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Neu Differentiation Factor Activation of ErbB-3 and ErbB-4 Is Cell Specific and Displays a Differential Requirement for ErbB-2

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Neu differentiation factor (NDF)-induced signaling involves the activation of members of the ErbB family of receptor tyrosine kinases. Although ectopic expression of recombinant ErbB receptors has yielded valuable insight into their signaling properties, the biological function and in vivo interplay of these receptors are still poorly understood. We addressed this issue by studying NDF signaling in various human cell lines expressing moderate levels of all known ErbB receptors. NDF-induced phosphorylation of ErbB-2 and ErbB-3 was found in the breast epithelial cell line MCF10A, the breast tumor cell lines T47D and MCF7, and the ovarian tumor cell line OVCAR3. Despite similar expression levels, NDF-induced phosphorylation of ErbB-4 was cell specific and only detected in T47D and OVCAR3 cells. Blocking cell surface expression of ErbB-2 by intracellular expression of a single-chain antibody revealed that in these two cell lines, ErbB-2 significantly enhanced phosphorylation of ErbB-4. Efficient NDF-induced phosphorylation of ErbB-3 was strictly ErbB-2 dependent in the breast tumor cell lines T47D and MCF7, while it was largely ErbB-2 independent in MCF10A and OVCAR3 cells. Consequently, NDF-stimulated intracellular signaling and induction of a biological response displayed a cell-specific requirement for ErbB-2. Thus, while ErbB-2 cooperates with NDF receptors in the breast tumor cell lines, ErbB-2 independent mechanisms seem to prevail in other cellular contexts.

Four members of the ErbB family of receptor tyrosine kinases (RTKs) are presently known: ErbB-1 (epidermal growth factor receptor [EGFR]), ErbB-2, ErbB-3, and ErbB-4 (33, 45, 59, 63). The overall homology in this receptor family is 40 to 50%, and all the members are characterized by two cysteine-rich regions in the extracellular domain. ErbB receptors are widely expressed in epithelial, mesenchymal, and neuronal tissues, and their aberrant expression is frequently observed in human malignancies (19, 26, 34). Numerous ligands binding members of the ErbB receptor family have been described. Growth factors that bind and activate EGFR include epidermal growth factor (EGF), transforming growth factor alpha, amphiregulin, and heparin-binding EGF-like growth factor (23, 37, 52, 53). Members of a new family of ligands for ErbB-3 and ErbB-4 (7, 46, 58) have been isolated by different groups. The polypeptides have various names, reflecting their source of isolation, including Neu differentiation factor (NDF), heregulin, acetylcholine receptor-inducing activity, and glial growth factor (14, 24, 35, 62). Although none of these factors directly bind ErbB-2, both EGF agonists and NDFs induce its tyrosine phosphorylation, presumably by ligand-driven heterodimerization and cross-phosphorylation (31, 46, 54). Interestingly, ErbB-2 confers high-affinity binding sites for EGF and NDF by heterodimerizing with EGFR and ErbB-3, respectively (54, 60). These observations demonstrate the importance of receptor heterodimerization in the ErbB receptor family and suggest that ErbB-2 is a key player.

The interaction of specific SH2- or phosphotyrosine-binding domain (PTB)-containing target proteins (30, 32) with tyrosine-phosphorylated residues on the intracellular domain of RTKs modulates intracellular signaling pathways. One of the best-characterized pathways is the one leading to activation of the mitogen-activated protein kinase (MAPK). RTKs are coupled to the MAPK pathway by binding and phosphorylating the SH2-PTB domain-containing adaptor protein Shc (43, 51). A distinct, MAPK-independent pathway which has been coupled to phosphatidylinositol (PtdIns) 3-kinase leads to the activation of $p70/p85^{S6K}$ (9). EGF and NDF receptors stimulate both of these pathways, and their activation is enhanced in the presence of increasing amounts of ErbB-2 (18, 29).

Ligand-induced activation of ErbB receptors and phosphorylation of intracellular substrates have predominantly been examined following their ectopic expression in cells lacking these receptors. Our goal has been to gain insight into the NDF-induced cooperative action of ErbB receptors in their natural setting as well as into the modulatory function of ErbB-2. The experimental approach is based on the intracellular expression of a recombinant single-chain antibody (scFv) which leads to the specific and stable loss of cell surface ErbB-2 (5). This technique has allowed us to study NDF-induced activation of NDF receptors and intracellular signaling in a natural cellular context in the presence and absence of ErbB-2. As model systems, we chose several cell lines of human origin: two mammary carcinoma cell lines (T47D and MCF7), one normal mammary epithelial cell line (MCF10A), and one ovarian carcinoma cell line (OVCAR3). Analysis of the NDF-induced activation of ErbB-2, ErbB-3, and ErbB-4, phosphorylation of intracellular substrates, and stimulation of MAPK and $p70/p85^{S6K}$ persuasively show a cell-specific requirement for ErbB-2 in NDF-induced signaling as well as imply the involvement of an as yet unidentified component(s) in ErbB-mediated signal transduction.

MATERIALS AND METHODS

Materials. Recombinant human EGF and bovine pancreatic insulin were purchased from Sigma. Recombinant human NDF isoforms were a generous gift from Amgen, Thousand Oaks, Calif. For all the experiments described in this

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TABLE 1. Expression levels and NDF-induced activation of ErbB receptors

Cell line	Type[a]	No. of receptors				NDF activation[e]			
		ErbB-2[b]	ErbB-3[c]	ErbB-4[c]	EGFR[d]	ErbB-2	ErbB-3	ErbB-4	EGFR
T47D	C	3×10^4	++++	+	7×10^3	++	+++	+++	−
	5R					−	+	++	−
MCF7	C	2×10^4	++++	+	3×10^3	+	+++	−	ND[f]
	5R					−	−	−	ND
MCF10A	C	2×10^4	+	+	3×10^5	+	++	−	−
	5R					−	++	−	−
OVCAR3	C	1×10^4	++	++	1×10^5	+	++	+++	−
	5R					−	++	++	ND

[a] C, control; 5R, scFv-5R expressing.
[b] Determined by Western blot analysis relative to SKBR3, with 10^6 receptors per cell.
[c] Relative amounts determined by Western blot analysis; see Fig. 1A. ++++, high level; +, low level.
[d] Data for T47D (18), MCF7 (50), and MCF10A (10) were reported previously. Data for OVCAR3 were determined by Western blot analysis relative to MCF10A.
[e] Relative values determined by analysis of NDF-induced tyrosine phosphorylation; see Fig. 3 and 4. +++, high level; +, low level; −, no induction.
[f] ND, not determined.

paper, the NDF-β3 isoform (amino acids 14 to 241) was used. The antibodies used were ErbB-2-specific antiserum 21N (25) and monoclonal antibody (MAb) FSP77 (22), ErbB-3-specific affinity-purified rabbit polyclonal antibody C17 (Santa Cruz Biotechnology), ErbB-4-specific affinity-purified rabbit polyclonal antibody C18 (Santa Cruz Biotechnology), Shc-specific rabbit immunoglobulin G (IgG) (UBI), p85-specific antiserum (UBI), ERK2-specific antiserum (38), phosphotyrosine-specific MAb (12), intracellular adhesion molecule 1 (ICAM-1)-specific MAb LB-2 (Becton Dickinson), and scFv-specific antiserum (5). The production of MAb 77 against the extracellular domain of human ErbB-4 will be described elsewhere.

Intracellular expression of ErbB-2-specific scFv. Amphotropic virus encoding the scFv-5R (5, 61) as well as empty vector control virus was used to infect the human mammary carcinoma cell line MCF7, the normal human mammary epithelial cell line MCF10A, and the ovarian carcinoma cell line OVCAR3. Two to 4 days after infection, the cells were subjected to selection in 1 to 2 μg of puromycin per ml for 7 to 14 days. Pools of puromycin-resistant cells were analyzed in all the experiments.

Cell culture. T47D, MCF7, and OVCAR3 sublines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS; Gibco BRL), insulin (5 μg/ml), and puromycin (1 μg/ml). MCF10A sublines were grown in DMEM/F12 medium supplemented with 10% FCS, EGF (10 ng/ml), insulin (5 μg/ml), 1 μM dexamethasone, and puromycin (1 μg/ml). Prior to growth factor stimulation, cells were starved for at least 24 h in serum-free medium (DMEM containing 1 mg of fetuin [Sigma] and 10 μg of transferrin [Sigma] per ml). To assess the biological effect of NDF, MCF7 control and scFv-5R-expressing cells were plated in DMEM containing 2.5% FCS plus either 2 nM NDF, 160 nM phorbol myristate acetate (PMA), or no factor (2×10^5 cells per well of a six-well dish). After 4 days, the cells were counted, photographed, or subjected to fluorescence-activated cell sorting (FACS) analysis. To measure growth stimulation of MCF10A cells by EGF and NDF, cells were plated in triplicate in serum-free medium (DMEM/F12 containing 1 mg of fetuin, 10 μg of transferrin, and 5 μg of insulin per ml plus 1 μM dexamethasone) supplemented with EGF (100 ng/ml), NDF (100 ng/ml), or no factor (2×10^3 cells per well of a 96-well dish). Growth was monitored after 3 days with the Cell Titer 96 AQ kit (Promega). All points were prepared in triplicate.

Flow cytometric analysis. Cells were trypsinized and washed with 2 ml of FACS buffer (phosphate-buffered saline [PBS] containing 0.1% sodium azide and 1% bovine serum albumin) prior to staining. A total of 10^6 cells were suspended in 200 μl of FACS buffer containing MAb FSP77, MAb 77, or MAb LB-2 (20 μg of each per ml) specific for, respectively, the ectodomain of human ErbB-2, ErbB-4, or ICAM-1. Following incubation on ice for 30 to 60 min, the cells were washed in 2 ml of FACS buffer. Bound MAb 77 was detected with phycoerythrin-linked and the other MAbs were detected with fluorescein-linked secondary antibodies for 30 min on ice. The cells were washed twice in 2 ml of FACS buffer, resuspended in 500 μl of buffer, and analyzed for their fluorescence in a Becton Dickinson FACScan.

Immunoprecipitation and Western (immunoblot) analysis. Cells were lysed in Triton extraction buffer (50 mM Tris [pH 7.5], 5 mM ethylene glycol tetraacetic acid [EGTA, pH 8.5], 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium molybdate, 20 μM phenylarsine oxide, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) for 10 min on ice. The lysates were clarified by centrifugation at 16,000 × *g* for 10 min. For immunoprecipitations, equal amounts of protein (usually 1 to 2 mg) were incubated with specific antibodies for 2 h on ice. Immune complexes were collected with protein A-Sepharose (Sigma) and washed three times with extraction buffer and once with TNE (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM EDTA). Bound proteins were released by heating for 10 min at 95°C in sample buffer. Total cell lysates or immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were blotted to polyvinylidenedifluoride 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 with the ECL detection system (Amersham). Filters were stripped in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 65°C, washed three times in TTBS, blocked, and reprobed with the indicated antibodies.

MAPK (ERK) assays. Cells were lysed in ERK lysis buffer (50 mM β-sodium glycerophosphate, 1.5 mM EGTA, 2 mM sodium orthovanadate, 1 mM dithiothreitol [DTT], 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, 1 mM benzamidine, and 1% Nonidet P-40) for 10 min on ice. The lysates were clarified by centrifugation at 16,000 × *g* for 15 min. ERK2 was immunoprecipitated from 200 μg of total lysate with 2 μl of specific antiserum (38) and protein A-Sepharose. The immunoprecipitates were washed three times with ERK lysis buffer and once with kinase buffer (30 mM Tris-HCl [pH 8.0], 20 mM $MnCl_2$, 2 mM $MgCl_2$). 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 myelin basic protein (MBP), 10 μM cold ATP, and 0.1 μM [γ-^{32}P]ATP (1,200 Ci/mmol). The reaction was stopped with sample buffer, proteins were subjected to SDS-PAGE and blotted, and the phosphorylation of MBP was quantitated with a PhosphorImager (Molecular Dynamics).

S6 kinase assays. Cells were lysed as above, and p70/p85^{S6K} activity was determined as follows. Crude lysate (0.4 μg) was incubated for 30 min at 37°C with 10 μg of 40S ribosomal subunit in a reaction buffer containing 100 mM MOPS (morpholinepropanesulfonic acid), 2 mM DTT, 20 mM $MgCl_2$, 20 mM *p*-nitrophenylphosphate, 60 μM unlabeled ATP, 0.05 μg cyclic AMP-dependent protein kinase inhibitor (Sigma), and 1 μCi of [γ-^{32}P]ATP (1,200 Ci/mmol). The reaction was stopped with sample buffer, proteins were subjected to SDS-PAGE and blotted, and phosphorylation of S6 ribosomal protein was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS

Antibody-mediated intracellular retention of ErbB-2. Inhibiting the transit of ErbB-2 through the endoplasmic reticulum (ER) by ER lumenal expression of the scFv-5R has proven to be a potent tool to specifically prevent cell surface localization and ligand-induced activation of ErbB-2 (5, 18). To investigate NDF-induced signaling in different cellular contexts as well as the involvement of ErbB-2 in this signaling, scFv-5R was expressed in the mammary carcinoma cell lines T47D (18) and MCF7, the normal mammary epithelial cell line MCF10A, and the ovarian carcinoma cell line OVCAR3. All the cell lines express each of the four ErbB receptors, and no one receptor appears to be dramatically overexpressed (Table 1). ErbB-3 is highest in T47D and MCF7 cells, while OVCAR3 and MCF10A have 5 to 15 times lower levels (Fig. 1A, left panel). Similar amounts of ErbB-4 are expressed in all cell lines (Fig. 1A, right panel).

The cell lines were infected with a recombinant retrovirus

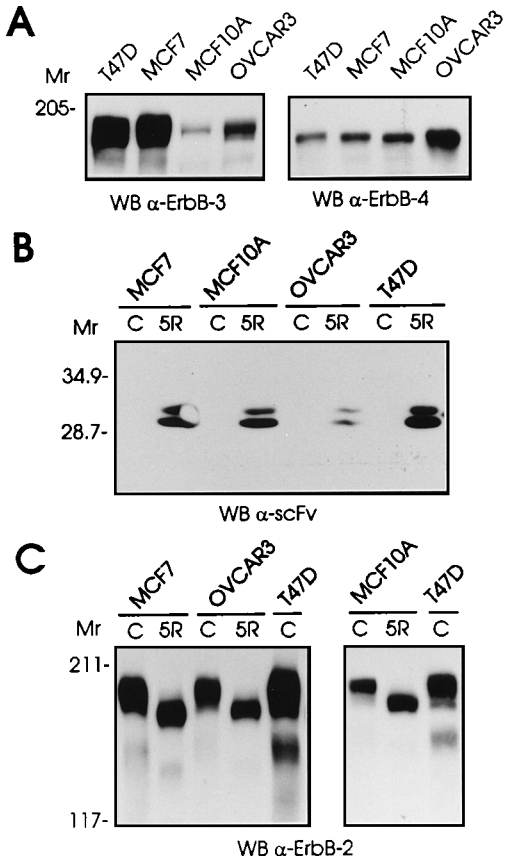

FIG. 1. Expression of scFv-5R and type I RTKs. (A) Left: ErbB-3 Western blot. Total protein (100 μg) from the indicated cell lines was subjected to SDS-PAGE (7.5% gel), blotted, and analyzed for ErbB-3 expression with antibody C17. Right: ErbB-4 Western blot. ErbB-4 was immunoprecipitated with antibody C18 from 1.2 mg of total protein of the indicated cell lines, subjected to SDS-PAGE (7.5% gel), blotted, and detected with antibody C18. (B) scFv-5R Western blot. Total protein (50 μg) from the indicated cell lines was subjected to SDS-PAGE (15% gel), blotted, and analyzed for scFv-5R expression with an scFv-specific antiserum. (C) ErbB-2 Western blot. Total protein (100 μg) from the indicated cell lines was subjected to SDS-PAGE (7.5% gel), blotted, and analyzed for ErbB-2 expression with the 21N antiserum. In this and subsequent figures, C indicates control cells and 5R indicates scFv-5R-expressing cells, and sizes are shown in kilodaltons.

encoding the scFv-5R, and the sublines T47D/5R (18), MCF7/5R, MCF10A/5R, and OVCAR3/5R as well as empty-virus control cell lines were established. Western blotting with an scFv-specific antiserum revealed high levels in MCF7, MCF10A, and T47D, while OVCAR3 contained less of the scFv protein (Fig. 1B). As previously observed (5, 18), the ErbB-2 protein in scFv-5R-expressing cells displayed a faster electrophoretic mobility than that in the control cells, which is likely due to underglycosylation of the ER-retained precursor (Fig. 1C). Intracellular retention of ErbB-2 was shown by flow cytometric analysis of intact cells stained with MAb FSP77, specific for the extracellular domain of the receptor (22). In all of the scFv-5R-expressing cells, ErbB-2 was essentially absent from the cell surface (Fig. 2) (18). This intracellular retention is specific for ErbB-2, since we have previously shown that trafficking of the related EGFR and ErbB-3 proteins was not affected (18). NDF induced tyrosine phosphorylation of ErbB-2 in each of the control cell lines (Table 1), in agreement with numerous previous reports (24, 41). As a consequence of its intracellular retention, no NDF-induced tyrosine phosphorylation of ErbB-2 was found in the scFv-5R-expressing cells (Table 1).

NDF-induced phosphorylation of ErbB-4. The binding of NDF to ErbB-4 leads to activation of ErbB-2 because of the formation of ErbB-2-containing heterodimers (46). Thus, we examined the NDF-induced activation of ErbB-4 and the effect of the presence of ErbB-2 in each of the cell lines. Cells were treated with NDF for 5 min, and the phosphotyrosine content of ErbB-4 was determined following its immunoprecipitation with a specific antibody. NDF efficiently activated ErbB-4 in T47D and OVCAR3 control and scFv-5R-expressing cells (Fig. 3 and Table 1), suggesting that ErbB-4 functions in a largely ErbB-2-independent manner. However, it is noteworthy that the level of NDF-induced ErbB-4 phosphorylation in T47D/5R and OVCAR3/5R cells was reduced by 40 and 15%, respectively, indicating that ErbB-4/ErbB-2 heterodimers do play a limited role in the activation of ErbB-4. Surprisingly, despite similar expression levels (Fig. 1A), no ErbB-4 phosphorylation was found in MCF7 and MCF10A cells (Fig. 3A and Table 1).

Flow cytometric analysis of intact cells with an antibody to the extracellular domain of ErbB-4 revealed that this receptor is present on the cell surface and thus available for ligand binding in each of the cell lines (Fig. 4). In fact, radiolabeled NDF can be covalently cross-linked to ErbB-4 in MCF7 cells (29). Thus, we attempted to achieve activation of ErbB-4 in

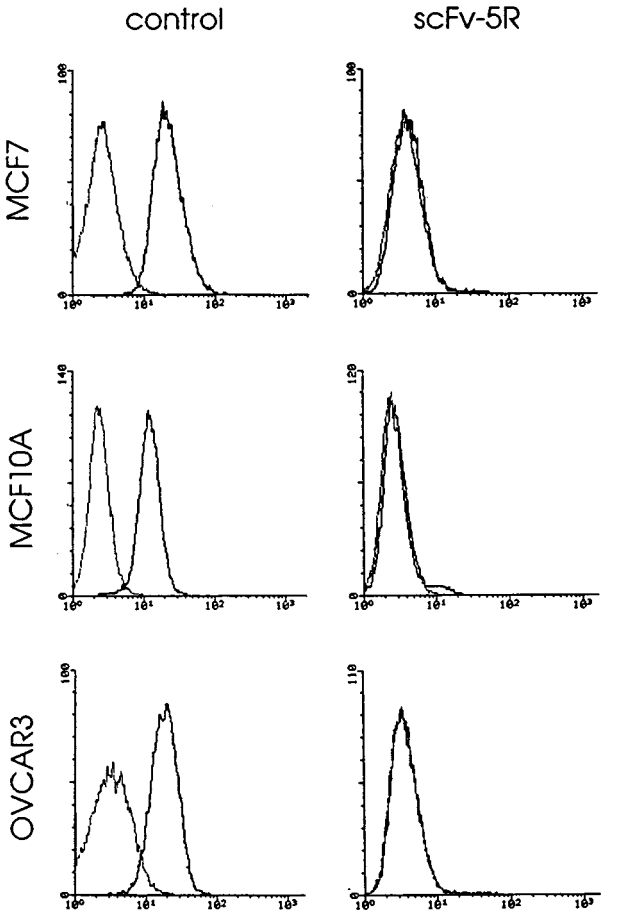

FIG. 2. Intracellular retention of ErbB-2. Intact control and scFv-5R virus-infected cells were stained with MAb FSP77 in combination with fluorescein-labeled anti-mouse IgG antibody and analyzed for their fluorescence in a FACScan. Right-hand curves, specific staining; left-hand curves, nonspecific staining (primary antibody omitted); ordinates, relative cell number; abscissas, log fluorescence.

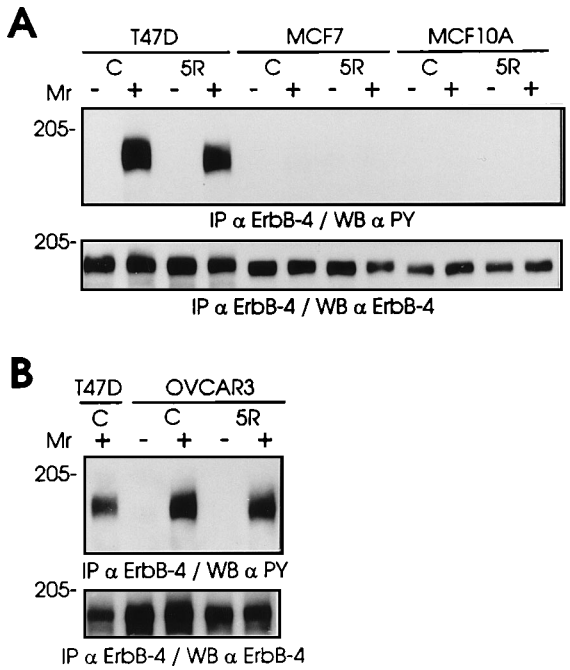

FIG. 3. NDF-induced phosphorylation of ErbB-4. The indicated cell lines were starved for 24 h in serum-free medium and either treated with NDF (50 ng/ml) (+) or left untreated (−) prior to lysis. ErbB-4 was immunoprecipitated from 2 mg of total protein, subjected to SDS-PAGE (8% gel), and analyzed by Western blotting with a phosphotyrosine-specific MAb (α PY). Filters were stripped and reprobed with ErbB-4-specific antibody.

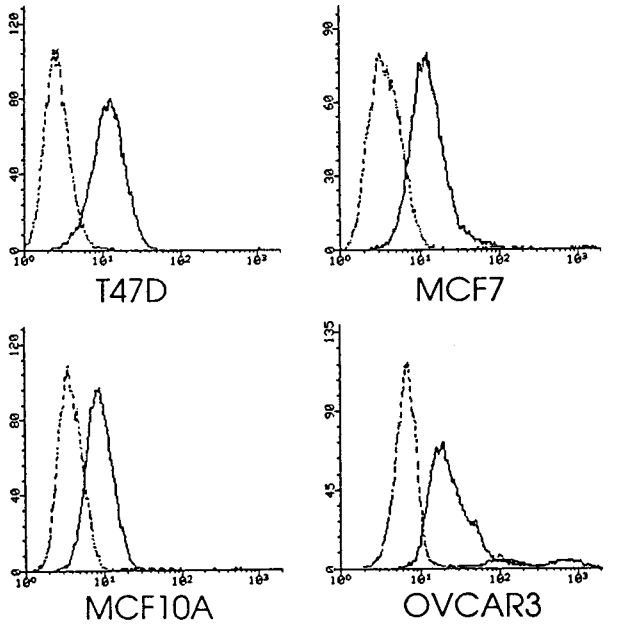

FIG. 4. Cell surface expression of ErbB-4. Intact control cells were stained with MAb 77 in combination with phycoerythrin-labeled anti-mouse IgG antibody and analyzed for their fluorescence in a FACScan. Right-hand curves, specific staining; left-hand curves, nonspecific staining (primary antibody omitted); ordinates, relative cell number; abscissas, log fluorescence.

MCF7 control cells by using different NDF isoforms (α1 or β1 instead of β3), altering the time of treatment (1 or 20 instead of 5 min), and treating the cells for 1 h with the tyrosine phosphatase inhibitor sodium orthovanadate prior to stimulation. However, none of these treatments led to an observable increase in tyrosine phosphorylation of ErbB-4 in MCF7 cells, while an increase in its phosphorylation was readily detected in T47D cells (not shown). Thus, NDF activation of ErbB-4 appears to be cell specific. The reason for the inability of NDF to activate ErbB-4 in MCF7 and MCF10A cells remains unclear.

NDF-induced phosphorylation of ErbB-3. The phosphotyrosine content of ErbB-3 was examined in each of the cell lines following NDF treatment. In the control cells, the relative level of NDF-induced ErbB-3 phosphorylation approximately reflected the receptor level (Fig. 5A and C). We have previously reported that in T47D cells, the NDF-induced phosphorylation of ErbB-3 is reduced by 80% if ErbB-2 is absent from the cell surface (Fig. 5A) (18). More strikingly, in MCF7/5R cells, there was no significant activation of ErbB-3 in the absence of ErbB-2 (Fig. 5A), demonstrating the importance of ErbB-2 in these cells. Indeed, we were able to show a physical interaction of the two receptors, since ErbB-2 could readily be detected in ErbB-3 immunoprecipitates (Fig. 5B). Surprisingly, no such ErbB-2 dependency was observed for the NDF-induced activation of ErbB-3 in MCF10A and OVCAR3 cells (Fig. 5C). Both in the control and in the scFv-5R-expressing cell lines, NDF induced ErbB-3 phosphorylation to a similar extent. Since some reports suggest that ErbB-3 is a mitigated kinase (20) which can only function as a heterodimer (54), it seemed possible that some other ErbB receptor is active in these cells. Because an involvement of ErbB-4 in MCF10A cells is unlikely (Fig. 3A), we analyzed NDF-induced activation of EGFR. However, no NDF-induced activation of EGFR was detectable in MCF10A control and MCF10A/5R cells (Fig. 5D and Table 1) or OVCAR3 cells (Table 1). Thus, while efficient activation of ErbB-3 is dependent on ErbB-2 in the breast tumor cell lines, it appears to be independent of any of the known ErbB receptors in the other cell lines.

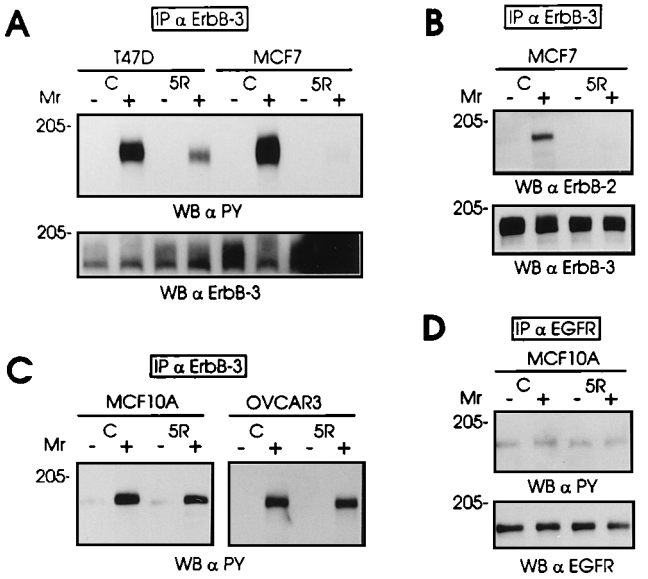

FIG. 5. NDF-induced phosphorylation of ErbB-3 but not EGFR. The indicated cell lines were starved for 24 h in serum-free medium and either treated with NDF (50 ng/ml) (+) or left untreated (−) prior to lysis. ErbB-3 (A, B, and C) or EGFR (D) was immunoprecipitated from equal amounts of total protein, subjected to SDS-PAGE (8% gel), and analyzed by Western blotting with a phosphotyrosine-specific MAb (A, C, and D) or an ErbB-2-specific antiserum (B). When possible, filters were stripped and reprobed with, respectively, ErbB-3- or EGFR-specific antibody.

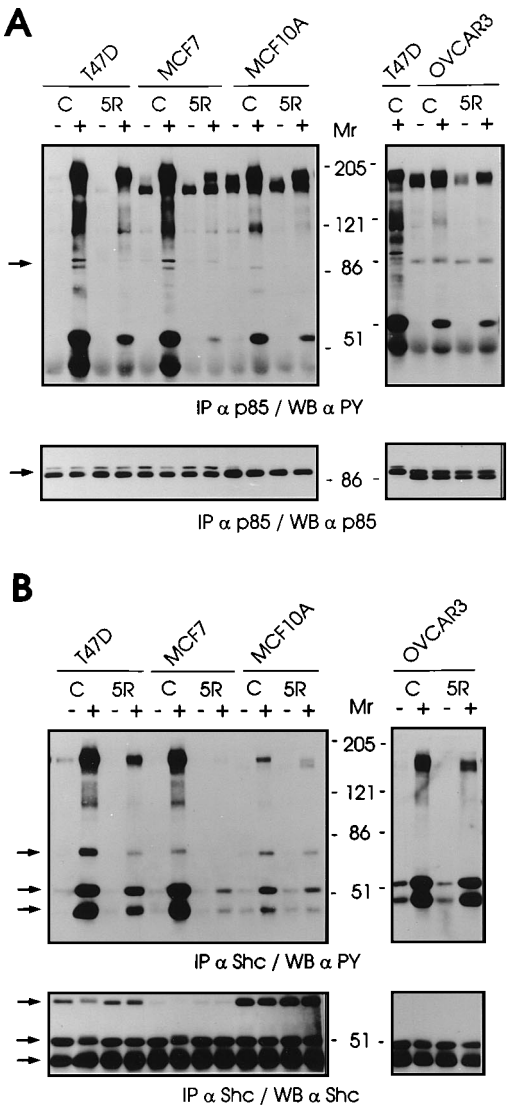

FIG. 6. NDF-induced substrate phosphorylation and association with ErbB receptors. The indicated cell lines were starved for 24 h in serum-free medium and either treated with NDF (50 ng/ml) for 5 min (+) or left untreated (−) prior to lysis. p85 (A) and Shc (B) were immunoprecipitated from 2 mg of total protein, subjected to SDS-PAGE (9% gel), and analyzed by Western blotting with a phosphotyrosine-specific MAb (upper panels). Filters were stripped and reprobed with, respectively, p85- and Shc-specific antibodies (lower panels). Arrows indicate the positions of the p85 isoforms (A) and of the 46-, 52-, and 66-kDa isoforms of Shc (B).

NDF-induced phosphorylation of p85 and association with ErbB receptors. PtdIns 3-kinase activity is increased in response to numerous growth factors. This is achieved following the binding of the SH2 domain-containing p85 subunit of PtdIns 3-kinase to a consensus phosphorylated Tyr-X-X-Met motif on the receptor, which brings the catalytic p110 subunit in close proximity to its substrate. Within the ErbB family of receptors, it has been shown that ErbB-3 associates efficiently with p85 as a result of the presence of at least two phosphorylated consensus motifs (16, 47). The apparent requirement for ErbB-2 for NDF-induced activation of ErbB-3 in some of the cell lines prompted us to analyze recruitment of p85 to NDF-activated receptors. p85 was immunoprecipitated from untreated and NDF-stimulated control and scFv-5R-expressing cell lines, and the precipitated proteins were analyzed by Western blotting with a phosphotyrosine-specific MAb (Fig. 6A). A total of three isoforms of p85 with similar but distinct electrophoretic mobilities were detected in the four cell lines (Fig. 6A, lower panel). In accordance with the observation that tyrosine phosphorylation of p85 occurs at a low stoichiometry (16), we observed only small amounts of phosphotyrosine associated with p85 following NDF treatment of the various cell lines. The least abundant isoform, detected only in T47D and MCF7 cells, underwent the most prominent phosphorylation. In contrast, the more abundant isoform expressed in all four cell lines underwent only a low level of phosphorylation in T47D, MCF7, and MCF10A cells. Significantly, NDF-induced tyrosine phosphorylation of p85 was dramatically reduced in cells lacking cell surface ErbB-2. No p85 phosphorylation was detected in OVCAR3 cells. The constitutively phosphorylated protein of approximately 86 kDa detected in OVCAR3 cells does not comigrate with either of the two isoforms expressed in these cells.

Several other tyrosine-phosphorylated proteins were coimmunoprecipitated with p85. The nature of the constitutively tyrosine phosphorylated protein(s) of approximately 160 to 180 kDa coimmunoprecipitated in MCF7, MCF10A, and OVCAR3 cells is unclear. The high-molecular-mass protein running at 180 to 190 kDa most probably corresponds to ErbB-3, since its molecular mass and level of phosphotyrosine correlate directly with those of ErbB-3 in the individual cell lines (Fig. 5). In addition, p85 is readily detectable in ErbB-3 immunoprecipitates from NDF-stimulated cells (not shown). In MCF7 cells, significant amounts of this receptor were coimmunoprecipitated with p85 only in the presence of cell surface ErbB-2. Thus, although ErbB-2 itself does not directly bind to p85 (55), its heterodimerization with and phosphorylation of ErbB-3 are essential for the association of ErbB-3 with p85. A similar, albeit less pronounced, effect was observed with T47D cells. In contrast, in MCF10A and OVCAR3 cells, ErbB-3/p85 complex formation was largely ErbB-2 independent, in agreement with the observed ErbB-2-independent activation of ErbB-3. In addition, similar amounts of p85 were found in ErbB-3 immunoprecipitates from NDF-treated MCF10A and OVCAR3 control and scFv-5R-expressing cells (not shown).

Two additional tyrosine-phosphorylated proteins of approximately 46 and 52 kDa were coimmunoprecipitated with p85 from NDF-stimulated cells. Reprobing of the filters revealed that these proteins are the p46 and p52 isoforms of Shc (not shown). A direct interaction of p85 with Shc has been reported recently for cells transformed by BCR/*abl* (21). However, because of the ability of phosphorylated ErbB-3 to associate with Shc (47), it is more likely that p85 and Shc are coimmunoprecipitated indirectly, through their presence in the same complex with ErbB-3. In summary, our results show a cell-specific requirement for ErbB-2 for efficient NDF-induced recruitment of p85 subunit of PtdIns 3-kinase to ErbB-3.

NDF-induced phosphorylation of Shc. The SH2-PTB domain-containing adaptor protein Shc is a key upstream component of the MAPK pathway, enabling growth factor receptors to activate Ras (51). The extent to which ErbB-2 affects NDF-induced Shc phosphorylation was evaluated by immunoprecipitating Shc from untreated and NDF-stimulated control and scFv-5R-expressing cell lines and analyzing its phosphotyrosine content by Western blotting (Fig. 6B). NDF induced the tyrosine phosphorylation of the 46- and 52-kDa isoforms of Shc to a similar extent in all the control cell lines except MCF10A cells. The low level of Shc phosphorylation in these cells correlates with the low ErbB-3 expression level and the

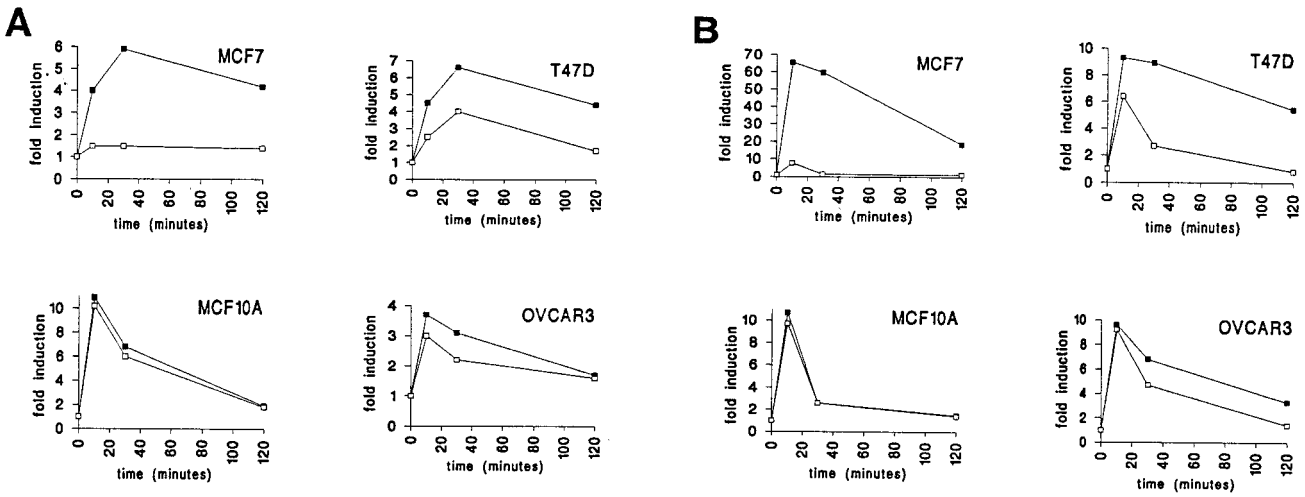

FIG. 7. (A) Time course of NDF-induced activation of p70/p85^{S6K}. MCF7, T47D, MCF10A, and OVCAR3 vector control (solid squares) and scFv-5R-expressing cells (open squares) were treated with 1 nM NDF for the indicated times. p70/p85^{S6K} activity was determined in cleared cell lysates by using purified 40S ribosomal subunit as a substrate. Proteins were subjected to SDS-PAGE and blotted, and phosphorylation of the S6 protein was quantitated with a PhosphorImager (Molecular Dynamics). (B) Time course of NDF-induced activation of ERK2. MCF7, T47D, MCF10A, and OVCAR3 vector control (solid squares) and scFv-5R-expressing cells (open squares) were treated with 1 nM NDF for the indicated times. ERK2 was immunoprecipitated, and immune complex assays with MBP as a substrate were carried out. Proteins were subjected to SDS-PAGE and blotted, and phosphorylation of MBP was quantitated with a PhosphorImager.

lack of detectable ErbB-4 activation. Expression of the 66-kDa isoform of Shc varied among the cell lines, and its phosphorylation reflected its amount. Compared with that in control T47D cells, an approximately fivefold reduction in NDF-induced Shc phosphorylation was observed in the T47D/5R cells. More dramatic results were obtained with MCF7 cells, in which intracellular retention of ErbB-2 almost completely abolished NDF-induced Shc phosphorylation. In contrast, in several independent experiments, the NDF-induced Shc phosphorylation was only reduced by 20 to 30% in MCF10A/5R and OVCAR3/5R cells compared with control cells.

With the exception of the MCF7/5R cells, tyrosine-phosphorylated proteins of 180 to 190 kDa were coimmunoprecipitated with Shc in all of the NDF-treated cell lines. In T47D and MCF7 control cells, we could detect ErbB-2 and ErbB-3 in this complex (not shown). In the T47D and OVCAR3 cells, ErbB-4 is likely to be in this complex as well. In the scFv-5R-expressing cells, no ErbB-2 was found complexed with Shc because of its absence from the cell surface. There was a dramatic reduction in the intensity of the 180- to 190-kDa band in the T47D/5R cells, and ErbB-3 association with Shc was reduced by fivefold in these cells (18). We were unable to detect ErbB-3 in Shc immunoprecipitates from MCF7/5R cells (not shown); in fact, there are no high-molecular-weight phosphotyrosine-containing proteins in these immunoprecipitates. In the MCF10A and OVCAR3 scFv-5R-expressing cells, the reduced intensity of the 180- to 190-kDa band is probably due to the loss of cell surface ErbB-2, since NDF treatment of scFv-5R-expressing cells did not dramatically affect the phosphorylation of ErbB-3 in the former and of ErbB-3 and ErbB-4 in the latter cells. Taken together, our observations demonstrate a cell-specific requirement for ErbB-2 for NDF-induced phosphorylation of Shc.

NDF-induced activation of MAPK and p70/p85^{S6K}. Activated RTKs stimulate MAPK via Shc coupling to the Ras/Raf/Mek pathway (51). There is evidence that RTK activation of p70/p85^{S6K} which is independent of the Ras-MAPK pathway requires PtdIns 3-kinase (9). The cell-specific requirement for ErbB-2 for NDF-induced receptor association of the p85 subunit of PtdIns 3-kinase and the phosphorylation of Shc prompted us to analyze the activation of S6 kinase (S6K) and MAPK in the different cell lines. p70/p85^{S6K} activity was measured in extracts of NDF-stimulated control and scFv-5R-expressing cell lines with 40S ribosomal subunit as a substrate (Fig. 7A). NDF activated p70/p85^{S6K} in all the control cell lines. However, little or no p70/p85^{S6K} activity was found in MCF7/5R cells, while its activity was reduced by approximately 40% in T47D/5R cells. In contrast, p70/p85^{S6K} activity was essentially unaffected by the lack of cell surface ErbB-2 in OVCAR3/5R and MCF10A/5R cells. These results correlate well with the fact that the p85 ErbB receptor association is mitigated in scFv-5R-expressing T47D and MCF7 cells, while its receptor association is only mildly affected in the other cells (Fig. 6A).

The p42 ERK2 isoform of MAPK was immunoprecipitated from control and scFv-5R-expressing cells treated for various times with NDF, and immune complex kinase assays were performed with MBP as a substrate (Fig. 7B). NDF rapidly stimulated the activity of ERK2 in all the control cells, with maximal activity at 10 min. The overall kinetics varied in the different cells. ERK2 activity was sustained for 2 h in T47D, MCF7, and OVCAR3 cells, while it was very transient in MCF10A cells. The maximal ERK2 activity was similar in T47D, MCF7, and OVCAR3 cells (not shown). However, because of different basal activities, the fold stimulation varied from 10- to 60-fold. ERK2 activity was 5- to 10-fold lower in MCF10A cells, paralleling the low level of NDF-induced Shc phosphorylation in these cells. In agreement with the observed cell-specific requirement for ErbB-2 for NDF-induced phosphorylation of Shc, intracellular retention of ErbB-2 differentially influenced the activation of MAPK in the four cell lines. Whereas the activity of MAPK was hardly affected in OVCAR3/5R and MCF10A/5R cells, maximal activity was reduced by approximately 30% in T47D/5R cells and 10-fold in MCF7/5R cells. In addition, the kinetics of ERK2 activity in T47D, MCF7, and OVCAR3 cells lacking cell surface ErbB-2 were more transient than in the control cells.

The dramatically reduced NDF-stimulatable MAPK activity in MCF7 cells lacking cell surface ErbB-2 prompted us to analyze the ability of an unrelated growth factor to activate this

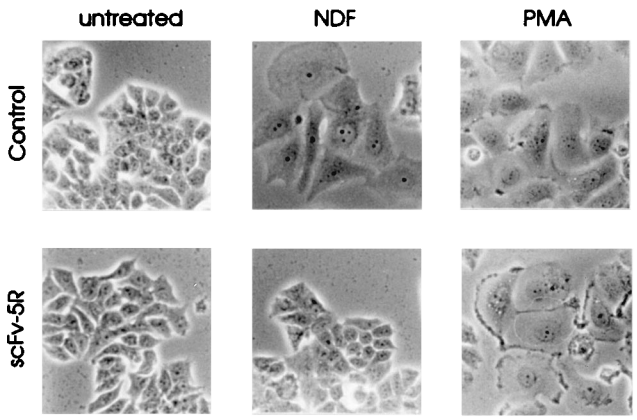

FIG. 8. Morphological differentiation of NDF- and PMA-treated MCF7 cells. MCF7 control and scFv-5R-expressing cells were plated in the presence and absence of 2 nM NDF or 160 nM PMA and incubated for 4 days.

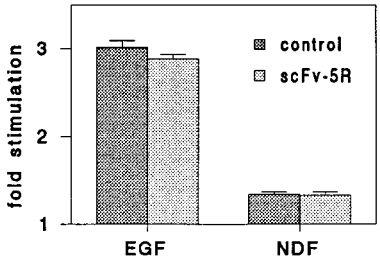

FIG. 9. Growth stimulation of MCF10A cells by EGF and NDF. Control and scFv-5R-expressing cells were plated in the presence and absence of NDF or EGF (100 ng/ml), and growth was monitored after 3 days with a Cell Titer AQ kit (Promega). Bars show fold growth stimulation in comparison to untreated cells; error bars indicate standard deviations.

pathway. Therefore, control and scFv-5R-expressing MCF7 cells were treated for 10 min with 1 nM basic fibroblast growth factor, and the activity of ERK2 was determined as described above. In both sublines, an approximately 50-fold stimulation was observed, indicating that the intracellular retention of ErbB-2 specifically abrogated NDF signaling without affecting signaling by other growth factors (not shown). Taken together, our results clearly show a cell-specific requirement for ErbB-2 for NDF-induced activation of two independent intracellular signaling pathways.

NDF- but not TPA-induced morphological differentiation of MCF7 cells is dependent on the presence of cell surface ErbB-2. NDF induces differentiation in some mammary epithelial cell lines, including morphological maturation, inhibition of cell growth, and synthesis of milk components (3, 41). In agreement with previous findings (3), we observed no significant effect of NDF treatment on the growth rate of MCF7 cells. However, MCF7 control cells cultured in 2 nM NDF for a prolonged time underwent phenotypic maturation and changed from a very compact shape to a more flattened shape, characterized by large nuclei and abundant cytoplasm (Fig. 8). Since it has been reported that some breast tumor cells display increased synthesis of ICAM-1 following treatment with NDF (2), we examined MCF7 cells for expression of this membrane glycoprotein. ICAM-1 synthesis was upregulated by almost twofold in NDF-treated MCF7 cells (Table 2). In contrast, no effect on morphology or ICAM-1 expression was observed with scFv-5R-expressing cells, indicating that in MCF7 cells, the NDF-induced morphological changes are strictly dependent on the presence of cell surface ErbB-2 (Fig. 8 and Table 2). Since phorbol esters also induce morphological differentiation of MCF7 cells (4), we analyzed the effect of phorbol myristate acetate (PMA) treatment on control and scFv-5R-expressing cells. When cultured in 160 nM PMA for 4 days, a growth inhibition of approximately 80%, paralleled by the acquisition of a mature phenotype, was observed with both cell lines (Fig. 8 and data not shown). Importantly, PMA induced a three- to fourfold increase in ICAM-1 expression in both cell lines (Table 2). Thus, the NDF unresponsiveness of the scFv-5R-expressing cells is due specifically to the absence of cell surface ErbB-2.

TABLE 2. Induction of ICAM-1 expression[a]

Treatment	MCF7		MCF7/5R	
	Fluorescence	Fold induction	Fluorescence	Fold induction
None	21.7		17.2	
NDF	41.3	1.9	17.1	1.0
PMA	71.2	3.3	73.6	4.3

[a] MCF7 control and scFv-5R-expressing cells were plated in 2.5% FCS in the presence or absence of 1 nM NDF or 160 nM PMA. After 4 days, the cells were trypsinized, stained with an antibody to the extracellular domain of ICAM-1, and analyzed for their fluorescence in a FACScan. Mean fluorescence (arbitrary units) and fold induction compared with induction in the untreated cells are given.

NDF-stimulated growth of MCF10A cells does not require ErbB-2. NDF stimulates the growth of some cells of mammary epithelial origin (24, 38). Since MCF10A cells require EGF for their growth (56), we evaluated the ability of NDF to replace EGF in the growth medium. NDF was found to stimulate the growth of MCF10A cells, although it was significantly less potent than EGF (Fig. 9). NDF-stimulated growth of MCF10A cells was similar to that seen for basic fibroblast growth factor (not shown). Significantly, NDF-stimulated growth of MCF10A cells was not reduced in the absence of cell surface ErbB-2 (34 versus 33% stimulation), in agreement with the fact that NDF-induced signaling is only mildly impaired in these cells (Fig. 9).

DISCUSSION

The study of signaling through cell surface receptors, such as the ErbB family of tyrosine kinases, is complicated by the extensive cross-talk between individual family members, making it difficult to assign a particular function to a specific receptor. The targeted inactivation of an individual receptor is a viable and promising approach to solving this problem. Experimental strategies for the targeted inactivation of ErbB receptors have been described, including the use of antisense oligonucleotides (11) and chemical inhibitors (6) as well as expression of dominant-negative mutants (40). Each approach offers advantages as well as disadvantages. In particular, a systematic analysis of the signaling ability of ErbB receptors with dominant-negative mutants is complicated by the fact that mutant growth factor receptors retain their ability to heterodimerize (48). Single-chain antibody-mediated intracellular retention of a growth factor receptor represents an interesting alternative (5). By combining the specificity and potency of intracellular antibody expression with the efficiency of retroviral gene transfer, a stable phenotypic receptor knockout in a given cell line can be achieved rapidly.

In this study, we have used the single-chain antibody-mediated intracellular retention of ErbB-2 to evaluate the involvement of this receptor in NDF-induced signaling in different cellular contexts. In previous studies with T47D cells, we ob-

served that ErbB-2 enhanced both EGF- and NDF-induced signaling (18). In addition, NDF-induced growth of T47D cells lacking cell surface ErbB-2 was dramatically reduced, paralleling a reduction in MAPK activity. We had anticipated that the removal of ErbB-2 from the cell surface might yield similar results in different cell lines. This does not appear to be the case. Analysis of the ligand-dependent phosphorylation of NDF receptors and the intracellular substrates Shc and the p85 subunit of PtdIns 3-kinase, as well as activation of the MAPK and p70/p85^{S6K} pathways, revealed a cell-specific requirement for ErbB-2. While NDF signaling in MCF7 cells is strictly ErbB-2 dependent, more so than in the T47D cells, it appears to be mainly ErbB-2 independent in MCF10A and OVCAR3 cells. Consequently, scFv-mediated intracellular retention of ErbB-2 abolishes NDF-induced morphological differentiation and upregulation of ICAM-1 expression of MCF7 cells, while it does not affect the NDF-induced proliferation of MCF10A cells. NDF had no measurable effect on either the morphology or the growth of OVCAR3 cells. However, it is worth mentioning here that our results show that NDF does bind and activate intracellular signaling in an ovarian cell line. Previous work showing that NDF does not bind to ovarian cells (42) should not be interpreted to mean that this factor does not play a role in ovarian cancer. It is possible that the cell lines examined previously do not express ErbB-3 or ErbB-4. Interestingly, ErbB-2 does not play as important a role in NDF-induced signaling in the ovarian tumor cell line as it appears to in the two breast tumor cell lines.

Expression of a transfected ErbB-4 alone yields a functional NDF receptor (46). In agreement with these findings, efficient NDF-stimulated phosphorylation of ErbB-4 in T47D and OVCAR3 was not dependent on the presence of cell surface ErbB-2, suggesting that ErbB-4 activation is predominantly mediated by homodimers (and possibly ErbB-4/ErbB-3 heterodimers). However, there is some evidence for the involvement of ErbB-4/ErbB-2 heterodimers, since the activation of ErbB-4 was mildly impaired in the scFv-5R-expressing cells. In striking contrast to the results obtained with T47D and OVCAR3 cells, we were unable to detect any NDF-induced ErbB-4 phosphorylation in MCF7 and MCF10A cells. This result was not expected, and on the basis of previous findings, no satisfying explanation can be given. The expression pattern of ErbB-4 is more restricted than that of EGFR and ErbB-2, and we are not aware of any studies on the activation of ErbB-4 in its natural, endogenous setting. It appears that control of its activation in MCF7 and MCF10A cells may be more complex than just the presence or absence of the receptor on the plasma membrane, and an additional component may be necessary to achieve its stimulation.

The observation that several amino acid residues which are conserved in the catalytic domain of protein kinases are altered in the ErbB-3 catalytic domain has raised the possibility that this receptor has an impaired kinase activity (44). Indeed, compared with EGFR, insect cell-expressed ErbB-3 has much lower kinase activity (20). ErbB-3 transfected on its own into COS-7 cells undergoes only a low level of NDF-induced phosphorylation. However, its coexpression with ErbB-2 reconstitutes a receptor, which is efficiently activated by NDF (54). On the basis of these observations, recent publications have emphasized the importance of ErbB-2/ErbB-3 heterodimerization in NDF-induced signaling (8, 27, 36) and in neoplastic transformation (1). In agreement with this view, the scFv-mediated intracellular retention of ErbB-2 almost completely abolished NDF-induced activation of ErbB-3 in MCF7 cells and dramatically reduced its phosphorylation in T47D cells. In contrast, the absence of cell surface ErbB-2 hardly affected NDF-induced phosphorylation of ErbB-3 in MCF10A and OVCAR3 cells. These results argue that, depending on the cellular context, NDF-induced formation of previously unreported ErbB-3-containing heterodimers may play as important a role as the ErbB-2/ErbB-3 heterodimers.

Theoretically, the formation of ErbB-3/EGFR or ErbB-3/ErbB-4 heterodimers could be envisioned to play a role in MCF10A and OVCAR3 cells. Such active heterodimers have been found in hematopoietic cells transfected with specific pairs of receptors (49). In our experiments, we have no evidence for NDF activation of EGFR. Our previous observation that NDF did not activate EGFR in T47D cells (18) could have been due to the low EGFR number ($<10^4$) in these cells. However, even in MCF10A cells, which express 3×10^5 EGFR (10), we could not detect NDF-induced EGFR activation. NDF-induced EGFR/ErbB-3 complexes were also not detected in a keratinocyte cell line (36). Using the more sensitive technique of cell surface linking radiolabeled NDF, it has been possible to find labeled EGFR (28, 58). However, the phosphotyrosine content of the EGFR was not examined in these experiments.

While an involvement of EGFR seems unlikely, ErbB-4 may be responsible for efficient ErbB-2-independent activation of ErbB-3 in OVCAR3 cells and also account for the residual NDF-induced ErbB-3 phosphorylation detected in T47D/5R cells. However, in MCF10A cells, ErbB-3/ErbB-4 heterodimer formation seems unlikely, since NDF appears not to activate ErbB-4 in these cells. Thus, our results in the MCF10A cells are compatible with either active ErbB-3 homodimers, a possibility which is at odds with most previous findings, or the existence of an additional activating component, such as a cytoplasmic tyrosine kinase (57) or another ErbB receptor family member.

Given the ErbB-2-independent activation of ErbB-3 in MCF10A/5R cells as well as the apparently inactive ErbB-4 expressed in these cells, one crucial question remains to be answered: how does ErbB-2 become activated in response to NDF in the control cells? At least two possibilities could explain NDF activation of ErbB-2 as well as efficient activation of ErbB-3 in the absence of ErbB-2. Both ErbB-2 and ErbB-3 could become activated through an additional ErbB receptor expressed in these cells. Alternatively, ErbB-3 could dimerize with ErbB-2 in the control cells while using an additional ErbB receptor as a substitute heterodimerization partner in the scFv-5R-expressing cells, in which ErbB-2 is not available. We favor the latter possibility because it is compatible with the formation of an ErbB-2/ErbB-3 heterodimeric high-affinity NDF receptor (53) in MCF10A control cells.

The ability of NDF to activate multiple receptors may help to explain the variety of its biological effects. It is likely that the specific subset of receptors expressed in a given cell defines a specific combination of intracellular signaling pathways activated by NDF. Thus, we attempted to correlate the individual ErbB receptors of the different cell lines with the efficiency of NDF-induced phosphorylation of specific substrates. No NDF-induced phosphorylation of phospholipase C-γ_1 (39), GTPase-activating protein, p60 and p190 GTPase-activating protein-associated proteins (13), p91 (17), or eps15 (15) was detected in any of the cell lines tested, irrespective of their ErbB-3 or ErbB-4 status (not shown). In contrast, NDF-induced phosphorylation of Shc was observed in all the cell lines. The efficiency of Shc phosphorylation in the individual cell lines did not reveal any receptor specificity but rather correlated with the overall ErbB receptor levels. However, in agreement with the observation that ErbB-3 is the only ErbB receptor capable

of coupling to PtdIns 3-kinase (55), p85 recruitment correlated with the ErbB-3 expression levels.

It seems likely that not only the presence or absence of a specific receptor in a given cell line influences the nature of intracellular signals generated by NDF. The fact that ErbB receptors undergo extensive heterodimerization potentially makes NDF-induced signaling even more complex. It is reasonable to assume that there are qualitative and quantitative differences in the signaling properties of different receptor heterodimers. In fact, our results support this idea. In MCF10A cells, the presence or absence of ErbB-2 hardly influences NDF-induced phosphorylation of ErbB-3 or the formation of ErbB-3/p85 complexes (see Fig. 5C and 6A), yet only in the presence of ErbB-2 does p85 become significantly phosphorylated (Fig. 6A). A very similar observation is made with a heavily tyrosine phosphorylated 121-kDa protein that is detected in p85 immunoprecipitates of NDF-treated control but not scFv-5R-expressing MCF10A cells (Fig. 6A). These observations are compatible with the activation of distinct ErbB-3-containing heterodimers in control versus scFv-5R-expressing cells.

In summary, this study represents the first analysis of the concerted action of all known ErbB receptors during NDF-induced signaling in various cellular contexts. The results presented provide first evidence for the existence of an additional, yet to be identified component(s) involved in NDF signaling. Importantly, the results also raise several questions concerning the regulation of ErbB-3 and ErbB-4 activity. Further work will be required to elucidate the molecular mechanisms of their regulation.

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