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Ligand-Independent Regulation of ErbB4 Receptor Phosphorylation by Activated Ras

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Abstract The ErbB family of receptor tyrosine kinases regulates cell growth, differentiation and survival. Activation of the receptors is induced by specific growth factors in an autocrine, paracrine or juxtacrine manner. The activated ErbB receptors turn on a large variety of signaling cascades, including the prominent Ras-dependent signaling pathways. The activated Ras can induce secretion of growth factors such as EGF and neuregulin, which activate their respective receptors. In the present study, we demonstrate for the first time that activated Ras can activate ErbB4 receptor in a ligand-independent manner. Expression of constitutively active H-Ras(12V), K-Ras(12V) or N-Ras(13V) in PC12-ErbB4 cells induced ErbB4-receptor phosphorylation, indicating that each of the most abundant Ras isoforms can induce receptor activation. NRG-induced phosphorylation of ErbB4 receptor was blocked by the soluble ErbB4 receptor, which had no effect on the Ras-induced receptor phosphorylation. Moreover, conditioned medium from H-Ras(12V)-transfected PC12-ErbB4 cells had no effect on receptor phosphorylation. It thus indicates that Ras induces ErbB4 phosphorylation in a ligand-independent manner. Each of the Ras effector domain mutants, H-Ras(12V)S35, H-Ras(12V)C40, and H-Ras(12V)G37, which respectively activate Raf1, PI3K, and RalGEF, induced a small but significant receptor phosphorylation. The PI3K inhibitor LY294002 and the MEK inhibitor PD98059 caused a partial inhibition of the Ras-induced ErbB4 receptor phosphorylation. Using a mutant ErbB4 receptor, which lacks kinase activity, we demonstrated that the Ras-mediated ErbB4 phosphorylation depends on the kinase activity of the receptor and facilitates ligand-independent neurite outgrowth in PC12-ErbB4 cells. These experiments demonstrate a novel mechanism controlling ErbB receptor activation. Ras induces ErbB4 receptor phosphorylation in a non-autocrine manner and this activation depends on multiple Ras effector pathways and on ErbB4 kinase activity. J. Cell. Biochem. 98: 1482–1494, 2006.

Key words: Ras; ErbB/HER family; ErbB4; HER4; neuregulin (NRG); tyrosine kinase

The ErbB subfamily of receptor tyrosine kinases (RTK) consists of four receptors: Epidermal growth factor receptor (EGFR, HER1, ErbB1), HER2 (ErbB2, neu), HER3 (ErbB3), and HER4 (ErbB4) [Yarden and Kelman, 1991]. ErbB4 is the more recently described member in this subfamily of receptors. ErbB4 binds a group of peptide growth factor isoforms termed neuregulins (NRGs), which activate the receptors [Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Plowman et al., 1993b]. ErbB4 may also be activated by three EGF-like growth factors: Epiregulin, betacellulin, and heparin-binding EGF (HB-EGF) which, unlike NRGs, are also ligands of the EGFR [Higashiyama et al., 1991; Riese et al., 1996; Shelly et al., 1998].

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; FTS, S-trans, trans farnezylthiosalicylic acid; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; NDF, Neu differentiation factor; NRG, neuregulin; PBS, phosphate buffered saline; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; RTK, receptor tyrosine kinase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Pazit Tal-Or, Shlomit Erlich, and Natalie Porat-Shliom contributed equally to this work.

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ErbB4 is normally expressed in a number of adult tissues including the heart, kidney, brain, and skeletal muscle, thus suggesting that this receptor has a significant role in the maintenance of adult organs [Plowman et al., 1993a; Carpenter, 2003]. Furthermore, ErbB4 has a critical role in development. This has been demonstrated in targeted null mice lacking ErbB4 [Gassmann et al., 1995]. The homozygous ErbB4 knockout mice die at embryonic day 10–11 due to cardiac malformation, and also exhibit neuronal defects. In addition, ErbB4 has been implicated in tumorigenesis, since a number of breast cancer cell lines overexpress the ErbB4 receptor [Plowman et al., 1993a].

Activation of ErbB4 in vitro turns on multiple signal transduction pathways that regulate cellular proliferation, survival, chemotaxis or differentiation (reviewed in [Carpenter, 2003]). Ligand binding to the ErbB4 receptor leads to receptor homodimerization and autophosphorylation in the absence of other ErbB receptors or to heterodimerization in their presence. Heterodimerization of ErbB4 with other ErbB family members diversifies the signaling outputs that lead to the biological responses mediated by the receptors [Graus-Porta et al., 1997; Riese et al., 1998; Shelly et al., 1998; Hobbs et al., 2002]. Among the signaling molecules activated by ErbB homo- or hetero-dimers are the Ras family of small GTPases [Ben-Levy et al., 1994]. The activated receptors recruit Ras specific guanine nucleotide exchange factors (Ras-GEFs), which induce GDP for GTP exchange on Ras [Reuther and Der, 2000]. The active Ras-GTP, in turn, activates multiple effector molecules including the Raf-1, phosphoinositide 3-kinase (PI3K), and RalGEF [Reuther and Der, 2000]. The ErbB mediated activation of the Ras/Raf-1/Mek/extracellular signal-regulated kinase (Erk) pathway and of the RalGEF pathway regulates cell proliferation or differentiation, depending on the cellular context, while receptor mediated activation of the Ras/PI3K pathway provides survival signals and is also associated with cytoskeleton reorganization [Culouscou et al., 1995; Sweeney and Carraway, 2000; Puricelli et al., 2002].

PC12 cells are extensively used to study RTKs and the signaling pathways triggered by these receptors to regulate cell growth, survival and differentiation manifested by neurite outgrowth [Greene and Tischler, 1976; Greene, 1978]. The involvement of Ras in PC12 cell differentiation has been demonstrated in earlier studies; constitutively active Ras mutants were found to induce differentiation independent of receptor activation [Bar-Sagi and Feramisco, 1985]. Other studies showed that in PC12 cells, activation of the EGFR induced a transient Ras/mitogen-activated protein kinase (MAPK) signal leading to cell proliferation, whereas NGF activation of the TrkA receptor induced a sustained Ras/MAPK signal leading to cell differentiation [Marshall, 1995]. More recent studies showed that Raf and PI3K mediate NGF-induced cell cycle arrest and neurite outgrowth, whereas RalGEF activity antagonizes this effect and promotes cell proliferation [Rusanescu et al., 2001]. This regulation of Ras signaling is promoted by protein kinase C (PKC) [Rusanescu et al., 2001]. In our own experiments, we focused on the outcome of ErbB4-mediated signaling in PC12 cells. We found that in PC12 cells expressing ErbB4 receptors, NRG induced neurite outgrowth and protected the PC12-ErbB4 cells from death caused by various apoptotic stimuli [Vaskovsky et al., 2000; Erlich et al., 2001; Goldshmit et al., 2001]. The differentiation induced by NRG required the MAPK pathway [Vaskovsky et al., 2000] whereas the survival effect of NRG required the PI3K/PKB pathway [Erlich et al., 2001; Goldshmit et al., 2001]. Importantly, PC12-ErbB4 cell differentiation induced by NRG was Ras dependent and oncogenic Ras mimicked the NRG effect [Goldsmit et al., 2001]. These experiments indicated that ErbB4, through the Ras/Raf-1/MEK/Erk pathway, induces PC12-ErbB4 differentiation. However, because activated Ras is known to induce the release of growth factors including EGF-like ligands such as amphiregulin, TGFα HB-EGF, and NRG [Yarden and Peles, 1991; Peles et al., 1992; Normanno and Ciardiello, 1997; He et al., 2000], we could not rule out the possibility that some of the effects of the oncogenic Ras were mediated by an autocrine loop.

In the present study, we examined whether oncogenic Ras induces NRG release from PC12-ErbB4 cells, and if this effect can explain the Ras-induced ErbB4 phosphorylation. We found that oncogenic Ras indeed induced ErbB4 phosphorylation and activation, yet this effect was not due to secreted NRG or other ErbB4 ligands. Apparently, the oncogenic Ras activated the ErbB4 receptors by intracellular

signal(s), leading to an increase in receptor phosphorylation. Our results established a new mechanism for a Ras-dependent positive feedback signal to activate the ErbB4 receptors.

MATERIALS AND METHODS

Materials and Buffers

The monoclonal anti-Ras antibodies were from Oncogene Research Products (Darmstadt, Germany). A monoclonal antiphosphotyrosine antibody (PY-20) and a rabbit anti-ErbB4 antibody were from Santa-Cruz Biotechnology (Santa-Cruz, CA). Monoclonal anti-NRG 1 antibodies were from NeoMarkers (Fermont, CA). Human recombinant NRGβ was purchased from R&D System Inc. (Oxon, UK). PD98059 was purchased from Promega (Madison, MI). LY294002 and PP2 were purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM disodium ethylenediaminetetra-acetic acid (EDTA), 1.5 mM MgCl2, 10% glycerol, 0.2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The extracellular domain of ErbB4 (IgB4) was a generous gift from Prof. Y. Yarden of the Weizmann Institute, Rehovot, Israel and was isolated as previously described [Dong et al., 1995].

Cell lines and tissue culture. The rat pheochromocytoma cells (PC12) that express the ErbB-4 receptor (JM-b isoform) were previously described [Vaskovsky et al., 2000]. For expression of ErbB4 with dead kinase (ErbB4DK), the expression vector LXSHD-ErbB4DK containing the coding region of ErbB4 with K751A substitution (ATP binding site) was introduced by infection into PC12 cells as previously described [Vaskovsky et al., 2000]. The histidinol resistant colonies were checked for ErbB4DK expression by Western blot, and one of the colonies was selected for further analysis. The two cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS) and 10% horse serum (HS). Cells were incubated at 37°C in 5% CO2 in air, and the medium was changed every 3–4 days. Cells were passaged when 90% confluent using 0.5 mM EDTA in phosphate buffered saline (PBS).

Transient Transfections

One day before transfection, PC12-ErbB4 cells were plated at a density of 10^6 cells/well in 6-well plates. To each well, DNA-lipofectamine mixture [2 μg of DNA and 15 μl of lipofectamine in 1 ml of OPTI-MEM (Gibco-BRL)] was added according to the manufacturer's instructions. Each transfection was performed in triplicates. Following incubation of cells for 6 h with the DNA lipofectamine mixture, 1 ml of DMEM supplemented with 7.5% FBS and 7.5% HS was added and incubation was continued. Twenty-four hours later, medium was replaced and 48 h post-transfection cells were collected for lysate analysis.

Lysate Preparation, Immunoprecipitation and Immunoblotting

Cells were exposed to the indicated stimuli. After treatment, cells were solubilized in lysis buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added to cell lysates. For other experiments, lysates were first subjected to immunoprecipitation with antiphosphotyrosine antibodies (PY-20). Antibodies were coupled to antimouse IgG agarose. The proteins in the lysate supernatant were immunoprecipitated with aliquots of the antimouse IgG agarose-antibody complexes for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, resolved by SDS–polyacrylamide gel electrophoresis (PAGE) through 7.5% gels and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 1 h in TBST buffer (0.02 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 6% milk, blotted with 1 μg/ml primary antibodies for 2 h, followed by 0.5 μg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp, Buckinghamshire, UK).

In Vitro Kinase Assay

To phosphorylate the receptors in vitro, cell lysates were immunoprecipitated with rabbit anti-ErbB4 antibodies (Santa Cruz). Immunoprecipitates were incubated for 15 min on ice in phosphorylation solution containing 5 mM

$MnCl_2$ and 0.01 μCi [γ-32P] ATP in HNTG. The reaction mixtures were washed three times with HNTG, eluted with sample buffer and resolved by SDS–PAGE.

Data Analysis

Quantification of the intensity of phosphorylated ErbB4 (pErbB4) and ErbB4 as obtained in three experiments was performed using Image J computer program. The data are presented as the relative fold induction of the ratio between pErbB4/ErbB4 over the control empty vector transfected cells and is the mean fold induction ± SD.

RESULTS

Oncogenic Ras Induces an Increase in Phosphorylated ErbB4 Receptors

We used PC12 cells stably expressing the ErbB4 receptor [Vaskovsky et al., 2000] to study the relationships between ErbB4 and activated Ras. Because receptor activation is followed by tyrosine phosphorylation, we first examined the

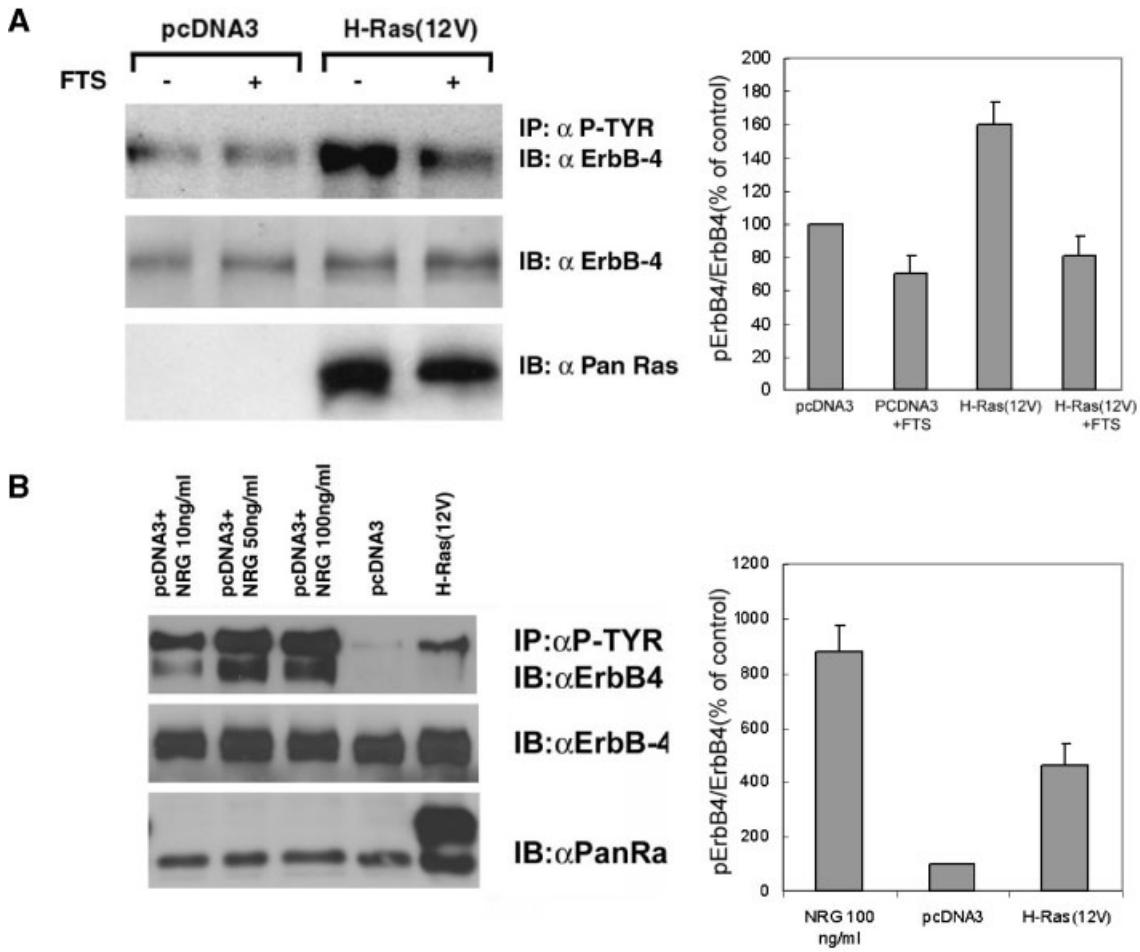

Fig. 1. H-Ras-induced ErbB4 phosphorylation in PC12-ErbB4. PC12-ErbB4 cells were transfected with either control pcDNA3 vector or H-Ras(12V) vector. Twenty-four hours following transfection, medium was replaced with DMEM containing 7.5% FCS and 7.5% HS and cells were incubated with or without FTS 50 μM. Whole cell lysates were prepared from cells at 48 h following transfection. **A**: In the **upper panel**, lysates that were immunoprecipitated with monoclonal antibody (mAb) PY-20 to phosphotyrosine. The immune complexes were resolved by gel electrophoresis and transferred to nitrocellulose filters. Immunoblotting (IB) was performed by using rabbit antiserum to ErbB4 (Santa Cruz). Total cell lysates were analyzed by IB with a mAb to Ras or a polyclonal Ab to ErbB4 (two **lower panels**). Densitometric analysis of three experiments is presented (right columns). Data are the mean (n = 3) ratio between pErbB4 and ErbB4 compared to the control pcDNA transfected cells. Bars = SD. **B**: Transfections were performed as described above. Forty-eight hours following transfection, cells were treated with or without the indicated NRG concentrations and lysates were either immunoprecipitated or immunoblotted as described in (A). Signal detection was performed by using a chemiluminescence kit. Densitometric analysis of three experiments is presented (right columns). Data are the mean (n = 3) ratio between pErbB4 and ErbB4 compared to the control pcDNA transfected cells. Bars = SD.

effect of Ras on the levels of phosphorylated ErbB4. PC12-ErbB4 cells were transiently transfected with H-Ras(12V) and the apparent amounts of phosphorylated ErbB4 were determined by immunoprecipitation with antiphosphotyrosine antibodies, followed by immunoblot with anti-ErbB4 antibodies. As shown in Figure 1, the oncogenic Ras induced a significant increase in the levels of tyrosine phosphorylated ErbB4, and the increase was attenuated by the Ras inhibitor S-trans, trans farnezylthiosalicylic acid (FTS) [Gana-Weisz et al., 1997]. The extent of Ras-induced increase in ErbB4 receptor phosphorylation was smaller than that induced by various concentrations of NRG (Fig. 1B). It, thus, seems that one of the effect of H-Ras(12V) was to induce receptor phosphorylation.

To examine whether the increase in ErbB4 phosphorylation was isoform specific, we transfected cells with three distinct constitutively active Ras isoforms and their wild-type counterparts (H-Ras, N-Ras, and K-Ras). As shown in Figure 2, all the constitutively Ras isoforms, namely, H-Ras(12V), N-Ras(V13), and K-Ras(V12), induced ErbB4 receptor phosphorylation. Their wild-type counterparts also induced a small increase in receptor phosphorylation. These results suggest that receptor phosphorylation may be due to activated GTP-bound Ras and that this effect is not isoform specific.

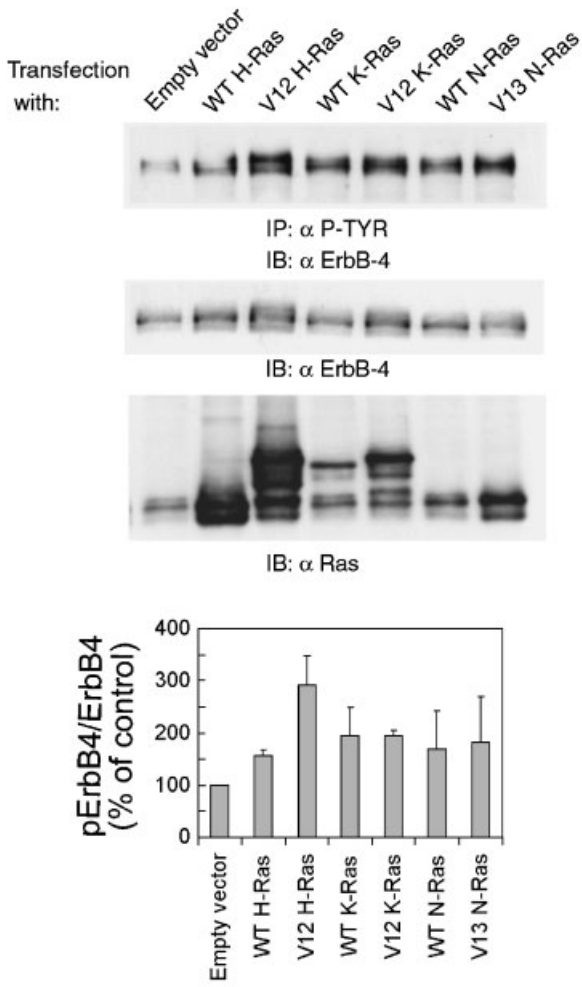

Fig. 2. Ras isoforms-induced ErbB4 phosphorylation in PC12-ErbB4. PC12-ErbB4 cells were transfected with control pcDNA3 vector or either H-Ras, H-Ras(12V), K-Ras, K-Ras(12V), N-Ras and N-Ras(13V) vectors. Twenty-four hours following transfection, medium was replaced and cells were incubated for additional 24 h. Whole cell lysates were prepared from the cells. In the **upper panel**, lysates were immunoprecipitated with mAb PY-20 to phosphotyrosine. The immune complexes were resolved by gel electrophoresis and transferred to nitrocellulose filters. IB was performed by using rabbit antiserum to ErbB4 (Santa Cruz). Total cell lysates were analyzed by IB with a mAb to Ras or a polyclonal Ab to ErbB4 (two **lower panels**). Signal detection was performed by using a chemiluminescence kit. Densitometric analysis of three experiments is presented (lower columns). Data are the mean (n = 3) ratio between pErbB4 and ErbB4 compared to the control pcDNA transfected cells. Bars = SD.

Ras-induced Increase in ErbB4 Receptor Phosphorylation is Independent of Autocrine Loops

The observed Ras-induced ErbB4 phosphorylation could be due to an autocrine loop, where expression of the activated Ras leads to secretion of ErbB4 ligands. This is a reasonable possibility, since it is well known that Ras activation can lead to the secretion of growth factors [Yarden and Peles, 1991; Peles et al., 1992; Normanno and Ciardiello, 1997; He et al., 2000]. In fact, the typical ErbB4 ligand, NRG, has been isolated from conditioned media of H-Ras transformed fibroblasts [Peles et al., 1992]. We tested this possibility in PC12-ErbB4 cells using the inhibitor of ErbB4 receptor, IgB4. This engineered soluble ErbB4 receptor acts as a scavenger of ErbB4 ligands, and thus prevents their binding to the cell membrane receptors [Dong et al., 1995]. We first induced ErbB4 receptor phosphorylation by exogenously added NRG, a specific ErbB4 receptor ligand. As expected, NRG induced ErbB4 receptor phosphorylation and IgB4 blocked this effect (Fig. 3A). However, H-Ras(12V)-induced phosphorylation of the ErbB4 receptor was not blocked by IgB4 (Fig. 3B). Moreover, conditioned medium

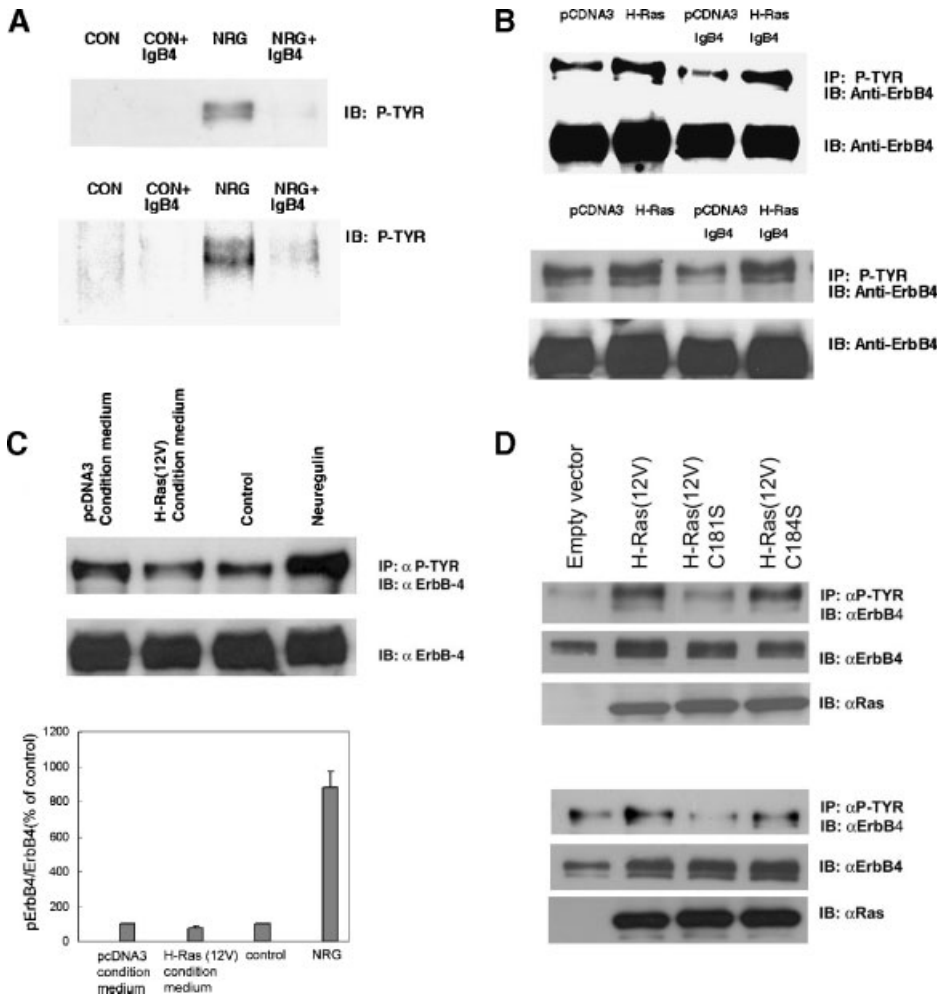

Fig. 3. H-Ras-induced ErbB4 phosphorylation is ligand independent. **A**: PC12-ErbB4 cells were incubated for 15 min with NRG (100 ng/ml) in the presence or absence of 4 μg/ml IgB4. Whole cell lysates were prepared and analyzed by IB with a monoclonal Ab to phosphotyrosine (PY-20). **B**: Cells transfected with either control pcDNA3 vector or H-Ras(12V) expression vector were treated or untreated with IgB4 for 48 h following transfection and subjected to immunoprecipitation and immunoblot analyses as described in Figure 1A. Typical duplicate immunoblots are presented. **C**: Cells transfected with either control pcDNA3 vector or H-Ras(12V) expression vector and conditioned medium were collected 48 h following transfection. PC12-ErbB4 cells were either treated with conditioned medium or with NRG 100 ng/ml or left untreated. Whole cell lysates were prepared and immunoprecipitated with mAb PY-20 to phosphotyrosine. The immune complexes were resolved by gel electrophoresis and transferred to nitrocellulose filters. IB was performed by using rabbit antiserum to ErbB4 (Santa Cruz). Total cell lysates were analyzed by IB with a rabbit antiserum to ErbB4 (**lower panel**). Typical duplicate immunoblots are presented. **D**: Cells transfected with either control pcDNA3 vector, H-Ras(12V)C181S, H-Ras(12V)C184S or H-Ras(12V) expression vectors. Lysates were subjected to immunoprecipitation and immunoblot analysis as described in Figure 1. Typical duplicate immunoblots are presented.

collected from H-Ras(12V)-transfected PC12-ErbB4 cells did not induce receptor phosphorylation in PC12-ErbB4 cells (Fig. 3C). Importantly, we have previously shown that exogenously added ligands of ErbB1 receptor (except for betacellulin) do not induce an increase in ErbB4 phosphorylation [Vaskovsky et al., 2000]. Betacellulin nonetheless, being also an ErbB4 ligand would bind to IgB4. Thus, the Ras-induced ErbB4 phosphorylation is not due to ligand dependent ErbB1/ErbB4 or ErbB3/ErbB4 heterodimers. In addition, we know from earlier studies that ErbB3 is not activated in PC12-ErbB4 cells [Vaskovsky et al., 2000]. Furthermore, we found that only Ras, that is targeted to the cell membrane, mediates ErbB4 receptor phosphorylation (Fig. 3D). In these experiments, we examined ErbB4 phosphorylation in PC12-ErbB4 cells transfected with H-Ras(12V)C184S which is targeted to the

plasma membrane, or with H-Ras(12V)C181S which is targeted to internal membranes [Roy et al., 2005]. As shown in Figure 3D, only H-Ras(12V)C184S could induce receptor phosphorylation and its effect was comparable with that of H-Ras(12V). We, therefore, conclude that the Ras-induced increase in ErbB4 receptor phosphorylation in PC-12-ErbB4 cells is ligand independent and occurs in the cell membrane. This effect of the activated Ras appears than to depend on intracellular Ras signals.

Intracellular Signaling That Mediates Ras-Induced Increase in ErbB4 Receptor Phosphorylation

In order to explore the signaling pathway that mediates Ras-induced receptor phosphorylation, we used effector-loop mutants of H-Ras that activate only one effector pathway: H-Ras(12V,35S) double mutant that activates Raf, H-Ras(12V,40C) that activates PI3K, and H-Ras(12V,37G) that activates RalGEF [Joneson et al., 1996]. PC12-ErbB4 cells were transfected with either H-Ras(12V) or each of its effector loop mutants and the levels of phosphorylated ErbB4 were then determined. Each of the Ras effector loop mutants induced a small but significant increase in ErbB4 phosphorylation (Fig. 4). These results suggest that Ras-induced ErbB4 phosphorylation is mediated in part by the three prominent Ras pathways. To further substantiate this conclusion, we used pharmacological inhibitors of PI3K (LY294002) or MEK (PD98059). As shown in Figure 5A and in line with the results described above, inhibition of each of these signaling pathways resulted in partial inhibition of Ras-mediated ErbB4 phosphorylation. Transient co-expression of H-Ras(12V) and dominant negative PI3K (Dp85) also resulted in partial inhibition of Ras-mediated ErbB4 phosphorylation (Fig. 5B). These results suggest that both PI3K and Erk signaling pathways are required for this effect.

Ras-induced Increase in ErbB4 Receptor Phosphorylation Requires ErbB4 Kinase but not Src Activity

To examine the possibility that ErbB4 phosphorylation induced by constitutively active Ras is mediated by Src, we used PP2, a pharmacological inhibitor of Src (Fig. 6). PP2 at low concentration had no effect on basal or Ras-mediated ErbB4 phosphorylation. At high concentration PP2 inhibited the basal ErbB4 phosphorylation but had no effect on Ras-mediated ErbB4 phosphorylation. In agreement with our results, recent experiments showed that activated Ras inhibits Src tyrosine kinase activity [Thornton et al., 2003]. Taken together, these data rule out the possibility that Ras-induced ErbB4 phosphorylation is mediated by Src, a prominent non-RTK.

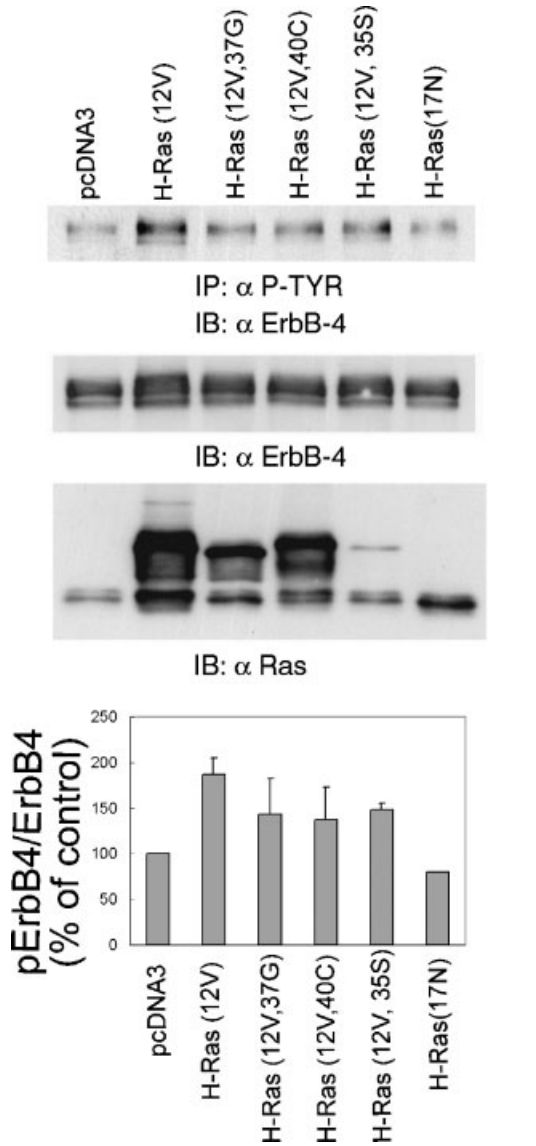

Fig. 4. The effect of H-Ras effector loop mutants on ErbB4 phosphorylation. PC12-ErbB4 cells were transfected with either control pcDNA3 vector or H-Ras(12V) and H-Ras(17N) vectors or effector-loop mutants of H-Ras that can activate a single signaling pathway: H-Ras(12V,35S) double mutant that activates Raf (the MAPK pathway), H-Ras(12V,40C) that activates PI3K and H-Ras(12V,37G) that activates RalGEF. Whole cell lysates were subjected to immunoprecipitation and immunoblot analyses as described in Figure 1. Densitometric analysis of three experiments is presented (lower columns). Data are the mean (n = 3) ratio between pErbB4 and ErbB4 compared to the control pcDNA transfected cells. Bars = SD.

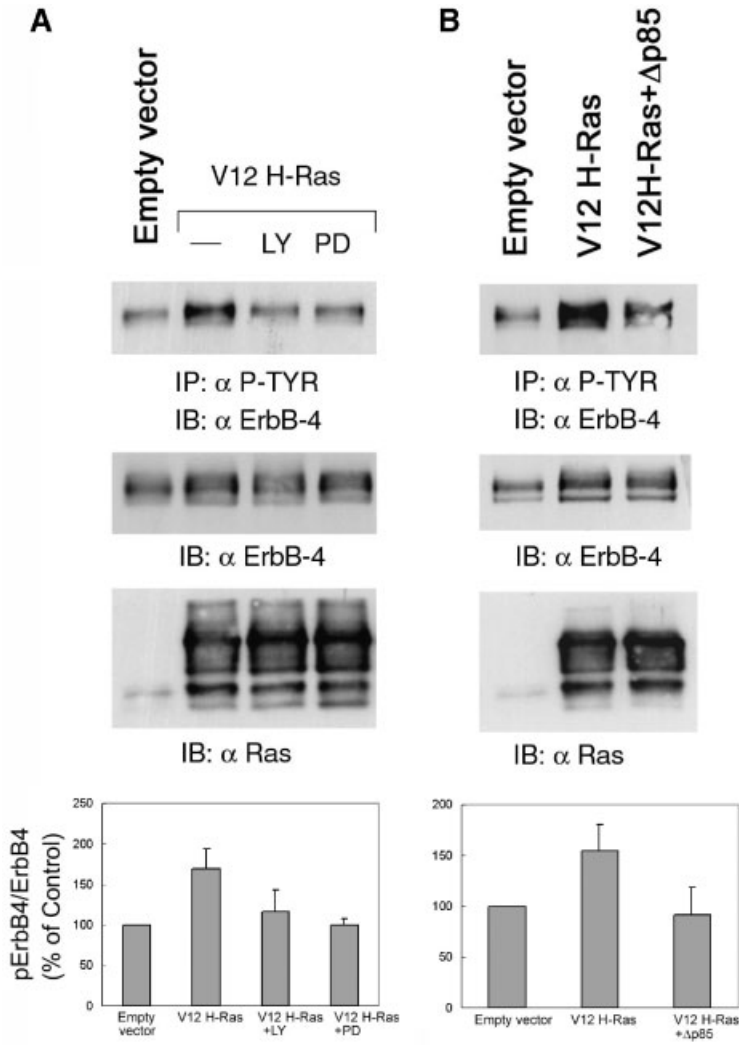

Fig. 5. The effect of PI3K and MAPK signaling pathways on H-Ras(12V)-induced ErbB4 phosphorylation. **A**: PC12-ErbB4 cells were transfected with either control pcDNA3 vector or H-Ras(12V). Six hours following transfection, medium was replaced to DMEM containing 7.5% FCS and 7.5% HS, and cells were incubated with or without 10 μM LY294002 (LY) or 20 μM PD98059 (PD). Forty-eight hours later, whole cell lysates were prepared and subjected to immunoprecipitation and immunoblot analyses as described in Figure 1. The inhibitors had only a minor effect on cell viability (not shown). **B**: PC12-ErbB4 cells were transfected with either control pcDNA3 vector or H-Ras(12V), with or without dominant negative p85 vector. Six hours following transfection, medium was replaced to DMEM containing 7.5% FCS and 7.5% HS and cells were incubated for 48 h. Whole cell lysates were prepared and subjected to immunoprecipitation and immunoblot analyses as described in Figure 1. The experiments were repeated three times with similar results. Densitometric analysis of three experiments is presented (lower columns). Data are the mean (n = 3) ratio between pErbB4 and ErbB4 compared to the control pcDNA transfected cells. Bars = SD.

In order to examine the possibility that Ras activation of ErbB4 is mediated by ErbB4 kinase activity, we constructed a new mutant ErbB4 expression vector with mutation in the ATP binding site of ErbB4 (lysine 751 to alanine substitution). The expression vector was termed LXSHD-ErbB4DK. This vector was used for retroviral infection of PC12 cells and stable clones were selected. In vitro kinase assay confirmed that the mutant receptor had no kinase activity (Fig. 7A). Ras(12V) did not increase ErbB4 phosphorylation in PC12-ErbB4DK cells indicating that the ErbB4 phosphorylation depends on the kinase activity of the receptor (Fig. 7B).

Ras-induced Increase in ErbB4 Receptor Phosphorylation Enhances Cell Differentiation

We next examined whether Ras-induced ErbB4 phosphorylation may have a biological

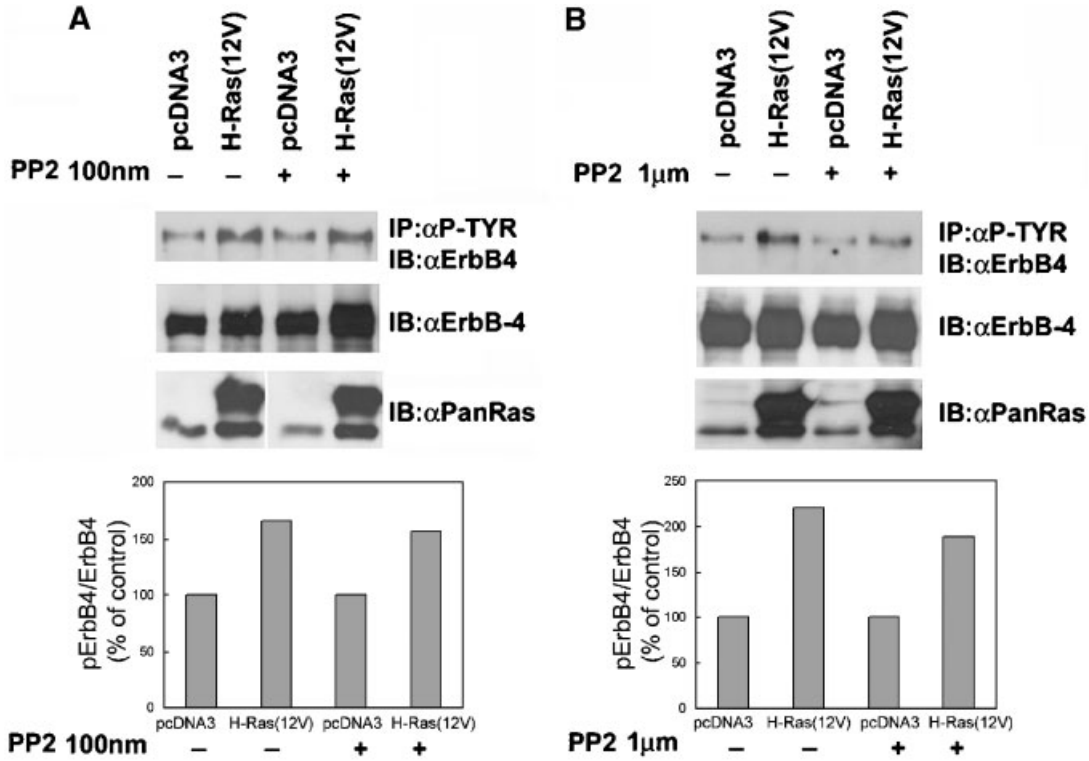

Fig. 6. The effect of Src inhibition on H-Ras induced ErbB4 phosphorylation. PC12-ErbB4 cells were transfected with either control pcDNA3 vector or H-Ras(12V). Six hours following transfection, medium was replaced with DMEM containing 7.5% FCS and 7.5% HS and cells were incubated with or without 100 nM PP2 (A) or 1 μM PP2. Forty-eight hours later, whole cell lysates were prepared and subjected to immunoprecipitation and immunoblot analyses as described in Figure 1. Densitometric analysis of the experiments is presented (lower columns).

outcome. As readout of biological activity, we used a cell differentiation assay, namely neurite outgrowth. Constitutively active Ras or ligand activated ErbB4 are known to induce neurite outgrowth in PC12 cells [Vaskovsky et al., 2000]. In the absence of ligands, ErbB4 does not induce neurite outgrowth in PC12-ErbB4 cells ([Vaskovsky et al., 2000], see also Fig. 8A). Ras(12V) induced strong neurite outgrowth in PC12-ErbB4, which was far more profound than that induced by Ras(12V) in PC12-ErbB4DK cells (Fig. 8A). The mean neurite length of PC12-ErbB4 cells was 45.9 ± 1.4 mm compared to 16 ± 0.6 mm in PC12-ErbB4DK cells. These results indicate that Ras-mediated ErbB4 phosphorylation in PC12-ErbB4 cells contributes to ligand-independent ErbB4 signaling, leading to cell differentiation.

DISCUSSION

Growth factor receptors, and especially the ErbB family and Ras are major contributors to normal cell growth and differentiation but also to oncogenic transformation [Carpenter, 2003; Cox and Der, 2003]. It is well known that Ras activation can lead to the secretion of growth factors [Ciardiello et al., 1990; Yarden and Peles, 1991; Normanno et al., 1994; Normanno and Ciardiello, 1997]. Thus, either active ErbB receptors or oncogenic Ras can facilitate cell growth or differentiation by autocrine loops. Accordingly, the effect of activated Ras on the levels of phosphorylated receptors could be associated with induced secretion of ErbB receptor ligands. In the present study, we demonstrate that constitutively active Ras induces ErbB4 receptor phosphorylation. This phosphorylation is ligand-independent, since soluble receptors that block ligand binding to the receptor did not inhibit Ras-mediated receptor phosphorylation. We also suggest that several signaling pathways are involved in Ras-mediated ErbB4 phosphorylation. Among these pathways are the prominent Ras-mediated signals; PI3K, Erk, and RalGEF.

Ligand binding to the extracellular part of tyrosine kinase receptors induces receptor

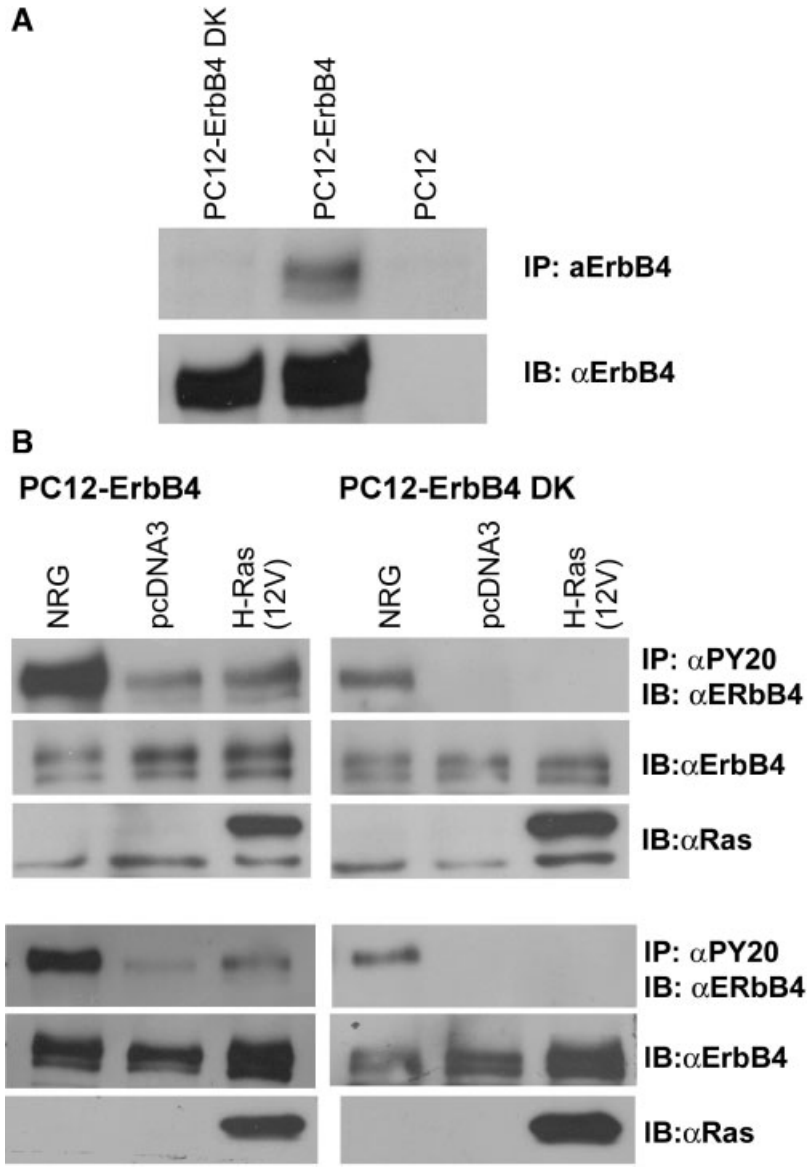

Fig. 7. The effect of ErbB4 kinase activity on H-Ras induced ErbB4 phosphorylation. **A**: Whole lysates were prepared from 10^7 PC12-ErbB4, PC12-ErbB4DK, and PC12 cells. ErbB4 receptor was immunoprecipitated using rabbit anti-ErbB4 antibodies (Santa Cruz). The immunoprecipitated receptors were subjected to in vitro *kinase* reactions, as described in Materials and Methods, and resolved by SDS–PAGE (7.5% acrylamide). The resulting autoradiograms (**upper panel**) and the immunoblot analysis of whole cell lysates of the samples (**lower panel**) are shown. **B**: PC12-ErbB4 and PC12-ErbB4DK were transfected with either control pcDNA3 vector or H-Ras(12V). Forty-eight hours following transfection, lysates were analyzed as described in Fig. 1. The results of two experiments are presented. It should be noted that H-Ras(12V) induced ErbB4 phosphorylation in PC12-ErbB4 cells but not in PC12-ErbB4DK cells.

dimerization, resulting in a signal transmitted across the membrane that activates the intracellular tyrosine kinase domain [Ullrich and Schlessinger, 1990; Yarden and Kelman, 1991]. This leads to autophosphorylation on tyrosine residues which function as docking sites for signal transducing molecules [Cantley et al., 1991]. The cytoplasmic tail of the receptors contains various phosphorylation sites that are responsible for the functional differences between the receptors [Di Fiore et al., 1992]. Several studies demonstrated that activated Ras induces the release of growth factors, including EGF-like ligands such as amphiregulin, TGFα HB-EGF, and NRG [Ciardiello et al., 1990; Yarden and Peles, 1991; Peles et al., 1992; Normanno and Ciardiello, 1997; He et al., 2000; Martinez-Lacaci et al., 2000]. Thus, activated

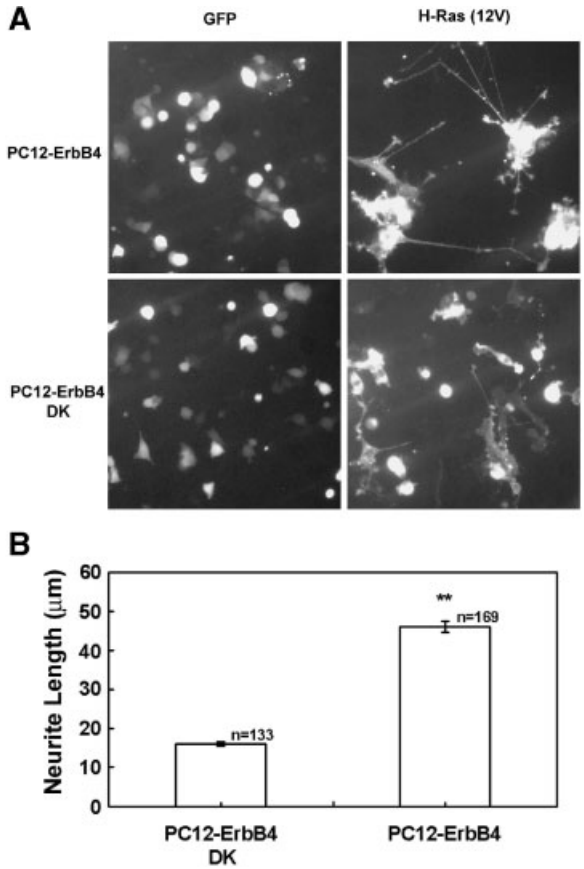

Fig. 8. Ras(12V) mediates enhanced neurite outgrowth in PC12-ErbB4 cells as compared to PC12DK cells. PC12-ErbB4 and PC12ErbB4DK cells were transfected with either control pEGFP vector or pEGFP-Ras(12V) vector. Twenty-four hours following transfections, cells were either treated with NRG 100 ng/ml or left untreated for an additional 24 h. At least 20 random fields of each treatment were photographed using Olympus optical inverted phase-contrast microscope Model IX70 (×20 magnification) and the lengths of the neurites were measured. **A**: **Panels** were randomly selected as representative fields. **B**: Quantification of the results. Data of the neurite length are presented as the mean ± SD of 20 randomly selected fields.

Ras can induce receptor phosphorylation via an autocrine loop. In our study, we demonstrate that expression of constitutively active Ras induces ErbB4 phosphorylation (Figs. 1 and 2). In order to test the possibility that Ras-mediated ErbB4 phosphorylation is not due to an autocrine loop, we used two approaches. First, using soluble ErbB4 receptor (IgB4), that blocks ligand binding to ErbB4, we demonstrated that oncogenic Ras-mediated receptor phosphorylation is unaffected by IgB4 (Fig. 3B). Second, conditioned medium from H-Ras(12V)-transfected PC12-ErbB4 cells did not induce receptor phosphorylation in PC12-ErbB4 cells (Fig. 3C). Thus, our results strongly suggest that the effect of H-Ras(12V) on ErbB4 phosphorylation is ligand-independent and involves intracellular signals. This effect of activated Ras can lead to receptor phosphorylation by an as yet unknown mechanism.

Ras activates several signaling pathways. The most prominent pathways are the RalGEF, MAPK, and PI3K signaling pathways [Joneson et al., 1996]. We found that effector loop mutants of Ras, that specifically activate each of these pathways, induced receptor phosphorylation (Fig. 4). In addition, pharmacological inhibitors of the PI3K and MAPK pathways reduced H-Ras(12V)-mediated ErbB4 phosphorylation (Fig. 5). These results suggest that several Ras signals, including the PI3K, Erk, and RalGEF pathways, are involved in the ligand-independent phosphorylation of ErbB4 receptors.

To examine the possibility that ErbB4 phosphorylation induced by constitutively active Ras is mediated by tyrosine kinase activity of the prominent non-RTK, Src, or by the kinase activity of the ErbB4 receptor, we perform two sets of experiments. Firstly, by using the Src inhibitor, we demonstrated that receptor phosphorylation is not mediated by Src. These results correlated with the previously described observation that activated Ras inhibits Src tyrosine kinase activity [Thornton et al., 2003]. Secondly, by using the mutant ErbB4 receptor that lacks kinase activity (Fig. 7A), we demonstrated that the kinase activity of the receptor is necessary for Ras-mediated ErbB4 phosphorylation. The mechanism of Ras-mediated

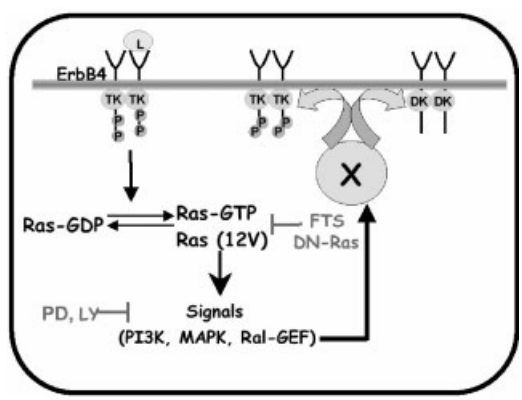

Fig. 9. A model of activated Ras mediated ErbB4 phosphorylation. The model depicts the signaling components that mediate Ras-dependent ErbB4 phosphorylation and the signaling events activated by Ras, and affects receptor phosphorylation.

receptor phosphorylation seems to be biologically relevant since Ras(12V) induced a larger increase in neurite length in cells expressing the ErbB4 receptors than in cells expressing ErbB4DK receptors.

Accordingly, we propose a model (Fig. 9) where, in addition to ligand-induced ErbB4 receptor autophosphorylation, constitutively active Ras increases receptor phosphorylation by intracellular signals that depend on the kinase activity of the receptor. These results are important in light of the high incidence of ErbB receptors' overexpression and Ras activation in human tumors.

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