Single-Chain Antibody-Mediated Intracellular Retention of ErbB-2 Impairs Neu Differentiation Factor and Epidermal Growth Factor Signaling

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ErbB-2 becomes rapidly phosphorylated and activated following treatment of many cell lines with epidermal growth factor (EGF) or Neu differentiation factor (NDF). However, these factors do not directly bind ErbB-2, and its activation is likely to be mediated via transmodulation by other members of the type I/EGF receptor (EGFR)-related family of receptor tyrosine kinases. The precise role of ErbB-2 in the transduction of the signals elicited by EGF and NDF is unclear. We have used a novel approach to study the role of ErbB-2 in signaling through this family of receptors. An ErbB-2-specific single-chain antibody, designed to prevent transit through the endoplasmic reticulum and cell surface localization of ErbB-2, has been expressed in T47D mammary carcinoma cells, which express all four known members of the EGFR family. We show that cell surface expression of ErbB-2 was selectively suppressed in these cells and that the activation of the mitogen-activated protein kinase pathway and p70/p85S6K, induction of c-fos expression, and stimulation of growth by NDF were dramatically impaired. Activation of mitogen-activated protein kinase and p70/p85S6K and induction of c-fos expression by EGF were also significantly reduced. We conclude that in T47D cells, ErbB-2 is a major NDF signal transducer and a potentiator of the EGF signal. Thus, our observations demonstrate that ErbB-2 plays a central role in the type I/EGFR-related family of receptors and that receptor transmodulation represents a crucial step in growth factor signaling.

Receptor tyrosine kinases (RTKs) are involved in the regulation of cell growth and differentiation. Ligand binding to the extracellular domain of these receptors induces dimerization, stimulation of the intrinsic kinase activity, and autophosphorylation. RTKs elicit their functions by binding and/or phosphorylating intracellular substrates (9, 48, 60). Tyrosine-phosphorylated sites in the activated receptors function as high-affinity binding sites for SH2 domain-containing proteins including Grb2, Shc, phospholipase Cγ, and the p85 subunit of phosphotyrosinolinositol 3-kinase (PtdIns 3-kinase), which couple RTKs to signal transduction pathways (19, 41, 49, 51, 66). The Ras-MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) cascade involves sequential kinase reactions which lead to the stimulation of MAPK and the induction of expression of transcription factors such as c-Jun and c-Fos (18, 35, 63). RTKs couple to the Ras-MAPK signaling network via the SH2-SH3 domain-containing adaptor protein Grb2 and/or the SH2 domain-containing protein Shc, which complexes with Grb2 following its phosphorylation on tyrosine (41, 51, 58). By binding Grb2, the Ras guanine nucleotide exchange factor Sos is brought within the vicinity of p21ras, which allows modulation of Ras (12, 40) and subsequently MAPK activity. Distinct intracellular pathways, such as the one leading to the activation of p70/s6k (4), whose link to RTKs appears to be mediated by PtdIns 3-kinase (13, 15), have been reported to exist.

Four members of the type I/epidermal growth factor receptor (EGFR)-related family of RTKs have been identified: ErbB/EGFR (69), ErbB-2 (76), ErbB-3 (37, 54), and ErbB-4 (52). The four proteins are normally coexpressed in various combinations in diverse tissues excluding the hematopoietic system. aberrant expression of EGFR has been observed in a variety of human tumors (24). ErbB-2 gene amplification and/or overexpression is frequently found in tumors arising at many sites, including breast and ovary (29). High levels of ErbB-3 have been found in a number of mammary tumor cell lines (36) as well as in primary breast cancer (39).

Ligands binding to different members of the type I RTKs have been identified. Several growth factors, including EGF (59), transforming growth factor α (43), and amphiregulin (62), bind and activate the EGFR. Recently, a number of peptide factors which were simultaneously identified by different groups were shown to induce tyrosine phosphorylation of ErbB-2, and thus they were described as putative ligands for this receptor. The names reflect the source of isolation and include Neu differentiation factor (NDF), heregulin, glial growth factor, and acetylcholine receptor-inducing activity (20, 26, 42, 50, 73). However, it has recently been shown that ErbB-2 does not directly bind NDF. Instead, ErbB-3 and ErbB-4 function as direct receptors for NDF (11, 53), and NDF-induced tyrosine phosphorylation of ErbB-2 seems to occur only in the presence of either ErbB-3 or ErbB-4, presumably by heterodimerization and transphosphorylation (10, 53, 64). Similarly, activation of EGFR by EGF and other agonists stimulates tyrosine phosphorylation of ErbB-2 by heterodimer formation (22, 31, 32). Although ErbB-2 alone cannot bind any of these ligands, it has been shown to modulate ligand affinities. ErbB-2 confers high-affinity binding sites for EGF by heterodimerizing with EGFR (70) as well as for NDF by heterodimerizing with ErbB-3 (64). It is likely that ErbB-2 also modulates intracellular signals elicited by EGF and NDF, since both factors cause its phosphorylation and activation.

We have used a novel approach to investigate the involvement of ErbB-2 in EGF- and NDF-induced signaling. We have recently described experimental strategies for the targeted inactivation of RTKs by using single-chain antibodies (scFv), recombinant proteins consisting of immunoglobulin heavy-
and light-chain variable domains linked over a short flexible linker peptide. A secreted form of an scFv comprising with EGF and FLP for binding to the EGFR has been expressed in EGF-transformed cells and was shown to inhibit receptor activity in an autocrine fashion (6). In an alternative approach, scFvs directed against the extracellular domain of ErbB-2 have been expressed intracellularly and targeted to the lumen of the endoplasmic reticulum (ER). By binding to the extracellular domain of newly synthesized ErbB-2, the scFvs prevented its transit through the ER to the cell surface, thereby causing its functional inactivation (7). In the present study, we used the same approach to inactivate ErbB-2 in T47D human mammary carcinoma cells. These cells express moderate levels of all known members of the EGFR family (30) and proliferate in response to NDF. We show that EGF- and NDF-induced activation of MAPK and p70/p85S6K as well as stimulation of growth by NDF is impaired in cells devoid of cell surface ErbB-2.

MATERIALS AND METHODS

Materials. Recombinant human EGF and bovine pancreatic insulin were from Sigma, recombinant human basic fibroblast growth factor was from Collaborative Biomedical Products, and recombinant human NDF isoforms were a generous gift of Dr. R. Weiss, Genentech, South San Francisco, Calif. For all of the experiments described in this report, the NDF-β3 isoform (amino acids 14 to 241) was used. Numbering is according to Wens et al. (74). Antibodies used were EGF-specific monoclonal antibody (MAb) EGFR/1 (Amersham), ErbB-2-specific antisera 21N (28) and MAb FSP77 (25), ErbB-3-specific affingly purified rabbit polyclonal antibodies C17 (Santa Cruz Biotechnology) and E38530 (Transduction Laboratories), Sc-specific rabbit immunoglobulin G (Upstate Biotechnology, Inc.), Grb2-specific antisera (Upstate Biotechnology, Inc.), ERK1- and ERK2-specific antisera (44), ERK2-specific MAb (Gibco BRL), phosphotyrosine-specific MAb (17), and scFv-specific antisera (7).

Expression of the ErbB-2-directed scFv in T47D cells. The heavy- and light-chain variable domains of MAB FRPs were cloned from hybridoma cells and used to construct a chimeric gene encoding the scFv FRP5 as described previously (72). For expression in mammalian cells and localization to the lumen of the ER, the scFv-5R cDNA was created as described elsewhere (7). Briefly, 0.14 μg of crude lysate was incubated for 30 min at 37°C with 10 μg of 40S ribosomal subunit in a reaction buffer containing 100 mM morpholinepropanesulfonic acid (MOPS), 2 mM diithothreitol, 20 mM MgCl₂, 20 mM n-nitrophenylphosphate, 50 μg of unlabelled ATP, 0.05 μg of MAb ERK1-2 specific protein A-antibody (Sigma), and 1 μCi of [γ-32P]ATP (1,200 Ci/mmol). The reaction was stopped with sample buffer, proteins were subjected to SDS-PAGE (15% gel) and blotted, and phosphorylation of S6 ribosomal protein was quantitated with a Molecular Dynamics densitometer.

Immunoprecipitation and Western blot (immunoblot) analysis. Cells were solubilized in Triton extraction buffer (50 mM Tris [pH 7.5], 5 mM EGTA [pH 8.5], 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium molybdate, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) except for MAPK and S6K assays, for which ERK lysis buffer (ELB; see below) was used. The lysates were clarified by centrifugation at 16,000 × g for 15 min. For immunoprecipitations, equal amounts of protein were precipitated with specific antibodies for 2 h at 4°C. Immunocomplexes were collected with protein A-Sepharose (Sigma) and washed three times with lysis buffer and once with TNE buffer (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM EDTA). For immunoprecipitation of EGFR, protein A-Sepharose was precoated with polyclonal rabbit anti-mouse immunoglobulin G (ICN Immunobiologicals). Bound proteins were released by boiling in sample buffer. Total cell lysates or immunoprecipitates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and proteins were blotted to polyvinylidene difluoride membranes. After blocking with 20% horse serum (Gibco BRL) in TTBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20), filters were probed with specific antibodies and proteins were visualized with peroxidase-coupled secondary antibody, using the ECL detection system (Amersham). In some experiments, filters were stripped in SDS buffer (30 mM Tris-HCl [pH 8.0], 20 mM MmcCl₂, and 2 mM MgCl₂). The activity of MAPK was then determined by incubating the immune complexes at 37°C for 30 min with 30 μl of kinase buffer containing 15 μg of myosin basic protein (MBP), 10 μM unlabeled ATP, and 0.1 μM [γ-32P]ATP (1,200 Ci/mmol). The reaction was stopped with sample buffer, proteins were subjected to SADS-PAGE (15% gel) and blotted, and the phosphorylation of MBP was quantitated with a Phosphorimag- ing Molecular Dynamics. To verify that equal amounts of ER were immunoprecipitated, a Western blot analysis with a specific MAB was carried out.

Northern (RNA) blotting. Total RNA was extracted from growth factor-treated cells by using Trizol (Gibco BRL). Ten micrograms of RNA was electrohoresed on a 0.66 M formaldehyde–1% agarose gel, transferred to a nylon membrane, and hybridized to a digoxigenin-labeled riboprobe encompassing the 1.3-kb BglII-PvuII fragment of the v-fos cDNA (8). The hybridized probe was detected by using alkaline phosphatase-conjugated antidigoxigenin F(ab)₂ fragments and the chemiluminesent substrate Lumigen PPD (Boehringer Mannheim).

Cell growth assays. Factor responsiveness for growth was examined under anchorage-independent conditions. Cells (3 × 10⁴) were plated in duplicate in 6-cm-diameter dishes in 6 ml of culture medium supplemented with 0.35% Noble agar. For growth in agar, 0.1% fetal bovine serum was added to the medium. The cells were incubated for 4 weeks at 37°C, after which colonies were stained by adding 2 ml of phosphate-buffered saline containing 0.5 mg of nitroblue tetrazolium per ml for 24 h. Colonies larger than 200 μm were counted with an Arttek 880 colony counter (DyneTech Laboratories, Inc.).

RESULTS

Intracellular retention of ErbB-2 in T47D cells. To investigate the involvement of ErbB-2 in EGF- and NDF-induced signaling, we have specifically inactivated ErbB-2 in T47D human mammary carcinoma cells. We have previously shown that an ER-retained variant of the ErbB-2-specific scFv FRP5, scFv-5R, specifically inhibits ER transit of ErbB-2 and leads to a loss of cell surface ErbB-2 (7). We therefore established a cell line, T47D/5R, which intracellularly expresses scFv-5R at high levels as judged by Western blotting (Fig. 1A). scFv-5R was detected as a double band, the upper of which corresponds to a N-glycosylated form (7). When analyzed by Western blotting, the ErbB-2 protein in the T47D/5R cells showed a faster electrophoretic mobility than the T47D/puro vector control cells (Fig. 1B). ErbB-2 is a glycoprotein, and its carbohydrate side chains undergo modification and extension in the Golgi complex (1). It is likely that ER retention of ErbB-2 causes nypglycosylation, which might explain the reduced apparent molecular weight.
molecular weight. Specific intracellular retention of ErbB-2 was demonstrated by flow cytometric analysis. Intact cells were stained for the presence of cell surface EGFR, ErbB-2, or ErbB-3 (Fig. 2). ErbB-2 was absent from the cell surface in T47D/5R cells but present on T47D/puro control cells. In contrast, the related EGFR and ErbB-3 proteins were expressed at equal levels on the cell surface of both cell lines, indicating that the scFv-5R-mediated abolishment of ErbB-2 was highly specific.

**EGF- and NDF-induced tyrosine phosphorylation of type I RTKs.** Following activation of EGFR by EGF, heterodimerization with ErbB-2 and cross-phosphorylation occur (22). It is likely that heterodimerization and receptor cross-phosphorylation of ErbB-2 with ErbB-3 and/or ErbB-4 follow NDF stimulation (53, 64). Thus, we investigated how the presence or absence of ErbB-2 influences the phosphorylation of other members of the EGFR family in response to EGF and NDF. T47D/puro and T47D/5R cells were treated with 50 ng of each factor per ml for 5 min, and the phosphotyrosine content of EGFR, ErbB-2, or ErbB-3 was analyzed (Fig. 3). The tyrosine phosphorylation of ErbB-2 dramatically increased in response to EGF and NDF in the T47D/puro control cells. In contrast, there was no increase in ErbB-2 tyrosine phosphorylation in the T47D/5R cells, likely because of the lack of cell surface ErbB-2 (Fig. 3B). In response to EGF, the EGFR was phosphorylated in both cell lines. However, in the T47D/5R cells, the EGFR phosphotyrosine content was reduced by 46% compared with the control cells. No tyrosine phosphorylation could be detected in response to NDF (Fig. 3A). NDF treatment of T47D/puro cells resulted in a strong tyrosine phosphorylation of ErbB-3. Remarkably, the phosphotyrosine content of ErbB-3 was reduced by 89% in the T47D/5R compared with the T47D/puro cells, suggesting that ErbB-2 contributes to a large extent to the stimulation of tyrosine phosphorylation of ErbB-3 triggered by NDF. No ErbB-3 phosphorylation was detected upon EGF treatment (Fig. 3C), which may be due to the low levels of EGFR in T47D cells (approximately $7 \times 10^5$ [27a]). It has been shown that in EGFR-overexpressing A431 cells, EGF induces tyrosine phosphorylation of ErbB-3 (65).
EGF- and NDF-induced tyrosine phosphorylation of cellular proteins. To assess the effect that loss of cell surface ErbB-2 has on EGF- and NDF-induced signaling through the type I RTKs, total cell lysates were initially analyzed by Western blotting with a phosphorysosine-specific MAb. Following EGF treatment (Fig. 4A), two proteins in the high-molecular-weight region of the gel (indicated with an arrow) rapidly became tyrosine phosphorylated in the T47D/puro control cells. In the T47D/5R cells, only the lower protein was phosphorylated. It is likely that the upper protein corresponds to ErbB-2 and that the lower protein corresponds to EGFR. Phosphorylation of several other proteins (indicated by arrowheads) with molecular masses of approximately 60, 48, 44, and 42 kDa was also induced by EGF treatment. While the 60- and the 48-kDa proteins have not yet been identified, the last two proteins were found to comigrate with ERK1 and ERK2, respectively. Phosphorylation of both ERKs remained high for 2 h in the T47D/puro cells, whereas it decreased to the basal level in the T47D/5R cells after 2 h. More striking differences between the two cell lines were observed when cells were stimulated with NDF (Fig. 4B). Rapid and strong phosphorylation of a protein(s) in the high-molecular-weight region of the gel (indicated with an arrow) was observed in T47D/puro control cells. This phosphorylation was dramatically reduced in the T47D/5R cells, presumably because of the lack of cell surface ErbB-2 and/or reduced ErbB-3/4 phosphorylation. As seen for EGF stimulation, phosphorylation of several other proteins with molecular masses of approximately 60, 48, 44, and 42 kDa was also induced. While phosphorylation of the 60-kDa protein was observed in both cell lines, phosphorylation of the 48-kDa protein was visible only in the T47D/puro cells. Strikingly, phosphorylation of the 44- and 42-kDa proteins, which comigrated with ERK1 and ERK2, respectively, remained high after 2 h of NDF treatment in the T47D/puro cells, whereas it started decreasing after 10 min in the T47D/5R cells.

Absence of cell surface ErbB-2 impairs EGF- and NDF-dependent activation of MAPK. The distinct time-dependent tyrosine phosphorylation of the two proteins comigrating with ERK1 and ERK2 prompted us to examine the activities of these two MAPK isozymes. In vitro kinase assays using MBP as a substrate were performed. Following EGF stimulation (Fig. 5A), ERK2 was rapidly activated with similar kinetics, and its activity declined slowly in both cell lines. However, throughout the time course, activation of ERK2 was always more pronounced in the T47D/puro cells. When cells were treated with NDF (Fig. 5B), ERK2 activity rapidly increased in both T47D/puro and T47D/5R cells, although again to a higher level in the former. Strikingly, in the T47D/puro cells, NDF-induced activation of ERK2 was sustained for 2 h, whereas in the cells lacking ErbB-2 on the cell surface, it was only transient and rapidly returned to the basal level. Activity of ERK1 in response to both EGF and NDF was also analyzed and found to follow the same pattern as the ERK2 activity (not shown). To show that the effects observed were specific for EGF and NDF, basic fibroblast growth factor, which interacts with a nonrelated receptor tyrosine kinase, was tested for its ability to stimulate MAPK. In both cell lines, the fold induction and kinetics of ERK2 activity (Fig. 5C) and ERK1 activity (not shown) followed the same pattern.

The EGF- and NDF-induced ERK1 and ERK2 stimulation in T47D/5R cells was always lower than that in T47D/puro cells. Therefore, different doses of the two factors were applied to the cells for 10 min. Similar results for EGF (Fig. 6A) and for NDF (Fig. 6B) were found: picomolar concentrations of both factors stimulated ERK2 activity in both cell lines, and even with saturating amounts, the activity in the T47D/5R cells was lower. For both growth factors, the same results were obtained with ERK1 (not shown). Therefore, we conclude that both EGF- and NDF-induced MAPK activation is impaired by the absence of cell surface ErbB-2.

EGF- and NDF-induced tyrosine phosphorylation of Shc, association with type I RTKs and Grb2. Activated type I RTKs are coupled to the Ras-MAPK cascade by binding to the SH2-SH3 domain-containing adaptor protein Grb2 and/or the SH2 domain-containing protein Shc, which is phosphorylated on tyrosine and then complexes with Grb2 (58). Given the evidence that EGF- and NDF-induced MAPK activation was diminished by the absence of cell surface ErbB-2 (Fig. 5), we reasoned that the stimulation of Shc tyrosine phosphorylation and Grb2 association might be affected. To test this hypothesis, cells were stimulated with 50 ng of EGF or NDF per ml for 5 min, proteins were extracted, and Shc was immunoprecipitated. A Western blot analysis showed that equal amounts of Shc proteins were immunoprecipitated from all samples (not shown). An analysis using a phosphotyrosine-specific antibody revealed that in T47D/puro cells, two of the three Shc isoforms, p46\textsuperscript{Shc} and p52\textsuperscript{Shc}, became highly phosphorylated in response to EGF and NDF. In the T47D/5R cells, the NDF-induced tyrosine phosphorylation of both proteins was reduced by 90% (Fig. 7A). EGF-induced Shc phosphorylation was also reduced in these cells, albeit to a lesser extent (63 and 41% for p46\textsuperscript{Shc} and p52\textsuperscript{Shc}, respectively). High-molecular-weight tyrosine-phosphorylated proteins (indicated by an arrow) were coimmunoprecipitated with the Shc proteins in growth factor-treated cells. A reprobing of the filter with different antibodies revealed that in T47D/puro but not T47D/5R cells, ErbB-2 was coimmunoprecipitated with Shc, both in response to EGF and

FIG. 4. Total phosphotyrosine blot of EGF- and NDF-induced T47D/puro and T47D/5R cells. Cells were stimulated with 0.5 nM EGF (A) or NDF (B) for the indicated times prior to lysis. One hundred micrograms of total protein was subjected to SDS-PAGE (9% gel), immunoblotted, and probed with a phospho-

FIG. 4. Total phosphotyrosine blot of EGF- and NDF-induced T47D/puro and T47D/5R cells. Cells were stimulated with 0.5 nM EGF (A) or NDF (B) for the indicated times prior to lysis. One hundred micrograms of total protein was subjected to SDS-PAGE (9% gel), immunoblotted, and probed with a phosphos-
in response to NDF (Fig. 7B). ErbB-3 was found to be complexed with Shc in both T47D/puro and T47D/5R cells in response to NDF. However, 81% less Shc-ErbB-3 complexes were found in T47D/5R cells, a finding that can be explained by the strongly reduced NDF-induced phosphorylation of ErbB-3 in these cells (Fig. 3C). No EGFR was detected in the Shc precipitates (not shown). However, this might be due to the low EGFR level in these cells. In fact, the high-molecular-weight tyrosine-phosphorylated protein that is coimmunoprecipitated with Shc in both EGF-treated T47D/puro and T47D/5R cells is likely to be EGFR (Fig. 7A). Grb2 was found to be associated with Shc in both EGF- and NDF-treated T47D/puro cells. This association was reduced by 24% in EGF-treated and by 76% in NDF-treated T47D/5R cells. These observations provide a molecular basis for the reduced activation of MAPK.

**FIG. 5.** Time course of ERK2 activation. T47D/puro (closed squares) and T47D/5R (open squares) cells were treated with 0.5 nM EGF (A), NDF (B), or basic fibroblast growth factor (C) for the indicated times. ERK2 was immuno-precipitated from 200 μg of cell lysate with a specific antibody (44), and kinase activity was determined by using MBP as a substrate. Proteins were subjected to SDS-PAGE (15% gel) and blotted, and phosphorylation of MBP was quantitated with a PhosphorImager (Molecular Dynamics).

**FIG. 6.** Dose response of ERK2 activation. T47D/puro (closed squares) and T47D/5R (open squares) cells were treated for 10 min with the indicated concentrations of EGF (A) or NDF (B). ERK2 was immuno-precipitated from 200 μg of cell lysate with a specific antibody (44), and kinase activity was determined by using MBP as a substrate. Proteins were subjected to SDS-PAGE (15% gel) and blotted, and phosphorylation of MBP was quantitated with a PhosphorImager (Molecular Dynamics).

EGF- and NDF-induced c-fos expression is impaired in cells lacking cell surface ErbB-2. c-fos expression can be induced by a variety of growth factors through the Ras-MAPK pathway (23, 34). Moreover, it was recently shown that ERK2 is directly involved in the activation of c-fos expression (34). Our results show that EGF- and NDF-induced activation of ERK2 is impaired in the T47D/5R cells; therefore, we evaluated whether c-fos expression was also affected. Cells were treated with 0.5 nM EGF and NDF for 30 min, and c-fos expression was examined by Northern blot analysis (Fig. 8). EGF and NDF induced the expression of c-fos in both cell lines. However, there were 23 and 81% reductions in, respectively, EGF- and NDF-treated T47D/5R cells. Our results suggest that the ultimate step in the activation of the Ras-MAPK pathway by ligands of type 1 RTKs, i.e., the phosphorylation of nuclear targets and induction of gene expression, is impaired by the lack of ErbB-2.

Absence of cell surface ErbB-2 impairs EGF- and NDF-dependent activation of p70/p85S6K. We have previously shown that NDF and EGF induce p70/p85S6K activity in T47D cells (44). Therefore, we investigated whether the activation of the
p70/p85S6K, which lies on a signaling pathway distinct from the Ras-MAPK pathway (4), is also affected by the loss of cell surface ErbB-2. p70/p85S6K activity was detected in total lysates, using 40S ribosomal subunits as a substrate. Under these experimental conditions, the predominant activity measured is contributed by p70/p85S6K, and the p90rsk activity is negligible (14). p70/p85S6K was rapidly stimulated by EGF in both cell lines. As seen for MAPK, the stimulation was always more pronounced in the T47D/puro cells (Fig. 9A). More notable was the result obtained with NDF, which led to a higher and much more sustained activation of p70/p85S6K in the T47D/puro cells than in the T47D/5R cells (Fig. 9B). To ascertain that these effects were specific for EGF and NDF, insulin-dependent activation of p70/p85S6K was analyzed. In both cell lines, the fold induction and kinetics of p70/p85S6K activity followed very similar patterns (Fig. 9C). In a dose-response assay for EGF and NDF, we observed that even with saturating amounts of each growth factor, p70/p85S6K activity was always lower in the T47D/5R cells (not shown).

Reduction of NDF-stimulated soft agar growth in cells lacking cell surface ErbB-2. With respect to morphology, anchorage-dependent growth, and anchorage-independent growth in serum-containing medium, the scFv-expressing cells were indistinguishable from the control cells (Table 1 and not shown). EGF and NDF responsiveness for growth was examined under anchorage-independent conditions (Table 1). Little or no growth stimulation was observed in response to EGF, while NDF induced soft agar growth of T47D/puro cells 2.8-fold. In T47D/5R cells, NDF stimulated growth only 1.5-fold, indicating that ErbB-2 activation by NDF is important for the transduction of a proliferative signal. These results support the view

FIG. 7. EGF- and NDF-induced tyrosine phosphorylation of Shc and complex formation of Shc with Grb2 and type I RTKs. (A) Phosphotyrosine blot. T47D/puro and T47D/5R cells were starved for 24 h and treated with EGF (50 ng/ml) or NDF (50 ng/ml) for 5 min or left untreated prior to lysis. Shc was immunoprecipitated from 2.5 mg of protein with a specific serum, subjected to SDS-PAGE (11% gel), and Western blotted (WB) with a phosphotyrosine-specific MAb (α-PY). The arrow indicates type I RTKs, and arrowheads indicate the different Shc isoforms. (B) The filter was stripped and reprobed with anti-ErbB-2 (α-ErbB-2) antibody 21N (28), anti-ErbB-3 (α-ErbB-3) antibody C17, and polyclonal rabbit anti-Grb2 (α-Grb2) antibody.

FIG. 8. Induction of c-fos mRNA by EGF and NDF. Cells were stimulated for 30 min with 0.5 nM each factor, and total RNA was extracted, electrophoresed, blotted, and probed with a c-fos-specific probe.

FIG. 9. Time course of p70/p85S6K activation. T47D/puro (closed squares) and T47D/5R (open squares) cells were treated with 0.5 nM EGF (A), NDF (B), or insulin (C) for the indicated times. p70/p85S6K activity was determined in crude lysate by using 40S ribosomal subunit as a substrate. Proteins were subjected to SDS-PAGE (15% gel) and blotted, and phosphorylation of the S6 ribosomal protein was quantitated with a PhosphorImager (Molecular Dynamics).
that ErbB-2 plays a central role in potentiating the signals triggered by ligands of other members of the EGFR family of RTKs.

**DISCUSSION**

In this study, we describe the application of a novel approach to investigate signaling by type I RTKs. The technique is based on the intracellular expression of an ErbB-2-specific single-chain antibody and results in the specific loss of cell surface ErbB-2. We have performed these experiments with T47D mammary carcinoma cells, which express all four members of the type I receptor family. Analysis of signals elicited by ligands of EGFR, ErbB-3, and ErbB-4 has revealed that in cells selectively lacking cell surface ErbB-2, phosphorylation of Shc, activation of MAPK, and p70S6K, and induction of c-fos expression in response to both NDF and EGF were impaired. NDF-induced stimulation of growth was also considerably reduced when ErbB-2 was absent from the cell surface. These results show that while neither NDF nor EGF directly binds ErbB-2, its coexpression with other members of the EGFR related family is important in intracellular signaling.

NDF is a ligand for ErbB-3 and ErbB-4, and these proteins become tyrosine phosphorylated following its binding (11, 53). The response of ErbB-2 to NDF is mediated by ErbB-3 and/or ErbB-4, presumably by ligand-induced heterodimerization and transphosphorylation (53, 64), in analogy to numerous studies showing EGF-induced stimulation of ErbB-2 tyrosine phosphorylation (32, 33, 67). More recently it has been reported that EGF stimulates the tyrosine phosphorylation of ErbB-3 in A431 cells (65). While studies on ErbB-4 phosphorylation following EGF treatment have not been published, it may be possible that heterodimerization between all four members of this receptor family occurs following EGF or NDF treatment.

In this study, we have examined three of the four type I RTKs in T47D cells following NDF and EGF treatment. It was previously reported that T47D cells express ErbB-4-specific mRNA (30, 52), but we have not been able to study the phosphorylation state of the protein because of a lack of sensitive ErbB-4-specific reagents. However, it is reasonable to assume that NDF also binds ErbB-4 in T47D cells. We detected tyrosine phosphorylation of ErbB-2 following EGF and NDF treatment of T47D control cells. However, ErbB-3 or EGF tyrosine phosphorylation was found only in response to NDF or EGF, respectively. T47D cells have low numbers of EGF receptor molecules, which might make it difficult to detect cross-phosphorylation between EGFR and ErbB-3. The NDF-induced ErbB-3 tyrosine phosphorylation was 10-fold stronger in T47D/puro cells than in T47D/5R cells, showing that a major part of the ErbB-3 phosphorylation is mediated by heterodimerization with ErbB-2. However, NDF also stimulated tyrosine phosphorylation of ErbB-3 in the absence of ErbB-2, making it likely that ErbB-3 homodimerization and/or heterodimerization with ErbB-4 occurs. Tyrosine phosphorylation of EGFR in response to EGF was observed in both cell lines but was reduced by 46% when ErbB-2 was absent from the cell surface, indicating that ErbB-2 also contributes to the EGF-induced tyrosine phosphorylation of EGFR.

There are numerous examples showing that the activation of RTKs leads to stimulation of various intracellular pathways, including the Ras-MAPK pathway and the independent cascade leading to p70S6K activation. The former pathway signals directly to the nucleus, appears to control transcription via phosphorylation of nuclear targets, and has been linked to cell proliferation. For example, inhibition of p70S6K by rapamycin blocks growth stimulation of Swiss 3T3 cells (16). A more general role for MAPK and p70S6K is proposed by the observation that both have been implicated not only in cell proliferation but also in cell differentiation (2, 75). EGF and NDF activate MAPK and p70S6K in T47D cells, and the presence of cell surface ErbB-2 potentiates this effect. ErbB-2 is involved in the formation of high-affinity binding sites for EGF and NDF (64, 70). However, this does not seem to be the reason for the observed enhancement of the signals since even at saturating ligand concentrations, the activities of both kinases were diminished in the absence of cell surface ErbB-2. Thus, it is likely that the participation of an active ErbB-2 in the intracellular signaling is responsible for potentiating the activation of MAPK and p70S6K.

It has recently been suggested that PtdIns 3-kinase provides the link between RTKs and p70S6K (13, 15) and that PtdIns 3-kinase is a target of Ras (57). Moreover, ErbB-3 possesses numerous binding sites for this protein (21, 65), suggesting that a direct ErbB-3/PtdIns 3-kinase/p70S6K pathway may exist, apart from a more general link via Ras. Thus, reduced activation of PtdIns 3-kinase due to diminished ErbB-3 tyrosine phosphorylation and/or impaired Ras activation via Shc/Grb2 might provide the explanation for the lower p70S6K activity in response to EGF and NDF in the cells lacking cell surface ErbB-2.

Shc has been shown to be phosphorylated by activated type I RTKs (5, 55, 61), and its subsequent association with Grb2 is one possible way by which the receptors couple to the MAPK pathway (58). We show that in T47D/5R cells, the EGF- and NDF-induced Shc tyrosine phosphorylation and complex formation with Grb2 are reduced, likely because ErbB-2 is not participating in the signaling. This may provide an explanation for the impaired activation of MAPK. However, in the case of NDF, the early activation of MAPK is reduced by only approximately 20% in the T47D/5R cells (Fig. 5B and 6B), while Shc phosphorylation and association with Grb2 are dramatically decreased (90 and 76%, respectively). This finding suggests that NDF activates MAPK also via an alternative, Shc-independent pathway. Since Grb2 can directly bind to activated RTKs (5, 41), and since ErbB4 has four potential Grb2 binding sites (10), one possible interpretation is that in T47D/5R cells, NDF stimulates the activation of MAPK through ErbB-4 associating with Grb2.

Strikingly, in T47D cells lacking cell surface ErbB-2, the NDF-induced activation of MAPK was transient, whereas in

**TABLE 1. Soft agar growth of T47D/puro and T47D/5R cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T47D/puro</th>
<th>T47D/5R</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of colonies (mean ± SD)</td>
<td>Fold stimulation</td>
<td>No. of colonies (mean ± SD)</td>
</tr>
<tr>
<td>None</td>
<td>130 ± 3.5</td>
<td>124 ± 2.5</td>
</tr>
<tr>
<td>EGF</td>
<td>139 ± 9.0</td>
<td>1.07</td>
</tr>
<tr>
<td>NDF</td>
<td>368 ± 15.5</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* Anchorage-independent growth was measured by placing cells in culture medium supplemented with 0.35% agar in the presence or absence of 1 nM EGF or NDF. After 4 weeks, viable cells were stained and colonies of ≥ 200 μm were counted with an Artek 880 colony counter. The colony numbers from duplicate media supplemented with 0.35% agar in the presence or absence of 1 nM EGF or NDF. After 4 weeks, viable cells were stained and colonies of ≥ 200 μm were counted with an Artek 880 colony counter. The colony numbers from duplicate
the T47D control cells, a more sustained activation was observed. Activated MAPK is phosphorylated on threonine and tyrosine, and its inactivation is brought about by dephosphorylation. A novel class of dual-specificity phosphatases, whose expression is induced upon mitogen stimulation, is involved in this process (68, 71). One possible explanation is that a phosphatase is induced more rapidly in the T47D/5R cells than in the T47D/puro cells, thereby counteracting sustained activity of MAPK. It will be of particular interest to elucidate the nature of this phosphatase(s) and how ErbB-2 is involved in modulating its expression.

Concomitant with the impaired activity of MAPK, the EGF- and NDF-induced expression of the immediate-early gene c-fos was reduced. A direct link between ERK2 and c-fos expression has been shown recently. The transcription factor TCF/Elk-1 is a substrate of ERK2 and binds the serum response element in the c-fos promoter, thereby activating transcription (34). Thus, it is likely that the impaired ERK2 activity is directly responsible for the reduced c-fos induction.

A strongly attenuated growth-stimulatory effect of NDF in the T47D/5R cells paralleled the impaired MAPK and p70/S6K activities. This finding suggests that the strength of the signals leading to the activation of these kinases, enhanced by the presence of activated ErbB-2, is important for the ultimate biological response. Overexpression of ErbB-2 leading to a constitutive activation of its kinase is observed in many types of human tumors (27) and frequently correlates with more malignant disease (29). Moreover, breast cancer cells often express ligands which bind the type I RTKs, including NDF, PDGF- and insulin-dependent pp70S6K activation mediated by phosphatidylinositol-3 kinase: a protein-tyrosine kinase inhibitor with selective for the epidermal growth factor receptor signal transduction pathway and potent in vivo antitumor activity. Proc. Natl. Acad. Sci. USA 91:2334–2338.


