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Cell-type specific phosphorylation of threonines T654 and T669 by PKD defines the signal capacity of the EGF receptor

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In Rat-1 fibroblasts epidermal growth factor (EGF), but not platelet-derived growth factor (PDGF) stimulates the activity of the c-Jun N-terminal kinase (JNK). Moreover, PDGF induced suppression of EGF-mediated JNK activation, apparently through protein kinase C (PKC) activation. Further analysis revealed that PKD was specifically activated by PDGF but not EGF in Rat-1 cells. In SF126 glioblastoma cells, however, EGF and PDGF synergistically activated JNK, while neither PDGF nor EGF stimulated PKD activity. In this cell line, overexpression of PKD blocked EGF- and PDGF-induced JNK activation. Mutational analysis further revealed that the EGFR mutant (T654/669E) was incapable of activating JNK and provided evidence that PKD-mediated dual phosphorylation of these critical threonine residues leads to suppression of EGF-induced JNK activation. Our results establish a novel crosstalk mechanism which allows signal integration and definition in cells with many different RTKs.
Keywords: c-Jun N-terminal kinase/epidermal growth factor receptor/platelet-derived growth factor receptor/protein kinase D/threonine phosphorylation

Introduction

Definition of cellular responses such as proliferation, differentiation or survival by a variety of external stimuli involves the regulation of transcriptional events in eukaryotic cells through intracellular signalling cascades, including pathways that activate kinases of the mitogen-activated protein kinase (MAPK) family (Treisman, 1996). Several subgroups of MAP kinases that differ in their substrate specificity and regulatory properties have been identified in mammalian cells. Members of the c-Jun N-terminal kinase (JNK) subgroup of MAP kinases are activated in response to diverse non-specific and specific extracellular stimuli, which include UV irradiation, proinflammatory cytokines and growth factors such as epidermal growth factor (EGF) (Sluss *et al*., 1994). The JNK subfamily includes products of the three related genes *jnk1* (Derijard *et al*., 1994), *jnk2* (Sluss *et al*., 1994) and *jnk3* (Gupta *et al*., 1996). Once activated, JNK translocates to the nucleus and forms a transient complex with the N-terminal activation domain of the transcription factor c-Jun, which results in phosphorylation of c-Jun serine residues S63 and S73. Phosphorylation of these sites dramatically stimulates c-Jun transcriptional activity (Minden *et al*., 1994). Other subgroups of MAP kinases were found to be incapable of efficiently phosphorylating these residues. c-Jun is a sequence-specific transcription factor whose function has been implicated in various cellular events ranging from proliferation and differentiation to neoplastic transformation (Angel and Karin, 1991).

The protein kinase C (PKC) family, a serine/threonine kinase family of 12 isoenzymes with distinct tissue-distribution characteristics, has been subdivided on the basis of different primary structures and enzymatic properties into conventional, novel and atypical PKCs (Hug and Sarre, 1993). Extensive evidence is available for their involvement in the control of proliferation, differentiation and motility (Clemens *et al.*, 1992). Protein kinase C-α (PKCα) has been reported to phosphorylate and activate c-Raf, which is the upstream regulator of the extracellular-regulated kinase kinase MEK. MEK itself is an activator of the extracellular-regulated kinase (ERK), another member of the MAP kinase family (Kolch *et al*., 1993). In contrast to the MAP kinase pathway, where PKC can activate ERKs, the role of PKC in regulating the JNK pathway is currently unclear. Recently, contradictory roles of PKC have been described. While simultaneous addition of Ca^{2+}-ionophore and phorbol ester markedly stimulated JNK activity in cardiac myocytes (Kudoh *et al*., 1997), the opposite effect was observed in Rat-1 cells, where activation of PKC inhibited JNK stimulation by endothelin (ET-1) and inhibition of PKC potentiated JNK activation by this ligand (Cadwallader *et al.*, 1997). Thus, the ERK and JNK signalling cascades are parallel pathways that can be differentially regulated by PKC.

Recently, protein kinase D (PKD)/PKCμ, a serine/threonine kinase distantly related to PKCs, has been identified (Johannes *et al*., 1994; Valverde *et al.*, 1994) and shown to be activated upon stimulation of cells with several mitogenic polypeptides such as bombesin, vasopressin, endothelin, bradykinin and platelet-derived growth factor (PDGF) (Zugaza *et al.*, 1997). PDGF stimulation of PKD involved the activation of phospholipase Cγ and PKC (Zugaza *et al.*, 1997; van Lint *et al.*, 1998). Moreover, PDGF-stimulation induced serine/threonine phosphorylation of PKD (van Lint *et al.*, 1998) and constitutively active mutants of PKCε and PKCη, but not PKCζ, caused PKD activation (Zugaza *et al.*, 1996). So far, however, a specific role of PKD in growth factor receptor signalling has not been described.

In addition to the influences on different MAP kinase

signalling pathways, PKC activation has been demonstrated to down-modulate the signalling potential of receptor tyrosine kinases like the EGF receptor (EGFR) and HER2/neu (Seedorf *et al.*, 1995a). For the EGFR, such negative regulation can occur through serine and/or threonine phosphorylation that is mediated by PMA-activated PKC, and causes a decrease of high-affinity receptor binding sites and inhibition of EGF-stimulated tyrosine kinase activity (Friedman *et al.*, 1984). Mutational analysis revealed two EGFR residues that were phosphorylated in response to phorbol esters: threonine T654, the target of protein kinase C, and threonine T669, a potential ERK substrate and the major site of EGFR phosphorylation (Hunter *et al.*, 1984; Morrison *et al*., 1993).

As shown by Morrison *et al.* (1993), EGFR mutants with either one of threonines T654 or T669 substituted by glutamic acid, in order to mimic the negative charge introduced by phosphorylation, had properties indistinguishable from the wild-type EGFR with regard to ligand-binding, EGF-stimulated tyrosine kinase activity and DNA synthesis. Furthermore, both mutants were still targets for inhibition by phorbol esters (Morrison *et al.*, 1993). Besides T654 and T669, four major serine phosphorylation sites on the EGFR have been mapped: serine S671 (Heisermann and Gill, 1988), serine S1002 (Kuppuswamy *et al.*, 1993) and serines S1046 and S1047 (Countaway *et al.*, 1992). While a role in EGFR desensitization had been implicated for S1002 as well as for SS1046/47 (Countaway *et al.*, 1992; Kuppuswamy *et al.*, 1993) and the latter has been connected to v-erbB transforming activity (Theroux *et al.*, 1992), the underlying mechanism is still not understood.

To date, no signalling pathway that is regulated by phosphorylation of the EGFR on serine and threonine residues has been characterized. In the present study, we show that in Rat-1 cells, phosphorylation on both threonines T654 and T669 in the juxtamembrane region of the EGFR results in downregulation of EGF-induced JNK activation while the MAPK pathway is unaffected. This pathway-specific signal modulation is enacted by PDGF and involves PKC and PKD activities. As a consequence, cellular responses such as proliferation and transformation involving transcriptional activation of specific genes are selectively downregulated. Thus, as shown here for the PDGFR and EGFR, members of the receptor tyrosine kinase (RTK) family may mutually influence their signalling capacity via PKD-mediated modification. This RTK crosstalk represents a mechanistic element that allows signal integration and definition in cells expressing a diversity of RTKs under varying environmental influences.

Results

PDGF costimulation suppresses EGF-induced JNK activation in Rat-1 fibroblasts

Growth factor receptors like the EGFR or the PDGFR can activate mitogen-activated protein kinases, including ERKs and JNKs. PDGF activation of the JNK pathway, however, appears to be regulated in a cell-type-characteristic manner. In NIH 3T3 fibroblasts, PDGF is able to stimulate JNK activity (Xie and Herschman, 1996), whereas in Chinese hamster embryo fibroblasts or human articular chondrocytes no JNK activation was observed

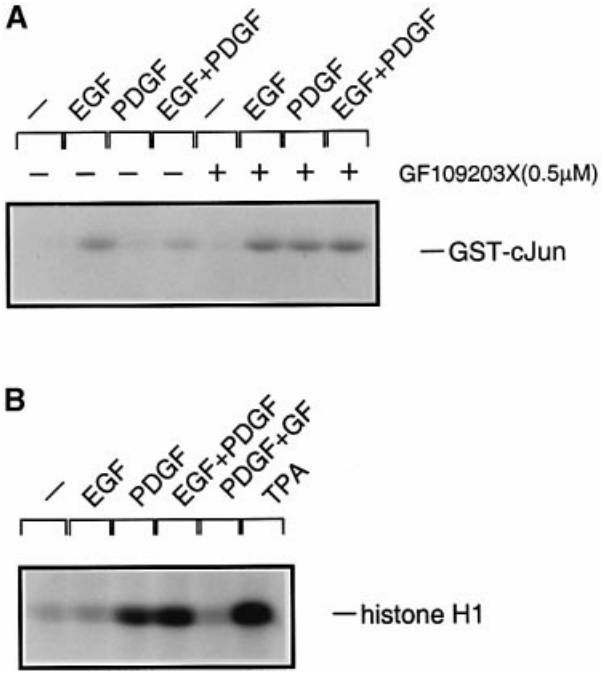

Fig. 1. (**A**) PDGF costimulation suppresses EGF-induced JNK activation mediated by PKC in Rat-1 fibroblasts. Quiescent cells were stimulated for 15 min with 100 ng/ml EGF or 25 ng/ml PDGF-BB, or costimulated either without or with pretreatment for 30 min with 0.5 μM GF109203X. Cell extracts were prepared and assayed for JNK activity by the solid-phase kinase assay with GST–c-Jun (1–79) as a substrate, described in Materials and methods. Phosphorylated substrate was visualized by autoradiography after gel electrophoresis. (**B**) Activation of PKD in Rat-1 fibroblasts. Following serum starvation for 24 h, cells were treated for 15 min with 100 ng/ml EGF, 25 ng/ml PDGF-BB, EGF+PDGF-BB, PDGF-BB+GF109203X (30 min pretreatment; 0.5 μM) or 1 μg/ml TPA. Lysates were used for immunoprecipitation with monoclonal anti-PKD antibody, and PKD activity was determined in an immunocomplex *in vitro* kinase assay using histone H1 as a substrate. Phosphorylated histone H1 was visualized by autoradiography.

upon PDGF stimulation (Geng *et al.*, 1996; Weber *et al.*, 1997). We found that in Rat-1 fibroblasts, as well as in the pancreatic tumour cell lines, Colo357, SW850 and PancTu 1, EGF, but not PDGF, was able to stimulate JNK activity. Interestingly, costimulation with EGF and PDGF-BB suppressed this effect (Figure 1A, first four lanes). In contrast, ERK activity was stimulated by both PDGF and EGF, and simultaneous treatment of Rat-1 cells with both ligands showed a synergistic effect (data not shown). To identify the mediators of this differential effect, we employed the PKC inhibitor GF109203X. Treatment of Rat-1 cells with GF109203X abolished the PDGF-induced suppression of EGF-stimulated JNK activity. Interestingly, upon PKC inhibition, PDGF was able to activate JNK (Figure 1A; last four lanes). A possible explanation for this differential signal control was that PDGF, by activating PKC, blocked its own JNK activation potential. Similar results were achieved by depletion of PKCs by long-term treatment of cells with TPA rather than inhibition with GF109203X (data not shown). We further investigated the activation of PKD, a PKC-activated serine/threonine kinase, in response to PDGF or EGF. PKD was activated by PDGF but not EGF in Rat-1 fibroblasts (Figure 1B), and PDGF-induced PKD activation was blocked upon

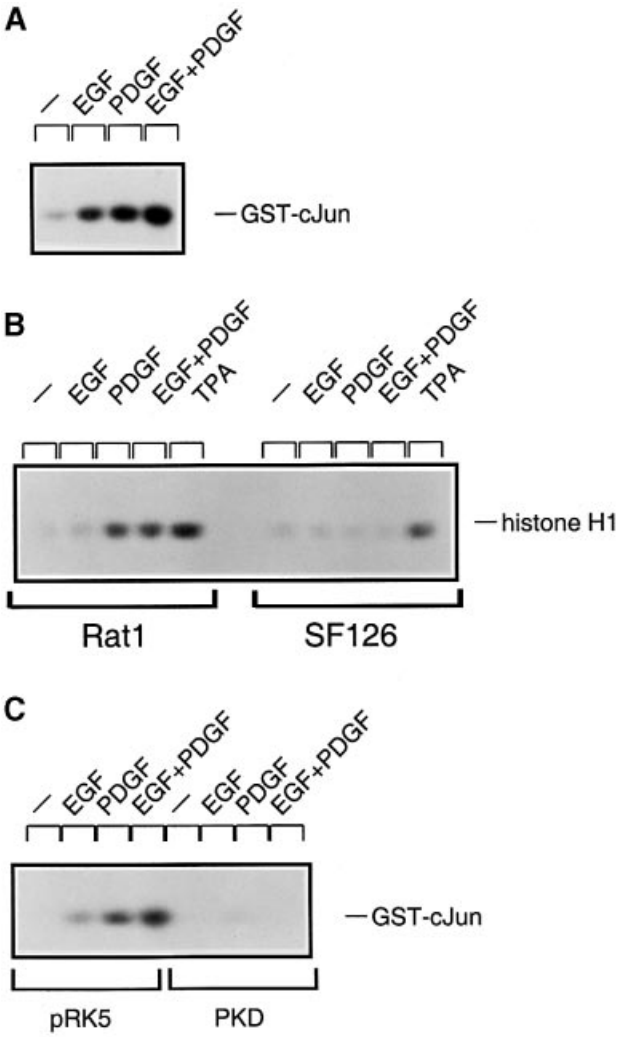

Fig. 2. (**A**) PDGF costimulation leads to a further increase in EGF-induced JNK activation in SF126 human glioblastoma cells. Starved cells were stimulated with 100 ng/ml EGF, 25 ng/ml PDGF-BB or both together. Lysates were used for measurement of JNK activity by a kinase assay using GST–c-Jun (1–79) as a substrate. Phosphorylated substrate was visualized by autoradiography after gel electrophoresis. (**B**) Comparison of PKD activation in Rat-1 or SF126 cells. Quiescent cells were treated for 15 min with 100 ng/ml EGF, 25 ng/ml PDGF-BB, 100 ng/ml EGF, and 25 ng/ml PDGF-BB or 1 μg/ml TPA. Lysates were used for a PKD *in vitro* kinase assay as described in Materials and methods. Phosphorylated histone H1 is shown on an autoradiogram after separation by SDS–PAGE. (**C**) PKD overexpression suppresses JNK activation. SF126 human glioblastoma cells transiently expressing JNK1 and PKD were stimulated with 100 ng/ml EGF, 25 ng/ml PDGF-BB or both. Control cells transfected with JNK1 and empty vector ('pRK5') were subjected to the same agonist stimulation for 15 min. After cell lysis and immunoprecipitation with a polyclonal anti-Flag antibody (Santa Cruz), an *in vitro* kinase assay was performed using GST–c-Jun (1–79) as a substrate. The autoradiogram shows the phosphorylated substrate after gel electrophoresis.

treatment with the PKC inhibitor GF109203X (Figure 1B, lane 5). These observations suggested a role for PKC in the differential definition of signals generated by distinct growth factor receptors and implicated PKD in the regulation of the JNK pathway.

Synergistic effect of EGF/PDGF costimulation on JNK activity in SF126 human glioblastoma cells

In contrast to Rat-1 cells, EGF and PDGF were both able to stimulate JNK activity in SF126 human glioblastoma cells and Calu-1 human lung carcinoma cells. Moreover, EGF and PDGF costimulation was synergistic (Figure 2A); however, neither PDGF nor EGF had an effect on PKD activity (Figure 2B, lanes 7 and 8). This was in contrast to Rat-1 cells, where PDGF treatment activated PKD (Figure 2B, lane 3). These observations indicate a negative role for PKD in growth-factor-induced JNK signalling. To address this issue further, we generated SF126 cells overexpressing PKD by transfection with a PKD expression vector (Figure 2C, lanes 5–8). Overexpression resulted in constitutive PKD activation (data not shown) and blockage of JNK induction by EGF and PDGF, confirming the negative regulatory function for PKD. These results identify PKD as a cell-type-specific negative regulatory element for the JNK MAP kinase pathway and shed new light on previous contradictory reports (Geng *et al.*, 1996; Xie and Herschman, 1996; Lopez-Ilasaca *et al.*, 1997; Weber *et al.*, 1997) regarding PDGF-induced JNK activation in different cell types.

Phosphorylation of threonines T654 and T669 in the EGFR juxtamembrane domain interferes with JNK activation

It has been shown previously that stimulation of Swiss 3T3 cells with PDGF leads to a decrease in high-affinity EGF-binding to cell-surface receptors and correlates with an increase in EGFR phosphoserine/phosphothreonine content (Collins *et al.*, 1983). This transmodulation was shown to involve the activation of PKC (Zachary and Rozengurt, 1985) and the phosphorylation of threonine residues T654 and T669 in the juxtamembrane domain of the EGFR (Hunter *et al.*, 1984; Countaway *et al.*, 1989). To investigate further the mechanism of PDGF-induced and PKC-mediated transmodulation of the EGFR and its significance for JNK pathway regulation, we used embryonic fibroblasts from EGFR gene-deficient mice, EF-HERc$^{-/-}$ cells, for further analysis (Sibilia and Wagner, 1995). We infected these cells with recombinant retroviruses containing either no insert, DNA sequences encoding the complete EGFR (HERc) or the juxtamembrane domain mutants HERcT654A, HERcT654E, HERcT669A, HERcT669E and HERcT654/669E (termed HERc2E). Our investigation of the ability of these EGFR mutants to activate JNK revealed that the HERc2E mutant, mimicking dual threonine phosphorylation, was incapable of stimulating JNK activity upon EGF treatment, whereas all other mutants were able to activate JNK upon EGF stimulation to a similar extent as the wild-type receptor (Figure 3A). In contrast, the HERc2E mutant was capable of activating the ERK pathway upon EGF treatment (Figure 3C), demonstrating that it was signalling competent for this pathway. The effect observed was not due to differential receptor expression levels, as shown in Figure 3B. These results demonstrate that phosphorylation of both T654 and T669 in the EGFR juxtamembrane domain is required for downregulation of the EGF-induced JNK signalling pathway.

PKD negatively regulates EGF-induced JNK activation

To assess further the necessity of dual phosphorylation of the EGFR positions T654 and T669 by PKD in order to block JNK pathway activation, we used transient co-

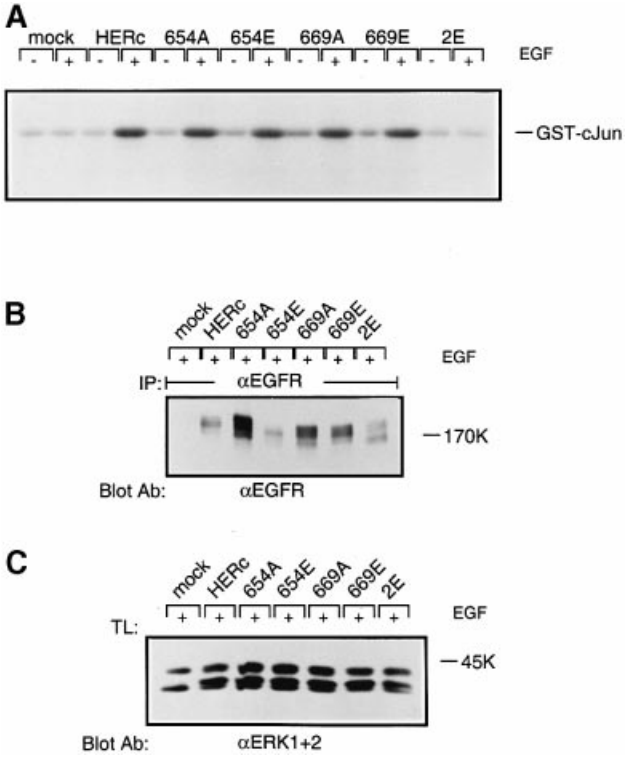

Fig. 3. (**A**) JNK activation by different EGFR mutants. Embryonic mouse fibroblast cells (HERc$^{-/-}$) were infected with recombinant retroviruses containing either wild-type HERc, HERcT654A, HERcT654E, HERcT669A, HERcT669E, HERcT654/669E (2E) or the control vector alone (mock). After selection for neomycin resistance with G418, polyclonal cells were subjected to a solid-phase kinase assay with GST–c-Jun (1–79) as a substrate. Each cell line was either unstimulated or stimulated for 15 min with 100 ng/ml EGF. Phosphorylated substrate was visualized on an autoradiogram. (**B**) EGFR expression in the polyclonal EF cell lines. The amount of EGFR in the polyclonal EF cell lines was analysed by immunoprecipitation of EGF-stimulated cells (100 ng/ml for 15 min) with a monoclonal anti-EGFR antibody (108.1) and immunoblotting following gel electrophoresis with polyclonal anti-EGFR antibody (SC-1005; Santa Cruz). Molecular size is indicated on the right. (**C**) Activation of ERKs in the polyclonal EF cell lines. MAP kinase gel-shift assays of polyclonal EF cell lines stimulated for 15 min with 100 ng/ml EGF. Total cell lysates were separated on 7.5% SDS–polyacrylamide gel and analysed by immunoblotting with anti-ERK1+ERK2 antibodies (Santa Cruz).

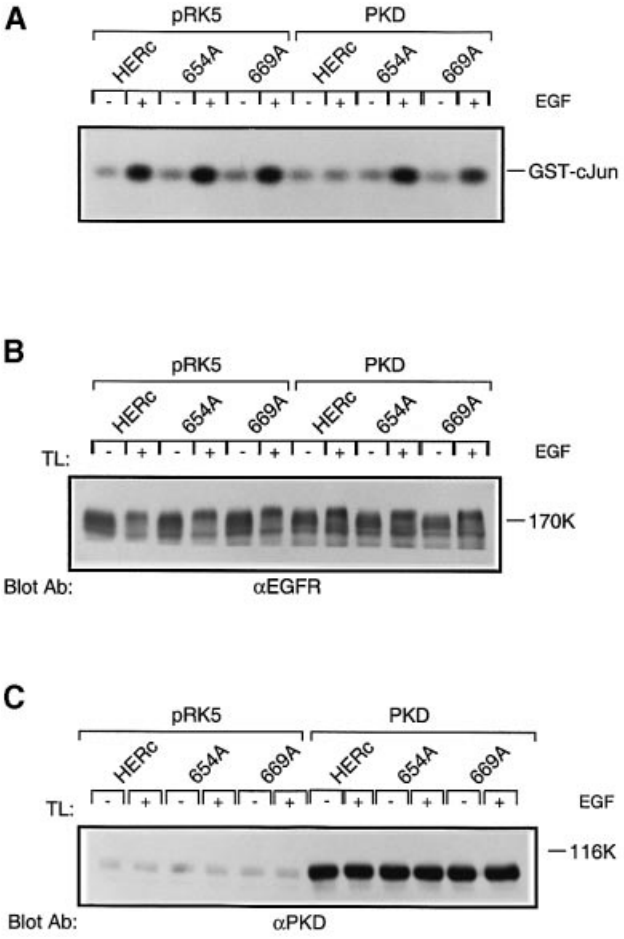

Fig. 4. (**A**) Negative regulation of EGF-induced JNK activation by PKD. 293 human kidney fibroblasts were transiently transfected with cDNAs encoding JNK + HERc wt, JNK + HERcT654A, JNK + HERcT669A either cotransfected with empty vector (pRK5) or PKD cDNA. After serum starvation for 24 h, EGF-stimulated (100 ng/ml; 15 min) and unstimulated cells were lysed and JNK activity was measured by immunoprecipitation with a polyclonal anti-Flag antibody (Santa Cruz) and an immunocomplex *in vitro* kinase assay using GST–c-Jun (1–79) as a substrate. The autoradiogram shows the phosphorylated substrate after separation by SDS–PAGE. (**B**) EGFR expression in transiently transfected 293 fibroblasts. Total cell lysates (TL) from the same experiment were separated by SDS–PAGE and immunoblotted with a polyclonal anti-EGFR antibody (SC-1005; Santa Cruz). (**C**) PKD expression in transiently transfected 293 fibroblasts. The amount of PKD expressed in the transfected 293 fibroblast cells was analysed by immunoblotting following SDS–PAGE with polyclonal anti-PKD antibody (D-20; Santa Cruz).

overexpression in 293 human embryo kidney fibroblasts. As in EF-HERc$^{-/-}$ cells (Figure 3), EGF-induced JNK activation was not affected in 293 cells co-overexpressing PKD with either of the mutants HERcT654A or HERcT669A, in contrast to cells cotransfected with wild-type EGFR and PKD (Figure 4A). The results confirmed that phosphorylation on both threonine residues is necessary for JNK pathway inactivation. As shown in Figure 4B and C, the extent of EGFR expression was not affected by high levels of PKD cotransfection and was comparable for wild-type and mutant EGFRs. Taken together, these results allow us to conclude that activation of PKD leads to phosphorylation of the two critical threonine residues T654 and T669 in the juxtamembrane region of the EGFR, causing the suppression of JNK activation by EGF.

Induction of c-Jun and c-Fos mRNA levels

c-Jun transcriptional activity is stimulated by phosphorylation at two N-terminal serine residues (S63 and S73) in response to a variety of extracellular stimuli, including growth factors, cytokines and UV irradiation (Minden *et al.*, 1994). Unlike JNKs, ERK1 and ERK2 do not phosphorylate these N-terminal serines: instead, they phosphorylate an inhibitory C-terminal residue (Minden *et al.*, 1994). On the other hand, ERKs are involved in the induction of c-Fos expression and thereby contribute to the stimulation of AP-1 (Karin, 1994). As shown in Figure 5, Northern blot analysis of c-Jun and c-Fos mRNA in Rat-1 fibroblasts showed that PDGF costimulation reduced EGF-induced c-Jun mRNA expression, whereas an additive effect was observed for c-Fos. The time course of both c-Jun and c-Fos mRNA induction was maximal after 30 min of ligand stimulation. PDGF costimulation caused either suppression of c-*jun* induction or, in the

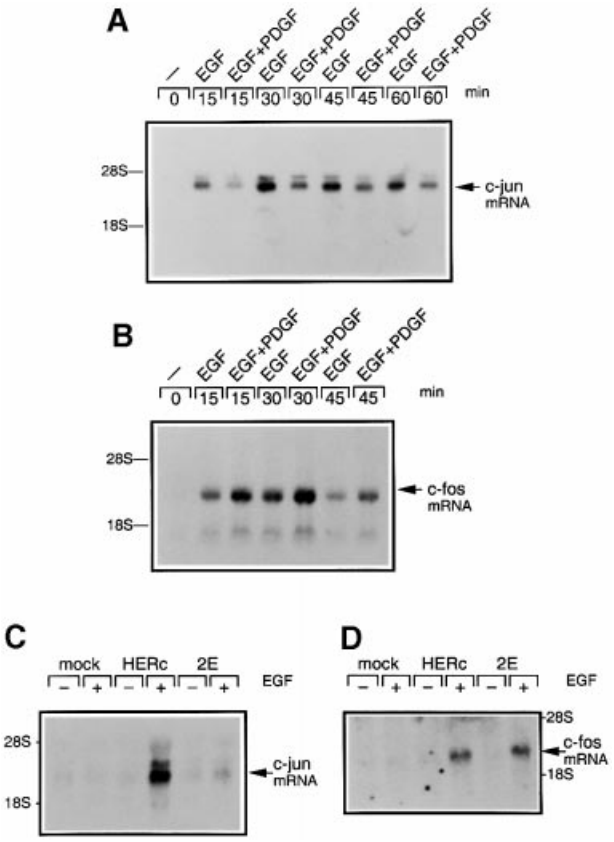

Fig. 5. Northern blot analysis of c-*jun* and c-*fos* induction. Total RNA was extracted after 30 min treatment with the appropriate ligand or after the indicated time points. Ten micrograms of each were separated on a 1.4% agarose–formaldehyde gel and transferred to nitrocellulose for hybridization with a ^{32}P-labelled cDNA probe of the c-Jun or c-Fos full coding sequence. Blots were exposed using an intensifying screen. (**A**) c-*jun* induction in Rat-1 fibroblast cells. (**B**) c-*fos* induction in Rat-1 fibroblast cells. (**C**) c-*jun* induction in polyclonal mouse embryonic fibroblast cell lines EF-HERc wt and EF-HERc 2E, and in EF cells infected with empty vector (MOCK). (**D**) c-*fos* induction in polyclonal mouse embryonic fibroblast cell lines EF-HERc wt, EF-HERc 2E and EF cells infected with empty vector (MOCK).

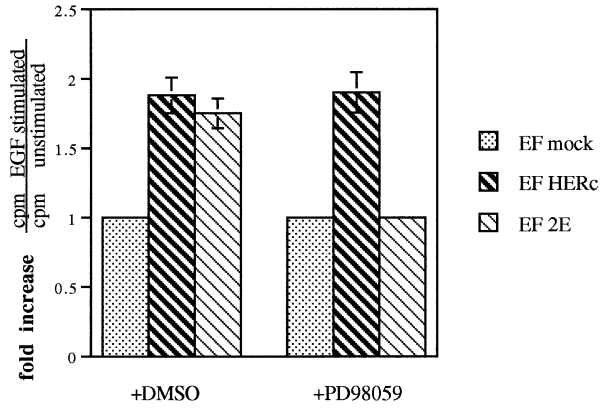

Fig. 6. Incorporation of [^{3}H]thymidine into DNA of embryonic fibroblasts. EF-mock, EF-HERc wt or EF-HERcT654/669E (2E) cells were seeded into 12-well plates (7.5×10^4 cells/well) and serum starved for 24 h. Cells were subjected to 30 min preincubation with 50 μM PD98059 (MEK1/2 inhibitor) or DMSO and were grown either without or with 100 ng/ml EGF. After 18 h incubation, cells were pulse-labelled with [^{3}H]thymidine for 4 h, and its incorporation was evaluated by trichloroacetic acid precipitation and subsequent liquid-scintillation counting. Results represent data of three independent experiments; values are means ± SD.

case of c-*fos*, an additive effect (Figure 5A and B). Moreover, in EF-HERc2E cells where JNK activation upon EGF-stimulation was abrogated due to the presence of negative charges in EGFR positions 654 and 669, EGF-induced c-Jun mRNA expression was dramatically reduced in comparison to EF-HERc wt cells (Figure 5C). On the other hand, EGF-induced c-Fos mRNA expression was not affected (Figure 5D). Our results suggest a strong correlation between PDGF-induced suppression of EGF-stimulated JNK activity and the inhibition of EGF-dependent c-Jun mRNA induction. In contrast, the synergistic effect of EGF and PDGF on ERK activation correlated with an additive effect on c-Fos mRNA induction. Furthermore, introduction of constitutively present negative charges in the HERc2E mutant caused impairment of c-Jun mRNA induction but did not affect EGF-stimulated c-Fos mRNA levels. The data shown in Figure 5 support a critical role of EGFR residues T654 and T669 in the regulation of parallel MAP kinase pathways and in the differential regulation of c-*fos* versus c-*jun* gene expression.

Stimulation of DNA synthesis in mouse embryonic fibroblasts

In human A549 lung carcinoma cells, the JNK signal is the dominant effector pathway for proliferation in response to EGF (Bost *et al*., 1997). To examine the importance of the JNK pathway for EGF-stimulated growth, we used the EGFR-reconstituted EF-HERc$^{-/-}$ cell system and investigated the incorporation of [^{3}H]thymidine into DNA. In EF-HERc wt cells, EGF treatment caused ERK- as well as JNK activation, suggesting that both pathways could contribute to proliferation. In contrast, stimulation of EF-HERc2E cells led only to ERK- but not to JNK activation (see above, Figure 3). In both EF-HERc and EF-HERc2E polyclonal cell lines, EGF stimulated [^{3}H]thymidine incorporation ~2-fold (Figure 6). In order to separate the DNA synthesis signal contributed by either ERK or JNK, we selectively blocked the former with the MEK1 inhibitor, PD98059. Under these conditions EF-HERc2E cells were unable to respond to EGF with enhanced [^{3}H]thymidine incorporation, while EF-HERc cells were unaffected in their DNA synthesis response. In conclusion, our data indicate that in mouse embryonic fibroblasts the JNK pathway represents an alternative to the ERK signal for promotion of DNA synthesis.

Role of threonines T654 and T669 in EGF-induced NIH 3T3 cell transformation

To investigate further the significance of the parallel JNK and ERK pathways for the definition of cell responses we investigated the EGFR mutant HERc2E, which was incapable of activating the JNK pathway upon EGF stimulation, for its ability to transform NIH 3T3 cells. As shown in Figure 7A, in comparison with the HERc wild-type receptor, the HERc2E mutant had a strongly reduced (>60%) transformation potency on NIH 3T3 cells, as measured by focus formation in cell monolayers upon infection with an equal m.o.i. of the respective recombinant retroviruses and stimulation with EGF. Furthermore, we determined the effect of simultaneous stimulation with

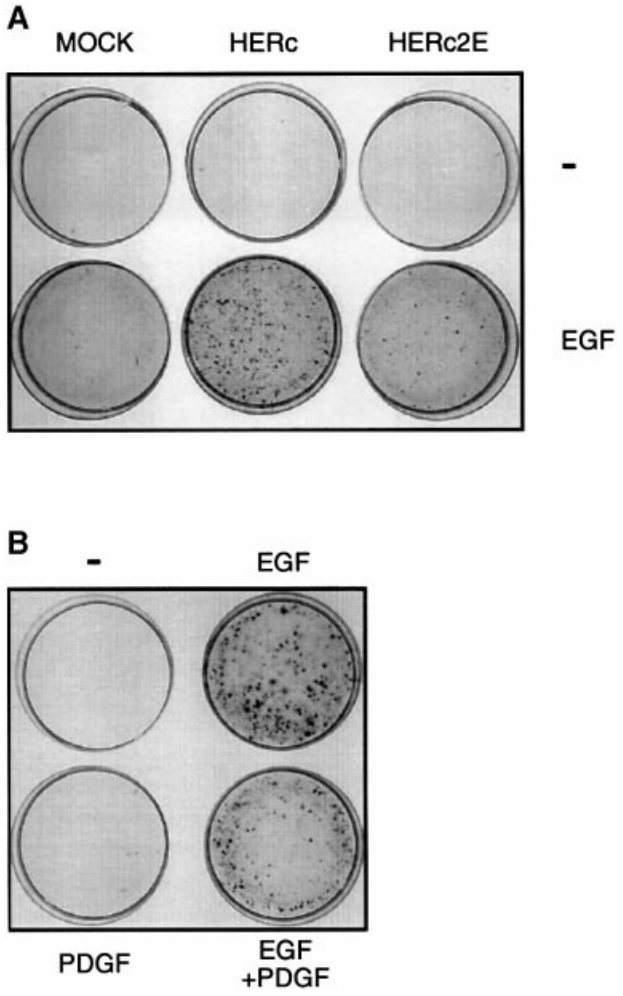

Fig. 7. EGF-dependent NIH 3T3 cell transformation. (**A**) Subconfluent NIH 3T3 cells (10^5 cells/6-cm dish) were infected with equal amounts of retrovirus containing the cDNAs of wild-type HERc, HERcT654/669E (2E) mutant or the empty vector (pLXSN, MOCK) (m.o.i. = 0.1). Cells were cultured in the presence of 4% FCS with or without addition of 50 ng/ml EGF. Medium was changed every second day and cell foci were stained after 14 days with crystal violet. (**B**) Subconfluent NIH 3T3 cells (10^5 cells/6-cm dish) were infected with retrovirus containing the cDNAs HERc wild type and PDGFR. Cells were cultured as above either unstimulated or stimulated with 50 ng/ml EGF, 25 ng/ml PDGF-BB or with 50 ng/ml EGF and 25 ng/ml PDGF-BB. Medium was changed every second day and cell foci were stained after 14 days with crystal violet. Results shown in (A) and (B) are representative experiments from three similar experiments, respectively.

EGF and PDGF on the transformation of NIH 3T3 cells upon coinfection with retroviruses conferring expression of wild-type EGFR and β-PDGFR. Simultaneous stimulation of double-infected cell monolayers with EGF and PDGF led to a strong reduction (~70%) of focus formation relative to the EGF-only control (Figure 7B). These results emphasize the important role of the JNK pathway in EGFR-dependent cell transformation.

Discussion

Recent findings support the general concept that the cellular communication network includes mechanisms which allow interactions and crosstalk between different signal transduction systems such as those employing G proteins or phosphotyrosine-containing elements (for review, see Hackel *et al.*, 1999). This enables cells to respond to varying and multiple environmental influences by using a limited repertoire of pathways. Similarly, one must consider interconnections in the network that allow crosstalk between individual signal emitters within one system such as that involving tyrosine phosphorylation and dephosphorylation of proteins.

Various members of the large PKC family of Ser/Thr kinases have been implicated in the regulation of signalling processes that are mediated by growth factor receptor tyrosine kinases (Hug and Sarre, 1993). Our previous work demonstrated that PKCα associates with different growth factor receptors such as those for EGF, insulin and PDGF, and we proposed that this interaction plays a role in the process of receptor signal modulation, internalization and degradation (Seedorf *et al.*, 1995a). Moreover, the distant PKC family members PKCε and PKCλ have been shown to become activated upon exposure of cells to EGF and PDGF through a signal mediated by PI3K (Yeo and Exton, 1995; Akimoto *et al.*, 1996; Moriya *et al.*, 1996). PKCs were also shown to be involved in a process termed transmodulation, where PDGF stimulation of Swiss 3T3 cells led to a decrease in high-affinity EGF binding sites and an increase in the serine/threonine phosphorylation state of the EGFR (Collins *et al.*, 1983; Zachary *et al.*, 1985). The initial observation that PDGF suppressed EGF-induced JNK activation via a PKC-dependent signal led to the search for the critical regulator of this signal transmodulation phenomenon. In transient 293 cell cotransfection experiments we employed a panel of PKC family members to identify the responsible PKC isoform, including the distant relative PKD, which had been shown to be activated by PDGF through a PKC-dependent pathway (Zugaza *et al.*, 1996). Our experiments identified PKD as a cell-type-characteristic, growth-factor-specific regulator of the JNK pathway and demonstrate the existence of a negative regulatory loop with PKD as a critical control element. In retrospect, these findings clarify a longstanding controversy regarding the ability of PDGF to utilize the JNK pathway (Geng *et al.*, 1996; Xie and Herschman, 1996; Weber *et al.*, 1997).

Another type of crosstalk was suggested earlier by Fenton and Sheffield (1997), who described that prolactin inhibits EGF-induced DNA synthesis and increases threonine phosphorylation of the EGFR in NMuMG mouse mammary carcinoma cells. Interestingly, similar to our findings, PKC inhibitors eliminated most of the inhibitory effect of prolactin on EGF-induced DNA synthesis. At present it is not known, however, whether the underlying mechanism responsible for prolactin-induced inhibition of EGF mitogenicity is identical to that elucidated in this study.

Some of the earliest structure–function studies that employed site-directed mutagenesis of the first cloned signal-generating cell-surface protein, the EGFR (Ullrich *et al.*, 1984), were aimed at PKC-mediated modulation of ligand binding affinity. In those studies the consequences of EGFR juxtamembrane domain phosphorylation on threonine residues T654 and T669 upon phorbol ester treatment were investigated (Hunter *et al.*, 1984; Doe *et al.*, 1988). Later, these phosphorylation sites were suggested to play a role in the PDGF-induced transmodula-

tion of the EGFR (Countaway *et al.*, 1989). However, until now mechanistic details of these phorbol ester- and PDGF-induced effects have remained unresolved. Our experiments clearly point to PKD as a critical element in the PDGF-mediated suppression of the EGFR signal leading to the activation of the JNK pathway. Evidence supporting such a mechanism is based on our mutational analysis of the EGFR juxtamembrane domain, which revealed that the effect of PKD on EGF-induced JNK activation is completely dependent on the two threonine residues T654 and T669 in the juxtamembrane region. Morrison *et al.* (1993) have shown previously that acidic residues replacing either T654 or T669 mimick the negative charge introduced by phosphorylation and modulate but do not block EGFR signalling. The EGF-binding properties of the mutant receptors were similar to those of the wild-type receptor, and EGF could stimulate tyrosine kinase activity and DNA synthesis in cells expressing both mutant receptors, indicating that both are essentially signalling competent and equivalent. At the onset of this study we concentrated on EGF-induced activation of JNKs and ERKs, and demonstrated that EGFR mutants with glutamate substitutions at either of residues 654 or 669 are capable of activating both pathways. A single phosphorylation event on these residues was insufficient to interfere with the EGFR signalling capacity. An EGFR mutant, however, in which both threonines were substituted by glutamic acid (HERc2E), was incapable of activating the JNK pathway. This demonstrated that phosphorylation of the two proximal threonine residues in the juxtamembrane region is a key regulatory event in the modulation of EGFR signal capacity. It remains to be established whether PKD alone or in conjunction with another kinase phosphorylates these threonine residues. Morrison *et al.* (1996) proposed that downregulation of EGFR tyrosine kinase activity by PKC involves activation of ERK by MEK. In that study, a specific MEK inhibitor prevented the decrease in EGFR tyrosine phosphorylation upon PKC activation. Thus, one must consider a scenario in which PKC-mediated activation of PKD, in conjunction with ERK activation, could lead to dual phosphorylation of the receptor. In fact, activators of PKC such as phorbol esters also activate ERKs, and T669 of the EGFR has been proposed to be a substrate of these kinases (Morrison *et al.*, 1993).

The HERc2E mutant was instrumental in the evaluation of the significance of dual phosphorylation in the juxtamembrane domain for modulating biological properties of the EGFR such as the transcriptional activation of specific genes. As a generally accepted mechanism, JNK is the major kinase responsible for phosphorylating and activating the transcription factor c-Jun which subsequently leads to transcription of the c-*jun* gene (Minden *et al.*, 1994). The role of JNK in the activation and induction of the transcription factor c-Fos is less clear and seems to involve co-operation with other factors (Cano *et al.*, 1995). For example, JNK is capable of activating the transcription factor Sap-1a, which can induce transcription of the c-*fos* gene in certain cell types, but there seemed to be a difference between Sap-1a activated by either JNK or ERK (Janknecht and Hunter, 1997). In this context, our findings with the EF-HERc$^{-/-}$ cell system clearly demonstrate that JNK and ERK pathways specifically induce c-*jun* or c-*fos* gene transcription, respectively. In other cell types, however, these signals may be regulated by mechanisms other than PKD-mediated phosphorylation of RTKs such as the EGFR.

Previous studies have demonstrated an important role for JNK in EGF-stimulated cell growth. For example, Bost *et al.* (1997), using A549 lung carcinoma cells, reported that the JNK pathway is the preferential effector pathway for EGF-induced growth by demonstrating that JNK1 and JNK2 antisense oligonucleotides completely blocked a proliferative response.

JNK has been implicated in EGF-dependent transformation and it has been shown that constitutive activation of JNK mediates oncogenic signals generated by an EGFR-variant type III mutant found in a variety of human tumours (Antonyak *et al.*, 1997). Moreover, as shown by Smeal *et al.* (1992), phosphorylation of the c-Jun protooncogene is required for cell transformation by activated Ras. We report here that the EGFR mutant HERc2E, which is incapable of stimulating JNK, showed a highly reduced capacity to transform NIH 3T3 cells. Interestingly, the same inhibitory effect is achieved by simultaneous stimulation with PDGF, which further emphasizes the important role of JNK in EGFR-dependent transformation. Thus, in addition to the previously established role of serine residues S1046 and S1047 (Theroux *et al.*, 1992), our findings demonstrate threonines T654 and T669 as additional negative regulatory residues of the EGFR and connect these determinants with normal and pathophysiological responses of the cell.

The exact mechanism by which dual threonine phosphorylation in the juxtamembrane region of the EGFR downregulates downstream processes is currently not understood. It might involve the subcellular localization and downregulation of the EGFR, as Seedorf *et al.* (1995b) had shown that coexpression of PKCα with EGFR, PDGFR and insulin receptor chimeras resulted in increased internalization and degradation of receptors upon PMA treatment. Thus, one might speculate that dual phosphorylation of the EGFR mediated by PKD could lead to accelerated degradation or altered internalization, which would uncouple the receptor from the JNK pathway. The fact that ERK activation is not affected by phosphorylation of the EGFR on T654 and T669 could be explained by differential time courses of the respective signals, with the ERK pathway already fully activated before the EGFR becomes transmodulated. This possibility is supported by the activation kinetics of JNK (maximum at 10 min) and ERK (maximum at 1 min) in Rat-1 cells (data not shown). Recently, a cytoplasmic and JNK-specific inhibitor, p21WAF1, has been described, which can block UV-induced JNK activation *in vivo* and *in vitro* (Shim *et al.*, 1996). Receptor threonine phosphorylation might trigger activation or recruitment of such inhibitory molecules that are specific for JNKs or may alter the influence of positive regulators. Heisermann *et al.* (1989) showed, for example, that mutation of threonine T669 and serine S671 of the EGFR results in impaired phosphorylation of an 85 kDa cellular substrate, indicating that the mutated region may specifically interact with this EGFR substrate. Another mechanism could involve a PDGF- and TPA-sensitive EGFR protease, as described for HER4, where PDGF or TPA treatment induces the proteolytic

cleavage through activation of PKC (Vecchi *et al.*, 1996). Threonine phosphorylation of the EGFR could render it susceptible to proteolytic degradation, thereby suppressing EGF-induced JNK activation.

In summary, dual threonine phosphorylation of the EGFR represents a potent mechanism for the selective control of specific signalling pathways and thereby contributes to the programming of the biological destiny of cells. Crosstalk between different signal-generating molecules such as the transmodulation of the EGFR by PDGF enables a cell to integrate signals from different growth factors, thereby coordinating and defining complex responses. Such mechanisms ultimately determine differential gene regulation in cellular processes such as growth, differentiation and survival, and may be critical in pathophysiological phenomena such as oncogenesis.

Materials and methods

Reagents, antibodies, cell lines and plasmids

NIH 3T3 clone 7 fibroblasts, human glioblastoma cells (SF126) and 293 fibroblasts were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma) at 37°C and 5% CO_2. Rat-1 fibroblast cells were a gift from R.Friedrich and were cultured in DMEM supplemented with 5% fetal calf serum. Embryonic fibroblast HERc$^{-/-}$ cells were a generous gift from E.F.Wagner (Sibilia and Wagner, 1995). Culture media were purchased from Gibco-BRL. Protein A–Sepharose was obtained from Pharmacia/Amersham Biotech Inc., and GF 109203X from Calbiochem. PDGF-BB was bought from Boehringer Mannheim. All other reagents were obtained from Sigma.

Polyclonal anti-flag, anti-EGFR, anti-PKD and anti-ERK1/2 antibodies were purchased from Santa Cruz (California). The human EGFR was precipitated with mouse monoclonal antibody 108.1 (Seedorf *et al.*, 1994); endogenous and exogenous PKD was immunoprecipitated with mouse monoclonal anti-PKD antibody (SUGEN, Inc.). The respective secondary antibodies were obtained from Bio-Rad or Dianova. For immunoblot detection, the ECL system from Pharmacia/Amersham was used. Stripping and reprobing of blots were performed according to the manufacturer's recommendations.

The plasmid encoding the GST–c-Jun fusion protein was cloned as described earlier (Hibi *et al.*, 1993) and its identity was verified by sequencing. All cDNAs used for transient transfections were cloned in cytomegalovirus-based expression plasmids. The respective DNA inserts coded for wild-type HERc (Ullrich *et al.*, 1984), HERcT654A, HERcT654E, HERcT669A, HERcT669E, HERcT654/669E (HERc2E) and wild-type PKD (Johannes *et al.*, 1994). pLXSN retroviral expression vectors containing the HERc cDNAs described above were generated by cloning the respective mutant PflMI fragments at the same site in pLXSN HERc wt (PflMI cut). Human JNK1 was tagged with a flag epitope by PCR (Derijard *et al.*, 1994).

Cell culture and transfections

Transient transfections for expression in mammalian cells were carried out using a modified calcium phosphate method (Weiss *et al.*, 1997). Six micrograms of expression plasmid were used for transfection of 1.8×10^6 cells per 10 cm dish. After incubation overnight in 3% CO_2, the medium was removed and cells were starved for 24 h in FCS-free DMEM.

Cell lysis, immunoprecipitation, association with fusion proteins and immunoblotting

Prior to lysis, cells grown to 80–90% confluence were treated with inhibitors and agonists as indicated, washed once with phosphate-buffered saline and then lysed for 10 min on ice in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin. Lysates were cleared by centrifugation at 13 000 r.p.m. for 10 min at 4°C. Supernatants were diluted with an equal volume of HNTG buffer (Seedorf *et al.*, 1994) and subsequently immunoprecipitated using the respective antibodies and 30 μl protein A–Sepharose for 4 h at 4°C. Alternatively, lysates were subjected to *in vitro* associations with 3 μg GST–c-Jun (1–79) prebound to 30 μl gluthathione–agarose beads. Precipitates were washed three times with 0.5 ml HNTG buffer, suspended in SDS sample buffer, boiled for 3 min and subjected to gel electrophoresis on 7.5% gels or 12.5% gels. Following SDS–PAGE, proteins were transferred to a nitrocellulose membrane and immunoblotted as described previously (Wallasch *et al.*, 1995). Detection was performed by addition of a peroxidase-coupled secondary antibody (Bio-Rad), followed by an enhanced chemiluminescence (Amersham) reaction, which was visualized on Kodak X-Omat film. Filters which were reprobed with different antibodies were stripped according to the manufacturer's protocol.

MAP kinase gel shift assay

Total cell lysates were separated on 7.5% SDS–polyacrylamide gel and analysed by immunoblotting with anti-ERK1+ERK2 antibodies. A peroxidase-coupled secondary antibody (Bio-Rad), followed by an enhanced chemiluminescence (Amersham) reaction, was used for detection.

Immunocomplex in vitro kinase assays

Epitope-tagged JNK1-flag was immunoprecipitated from lysates obtained from 6-cm dishes using 2.5 μg polyclonal anti-flag antibody; endogenous PKD was immunoprecipitated from 10-cm dishes using 2 μg monoclonal anti-PKD antibody. Immunoprecipitates were washed three times with 0.25× HNTG buffer and once with 0.4 ml of kinase buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM sodium orthovanadate). Subsequently, kinase reactions were performed in 30 μl kinase buffer supplemented with 3 μg GST–c-Jun (1–79) (JNK assay) or 0.5 mg/ml histone H1 (PKD assay), and 50 μM ATP and 1 μCi [γ-^{32}P]ATP (6000 Ci/mmol) for 20 min at 30°C. Reactions were stopped by addition of 30 μl of 2× SDS sample buffer and subjected to gel electrophoresis on 12.5% gels. Phosphorylated substrates were visualized by autoradiography on Kodak X-Omat films. Quantitative analysis was performed with a Phosphoimager (Fuji).

Solid-phase protein kinase assay

Solid-phase protein kinase assays were performed as described by Hibi *et al*. (1993). Clarified cell extracts were incubated with GST–c-Jun (1–79) fusion proteins immobilized on GSH–agarose beads. After 3 h at 4°C, the beads were washed three times with 0.25 HNTG buffer and once with 0.4 ml kinase buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 μM sodium orthovanadate). The bound JNK activity was detected by the addition of 1 μCi [γ-^{32}P]ATP (6000 Ci/mmol). The reaction was terminated after 20 min at 30°C and the products were resolved by SDS–PAGE. The incorporation of [^{32}P]phosphate was visualized by autoradiography.

RNA extraction and Northern blot analysis

Rat-1 or EF cells were subjected to various treatments as indicated. Total cytoplasmic RNA was extracted as described (Ullrich *et al.*, 1984). RNA samples (10 μg) were separated on a 1.4% agarose–formaldehyde gel and transferred to nitro-cellulose for hybridization with α-^{32}P-labelled cDNA probe of c-Jun or c-Fos full coding sequence. Blots were exposed using an intensifying screen.

[^{3}H]thymidine incorporation into DNA

Polyclonal murine embryonic fibroblast (EF) cells were seeded into 12-well plates (7.5×10^4 cells/well) and serum-starved for 24 h. Cells were subjected to 30 min preincubation with 50 μM PD98059 (MEK1 and 2 inhibitor) or dimethylsulfoxide (DMSO) and were grown either without or with 100 ng/ml EGF. After 18 h incubation, cells were pulse-labelled with [^{3}H]thymidine for 4 h (0.5 μCi/well) and its incorporation into DNA was determined as described earlier (Redemann *et al*., 1992).

Retrovirus-mediated gene transfer and focus formation assay

To produce virus-encoding wild-type or mutant HERc cDNAs, BOSC23 cells were transiently transfected with pLXSN retroviral expression plasmids as described by Pear *et al*. (1993). The virus-containing supernatants of the transfected cells were used to infect subconfluent NIH 3T3 cells (10^5 cells/6-cm dish) for 4 h in the presence of polybrene (4 μg/ml). Equal amounts of retrovirus containing the cDNAs of wild-type HERc, HERc2E or the vector (pLXSN) (m.o.i. = 0.1) were utilized for infection. Cells were cultured in 4% FCS with or without addition of 50 ng/ml EGF. Medium was changed every second day and cell foci

were stained after 14 days with crystal violet (0.1% crystal violet, 30% methanol).

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