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Molecular requirements for the effect of neuregulin on cell spreading, motility and colony organization

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Neuregulin can trigger morphogenetic signals in cells both *in vivo* and in culture through the activation of receptors from the ErbB family. We have ectopically expressed various ErbB-receptors in 32D myeloid cells lacking endogenous ErbB-proteins, and in CHO cells, which express only ErbB-2. We show here that activation of ErbB-3/ErbB-2 heterodimeric receptors triggers PI3-kinase-dependent lamellipodia formation and spreading, while individual ErbB-receptor homodimers as well as ErbB-3/ErbB-1 heterodimers are much less effective. CHO cells expressing ErB-3/ErbB-2 together with N-cadherin, an adhesion receptor, form epithelioid colonies. Neuregulin activates cell motility leading to transition of these colonies into ring-shaped multicellular arrays, similar to those induced by neuregulin in epithelial cells of different types (Chausovsky *et al.*, 1998). This process requires both PI3-kinase and MAP kinase kinase activity and depends on coordinated changes in the actin- and microtubule-based cytoskeleton. Transactivation of ErbB-2 is not sufficient for the activation of cell motility and ring formation, and the C-terminal domain of ErbB-3 bearing the docking sites for the p85 subunit of PI3-kinase is essential for these morphogenetic effects. Thus, ErbB-3 in conjunction with ErbB-2 mediates, via its C-terminal domain, cytoskeletal and adhesion alterations which activate cell spreading and motility, leading to the formation of complex structures such as multicellular rings. *Oncogene* (2000) **19,** 878–888.

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Introduction

Morphogenetic processes during embryonic development, are precisely coordinated in time and space (reviewed in Trinkaus, 1984; Edelman *et al.*, 1990; Bray, 1992; Hay, 1995; Gumbiner, 1996). Soluble ligands (growth factors) were shown to be essential for the control of morphogenesis both *in vivo* and *in vitro*. For example, hepatocyte growth factor/scatter factor (HGF/SF), which operates by the activation of its receptor, c-Met (Stoker *et al.*, 1987; Bottaro *et al.*, 1991; Naldini *et al.*, 1991; Gherardi *et al.*, 1993; Jeffers *et al.*, 1996), induces scattering of epithelial colonies grown on two-dimensional substrates *in vitro* (Stoker *et al.*, 1987; Gherardi *et al.*, 1993), and formation of branching tubular structures by cells grown in a three-dimensional collagen gel (Montesano *et al.*, 1991; Soriano *et al.*, 1995). Knockout of either HGF/SF or c-Met results in embryonic lethality due to disturbed placenta and liver development and an apparent defect in the migration of muscle precursor cells (Birchmeier *et al.*, 1997).

Another group of ligands, the neuregulins (also known as NDF, or heregulin) (Burden and Yarden, 1997) are involved in morphogenetic processes including the morphogenesis of heart trabeculae (Meyer and Birchmeier, 1995; Kramer *et al*, 1996), the migration of neural crest cells (Britsch *et al.*, 1998), and the formation of alveolar structures in mammary gland development (Yang *et al.*, 1995). All neuregulin isoforms bind directly to the ErbB-3 and ErbB-4 receptors, which cooperate with ErbB-1 (EGF receptor) or the 'orphan' ErbB-2 receptor in the generation of a transmembrane signal (Burden and Yarden, 1997; Tzahar and Yarden, 1998). The ErbB-1 and ErbB-2 co-receptors are essential for ErbB-3-mediated signaling since this receptor has little or no intrinsic tyrosine kinase activity (Guy *et al.*, 1994).

We have recently shown that stimulation of cultured epithelial cells by neuregulin leads to a unique morphogenetic response, manifested by the formation of ring-shaped multicellular arrays with an internal 'lumen'. This morphogenetic reaction appeared to depend on neuregulin-mediated stimulation of ErbB-3/ErbB-2 receptor heterodimers (Chausovsky *et al.*, 1998).

In the present study we investigated the specific roles of ErbB-3 and ErbB-2 in the various manifestations of this morphogenetic response. For this purpose two model systems were used: the first is 32D hematopoietic cells, which lack endogenous ErbB family receptors (Pinkas-Kramarski *et al.*, 1996). The 32D system enabled us to study the effect of different combinations of ErbB receptors on cell attachment, lamellipodia protrusion and spreading. We show here that only the combination of ErbB-2 and ErbB-3 induces significant spreading of 32D cells, which normally grow in suspension.

The formation of more complex morphological structures was investigated in ErbB-3 transfected CHO cells. These cells express endogenous ErbB-2, but no other ErbB family member (Tzahar *et al.*, 1996). Since CHO cells form only weak intercellular adhesions, we transfected them with N-cadherin cDNA prior to ErbB-3 transfection. The transfected cells form adherens junctions and grow as epithelioid colonies

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(Levenberg *et al*., 1998). The CHO line, expressing a combination of ErbB-2/ErbB-3 and N-cadherin acquired the ability to demonstrate a profound morphogenetic response to neuregulin stimulation, manifested by the formation of multicellular ring-shaped structures. This reaction involved coordinated reorganization of the actin cytoskeleton, focal adhesions, and the microtubular system. We show here that the MAP kinase and PI3-kinase pathways are both required for the process of ring formation, and that the C-terminal part of the ErbB-3 receptor, bearing its major cytoplasmic docking sites, is essential for this morphogenetic response.

Results

ErbB-2/ErbB-3 heterodimers are required for activation of lamellipodial activity and cell spreading in 32D cells

To investigate the role of specific ErbB receptors in cellular morphogenesis we used 32D hematopoietic cells, stably transfected with various ErbB receptors. The parental 32D cells do not express any ErbB receptors and fail to spread on glass or plastic surfaces.

Activation of individual receptors of the ErbB-family by their specific ligands did not induce significant cell spreading, though in some cases induction of actin containing protrusions was observed. For example, activation of 32D cells transfected with ErbB-1 by EGF (20 ng/ml) enhanced formation of ruffles on the surface of the spherical cells, and activation of ErbB-2-expressing cells by a specific activating antibody (1–10 μg/ml) induced formation of narrow processes in approximately 10% of the cells (not shown) without inducing cell spreading on the substrate. Activation of ErbB-4 by addition of neuregulin also did not induce spreading, although formation of brightly stained actin 'paracrystals' was noticed (not shown). Addition of neuregulin to 32D cells expressing ErbB-3 did not induce any changes in cell adhesion, morphology or in the actin cytoskeleton (not shown). This absence of a morphogenetic response could be explained by the impaired kinase activity of the ErbB-3 receptor (Guy *et al*., 1994).

However, stimulation of 32D cells expressing ErbB-3 together with ErbB-2 (32D-ErbB-2/ErbB-3 line) induces a strong morphogenetic response to neuregulin (Figures 1a,b; 2a,b) manifested by increased lamellipodial activity and formation of numerous actin rich peripheral ruffles after 20 min of incubation with neuregulin. This process was accompanied by cell adhesion and spreading, leading to a major increase in the projected cell area after 1 h incubation with the ligand. A similar response was obtained after treatment of 32D-ErbB-2/ErbB-3 cells with activating antibodies which engaged either the ErbB-2 (Figure 1d) or ErbB-3 (Figure 1e) receptor subunits, while antibody to ErbB-1 was inactive (Figure 1c).

ErbB-1 appeared to be much less potent as a co-receptor for ErbB-3 as compared to ErbB-2 in the induction of this morphogenetic response (Figure 2). 32D cells co-transfected with ErbB-1 and ErbB-3 (32D-ErbB-1/ErbB-3) exhibited slightly increased ruffling after treatment by either EGF or neuregulin, but did

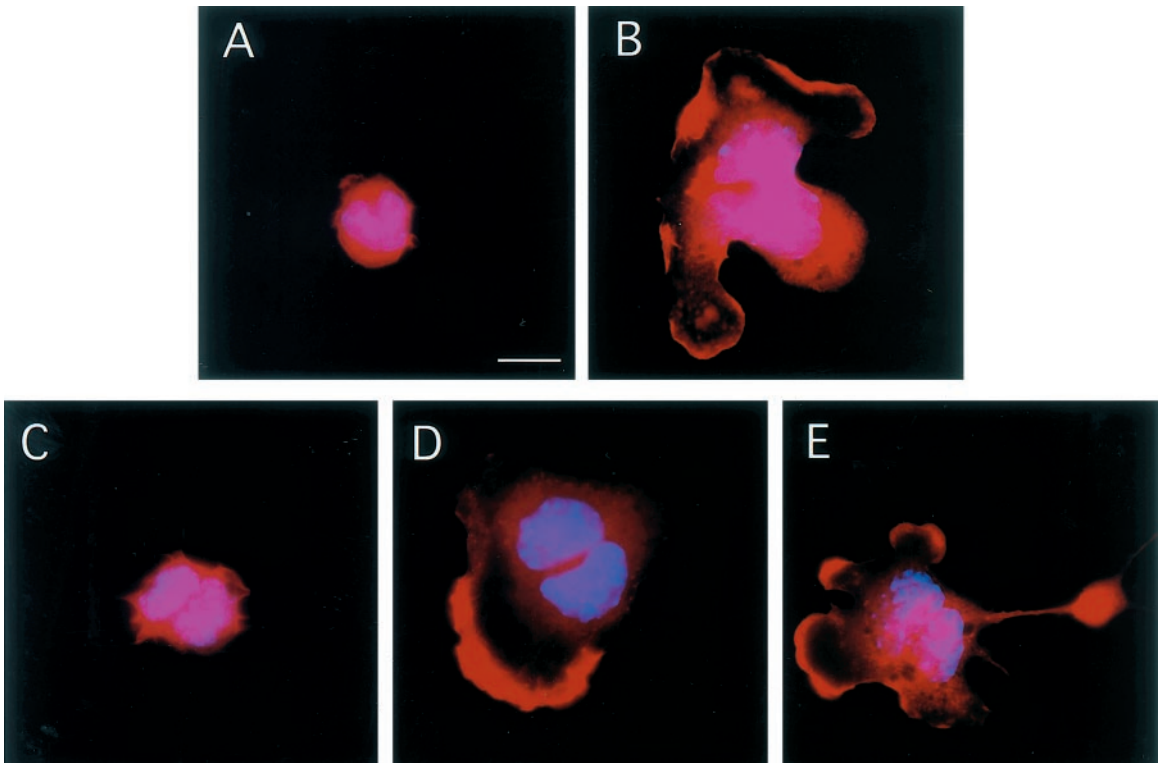

Figure 1 ErbB-2/ErbB-3 mediated signaling induces increased lamellipodial activity and spreading in 32D cells. 32D cells stably transfected with both ErbB-2 and ErbB-3 were incubated for 60 min in serum-free medium without additions (**a**), with 20 ng/ml of neuregulin (**b**), or with 10 μg/ml of anti ErbB-1 (**c**), anti-ErbB-2 (**d**) or anti-ErbB-3 (**e**) antibodies. Double staining with phalloidin (red) and DAPI (blue). Scale bar, 10 μm. Note significant spreading and formation of actin-rich ruffles by the cells treated either with neuregulin, or with antibodies to ErbB-2 and ErbB-3

not show a significant increase in the projected cell area (Figure 2d,e).

Formation of multicellular rings by neuregulin-responsive cells

To study the role of ErbB-2/ErbB-3 receptors in the induction of more complex morphogenesis, we stably transfected ErbB-3 into CHO cells that express chicken N-cadherin. This cell line (clone FL4) forms epithelial islands with cell–cell adherens junctions and, like wild-type CHO, expresses endogenous ErbB-2, but no other members of the ErbB family (not shown). Stable expression of ErbB-3 in FL4 cells enabled neuregulin binding by these cells (Figure 3).

Stimulation by neuregulin significantly enhanced cell motility as revealed by the phagokinetic track assay (Figure 3d–f). Furthermore, addition of neuregulin to the cell colonies led to a dramatic reorganization of their architecture and formation of ring-shaped multicellular arrays (Figure 4). Ring formation was rapid, and within 1.5–2 h of incubation with neuregulin, approximately 90% of colonies consisting of more than four cells, formed rings. The effect required the continuous presence of neuregulin in the medium during the period of ring formation, and the newly formed rings were stable for at least 4 h in the presence of the ligand.

Examination of the ring formation process, using time-lapse video microscopy (Figure 4), indicated that neuregulin induced a major increase in lamellipodial activity at the periphery of the cell colonies. Ruffling activity became visible within 5 min of neuregulin treatment and was maximal following 20–30 min of incubation. The increase in ruffling was accompanied by rapid spreading of the entire colony, manifested by a twofold increase in its projected area within 60 min of ligand addition.

Usually, within 30–60 min after addition of neuregulin, a gap was formed inside the spreading colony. This gap rapidly increased in size, apparently due to contractions of centrally located cells and their outward movement (Figure 4). Ring formation was often accompanied by re-alignment of the peripheral cells: they first moved radially, and then re-oriented themselves tangentially, developing a long axis roughly parallel to the outer edge of the ring (Figure 4, 60–70 min).

The morphogenetic response of FL4-ErbB-3 cells to neuregulin was dependent on the presence of cadherin-mediated cell–cell adhesions. Disruption of these contacts using a specific antibody against the extracellular domain of N-cadherin induced complete scattering of colonies both in the absence and in the presence of neuregulin (not shown).

Neuregulin-induced cytoskeletal reorganization

Fluorescence microscopy revealed that the increase in lamellipodial activity induced by activation of ErbB-3/ErbB-2 in either 32D (Figure 1) or FL4 cells (Figure 5b) was accompanied by an increase in the concentra-

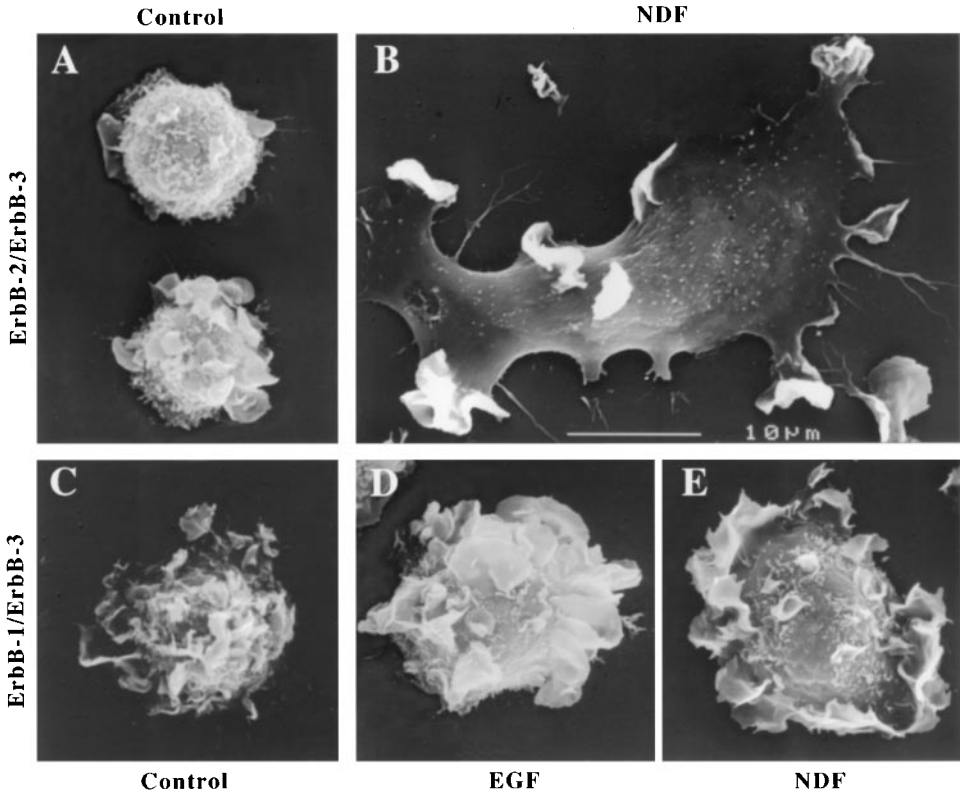

Figure 2 ErbB-2 is a more potent co-receptor for ErbB-3 than ErbB-1 in induction of lamellipodial activity and spreading. 32D cells transfected with either a combination of ErbB-2 and ErbB-3 (**a**, **b**) or ErbB-1 and ErbB-3 (**c**, **d**, **e**) were stimulated with 20 ng/ml of neuregulin (NDF) (**b**, **e**), with 20 ng/ml of EGF (**d**), or left untreated (**a**, **c**) and then processed for scanning electron microscopy. While activation of 32D cells expressing ErbB-2/ErbB-3 by neuregulin induced significant spreading (**b**), activation of the 32D cells expressing ErbB-1/ErbB-3 by either neuregulin or EGF induced only minor spreading and some ruffling activity at the periphery (**d**, **e**). All photographs were taken with the same magnification, indicated by the scale bar (**b**)

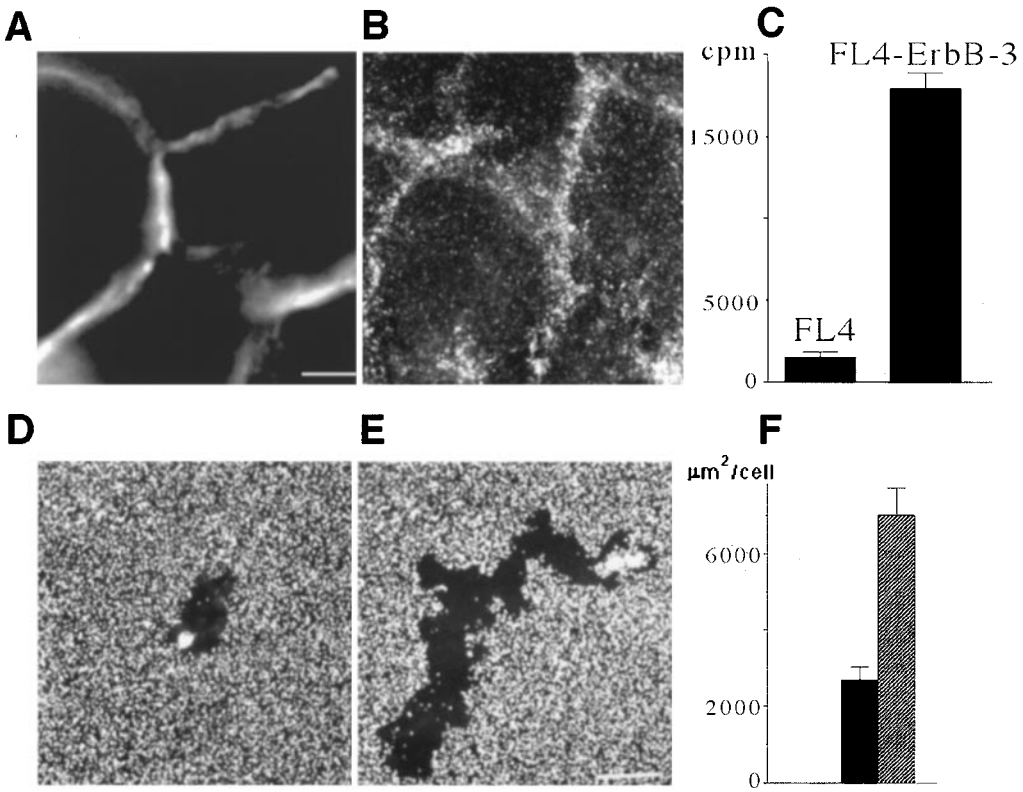

Figure 3 FL4-ErbB-3 cells express both N-cadherin and ErbB-3 and exhibit increased motility upon neuregulin (NDF) stimulation. Immunofluorescent localization of N-cadherin (**a**) and ErbB-3 receptors (**b**) in FL4-ErbB-3 cells. Scale bar, 5 μm. (**c**) Binding of ^{125}I-NDF to FL4 and FL4-ErbB-3 cells, showing that only FL4-ErbB-3 cells specifically bind neuregulin. (**d** and **e**) Phagokinetic tracks of FL4-ErbB-3 cells without (**d**) and with (**e**) neuregulin stimulation, showing the effect of neuregulin on cell motility. Scale bar, 50 μm. (**f**) Calculated areas (mean$\pm$s.e.m.) of phagokinetic tracks after overnight incubation with or without 20 ng/ml of β4 NDF

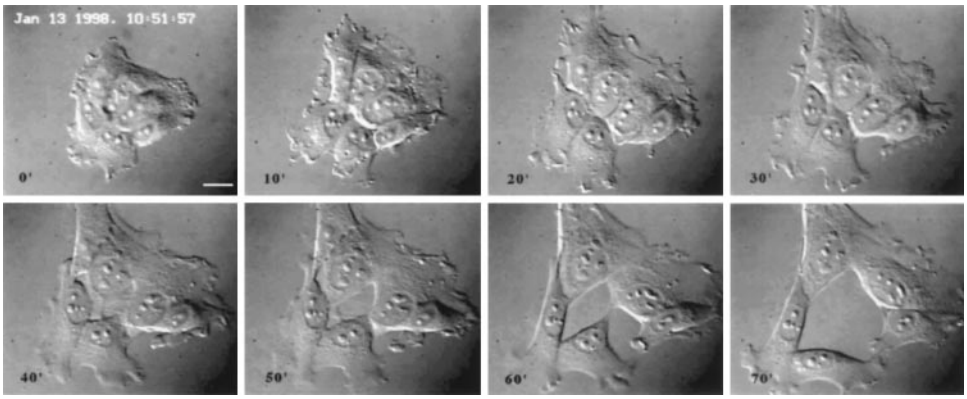

Figure 4 Differential interference contrast (DIC) images showing the sequence of neuregulin-induced morphogenetic reactions of FL4-ErbB-3 cells leading to the formation of multicellular rings. Note the increase in lamellipodial activity 10 min after neuregulin addition, the formation of a central gap 30 min thereafter, and cell polarization at later stages of the process. Scale bar, 15 μm

tion of F-actin in the ruffles and lamellipodia. In non-stimulated colonies of FL4 cells, actin staining was mainly observed along cell–cell contacts and co-localized with the N-cadherin-rich adhesion belt (Figure 5a). Following neuregulin stimulation, adherens junctions were largely preserved, yet they gradually transformed from a continuous belt into an array of streak-like structures, oriented perpendicularly to cell–cell borders (Figure 5c).

Ring formation was also accompanied by significant changes in cell-substrate contacts. Non-stimulated cells displayed small and sparse phosphotyrosine-containing focal contacts, at the periphery of the colonies (Figure 5g). Following 20 min of neuregulin treatment, there was a major increase in the number and intensity of these adhesions (Figure 5h), which progressively developed into mature, elongated focal contacts associated with actin bundles (Figure 5c,i). Similar changes were observed using anti-vinculin staining (not shown).

Formation of lamellipodia and colony spreading were accompanied by a radial extension of microtubules towards the periphery of the colony; microtubule arrays in mature rings were always oriented outwards (Figure 5d–f). Centrosomes were also reoriented toward the periphery (Figure 5e,f). Microtubule reorganization appears to be required for ring formation, since pretreatment of the cells with agents that depolymerize microtubules (nocodazole) or sup-

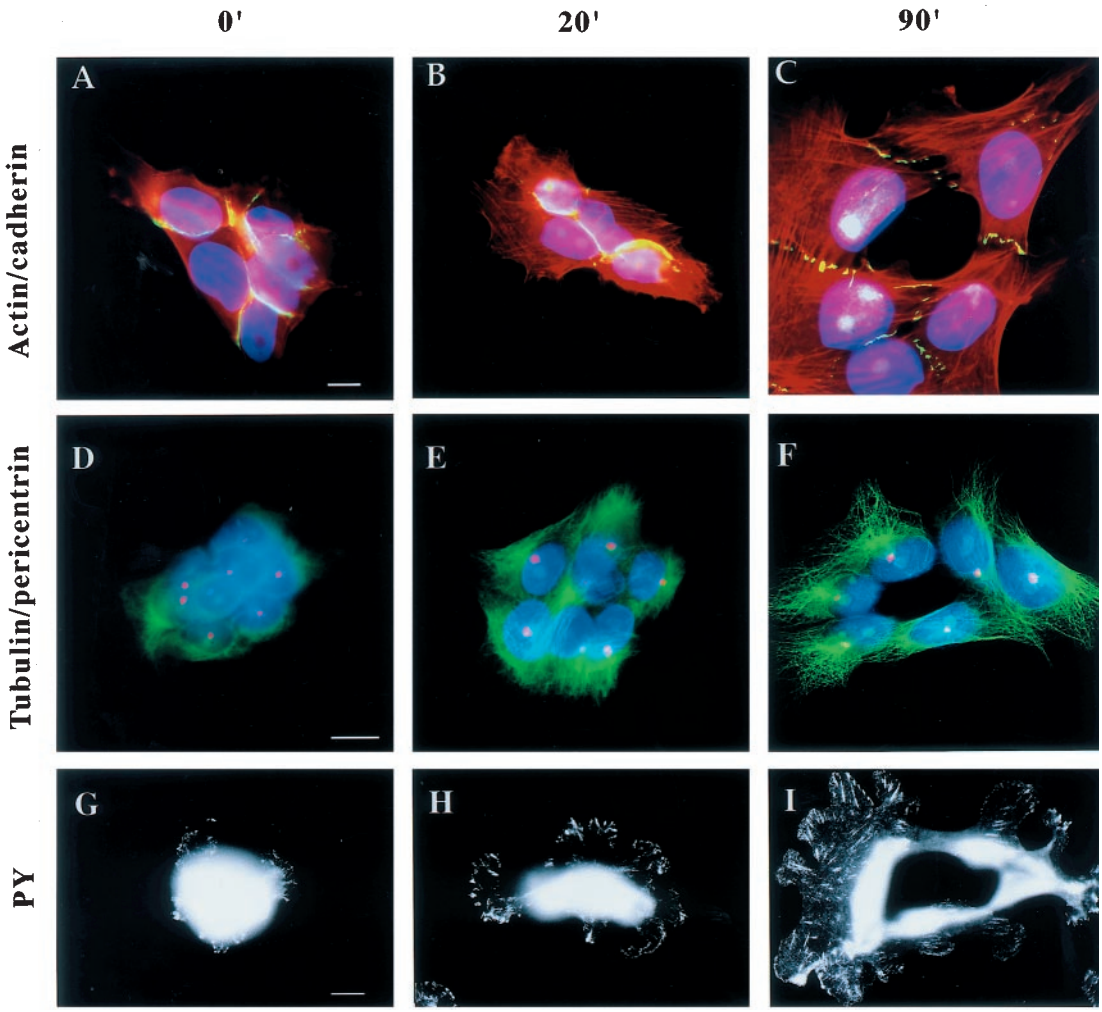

Figure 5 Neuregulin-induced cytoskeletal reorganization. (**a**, **b**, **c**). Triple fluorescence staining of neuregulin-stimulated FL4-ErbB-3 cells with anti-cadherin (green), TRITC-phalloidin (red) and DAPI (blue). Cell–cell junctions containing both N-cadherin and actin are seen as yellow lines due to superposition of red actin and green cadherin staining. Note numerous actin-containing ruffles (**b**) and stress fibers (**c**) in the neuregulin-treated cells. Following stimulation, cadherin-containing junctions are converted into the streak-like structures at the border between adjacent cells. Scale bar 8 μm. (**d**, **e**, **f**). Visualization of microtubules using anti-tubulin antibodies (green), centrosomes, using anti-pericentrin antibodies (red), and nuclei (DAPI, blue). Note the neuregulin-induced outward extension of the microtubules, and a shift in the position of the centrosomes towards the periphery of the ring (**e**, **f**). Bar, 15 μm. (**g**, **h**, **i**) Visualization of the focal adhesions with anti-phosphotyrosine antibodies. Note numerous dot-like focal contacts formed within 20 min after addition of neuregulin (**h**), and mature focal adhesions after 60 min of treatment (**i**). Scale bar, 15 μm

press their dynamics (taxol) hindered this process (Table 1). Neuregulin-induced lamellipodia formation and colony spreading were inhibited in both the taxol- and nocodazole-treated cells. However, nocodazole apparently did not prevent contraction of centrally located cells and formation of gaps in the neuregulin-treated colonies; as a result, defective rings with small diameters sometimes appeared even in the presence of this drug (Table 1).

Thus, formation of actin-rich lamellipodia enhanced by the dynamic microtubular system apparently plays a critical role in neuregulin-induced morphogenesis.

Involvement of the PI3-kinase and MAP kinase pathways in neuregulin-induced morphogenesis

It was recently shown that actin polymerization and formation of lamellipodia depend on PI3-kinase and can be blocked by inhibitors of this signaling enzyme (Kotani *et al*., 1994; Keely *et al*., 1997). Indeed, LY294002, a specific inhibitor of PI3-kinase (Vlahos *et al*., 1994) blocked neuregulin-induced spreading in 32D-ErbB-2/ErbB-3 cells (Figure 6c,d), as well as in FL4-ErbB-3 cells stimulated with neuregulin (not shown).

On the other hand, PD98059, an inhibitor of mitogen-activated protein kinase (MAP kinase) pathway (Dudley *et al*., 1995) which selectively inhibits the MAP kinase activating enzyme, MAP kinase kinase (MEK), and prevents MAP kinase activation by neuregulin (Figure 6g), reduced the lamellipodial activity and cell spreading only slightly, if at all (Figure 6e,f). Nevertheless both LY294002 and PD98059 were highly efficient in preventing the formation of neuregulin-induced rings in FL4-ErbB-3 cells (Table 1).

The specific role of ErbB-3 in neuregulin-induced morphogenesis

ErbB-3 binds neuregulin, but has an impaired kinase activity, while ErbB-2 is an efficient tyrosine kinase, but cannot directly bind neuregulin, or any other known ligand (Burden and Yarden, 1997). To elucidate the

specific roles of ErbB-3 and ErbB-2 in multicellular ring formation, we transfected FL4 cells with several chimeric and truncated receptors (Figure 7). To determine whether ErbB-3 is essential only for ligand binding and transactivation of ErbB-2, or has some additional independent role(s) in the transduction of morphogenetic signals, we have stably expressed in FL4 cells a chimeric receptor (NEC) comprised of the cytoplasmic and transmembrane domains of ErbB-2 and the extracellular domain of the EGF receptor (Peles *et al*., 1992). The FL4-NEC cells bound EGF (Figure 8a), and responded to it by auto-phosphorylation of the NEC receptor, and MAP kinase activation (Figure 8b). However, no morphogenetic response similar to that induced by neuregulin in FL4-ErbB-3 cells was noted (compare Figure 9b and d). Moreover, while neuregulin stimulation of FL4-ErbB-3 led to a major increase in cell motility (Figure 3d–f), FL4-NEC cells did not undergo a significant change in their lamellipodial activity (Figure 9d) or motility (not shown) following EGF stimulation. These data are in agreement with the absence of strong spreading and lamellipodial activity in stimulated 32D-Erb-2 cells, as described above.

The ErbB-3 receptor is distinct from other ErbB family members in that its C-terminal segment downstream from the impaired tyrosine kinase domain contains unique docking sites that interact with the SH2 domains of various signal transducing proteins. In particular, ErbB-3 is the only ErbB family member that contains six consensus binding sites for the regulatory subunit of PI3-kinase (Hellyer *et al*., 1998). To test whether these docking sites are involved in the morphogenetic response to neuregulin, FL4 cells were stably transfected with a chimeric receptor, 1T3, which consists of the ErbB-1 (EGF receptor), containing a C-terminus derived from ErbB-3 (Figure 7). As shown in Figure 9h, EGF stimulation of these cells resulted in ring formation. We also expressed in FL4 cells a truncated variant of the ErbB-3 receptor, ΔCT-ErbB-3, lacking the C-terminal segment which follows the kinase domain (Figure 7). We found that such cells bound neuregulin (Figure 8c) and underwent MAP kinase activation, similar to cells expressing full-length ErbB-3 (Figure 8d). However, the signaling via ΔCT-ErbB-3 did not induce a morphogenetic response (Figure 9f), suggesting that the C-terminal domain of

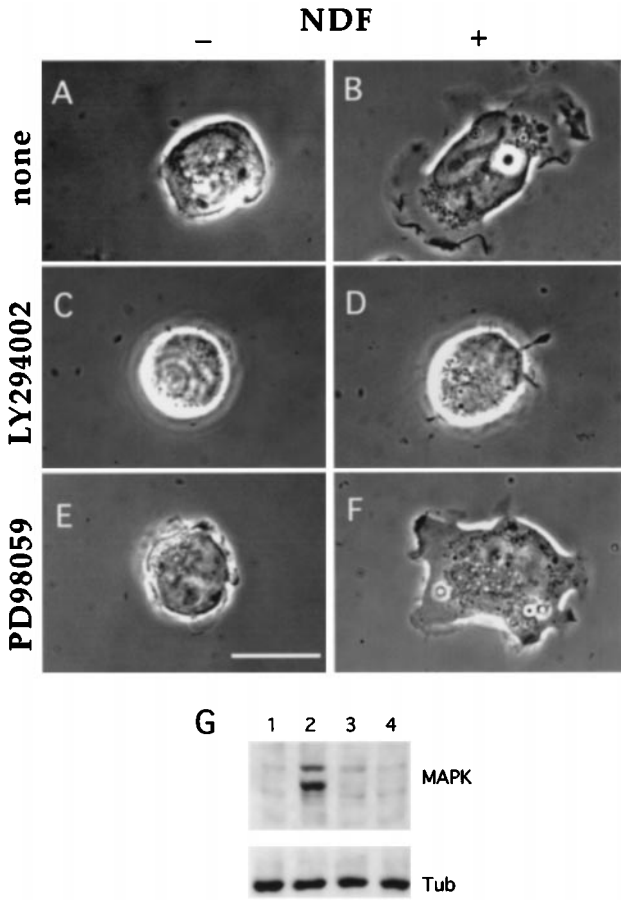

Figure 6 Effect of inhibitors of PI3-kinase and MAPK kinase on neuregulin-induced activation of lamellipodial activity and spreading. 32D-ErbB-2/ErbB-3 cells were left untreated (**a**, **b**) or pretreated with the PI3-kinase inhibitor LY294002 (**c**, **d**), or the MAPK kinase inhibitor PD98059 (**e**, **f**). After 30 min of preincubation, 20 ng/ml of neuregulin (NDF) was added (**b**, **d**, **f**). Cells treated with LY294002 did not demonstrate any increase in lamellipodial activity (**d**). Increased lamellipodial activity upon neuregulin treatment was observed in control cells (**b**) and in PD98059 treated cells (**f**). Phase contrast microscopy. Scale bar, 12 μm. (**g**) Western blot of total 32D-ErbB2/ErbB-3 cell lysates stained with anti-activated MAP kinase antibody. Lane 1, control cells, lane 2, cells treated with neuregulin for 10 min, lane 3, cells pretreated with 100 μm PD98059 for 30 min and incubated with neuregulin in the presence of PD98059 for additional 10 min, lane 4, cells incubated with PD978059 for 40 min. Neuregulin stimulation induced activation of MAP kinase (MAPK), while inhibitor treatment completely prevented this effect. Lower panel–control of protein loading by staining of α-tubulin (Tub) in the same blot

Table 1 Effect of inhibitors on the neuregulin-induced transition of FL4-ErbB-3 cell colonies into ring-shaped multicellular arrays

Inhibitor	*β4-NDF (20 ng/ml)*	*Per cent of colonies with lumen*[a]
–	–	1.2
	+	60.4
Taxol	–	0
(20 μM)	+	0
Nocodazole	–	1.6
(10 μM)	+	30.5[b]
PD98059	–	0.6
(100 μM)	+	4.1
LY294002	–	0.7
(25 μM)	+	0.7

[a]For each treatment, 150–200 colonies were scored. Cells were preincubated with inhibitors for 30 min and then neuregulin was added for an additional 90 min. [b]The colony spreading was suppressed and lumen diameters did not exceed several microns in nocodazole-treated culture

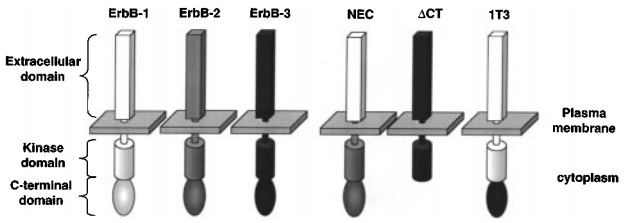

Figure 7 A scheme depicting domain organization of various ErbB family receptors and their chimeric and mutant derivatives used in the present study. ErbB-1 (EGF receptor) and its domains are shown in white, ErbB-2 in gray, and ErbB-3 in black. Extracellular domains of all the receptors are denoted by rectangular boxes, kinase domains by cylinders, and C-terminal domains by ellipsoids

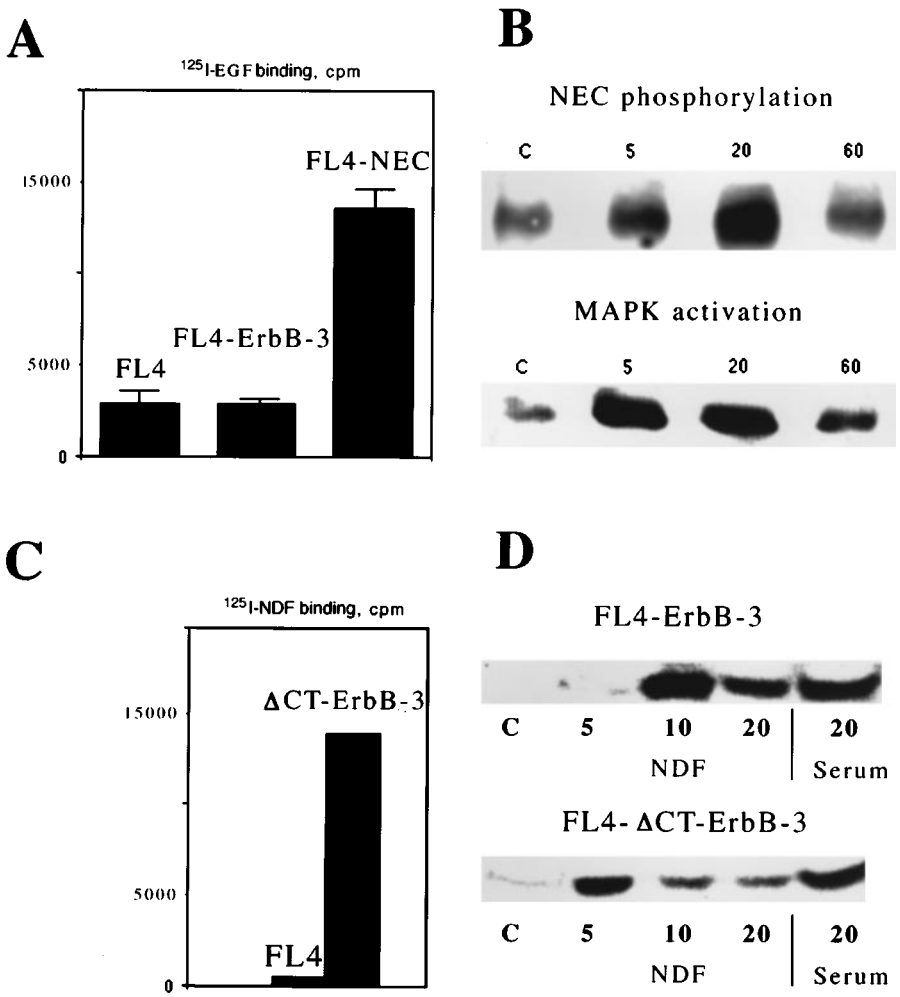

Figure 8 Effect of activation of different ErbB receptor variants on MAP kinase activation. (**a**) Binding of ^{125}I-EGF to parental FL4 cells, FL4-ErbB-3, and FL4-NEC. Only cells expressing NEC (ErbB-1/ErbB-2 chimeric receptor) specifically bind EGF. (**b**) EGF-induced tyrosine phosphorylation of the NEC chimeric receptor (upper panel) and MAPK activation (lower panel) in FL4-NEC cells. Lane c (control), non-stimulated cells; numbers indicate time after stimulation in minutes. (**c**) Binding of ^{125}I-NDF to parental FL4 cells and FL4 cells stably transfected with a truncated variant of ErbB-3 (ΔCT-ErbB-3). (**d**) Neuregulin and serum activation of MAP kinase in FL4 cells transfected with the full-length ErbB-3 receptor (upper panel) or the truncated ΔCT-ErbB-3 variant (lower panel)

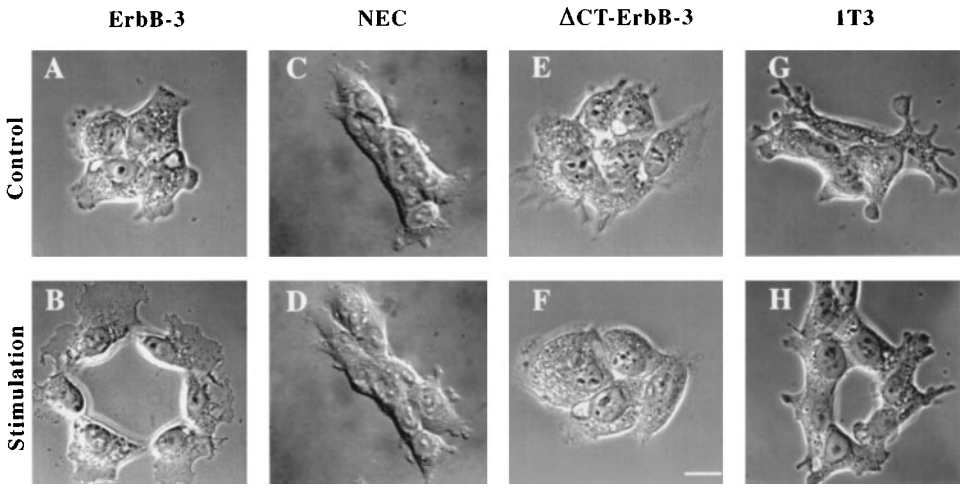

Figure 9 Effect of receptor stimulation on distribution and morphogenetic behavior of transfected FL4 cells. (**a**, **b**) FL4 cells transfected with full-length ErbB-3. (**c**, **d**) FL4 cells transfected with the NEC chimeric receptor. (**e**, **f**) FL4 cells transfected with the ΔCT-ErbB-3 receptor. (**g**,**h**) FL4 cells transfected with the 1T3 chimeric receptor. The cells were either examined in a non-stimulated state (**a**, **c**, **e**, **g**), or stimulated by the corresponding ligands, NDF (**b**, **f**) or EGF (**d**, **h**). FL4 cells transfected with either full-length ErbB-3 or with the 1T3 chimeric receptor but not those transfected with NEC or ΔCT-ErbB-3 formed ring-shaped structures upon receptor stimulation (**b**, **d**, **f** and **h**). Scale bar, 12 μm

the ErbB-3 receptor is essential for neuregulin-induced morphogenetic signaling.

Discussion

In this study we explored the molecular mechanisms involved in neuregulin-induced morphogenesis. We demonstrated that co-expression of the high affinity neuregulin receptor ErbB-3 with the 'orphan' co-receptor ErbB-2, induced – upon neuregulin stimulation – a dramatic increase in adhesion and cell spreading in weakly adherent 32D cells. Neither individual ErbB family receptors, nor co-expression of ErbB-3 with ErbB-1 induced this dramatic response. Moreover, the expression of ErbB-2/ErbB-3 together with the adhesion receptor, N-cadherin, in CHO cells appeared to be both essential and sufficient to reconstitute a system demonstrating ring formation, a rather complex morphogenetic behaviour, in response to neuregulin signaling.

The morphogenetic behavior induced by neuregulin in cells expressing ErbB-3/ErbB-2 and N-cadherin, manifested by the formation of organized multicellular structures with an internal lumen, appears to be a process that depends on a temporally and spatially coordinated interplay between cell adhesion and motility. This process can be subdivided into three sequential stages: (1) the rapid activation of polar lamellipodial extensions and cell spreading; (2) outward cell motility without loss of cell–cell adhesion; and (3) cell contraction and formation of an internal lumen. N-cadherin mediated cell–cell adhesion is an obvious prerequisite for such morphogenesis, since it is essential for the formation of the epithelioid islands. Consequently, anti-N-cadherin antibodies that blocked the homophilic adhesive interactions induced complete cell scattering rather than ring formation. The driving force for ring formation was an apparent activation of cell motility demonstrated on isolated cells by the phagokinetic track assay.

Thus, formation of ring-shaped colonies following neuregulin stimulation depends on a balance between motility and adhesion. This balance must be accurately regulated to allow sufficient locomotory activity to drive cells outwards, yet retain sufficient cell–cell adhesion to prevent complete scattering. Maintenance of such a balance in the system described here, or in epithelial cells at large (Chausovsky *et al*., 1998), appears to be essential characteristics of the morphogenetic response to neuregulin. In contrast, classical scatter factor HGF/SF causes not only strong locomotory stimulus, but also a significant reduction of cell–cell adhesion (Potempa and Ridley, 1998) resulting in complete scattering of cell colonies in two dimensional culture.

Reorganization of both actin and the microtubule system appear to be important for neuregulin-induced multicellular ring formation. Lamellipodial extension and cell spreading are accompanied, as expected, by F-actin enrichment in the lamellipodia and ruffles. At the same time, a massive and polarized peripheral outgrowth of the microtubules and shift of centrosome positions occurs. At the stage of lumen formation, cell contractility is activated as can be seen from the rapid augmentation of stress fibers and focal adhesions (Bershadsky *et al*., 1996; Chrzanowska-Wodnicka and Burridge, 1996).

Experiments with specific inhibitors demonstrate that each of these cytoskeletal events is required for the complete morphogenetic response to neuregulin. In particular, both hyperstabilization of microtubules by taxol or their disruption by nocodazole inhibits neuregulin-induced ring formation by interfering with the first stage of the morphogenetic response, namely the formation of polarized lamellipodia. This is consistent with a number of previous studies demonstrating that dynamic and properly oriented microtubules are essential for polarized formation of lamellipodial extensions (Bershadsky and Vasiliev, 1993; Elbaum *et al*., 1998).

We have also demonstrated here that the lamellipodial activity induced by neuregulin critically depends on the activity of PI3-kinase, as was shown previously for several other inducers (Kotani *et al*., 1994; Adam *et al*., 1998; Khwaja *et al*., 1998). The PI3-kinase inhibitor, LY294002, blocked not only lamellipodia formation and spreading, but also ring formation, confirming the essential role of lamellipodia activity in this morphogenetic process. The mechanism of PI3-kinase involvement in the induction of lamellipodial activity is still unclear, yet it appears to cooperate with the small GTPases, Rac and Cdc42, and with p21-activated kinase (PAK) in the formation of actin-rich extensions via stimulation of actin polymerization (Reif *et al*., 1996; Carpenter *et al*., 1997; Keely *et al*., 1997; Adam *et al*., 1998). There is also evidence for the association of PI3-kinase with microtubules (Kapeller *et al*., 1995), and for the involvement of the microtubule system in the outgrowth of cell processes induced by PI3-kinase (Kobayashi *et al*., 1997; Kita *et al*., 1998). Irrespective of the exact mechanism, the involvement of PI3-kinase suggests an essential role for the ErbB-3 receptor, since this receptor is the only member of the ErbB family that contains consensus binding sites for PI3-kinase recruitment.

The mechanism of ErbB-3 involvement in the transduction of morphogenetic signal deserves special discussion. The ErbB-3 receptor is unique in that it is devoid of intrinsic protein kinase activity (Guy *et al*., 1994; Sierke *et al*., 1997). In fact, our experiments with 32D cells expressing ErbB-3 alone showed that this receptor could not itself trigger the morphogenetic response. However, ErbB-3 can be phosphorylated upon ligand-induced heterodimerization of ErbB-3 with either ErbB-1 or ErbB-2 (Soltoff *et al*., 1994; Carraway *et al*., 1995; Kim *et al*., 1998). We demonstrated that the combination of ErbB-3 with ErbB-2 was much more potent in the induction of cell spreading than ErbB-3/ErbB-1. ErbB-3 can efficiently transactivate the kinase activity of the 'orphan' receptor ErbB-2; a specific motif, LVI, localized at the impaired kinase domain of ErbB-3 is required for this transactivation (Schaefer *et al*., 1999). Truncated ErbB-3 receptors, preserving this LVI motif can transactivate ErbB-2, which in turn leads to phosphorylation of ErbB-3 receptor itself (Schaefer *et al*., 1999). In our experiments we observed that the ΔCT-ErbB-3 receptor, lacking the C-terminal region downstream from the kinase domain but preserving LVI motif, can mediate some signaling effects, in particular, MAP kinase activation. This receptor however was not able

to induce morphogenetic effects, such as cell spreading and ring formation. Thus, transactivation of ErbB-2 and even MAP kinase stimulation is not sufficient for morphogenetic effects. We confirmed this by showing that the direct activation of ErbB-2, using a chimeric receptor that consists of the extracellular region of the EGF receptor and the transmembrane and intracellular domains of ErbB-2 does not product any morphological effects. On the other hand, activation of a chimeric ErbB-1 receptor, bearing an ErbB-3-derived C-terminus (1T3) was shown to induce ring formation in FL4 cells. Thus, one role of transactivation of ErbB-2 is to phosphorylate the C-terminal domain of ErbB-3 thereby creating new docking sites necessary for morphogenetic signaling. Other functions of ErbB-2 cannot be excluded, however, since 1T3 transfection of 32D cells (lacking endogenous ErbB-2) was insufficient to induce morphological changes in response to EGF (our unpublished results).

Among the docking sites uniquely present on the C-terminal tail of ErbB-3 are the docking sites for the p85 subunit of PI3 kinase. The amino acid sequence motif (phospho)Tyr-X_1-X_2-Met (pYXXM), has been shown to specifically associate with the SH2 domains of p85, the PI3-kinase regulatory subunit (McGlade *et al*., 1992; Prigent and Gullick, 1994). The C-terminus of ErbB-3 contains six YXXM sites and several of these are spaced suitably to permit tandem binding of two SH2 domains of p85, while others may also cooperate in p85 binding (Hellyer *et al*., 1998). These structural features make ErbB-3 particularly potent in the signal-induced activation of PI3-kinase (Prigent and Gullick, 1994).

The essential role of PI3-kinase explains why activation of MAP kinase is not sufficient for the complete morphogenetic response. Moreover, an inhibitor of the MAP kinase pathway, PD98059, did not interfere with lamellipodia formation and cell spreading. This inhibitor, however, still prevented the assembly of the ring-shaped structures. The role of MAP kinase signaling therefore appears to be important in the events following the induction of spreading. Several recent studies demonstrated the involvement of the MAP kinase pathway in various aspects of cell motility (Klemke *et al*., 1997; Khwaja *et al*., 1998). Moreover, it was shown recently that neuregulin-induced alveolar morphogenesis in Matrigel-based 3-D culture system was inhibited by PD98059 (Niemann *et al*., 1998). The possible function of MAP kinase signaling in the organization of multicellular rings requires further investigation.

Based on our studies, a likely scenario for the neuregulin-induced morphogenetic response includes the following events: (a) neuregulin stimulation of ErbB-3/ErbB-2 dimer formation and transactivation of ErbB-2; (b) ErbB-2-mediated phosphorylation of specific tyrosine residues at the C-terminus of the ErbB-3, including consensus sites for PI3-kinase binding; (c) PI3-kinase recruitment (and probably recruitment of other downstream elements) and triggering of actin- and microtubule-dependent activation of lamellipodial extension and cell spreading, without concomitant disruption of the adherents junctions; (d) MAP-kinase-dependent outward cell movement and contraction, to convert cell colonies into multicellular rings containing a lumen-like gap.

Thus, ErbB-3/ErbB-2 receptor dimers appear to mediate the neuregulin-induced morphogenetic signal by coordinate activation of both the PI3-kinase and MAP kinase pathways. Further studies are necessary to elucidate the interplay between the signaling events and the subsequent changes in the cytoskeletal machinery, which are responsible for the performing of this simplified but instructive morphogenetic process.

Materials and methods

Cell culture, transfections, and ligand treatment

Sublines of the 32D murine hematopoietic progenitor cell line (Greenberger *et al*., 1983) that express various ErbB-proteins were established through a two-step transfection protocol with ErbB-expression vectors, as described (Pinkas-Kramarski *et al*., 1996). Both the original 32D cells and their stable transfectants were routinely cultivated in RPMI medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum and 0.1% medium that was conditioned by IL-3-producing cells. 32D cells do not attach and spread on tissue culture dishes or on glass coverslips. Preliminary experiments showed that binuclear cells are much more sensitive to stimulants inducing attachment and spreading as compared to mononuclear cells, therefore binuclear cells were obtained by pretreatment with cytochalasin D, which interferes with cytokinesis, but does not affect nuclear division. The cells were preincubated in growth medium containing 0.3 μg/ml cytochalasin D for 16 h before each experiment. After this preincubation, about 90% of the cells were binuclear. The cells were then washed twice and incubated in serum-free medium without cytochalasin D for 1 h. The various ligands (see below) were added to the cultures for 1 h and the cells fixed.

To produce cells expressing both N-cadherin and either ErbB-3, or chimeric or truncated receptors, the FL4 subline was used (Levenberg *et al*., 1998). These cells were produced by transfection of wild-type CHO cells with a vector encoding chicken N-cadherin. FL4 cells were stably co-transfected with the PCDNA-3 expression vectors containing ErbB-3, ΔCT-ErbB-3, 1T3 or NEC c-DNA together with a puromycin-resistance gene. NEC is a chimeric receptor consisting of the extracellular part of the EGF receptor (ErbB-1) and the transmembrane and cytoplasmic portions of the Neu (ErbB-2) receptor (Peles *et al*., 1992). ΔCT-ErbB-3 receptor is a deletion mutant of ErbB-3, lacking the C-terminal cytoplasmic region, immediately downstream of the tyrosine kinase domain. The 1T3 chimeric receptor consists of the ErbB-1 receptor with its C-terminal region exchanged for that of ErbB-3 receptor. The various chimeric constructs used are schematically depicted in Figure 7. Cells were cultured as previously described (Sliwkowski *et al*., 1994).

For microscopic observations of FL4 cell derivatives, 5×10^4 cells were plated on 18 mm coverslips, maintained for 24 h in DMEM with 10% bovine calf serum, washed once with serum-free DMEM and incubated for an additional 24 h. At that time, compact cell colonies were formed. Neuregulin (20 ng/ml) or EGF (20 ng/ml) was added in serum-free DMEM for an additional 1–3 h. Experiments with 1T3 cells were performed in DMEM containing 1% bovine calf serum. Recombinant rat neuregulin (NDF β4) expressed in CHO cells (Amgen Co, USA) was used in all experiments. EGF was purchased from Sigma Israel (Cat. No E-1257). Antibodies against ErbB-1, ErbB-2, and ErbB-3 were described previously (Pinkas-Kramarski *et al*., 1996). The PI3-kinase inhibitor LY294002 and the MAP kinase kinase (MEK) inhibitor PD98059 (Calbiochem, La Jolla, CA, USA) were used at a final concentration of 25 and 100 μM respectively. Taxol (paclitaxel) and nocodazole (methyl-[5-(2-

thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate) obtained from Sigma Israel were used at a final concentration 20 μM (taxol) and 10 μM (nocodazole).

Biochemical and immunochemical procedures

Immunoprecipitation and Western blotting were performed as previously described (Bershadsky *et al*., 1996). Anti-ErbB-1 (Tzahar *et al*., 1996), anti-PY (PT-66, Sigma, Israel), or anti-activated MAP kinase (Gabay *et al*., 1997; kindly provided by Dr Rony Seger, Weizmann Institute of Science), were used as primary antibodies. The secondary antibody used was horseradish peroxidase-labeled anti-mouse IgG (Amersham, UK). Radiolabeling of ligands, covalent cross-linking and ligand binding assays were done according to Tzahar *et al*. (1996). In all experiments the protein loading was controlled by the staining of the same blots with the anti-α-tubulin antibody (clone DM-1A, Sigma, Israel).

Microscopy and immunofluorescence

For light microscopic examination of living cells, the cells were plated and cultured overnight in serum-containing DMEM and for 24 h in serum-free DMEM. The medium was changed to serum-free L15 medium for 1 h. The dish was then transferred to an inverted microscope (Zeiss Axiovert 35) equipped with DIC optics and red light illumination. The objective (Zeiss Fluar 100×1.3) was maintained at 37°C by a regulated electric heater (Bioptechs Ltd, USA). Images were taken with a CCD video camera (iSight, Israel) and recorded on S-VHS cassettes.

Immunofluorescence staining was performed as described (Bershadsky *et al*., 1996). The following primary antibodies were used: rabbit anti-pan cadherin (C3678, Sigma, Israel), monoclonal anti-phosphotyrosine (clone PT66, Sigma, Israel), monoclonal anti-α tubulin (clone DM1A, Sigma, Israel), and rabbit anti-pericentrin (kindly provided by Dr SJ Doxey, University of Massachusetts, USA). FITC- or TRITC-labeled goat antibodies to mouse and rabbit immunoglobulins (Jackson Laboratories, West Grove, PA, USA) were used as secondary antibodies. Actin was stained with FITC- or TRITC-labeled phalloidin (Sigma, Israel) and nuclei were stained with DAPI. The specimens were examined by fluorescence microscopy using a Zeiss Axiophot microscope (Zeiss, Germany) equipped with a 100×1.3 Planapochromat objective, a CCD camera (Photometrics, USA), and the Priism software package (Applied Precision, Issaquah, WA, USA) on a Silicon Graphics computer. The Priism software was used for superposition of triple labeling onto a single color picture. Scanning electron microscopy was performed as described previously (Levenberg *et al*., 1998).

Cell motility measurements

To assess the rate of cell motility, the phagokinetic track method was used (Albrech-Buehler, 1977). Cells were seeded on 18 mm coverslips coated with colloidal gold at a density of 2×10^3 per coverslip and incubated for 4 h. The medium was then changed to serum-free medium, with or without β4 NDF (20 ng/ml), and the cells were incubated overnight (20 h). Tracks were examined by dark field microscopy using a $10\times$ objective. The areas of 40–50 randomly selected tracks were measured for each determination.

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