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Truncated, desensitization-defective neurokinin receptors mediate sustained MAP kinase activation, cell growth and transformation by a Ras-independent mechanism

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We have used the neurokinin NK-2 receptor as a model to examine how receptor desensitization affects cellular responses. The liganded receptor transiently activates phospholipase C (PLC) and is rapidly phosphorylated on Ser/Thr residues in its C-terminal domain. Mutant receptors lacking this domain mediate persistent activation of PLC. We now show that, in transfected Rat-1 cells, mutant receptor mediates ligand-induced DNA synthesis, morphological transformation and growth in soft agar, whereas wild-type (wt) receptor does not. Wt receptor causes only transient MAP kinase activation. In contrast, MAP kinase activation by mutant receptor is sustained for >4 h. Neither wt nor mutant receptor couples to Ras activation. Down-regulation of protein kinase C (PKC) has little effect on MAP kinase activation, DNA synthesis and transformation. Mutant receptors also promote stronger protein tyrosine phosphorylation and stress fibre formation than does wt receptor. Thus, C-terminal truncation allows the NK-2 receptor to signal sustained MAP kinase activation, cell growth and transformation by a Ras- and PKC-independent mechanism. Our results reveal the importance of the C-terminal 'desensitization domain' in suppressing the oncogenic potential of a prototypic PLC-coupled receptor.

Keywords: G protein-coupled receptor/MAP kinase/mitogenesis/phospholipase C/transformation

Introduction

There is currently much interest in elucidating the principles of mitogenic signalling by G protein-coupled receptors. It has been established recently that certain G protein-linked receptors share common signalling pathways with receptor tyrosine kinases to stimulate cell proliferation. In particular, agonists acting on receptors that couple to pertussis toxin (PTX)-sensitive G_i proteins mimic peptide growth factors in rapidly stimulating Ras-GTP accumulation to activate the mitogen-activated-protein (MAP) kinase cascade leading to induction of DNA synthesis (Alblas *et al.*, 1993; Cook *et al.*, 1993; Howe and Marshall, 1993; van Corven *et al.*, 1993; Crespo *et al.*, 1994; van Biesen *et al.*, 1995). In contrast, receptors that act through PTX-insensitive G_q proteins, which activate the phospholipase C (PLC)–protein kinase C (PKC) pathway, are usually not mitogenic by themselves; for example, in the absence of additional growth factors, agonists like bradykinin, endothelin, serotonin or carbachol acting on their cognate G_q–PLC-coupled receptors fail to signal fibroblast proliferation (for review, see Moolenaar, 1991; Pouysségur and Seuwen, 1992). Given the ability of PKC to bypass Ras to activate the MAP kinase cascade (Blumer and Johnson, 1994; Marshall,1994; Cobb and Goldsmith, 1995), it is somewhat surprising that PLC-activating receptors are not mitogenic.

A common property of G protein-linked receptors is that prolonged or repeated agonist exposure leads to signal attenuation and loss of cellular responses over time ('desensitization'). One obvious function of desensitization is to prevent generation of constitutive signals that could disrupt normal cell behaviour. Thus, from a mitogenic signalling point of view, receptor-mediated signal shutoff may serve to prevent quiescent cells from unscheduled entry into the cell cycle. The molecular basis of desensitization is complex and has been best studied in the β-adrenergic receptor. One major mechanism involves ligand-induced phosphorylation of the receptor's cytoplasmic tail by various Ser/Thr kinases, including PKC, protein kinase A and specific receptor kinases (Premont *et al.*, 1995). The available data support a model in which ligand-induced phosphorylation of the C-terminal domain interferes, either directly or indirectly, with the coupling of the receptor to the relevant G protein(s) and thereby shuts off signalling.

Recently, we set out to examine how signal transduction via PLC-coupled receptors is altered when their putative 'desensitization domain' is deleted. Our approach makes use of the human NK-2 receptor for the decapeptide neurokinin A (NKA, or substance K). We showed that this receptor undergoes rapid ligand-induced Ser/Thr phosphorylation and is subject to desensitization (Alblas *et al.*, 1995). Removal of the last 60 or 70 residues from the receptor's tail does not affect ligand binding but abolishes ligand-induced receptor phosphorylation and signal attenuation; when expressed in Rat-1 cells, these truncated receptors mediate persistent rather than transient activation of PLC (Alblas *et al.*, 1995).

Here, we have examined the long-term cellular consequences of defective receptor desensitization. We find that C-terminal truncation confers dramatic mitogenic and transforming properties on the resulting mutant receptor. Receptor-mediated mitogenesis is attributable to sustained activation of MAP kinase in a Ras- and PKC-independent manner, whereas oncogenic transformation proceeds via a different pathway. Our results highlight the critical role of the C-terminal desensitization domain of a prototypic PLC-coupled receptor in regulating the duration of MAP kinase activation and in suppressing the oncogenic potential of such receptors.

Results

Stimulation of DNA synthesis by truncated but not wild-type NK-2 receptor

We have generated Rat-1 fibroblasts that stably express either wild-type (wt) or C-terminally truncated NK-2 receptors at modest densities (~10 000 receptors/cell). Mutant and wt receptors show similar affinities for NKA (Alblas *et al.*, 1995).

Serum-starved transfectants were tested for their mitogenic responsiveness to NKA. Figure 1 shows that activation of wt receptor fails to stimulate DNA synthesis under conditions where epidermal growth factor (EGF) evokes a strong response. In marked contrast, addition of NKA (1 μM) to cells expressing the truncated Δ328 receptor (Δ328 cells), lacking 70 C-terminal residues (Alblas *et al.*, 1995), results in significant stimulation of DNA synthesis. The mitogenic response to NKA is about equal to that observed with EGF (10 ng/ml), lysophosphatidic acid (LPA; 50 μM) or serum (Figure 1 and results not shown). Qualitatively similar effects were observed in cells expressing another deletion mutant of the NK-2 receptor (Δ338), which lacks 60 C-terminal residues (Alblas *et al.*, 1995). The latter transfectants have a somewhat elevated receptor density (~26 000/cell). In our further experiments, we focused mainly on wt and Δ328 receptors because of their comparable expression levels in Rat-1 cells; yet, where tested, mutant Δ338 behaved in a qualitatively similar manner to Δ328.

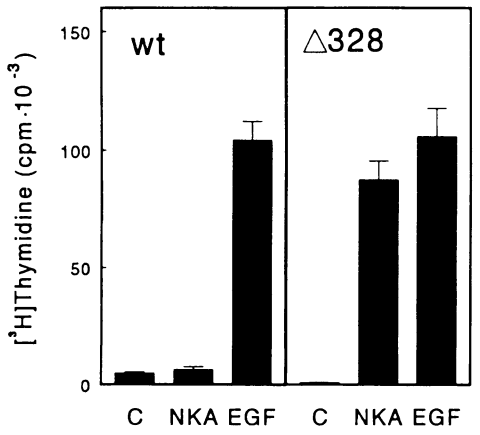

Fig. 1. Agonist-induced DNA synthesis in Rat-1 cells expressing either wt or truncated Δ328 NK-2 receptor. Serum-starved cells were stimulated for 26 h with NKA (1 μM) or EGF (10 ng/ml), and [^{3}H]thymidine incorporation was measured as described in Materials and methods.

NKA-induced DNA synthesis in Δ328 cells was found to be completely insensitive to PTX (100 ng/ml), indicating that G_i-coupled effector pathways are not involved.

Truncated receptor causes phenotypic transformation in response to NKA

In the absence of NKA, confluent Rat-1 cells expressing either wt or truncated NK-2 receptors show a flattened, contact-inhibited morphology indistinguishable from that of non-transfected cells. Remarkably, prolonged (>10 h) NKA treatment of Δ328 (or Δ338) cells results in dramatic morphological changes, which are not observed in cells expressing wt receptor. NKA-treated Δ328 cells show many hallmarks of transformation, including a refractile spindle-like morphology, loss of contact inhibition and an unorganized criss-cross growth pattern (Figure 2). Small foci of rounded cells were often observed. Furthermore, NKA-treated Δ328 cells detached much more easily from the dish than did control cells. These morphological changes were not observed in cultures treated with EGF or LPA (although EGF induced some criss-cross growth, but not focus formation).

The first signs of morphological transformation were observed at 10–12 h after NKA addition. Full phenotypic transformation persisted for at least 48 h. The mutant receptor-induced phenotype required the continuous presence of ligand: wash-out of NKA resulted in gradual reversal of transformation; furthermore, when focus-forming cells were replated in fresh medium without NKA, they adopted their normal morphology.

Rat-1 cells expressing Δ328 receptors also produced colonies in soft agar in a strictly ligand-dependent manner, as shown for three different transfectants in Figure 3. Under the same experimental conditions, wt receptor failed to mediate colony formation in soft agar (Figure 3).

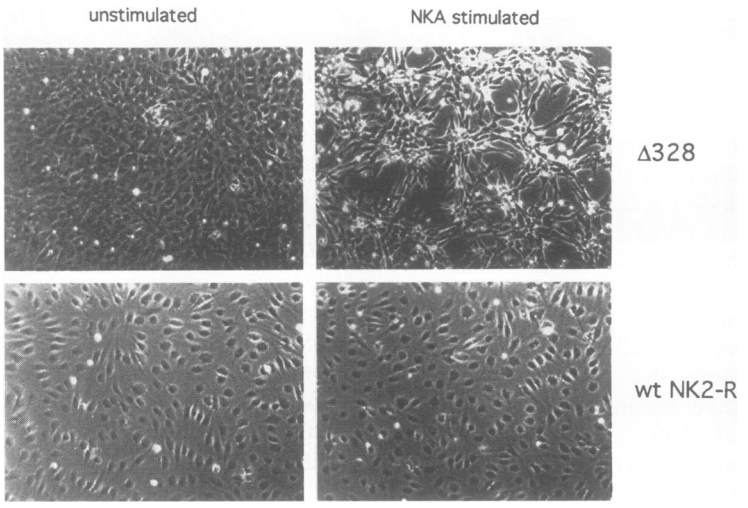

Fig. 2. Long-term morphological changes mediated by truncated NK-2 receptor in response to ligand. Rat-1 cells expressing either wt or Δ328 receptor were grown to confluency, serum starved for 16 h and then stimulated with NKA (1 μM). Cells were photographed after 18 h of NKA addition. Pictures shown are representative of numerous experiments using independently derived single-cell clones of Δ328 transfectants.

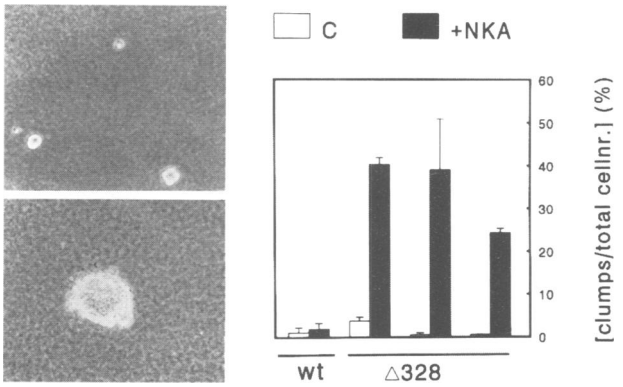

Fig. 3. NKA-induced cell proliferation in soft agar. Rat-1 cells expressing wt or mutant Δ328 NK-2 receptor were seeded and grown in complete, agar-containing medium in the presence or absence of NKA, as described in Materials and methods. After 3 weeks, cells grown to clumps larger than 10 cells were scored, photographed and given as percentage of total number of cells. Upper photograph (left panel), typical Δ328 cell culture in the absence of NKA; clumps shown contain <10 cells. Lower photograph, NKA-induced formation of colony (>10 cells) of Δ328 cells. The diagram (right panel) represents colony formation in soft agar; cells expressing either wt or Δ328 receptor were grown in the absence (white bars) or presence of NKA (black bars). For Δ328 cells, three independently derived cell clones were examined. Experiments were performed in duplicate.

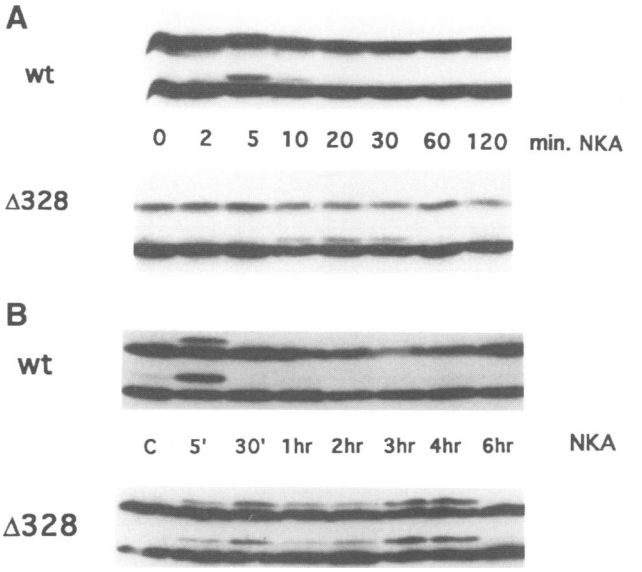

Fig. 4. Time course of MAP kinase activation in Rat-1 transfectants in response to NKA. Cells expressing wt NK-2 receptor or mutant Δ328 were stimulated for the indicated periods of time with NKA (1 μM) and assayed for reduced mobility of p42/p44 MAP kinase by immunoblotting. (**A**) Early (<2 h) activation induced by NKA. (**B**) Prolonged phase of activation (0–6 h) in response to NKA. See text for details.

Transient versus sustained activation of MAP kinase

Sustained activation of MAP kinase is critical for growth factor-induced fibroblast proliferation (Meloche *et al.*, 1992; Pages *et al.*, 1993; Marshall, 1994). We examined the kinetics of MAP kinase activation mediated by both wt and truncated NK-2 receptor in Rat-1 cells, using anti-MAP kinase immunoblots where the activated, phosphorylated p42/p44 MAP kinases display a reduced electrophoretic mobility.

Wt receptor mediates rapid but transient activation of MAP kinase, peaking at ~5 min and lasting <20 min (Figure 4A and B). However, MAP kinase activation mediated by Δ328 receptor shows markedly different kinetics. In the continuous presence of NKA, MAP kinase activation is seen to be biphasic: an early transient phase is followed by a second activation phase which is sustained for at least 4 h (Figure 4B); thereafter, at ~6 h, MAP kinase activity returns to control levels. Such a prolonged phase of MAP kinase activity was also seen when cells were stimulated with EGF or LPA (results not shown). We note that, in ~50% of the experiments, the early transient phase of MAP kinase activation was delayed by 15–20 min when compared with wt receptor (for example see Figure 4A). We attribute this temporary inhibition to the rapid elevation of intracellular cAMP levels that occurs in NKA-treated Δ328 cells (Alblas *et al.*, 1995; see also below).

Neither wt nor mutant receptor couples to Ras activation

Mitogenic receptors couple to the MAP kinase cascade via activation of the small GTP binding protein Ras. In common with receptor tyrosine kinases, mitogenic G_i-coupled receptors, such as those for LPA and thrombin, mediate rapid accumulation of Ras-GTP in quiescent fibroblasts (van Corven *et al.*, 1993). Also, α_2-adrenergic and muscarinic-m2 receptors activate Ras in a fibroblast context (Alblas *et al.*, 1993; Winitz *et al.*, 1993). We examined the ability of both wt and Δ328 receptor to activate Ras by immunoprecipitating the protein from ^{32}P-labelled cells and determining the relative amounts of GTP versus GDP bound. Figure 5A and B shows that NKA stimulation of wt receptor leaves Ras-GTP levels unaltered, whereas EGF evokes a significant response. Similarly, stimulation of Δ328 cells did not result in detectable Ras-GTP accumulation, as measured after either 5 min or 3 h of NKA addition (Figure 5A and B).

We also examined whether dominant-negative Ras interferes with MAP kinase activation by mutant receptor. To this end, we co-transfected COS-7 cells with cDNA encoding NK-2 receptor along with Myc epitope-tagged p42 MAP kinase (Howe and Marshall, 1993). Activation of MAP kinase by the transfected NK-2 receptor or by endogenous EGF or LPA receptors is readily detectable by *in vitro* myelin basic protein (MBP) phosphorylation (Figure 6, upper panel). Co-expression of dominant-negative Ras (N17Ras) inhibited EGF- and LPA-induced MAP kinase activation, but failed to affect the NK-2 receptor-mediated response (Figure 6, upper panel). It thus appears that the NK-2 receptor bypasses Ras to activate MAP kinase.

Involvement of Raf-1

Having shown that Ras is not required for MAP kinase activation by Δ328 receptor, we investigated whether the $p74^{raf-1}$ kinase (Raf) is involved in this pathway. To this end, we co-transfected into COS cells a dominant-negative Raf construct encoding the N-terminal region of Raf (NΔRaf; Schaap *et al.*, 1993). Figure 6 (lower panel) shows that expression of dominant-negative Raf inhibits MAP kinase activation by NK-2 receptor as well as the

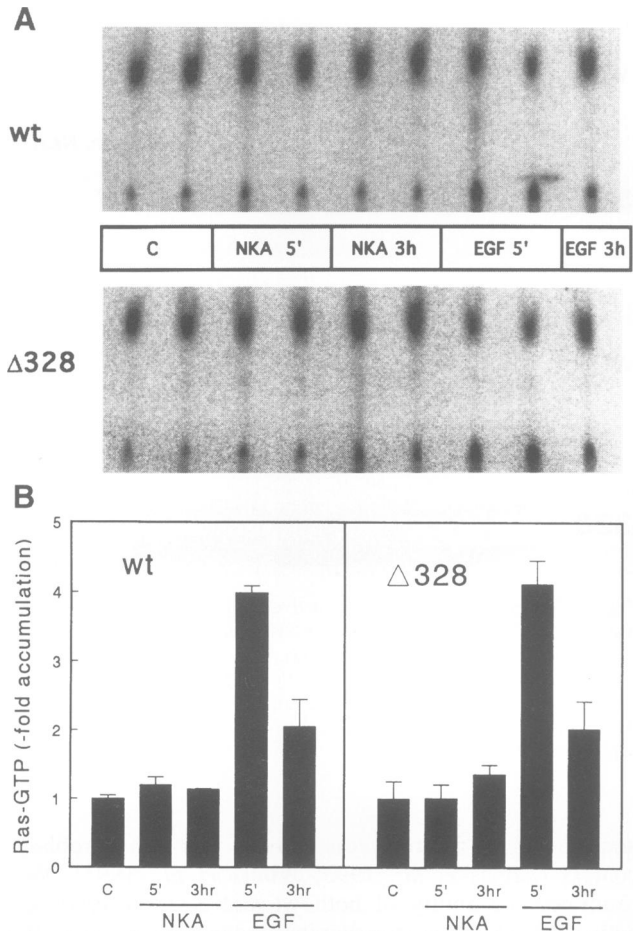

Fig. 5. Agonist-induced Ras activation. Rat-1 cells expressing either wt NK-2 receptor or Δ328 were assayed for GTP-loading of $p21^{ras}$ (Ras) after stimulation of $^{32}P_i$-labelled cells with NKA (1 μM) or EGF (10 ng/ml) for 5 min or 3 h, as indicated. Ras was immunoprecipitated and relative amounts of GTP and GDP content were determined (see Materials and methods). (**A**) Typical PhosphoImager autoradiograph of ^{32}P-labelled GDP and GTP separated by TLC. (**B**) Quantitation of the relative amount of ^{32}P in Ras-GTP (normalized to 1.0). The fold-increase in Ras-GTP levels shown represents the mean and standard error (bars) determined for three separate experiments.

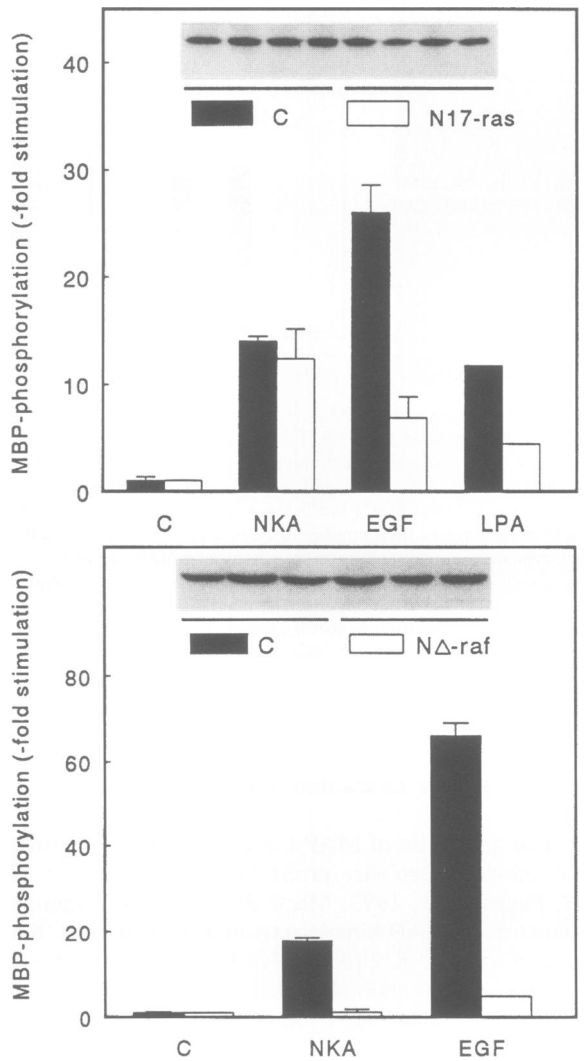

Fig. 6. (Upper panel) Effect of dominant-negative Ras (N17ras) on MAP kinase activation. COS-7 cells were transfected with an epitope-tagged MAP kinase construct together with NK-2 receptor and either N17ras or control plasmid. After serum starvation, cells were stimulated for 10 min with NKA (1 μM), EGF (10 ng/ml) or LPA (10 μM). Following immunoprecipitation, epitope-tagged MAP kinase was subjected to *in vitro* kinase assays using MBP as a substrate (see Materials and methods). Inset: Western blot of transfected p42 MAP kinase, showing that MAP kinase protein levels are comparable in all experiments. Bars represent the amount of ^{32}P incorporated into MBP. Experiments were performed in duplicate. (Lower panel) Effect of dominant-negative Raf (NΔraf) on agonist-induced MAP kinase activation. Experimental conditions as in upper panel. Inset: Western blot of transfected p42 MAP kinase, showing equal protein levels.

EGF receptor. Furthermore, when NΔRaf was transiently transfected into Δ328-Rat-1 cells, NKA-induced DNA synthesis in the transfected cell population was inhibited, as it was in EGF-treated cells (Figure 7). From these data, we conclude that Raf-1 is required for mitogenic signalling by the Δ328 receptor. Support for this notion comes from experiments using 8Br-cAMP, which inhibits the MAP kinase cascade at the level of Raf-1 (see below).

Correlation between phosphoinositide hydrolysis and MAP kinase activation

In NKA-stimulated Δ328 cells, sustained MAP kinase activation correlates with prolonged activation of PLC. However, the persistent formation of inositol phosphates in these cells does not lead to changes in the kinetics of the receptor-mediated rise in cytosolic Ca^{2+} (Alblas *et al.*, 1995). This eliminates the possibility that Ca^{2+} signalling contributes to prolonged MAP kinase activation. One would rather predict that activation of PKC provides the signal. To test the possible involvement of PKC, we down-regulated PKC by long-term (24 h) treatment of the cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Immunoblot analysis confirmed that this treatment results in the disappearance of at least two major PKC subtypes (α- and ε- isoforms, but not the TPA-insensitive ζ isotype; not shown). However, both the transient and the sustained phase of MAP kinase activation were only slightly affected by PKC down-regulation (Figure 8A and B). Also, NKA-induced DNA synthesis was not significantly affected by PKC down-regulation, neither were the long-term morphological alterations.

Addition of the PKC inhibitor Ro-31-8220 (3 μM), which inhibits the activity of all PKC isotypes (Davis

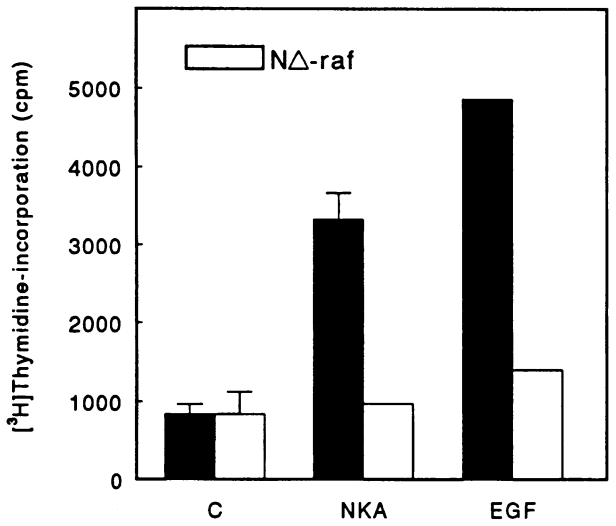

Fig. 7. Effect of dominant-negative Raf on induction of DNA synthesis. Δ328 cells transiently transfected with NΔraf and CD8 or control plasmid were stained with antibody against CD8. The CD8-positive cell population was sorted by FACS and then replated, serum starved for 24 h, stimulated with NKA (1 μM) or EGF (25 ng/ml) for 24 h and then assayed for [^{3}H]thymidine incorporation (see Materials and methods).

et al., 1989), has only a minor effect on NKA-induced MAP kinase activation (Figure 8B). Taken together, these results argue against a critical role for PKC in mediating MAP kinase activation.

Mutant receptor mediates enhanced protein tyrosine phosphorylation and stress fibre formation

G protein-coupled receptors that activate PLC trigger rapid tyrosine phosphorylation of cytoskeletal proteins, such as the 125 kDa focal adhesion kinase (FAK) and paxillin (68 kDa; Zachary *et al.*, 1993; Hordijk *et al.*, 1994a; Seufferlein and Rozengurt, 1994). Correlating with these tyrosine phosphorylations is the rapid formation of focal adhesions and actin stress fibres (Ridley and Hall, 1994). We found that while wt NK-2 receptor stimulation causes little increase in phosphotyrosine levels, the activated Δ328 receptor induces significant tyrosine phosphorylation, particularly of proteins in the regions 110–130 and 60–75 kDa comprising FAK and paxillin, respectively (Figure 9). Increased tyrosine phosphorylation persisted for at least a few hours. A similar tyrosine phosphorylation pattern is observed in LPA- or endothelin-treated Rat-1 cells, but there the phosphorylation kinetics are very transient (Hordijk *et al.*, 1994a).

In common with other PLC-coupled receptors, wt NK-2 receptor mediates rapid formation of actin stress fibres in serum-starved Rat-1 cells (Figure 10; Postma *et al.*, 1996), although the response is less pronounced than observed with LPA or endothelin. However, stress fibres formed through the activated mutant receptor are much more abundant and prominent than those induced by wt receptor (Figure 10). Thus, there is a quantitative correlation between the degree and duration of phosphoinositide breakdown, protein tyrosine phosphorylation and concomitant stress fibre formation.

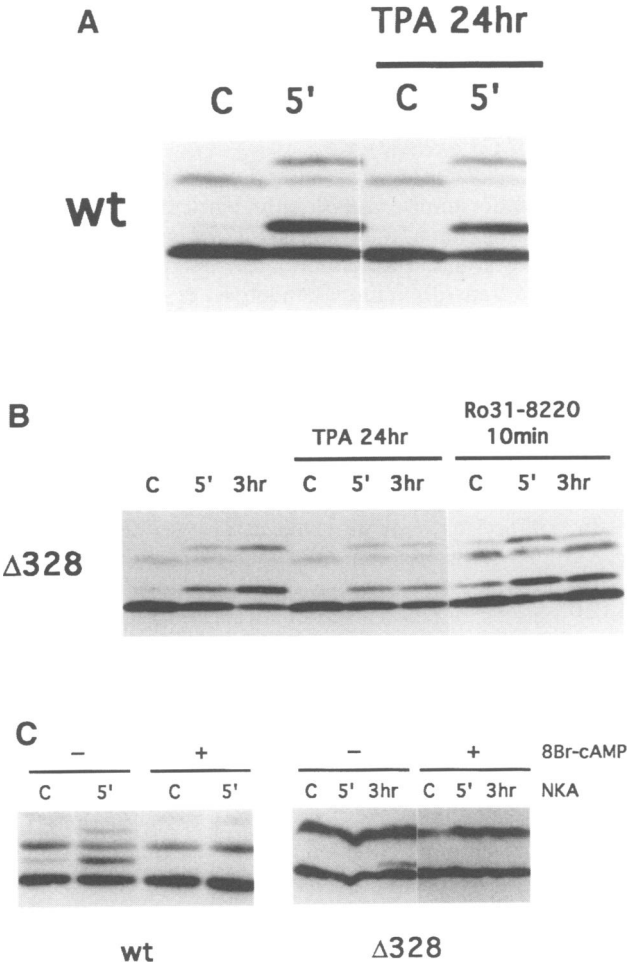

Fig. 8. (**A** and **B**). Effects of down-regulation or inhibition of PKC on MAP kinase activation. Where indicated, wt receptor-expressing cells (A) or Δ328 cells (B) were pre-treated for 24 h with TPA (100 ng/ml) to down-regulate conventional PKC isotypes. Ro-31-8220 (3 μM, 10 min treatment) was used as an inhibitor of all known PKC isotypes. (**C**) Inhibitory effect of 8Br-cAMP (1 mM) on NKA-induced MAP kinase activation. Cells were stimulated with NKA for 5 min or 3 h, and MAP kinase activation was detected by immunoblotting.

Morphological transformation by mutant receptor is uncoupled from sustained MAP kinase activation

Sustained activation of the MAP kinase cascade is thought to be necessary and sufficient not only for mitogenesis but also for oncogenic transformation (Cowley *et al.*, 1994; Mansour *et al.*, 1994). We used 8Br-cAMP to examine whether MAP kinase activation is required for morphological transformation of NKA-treated Δ328 cells. We found that 8Br-cAMP (1 mM) blocks the early transient as well as the sustained phase of MAP kinase activation in both wt and Δ328 receptor-expressing cells, respectively (Figure 8C). MAP kinase inhibition by cAMP occurs at the level of Raf kinase activation, although the precise mechanism remains to be elucidated (Cook and McCormick, 1993; Hordijk *et al.*, 1994b; for review, see Burgering and Bos, 1995). As expected, 8Br-cAMP (1 mM) also blocks NKA-induced mitogenesis in Δ328 cells (not shown). However, 8Br-cAMP had no detectable effect on NKA-induced morphological changes in Δ328 cells (focus formation, cell rounding, criss-cross growth).

Thus, it appears that morphological transformation induced by mutant NK-2 receptor is uncoupled from sustained MAP kinase activation.

Effects of C3 exoenzyme

Receptor-mediated changes in cell morphology are regulated by Rho family GTPases. In particular, active Rho triggers formation of focal adhesions and actin stress fibres (Hall, 1994) and, furthermore, has been implicated in oncogenic transformation (Prendergast *et al.*, 1995). Rho signalling is inhibited by ADP ribosylation using the *Clostridium botulinum* C3 exoenzyme. While C3 pre-treatment (30 μg/ml; 4 h) abolished NKA-induced stress fibre formation, it did not inhibit long-term morphological changes, focus formation and criss-cross growth (not shown), indicating that phenotypic transformation of Δ328 cells does not depend on functional Rho.

In addition to regulating the actin cytoskeleton, Rho can trigger transcriptional events and is required for cell cycle progression through G_1 independent of the p42/p44 MAP kinases, at least in serum-stimulated 3T3 cells (Hill *et al.*, 1995; Olson *et al.*, 1995). Consistent with this notion, treatment of Δ328 cells with C3 exoenzyme (30 μg/ml; 4 h pre-treatment) leads to a 30–40% inhibition of NKA-induced DNA synthesis (Figure 11).

Discussion

We have examined how disruption of receptor desensitization affects G protein-mediated signalling and cellular phenotype, using the G_q–PLC-coupled NK-2 receptor as a model. We recently showed that deletion of the phosphorylatable tail causes the receptor to couple to persistent activation of PLC in response to ligand (Alblas *et al.*, 1995). Here we show that such desensitization-defective receptors have acquired dramatic mitogenic and transforming properties. This gain-of-function is attributable, at least in part, to the mutant receptor's ability to activate MAP kinase in a sustained rather than transient manner. Furthermore, we show that impaired receptor desensitization results in enhanced agonist-induced protein tyrosine phosphorylation and cytoskeletal changes.

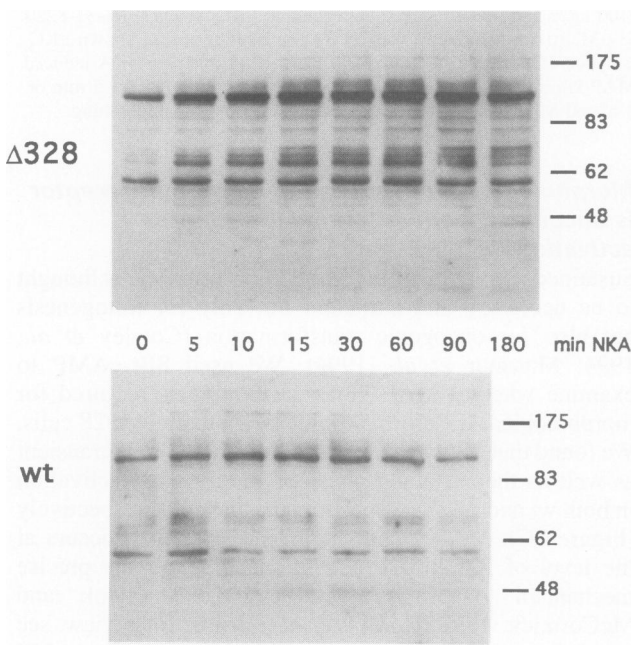

Fig. 9. Induction of protein tyrosine phosphorylation. Rat-1 cells expressing Δ328 or wt NK-2 receptor were stimulated with NKA (1 μM) for the indicated periods of time. Cell lysates were immunoblotted with monoclonal antibody 4G10. The positions of the molecular weight standards (kDa) are shown on the right.

Sustained MAP kinase activation and mitogenesis

There is increasing evidence that the duration of p42/p44 MAP kinase activation determines long-term cellular responses (reviewed by Marshall, 1995). For example, in PC12 cells, prolonged MAP kinase activity [induced by nerve growth factor (NGF)] is thought to direct neurite outgrowth and differentiation, whereas transient activation (by EGF) represents a mitogenic signal. In quiescent fibroblasts, on the other hand, sustained p42/p44 MAP kinase activation is essential for growth factor-induced stimulation of DNA synthesis (Meloche *et al.*, 1992; Pages *et al.*, 1994; Marshall, 1995). The latter notion is fully supported by the present study, although sustained p42/p44 MAP kinase activation is not necessarily sufficient for a full mitogenic response, as the Rho GTPase contributes to stimulation of DNA synthesis (cf. Figure 11) through a pathway independent of the p42/p44 MAP kinases (Hill *et al.*, 1995; Olson *et al.*, 1995).

Defective desensitization at the receptor level, as reported here, is a hitherto unrecognized way for receptors to increase the duration of p42/p44 MAP kinase activation in response to ligand. One known pathway for receptors to signal prolonged MAP kinase activation in fibroblasts involves prior activation of Ras, as observed with both receptor tyrosine kinases and G_i-coupled receptors. In contrast, sustained MAP kinase activation by desensitization-defective NK-2 receptors in Rat-1 cells is not preceded by Ras activation, nor is it G_i mediated; furthermore, dominant-negative Ras does not prevent MAP kinase activation by such receptors, at least in COS cells. While Ras is bypassed, the downstream Raf-1 kinase is clearly required for mitogenic signalling, as shown by experiments using dominant-negative Raf-1 (Figures 6 and 7) and supported by the inhibitory effects of 8Br-cAMP on MAP kinase activation (Figure 8C).

If Ras signalling is not involved, how then does mutant NK-2 receptor activate the Raf-MAP kinase cascade to stimulate DNA synthesis? Given the receptor's ability to persistently activate PLC, one would predict that prolonged PKC activation underlies sustained MAP kinase activation. However, while the kinetics of PLC activation correlate with those of MAP kinase activation, Ca^{2+}/PKC signalling cannot account for sustained MAP kinase activation, as down-regulation or inactivation of PKC has only minor effects and Ca^{2+} signalling is very short-lived (Alblas *et al.*, 1995). Furthermore, PKC-activating phorbol ester has little effect on MAP kinase activity in Rat-1 cells (Hordijk *et al.*, 1994a). This suggests that other phosphoinositide-derived signals are involved or, alternatively, that the decrease in phosphoinositide levels (or their continuous resynthesis) provides the signal. On the other hand, there is the puzzling observation that strong stimulation of PLC in Rat-1 cells, as induced by endothelin (Hordijk *et al.*, 1994a), leads to only very weak MAP kinase activation. This cautions against a simple correlation between PLC

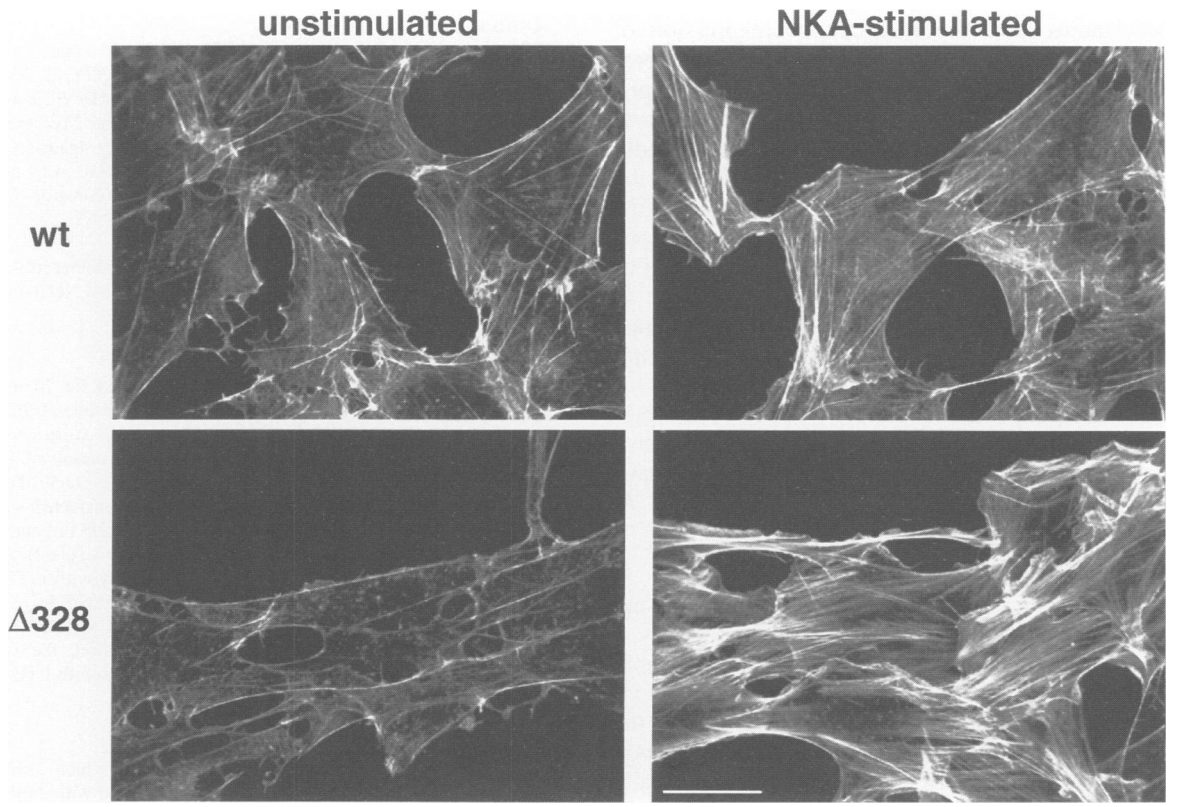

Fig. 10. NKA-induced stress fibre formation in Rat-1 cels. Cells expressing either wt or Δ328 NK-2 receptor were stimulated for 10 min with NKA (1 μM) or vehicle, fixed and stained for F-actin using NBD–phalloidin, as decribed in Materials and methods. F-actin staining was visualized by confocal microscopy. Bar, 25 μm.

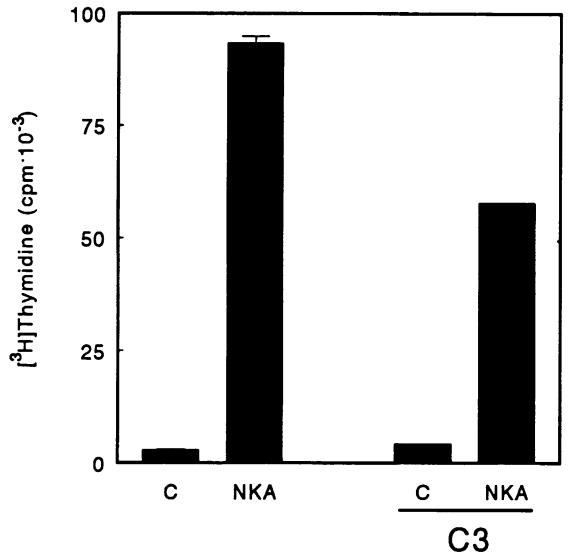

Fig. 11. Effect of C3 exoenzyme on NKA-induced DNA synthesis. Δ328 cells were pre-treated for 4 h with either C3 (30 μg/ml) or vehicle and then assayed for NKA-induced [^{3}H]thymidine incorporation (see Materials and methods).

activity and p42/p44 MAP kinase activation. Seufferlein *et al.* (1995) reported that, in transfected Rat-1 cells, MAP kinase activation by the PLC-coupled gastrin receptor also occurs through a pathway that is neither dependent on PKC nor on G_i–Ras signalling. Clearly, elucidation of this Ras- and PKC-independent pathway awaits further studies.

Cytoskeletal responses and oncogenic transformation

Our results obtained with mutant versus wt NK-2 receptors reveal a close correlation between the degree of phosphoinositide hydrolysis and early cytoskeletal responses. Phosphoinositides are thought to play a critical role in regulating the actin cytoskeleton. In particular, newly synthesized PIP and PIP_2 in stimulated cells may help regulate actin polymerization by interacting with actin binding proteins (for example, see Apgar, 1995; Hartwig *et al.*, 1995). Furthermore, rapid actin remodelling in response to receptor stimulation is regulated by Rho family GTPases (Hall, 1994), but the biochemical details of the putative phosphoinositide–Rho–cytoskeleton connection are currently not understood.

We note that oncogenic transformation by mutant NK-2 receptor, as evidenced by long-term morphological changes, focus formation and growth in soft agar, does not correlate simply with the early cytoskeletal events. The latter response is inhibited by the C3 ADP-ribosyltransferase, whereas long-term transformation is not. This argues against a role for the Rho GTPase in mediating the observed phenotypic transformation. Morphological transformation induced by mutant NK-2 receptor does not rely on p42/p44 MAP kinase activation either, as shown by the experiments with 8Br-cAMP. The nature of the MAP kinase-independent pathway leading to transformation remains to be established; preliminary experiments suggest that it involves receptor-mediated release of autocrine 'morphoregulatory' factors (J.Alblas and W.H. Moolenaar, unpublished results).

Earlier studies have reported that overexpression of certain PLC-coupled receptors promotes fibroblast proliferation and transformation in the presence of agonist (Julius *et al.*, 1989; Gutkind *et al.*, 1991). The ability of such receptors to induce transformation is undoubtedly due to a combination of high expression levels, the presence of additional serum factors and, in particular, the use of cell lines that are highly susceptible to spontaneous transformation (for discussion, see Moolenaar, 1991). For example, overexpression of the PLC-coupled serotonin 1c receptor in NIH 3T3 cells has been reported to generate transformed foci in the presence of ligand (Julius *et al.*, 1989). However, agonist-induced focus formation is dependent on serum factors, and the transfected receptor is no longer required once the focus-forming cells have been passaged as tumours, indicating that such cells have acquired additional genetic alterations.

Concluding remarks

Our studies support a model in which the C-terminal tail of a prototypic PLC-coupled receptor functions as a regulatory 'desensitization domain' that undergoes rapid ligand-induced phosphorylation leading to receptor–effector uncoupling. Deletion of this domain allows the receptor to signal fibroblast proliferation and transformation. In other words, the desensitization domain normally acts to suppress the receptor's oncogenic potential.

While few studies have addressed the role of the C-terminal tail in modulating receptor–PLC coupling, the long-term cellular consequences of deregulated signalling have largely been ignored. For example, C-terminal truncation of the receptor for platelet-activating factor expressed in CHO cells leads to enhanced phosphoinositide hydrolysis (Takano *et al.*, 1994), but long-term effects on cellular function have not been reported. C-terminal truncation of the α_1-adrenergic receptor overexpressed in COS cells does not seem to affect the degree of PLC activation when compared with the native receptor (Cotecchia *et al.*, 1990), but signal attenuation over time was not investigated.

Interestingly, various human diseases have been linked to specific alterations in G protein-coupled receptors, particularly point mutations that cause constitutive receptor activation in the absence of ligand (for review, see Clapham, 1993; Lefkowitz, 1993; Coughlin, 1994; see also Allen *et al.*, 1991). To our knowledge, defects in ligand-induced receptor phosphorylation and desensitization have not yet been linked to human pathologies. In view of the present findings, it will be of interest to examine G protein-coupled receptors for disease-associated mutations in their phosphorylatable tail.

Materials and methods

Materials

Protein A–Sepharose (CL-4B) beads were from Pharmacia Biotech Inc. Expression plasmid pEXV-ERK2tag was a kind gift of C.Marshall; pCMUIV-CD8 was kindly provided by Dr J.J.Neefjes; EGF was from Collaborative Research; pertussis toxin was from List Biological Laboratories (Campbell, CA); C3 exoenzyme was a kind gift of Dr S.Narumiya; Ro-31-8220 was from Roche Research Centre (Welwyn Garden City, UK); $^{32}P_i$ (10 mCi/ml), [τ-^{32}P]ATP (10 mCi/ml), [^{3}H]thymidine (1 mCi/ml) and the ECL kit were from Amersham. All other chemicals were from Sigma.

Antibodies

Polyclonal antiserum recognizing p42/p44 MAP kinase was raised against the C-terminal ERK-2 peptide EETARFQPGYRS. Monoclonal anti-Ras antibody Y13-259 was kindly provided by Dr J.L.Bos (Utrecht University). Monoclonal antibody 9E10 against the Myc epitope was obtained from Dr R.Bernards and OKT8 antibody against CD8 from Dr J.J.Neefjes. Anti-phosphotyrosine antibody 4G10 was from ICN. Peroxidase-conjugated antibodies were from Dako (Glostrup, Denmark).

Cell culture

Rat-1 cells and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum and antibiotics.

DNA synthesis

Cells were grown in 24-well plates, serum starved for 24 h and then stimulated with agonist for another 24 h. [^{3}H]Thymidine (1 μCi/ml) was allowed to incorporate during the last 4–6 h. Cells were washed with phosphate-buffered saline (PBS) and methanol, dissolved in 0.1 M NaOH and radioactivity was counted. To examine the effects of dominant-negative Raf, Δ328 cells in 10 cm dishes were transfected with 10 μg of pMT2-NΔraf together with 10 μg of pCMUIV-CD8 or control vector. At 24 h after transfection, cells were stained for expression of CD8 using monoclonal antibody OKT8 and subsequently with FITC-labelled goat-anti-mouse IgG. CD8-positive cells were sorted by a FACStar apparatus (Becton Dickinson, San Jose, CA) and replated in 96-well plates (~10 000 cells/well). After serum starvation, the cells were stimulated with agonist for 24 h and pulse-labelled with [^{3}H]thymidine (10 μCi/ml) for 6 h.

Growth in soft agar

Soft agar growth was assayed in 6 cm dishes, in which 2 ml of 0.5% agar noble (Difco) in complete medium was overlaid with 2 ml of 0.37% agar in serum-supplemented DMEM containing 2×10^4 cells. Half of the dishes received 1 μM NKA. After 3 weeks, cells growing in clumps (>10 cells) were scored in randomly selected areas per dish and given as percentage of total cell number.

Ras activation

Nearly confluent cells in 6-well tissue culture plates were serum starved overnight, labelled for 3 h in phosphate-free Eagle's minimum essential medium (ICN Biomedicals) supplemented with 100 μCi/ml $^{32}P_i$, stimulated for the indicated periods with agonists and washed with ice-cold PBS. Cells were lysed in 1% Triton X-114 buffer, and $p21^{ras}$ was immunoprecipitated with monoclonal Y13-259 pre-coupled via rabbit-anti-rat IgG to protein A–Sepharose and then assayed for bound guanine nucleotides by TLC separation using polyethyleneimine–cellulose plates as described by van Corven *et al.* (1993). The amount of ^{32}P in the GDP and GTP spots was quantified using a PhosphoImager.

MAP kinase mobility shift assays

Phosphorylation of $p42^{MAPK}$ and $p44^{MAPK}$ was determined by the mobility shift assay; serum-starved cells were stimulated, lysed in SDS sample buffer and proteins were separated on a 10% polyacrylamide–0.1% bisacrylamide gel and transferred to nitrocellulose. The Western blot was blocked with 5% dried non-fat milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20; also used for all incubations and washing steps) and incubated with a rabbit polyclonal antibody (1:5000) for 1 h, followed by extensive washing. The blots subsequently were incubated with peroxidase-conjugated swine anti-rabbit IgG (1:7500) for 30 min, washed and subjected to the enhanced chemiluminescence (ECL) procedure.

COS cell experiments

For transient expression studies, COS-7 cells were transfected with a modified pMT2 vector containing wt NK-2 receptor (0.5 μg/6 cm dish) together with pEXV-ERK2tag (Howe and Marshall, 1993) (2 μg/dish) and either pMT2-N17ras (0.7 μg/dish), pMT2-NΔraf (0.7 μg/dish; Schaap *et al.*, 1993) or control plasmid, using the DEAE-dextran method. At 24 h after transfection, cells were serum starved for another 24 h and then stimulated with the indicated ligands for 10 min, washed with ice-cold PBS and lysed in 0.5 ml of lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, 10 mM $Na_2P_2O_7$, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were pre-cleared twice with normal mouse serum pre-coupled to protein A–Sepharose beads (30 min, 4°C). Precipitation of the ERK2 protein

was performed with monoclonal antibody 9E10 recognizing the myc tag (1 h, 4°C). The precipitates were washed four times with lysis buffer and once with kinase buffer [20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM pNPP]. Immunoprecipitated kinase activity towards MBP was assayed in kinase buffer supplemented with 0.25 mg/ml MBP and 20 μM ATP (including 0.5 mCi/ml [γ-^{32}P]ATP) for 10 min at 30°C. Reactions were stopped by addition of 1 M formic acid. Supernatants were then spotted on P81 cellulose phosphate paper (Whatmann), immersed in 150 mM H_3PO_4, washed three times with the same solution, dried and scintillation counted. Control precipitations using normal mouse serum or untransfected cells were also subjected to the kinase assay; their MBP phosphorylation activity was subtracted from the specific 9E10-precipitated signal.

Protein tyrosine phosphorylation

Serum-starved cells were treated with agonist for various times and then taken up in SDS sample buffer, and proteins were analysed by SDS–PAGE using a 10% gel. Proteins were transferred to nitrocellulose, incubated with 4G10 antibody followed by peroxidase-labelled second antibody. Immunostained proteins were visualized using the ECL system.

Formation of actin stress fibres

Cells were grown on glass coverslips, serum starved for 12–24 h, stimulated with agonist and then washed with PBS and fixed with 4% formaldehyde in PBS for 10 min. Cells were washed thoroughly with PBS–0.1% bovine serum albumin (BSA) and then incubated in NBD–phalloidin (2 U/ml in PBS/BSA) for 30 min. The coverslips were washed again, embedded in Vectashield and analysed using a Bio-Rad confocal microscope.

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