold) is already sufficient for enhancement of ligand binding (Figure 4). In line with ErbB-2 being a shared subunit of the high affinity NDF and EGF receptors, we observed in various tumor cells an NDF-induced reduction in EGF binding affinity (Karunagaran *et al.*, 1995). Moreover, overexpression of ErbB-2 in these cells prevented the *trans*-inhibitory effect of NDF, as expected by a heterodimerization model. It is noteworthy that the function of ErbB-2 as a component of NDF and EGF receptors, that acts by slowing ligand dissociation, is analogous to non-binding components of interleukin (IL) receptors. For example, gp130 is shared by the receptors for IL-6, leukemia inhibitory factor, oncostatin M, IL-11 and ciliary neurotrophic factor (Hilton *et al.*, 1994, and references therein). Likewise, signaling by IL-2, IL-4, IL-7 and IL-9 depends on the formation of heterodimers between specific β subunits and a common γ subunit (Kawahara *et al.*, 1994). One important implication of our results is that the physiological receptors for NDF and EGF are in fact heterodimers that contain ErbB-2. This realization may explain why detergent solubilization of EGF receptors involves 10-fold reduction in binding affinity (Carpenter, 1987), and how mutants of the EGF receptor that are defective in kinase activity or tyrosine autophosphorylation sites are still able to transmit some biochemical signals (Selva *et al.*, 1993; Gotoh *et al.*, 1994). An inevitable

question is how this aspect has so far escaped detection. This may be due to the generally broader expression of ErbB-2, as compared with ErbB-1, ErbB-3 and ErbB-4. In other words, virtually all of the EGF or NDF receptor positive cell lines also express ErbB-2, so that analysis of ligand binding to the individual receptors in their natural cell context is practically impossible.

Signal amplification by ErbB-2 may explain its oncogenic activity

Removal of ErbB-2 from the cell surface has been previously shown to reduce various aspects of the signaling by NDF and EGF, including tyrosine phosphorylation of cytoplasmic substrates, and the activation of MAP kinase and ribosomal protein S6 kinase (Graus-Porta *et al.*, 1995). In the present study, we extended these observations to the activation of another protein kinase cascade, namely JNK/SAPK, and concentrated on the effect of a relatively moderate ErbB-2 overexpression. This level of overexpression is comparable to, or smaller than, the extent of overexpression that is frequently observed in breast and in other human adenocarcinomas (Stancovski *et al.*, 1994). In analogy to the trans-stimulatory action of an overexpressed ErbB-2 that we observed in mammary cells (Figure 7), it has been recently shown that overexpression of either EGF or insulin receptor can prolong MAP kinase activation by the respective ligands (Dikic *et al.*, 1994; Traverse *et al.*, 1994). Our studies with MCF-7 cells indicate that even small changes in the level of ErbB-2 expression can profoundly affect signaling by NDF and EGF. One explanation for the enhancement and prolongation effects on MAP kinase, as well as on c-Jun kinase, is that they are solely due to the ErbB-2-mediated extension of the half-lives of ligand–receptor complexes (Figure 5). However, because it has been previously shown that ErbB-2 efficiently stimulates the MAP kinase pathway (Ben-Levy *et al.*, 1994), it is likely that this receptor functions not only as a common binding component but also as a shared signaling subunit of NDF and EGF receptors. For example, ErbB-2, by means of its own set of docking sites for Src homology 2 (SH-2) proteins, may diversify the nature of the intracellular signals that are generated by NDF and EGF (Carraway and Cantley, 1994). Because sustained activation of MAP kinase leads to either transformation or differentiation (Cowley *et al.*, 1994), our observations may explain, on the one hand, how ErbB-2 alone (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), or in combination with ErbB-1 (Kokai *et al.*, 1989), transforms fibroblasts and, on the other hand, how NDF affects both mitogenesis (Holmes *et al.*, 1992) and differentiation (Peles *et al.*, 1992) in different mammary tumor cells. Taken together, our results imply that by overexpression of ErbB-2, tumor cells may gain a selective advantage because they can now respond with higher potency to relatively low concentrations of EGF-like growth factors. In the light of this finding, various biological aspects of *erbB-2*-overexpressing tumors, such as high incidence in ductal carcinoma *in situ* (Barnes *et al.*, 1992) and the response of *erbB-2*-overexpressing tumors to chemotherapy (Gusterson *et al.*, 1992; Wright *et al.*, 1992; Muss *et al.*, 1994) may be considered as reflections of increased sensitivity to growth factors. In addition, the proposed model predicts that co-expression of ErbB-2 with other ErbB proteins, or with their corresponding ligands, may bear prognostic or therapeutic significance.

Lastly, lessons learned with the ErbB family may be relevant to other subgroups of growth factor receptors. Conceivably, the multiplicity of homologous receptors that preserved heterodimerizing capability can confer to mammalian cells an additional level of combinatorial complexity. Presumably, an internal hierarchy exists in each receptor family and this, in conjunction with spatial and temporal control of receptor co-expression, may determine the potency and diversity of intracellular signals.

Materials and methods

Materials and cell culture

EGF (human, recombinant) was purchased from Boehringer Mannheim and recombinant NDF preparations were from Amgen (Thousand Oaks, California). Radioactive materials were obtained from Amersham (Buckinghamshire, UK). Protein A coupled to Sepharose was obtained from Pharmacia (Uppsala, Sweden) or prepared in our laboratory. Molecular weight standards for gel electrophoresis were from Bio-Rad or from Sigma. Iodogen and BS^3 were from Pierce. All other chemicals were purchased from Sigma unless otherwise indicated. Polyclonal antibodies against the C-terminal portions of ErbB-1, ErbB-2 and ErbB-3 proteins (14 amino acid-long synthetic peptides) were generated in rabbits as described (Peles *et al.*, 1991) or were purchased from Santa Cruz Biotechnology (SC285, an anti-ErbB-3 antibody). A murine monoclonal antibody to ErbB-4 was generated in mice that were immunized with a recombinant extracellular portion of human ErbB-4, and it will be described elsewhere. As control we used antibodies to the fibroblast growth factor receptor (from Santa Cruz Biotechnology). Anti-ERK2 antiserum was raised in rabbits that were injected with a synthetic peptide (residues 346–358) as described (Marte *et al.*, 1995).

Establishment of ErbB-expressing CHO cell lines

CHO cells were transfected with expression vectors that direct expression of *erbB-1*, *erbB-3* and *erbB-4* as previously described (Tzahar *et al.*, 1994), except that the *erbB-1* expression vector that we used was pcDNA3/*erbB-1* (Karunagaran *et al.*, 1995). Clones of stably expressing cells were selected in the presence of G418 (Gibco) and assayed for EGF and NDF binding.

Buffered solutions

Binding buffer contained Dulbecco modified Eagle's medium (DMEM) with 0.1% bovine serum albumin (BSA). HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5 mM EGTA, 1.5 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.15 trypsin inhibitor unit/ml) and 10 μg/ml leupeptin.

Radiolabeling of ligands, covalent crosslinking and ligand binding analyses

Human recombinant EGF and human recombinant NDF-$\beta1_{177-246}$ were labeled with Iodogen (Pierce) as follows: 5 μg protein in phosphate buffered saline (PBS) was mixed in an Iodogen-coated (1μg reagent) tube with $Na^{125}I$ (1 mCi). Following 10 min at 23°C, tyrosine was added to a final concentration of 0.1 mg/ml and the mixture was separated on a column of Excellulose GF-5 (Pierce). The specific activity was determined by counting γ-radioactivity before and after separation on the column. The range of specific activity varied between 2 and 5×10^5 c.p.m./ng. For ligand displacement analyses, monolayers of cells ($1–2 \times 10^5$ cells/well) in 48-well dishes were washed once with binding buffer and then incubated with 5 ng/ml of [^{125}I]EGF in the same buffer. The unlabeled ligand, at different concentrations, was co-incubated with the radiolabeled ligand for 2 h at 4°C, and the cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in 0.5 ml of 0.1 N NaOH containing 0.1% SDS for 15 min at 37°C and the radioactivity was determined by using a γ-counter. For Scatchard analysis, the indicated cell lines were incubated for 2 h at 4°C with different concentrations of either ^{125}I-labeled NDF-$\beta1_{177-246}$ or [^{125}I]EGF. Non-specific binding was determined by the addition of 100-fold excess of

the unlabeled ligand together with the corresponding labeled ligand in the same experiment. Scatchard analysis was performed by using the computerized program LIGAND (Munson and Rodbard, 1980). For covalent crosslinking analysis, monolayers (10^6 cells) of cells were incubated on ice for 2 h with either [^{125}I]EGF (20 ng/ml) or [^{125}I]NDF-$\beta1_{177-246}$ (10 ng/ml). The chemical crosslinking reagent BS^3 was then added (1 mM) and after 45 min on ice cells were washed with PBS. Cell lysates were prepared and analyzed by gel electrophoresis.

Lysate preparation and immunoprecipitation

For analysis of total cell lysates, gel sample buffer was directly added to cell monolayers. For other experiments, solubilization buffer was added to the monolayer of cells on ice. Cells were scraped with a rubber policeman into 1 ml of the buffer, transferred to microtubes, vortexed harshly, and centrifuged (10 000 *g*, 10 min at 4°C). Rabbit antibodies were coupled directly to protein A–Sepharose while shaking for 20 min. Mouse antibodies were first coupled to rabbit anti-mouse immunoglobulin G and then to protein A–Sepharose by the same procedure. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A–Sepharose–antibody complex for 1 h at 4°C. Immunoprecipitates were then washed three times with HNTG (1 ml each wash).

Analysis of ligand dissociation

The cells were first incubated with 5 ng/ml [^{125}I]EGF or [^{125}I]NDF-$\beta1_{177-246}$ for 2 h at 4°C and then washed three times. Dissociation was monitored by incubating the cells in binding buffer with the corresponding unlabeled ligand (100 ng/ml) for various periods of time at 4°C. Non-specific binding and release of radiolabeled ligand were determined in a parallel experiment and subtracted from the total amount of bound ligand at each time point. The data were analyzed by plotting the natural logarithm of B_t/B_0 versus time, where B_t is the concentration of ligand bound at time t and B_0 is the same at the starting time of dissociation.

Biosynthetic labeling

Biosynthetic labeling was performed essentially as described (Ben-Levy *et al.*, 1994). Subconfluent cells in 10 cm dishes were washed with methionine-free DMEM and grown for 16 h in the same medium supplemented with 2% dialyzed calf serum containing 50 μCi/ml of [^{35}S]methionine. The cells were washed three times with PBS and then scraped into 1 ml of solubilization buffer. The lysates were spun for 10 min at 4°C (10 000 *g*) and the supernatants were used for immunoprecipitation as described earlier. Equal amounts of trichloroacetic acid precipitable radioactivity were subjected to immunoprecipitation in each lane.

Determination of MAP kinase activity

Cells were grown in DMEM with 10% calf serum, serum-starved for 1–2 days in 0.5% calf serum in DMEM, and then stimulated with growth factors. Three different assays were used to determine MAP kinase activity with MBP as a substrate.

Mini-columns. Cell lysates were prepared by sonication (7 s at 50 W, twice) in lysis buffer (40 mM HEPES at pH 7.5, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM benzamidine, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 μg/ml pepstatin A) and cleared by centrifugation. The lysates were fractionated on DEAE cellulose minicolumns (0.4 ml, equilibrated in 50 mM β-glycerophosphate at pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT and 0.1 mM sodium orthovanadate) and the MAP kinase-containing fraction (0.22 M NaCl) assayed for phosphate incorporation into myelin basic protein as described (Seger *et al.*, 1994). The assayed fraction contained both ERK1 and ERK2 and >85% of MAP kinase activity.

Immunoprecipitation assay. The previously described method (Graus-Porta *et al.*, 1995) was used with no modifications.

Kinase assay in MBP-containing polyacrylamide gels. Cell lysates were prepared as described above and aliquots were subjected to electrophoresis in SDS–polyacrylamide gels that were polymerized in the presence of 0.5 mg/ml MBP. The protocol used for in-gel MAP kinase assay was essentially as described (Tobe *et al.*, 1991), with some modifications. SDS was removed from the gel by washing twice for 1 h in 100 ml of 20% isopropanol in 50 mM Tris–HCl (pH 8.0), followed by three washes in 50 ml buffer A (5 mM β-mercaptoethanol in 50 mM HEPES pH 7.6). Protein denaturation was performed by incubating the gel for 15 min in buffer A containing 6 M urea. This step was repeated and the gel subjected to renaturation by washing at 4°C in decreasing concentrations of urea (3 M, 1.5 M and 0.75 M, 15 min each) in buffer A that contained also 0.05% Tween-20. Finally, the gel was washed thrice in the same buffer, but without urea, and left overnight in the last washing solution. The next day the gel was incubated for 30 min at 30°C in 30 ml kinase buffer (20 mM HEPES pH 7.6, containing 20 mM $MgCl_2$). This buffer was replaced with kinase buffer that contained in addition 2 mM DTT, 20 μM ATP and 100 μCi [γ-^{32}P]ATP. After 2 h at 30°C, the gel was washed in washing solution [50 ml of trichloroacetic acid (5% w/v), 1% Na-pyrophosphate] until radioactivity of the solution became negligible. The washed gel was dried and then subjected to autoradiography.

Determination of c-Jun kinase activity

JNK activity was determined in cell lysates that were prepared as described above for MAP kinase (protocol 1). Lysates (0.15 ml) were mixed with 20 μl GSH–agarose suspension (Sigma) to which GST–Jun (10 μg) was bound and 30 μl of 10-fold concentrated binding buffer (220 mM HEPES pH 7.7, 20 mM $MgCl_2$, 1 mM EDTA, 0.75% Triton X-100, 400 mM β-glycerophosphate, 1 mM sodium orthovanadate and protease inhibitors) was added. The mixture (0.3 ml) was incubated at 4°C for 2 h (Hibi *et al.*, 1993). The beads were pelleted, washed in HNTG and resuspended in 30 μl of kinase buffer (20 mM HEPES buffer at pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate and 0.1 mM Na_3VO_4) containing 20 μM ATP and 2 μCi of [γ-^{32}P]ATP. The reaction was terminated after 20 min at 30°C by adding Laemmli sample buffer and electrophoresis on SDS–polyacrylamide gels (10% acrylamide). Autoradiograms were scanned after fixation and gel staining.

Acknowledgements

We thank Sara Lavi for technical assistance. This work was supported by grants from the National Cancer Institute, The Israel Science Foundation administered by The Israel Academy of Sciences and Humanities (to Y.Y.) and the Israel Cancer Association (to R.S.). R.S. is an incumbent of the Samuel and Isabelle Friedman Career Development Chair and Y.Y. is a recipient of a Research Career Development Award from the Israel Cancer Research Fund.

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Received on April 18, 1995; revised on September 18, 1995