Analysis of R-Ras signalling pathways

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SUMMARY

R-Ras has a high degree of sequence homology to Ras and to other members of the Ras subfamily including Rap, TC21 and M-Ras. Activated versions of Ras and TC21 are highly transforming in a variety of cell lines and mutated forms of both proteins have been found in human tumours. R-Ras interacts with many of the same proteins as Ras and TC21, including c-Raf1, and can induce transformed foci, although this activity is weak compared to Ras and appears to be cell-type specific. Here, we have investigated R-Ras signalling pathways in a variety of cell types. We find that microinjection of activated R-Ras into quiescent fibroblasts stimulates cell cycle progression through G_1 phase and subsequent DNA synthesis. However, unlike Ras, R-Ras does not activate the ERK MAP kinase pathway nor does it activate the JNK or p38/Mpk2 MAP kinase pathways. Microinjection of R-Ras into PC12 cells does not induce terminal differentiation, but instead causes extensive cell spreading, consistent with R-Ras having a role in integrin activation. Finally, in a macrophage cell line, R-Ras activates the $\alpha_M\beta_2$ integrin via the small GTPase Rap1, leading to phagocytosis of opsonized red blood cells, whereas Ras does not. These results indicate that R-Ras has an important role in the regulation of cell growth and adhesion, but that this is mediated through downstream signals distinct from those used by Ras.

Key words: R-Ras, DNA synthesis, ERK MAP kinase, Integrin

INTRODUCTION

R-Ras is a member of the superfamily of small GTPases, originally cloned through its homology to Ras (Lowe and Goeddel, 1987). The R-Ras protein is 55% identical to Ras and has a 26-amino-acid N-terminal extension. Its minimal effector region (amino acids 30-40) is identical to Ras, but the protein differs at nine amino acids within the extended effector region comprising amino acids 23-46 (Self et al., 1993). R-Ras interacts in vitro with many of the same proteins as Ras including the two GAPs, p120RasGAP and neurofibromin, the exchange factor RasGRF and the three downstream effector proteins Raf, PI3-kinase and RalGDS, but it does not interact with the Ras exchange factors SOS1 or RasGRP (Buday and Downward, 1993; Rey et al., 1994; Spaargaren and Bischoff, 1994; Spaargaren et al., 1994; Marte et al., 1996; Gotoh et al., 1997; Ebinu et al., 1998). These similarities have generated speculation that, like Ras, R-Ras might activate similar signal transduction pathways and regulate important aspects of growth control.

The intrinsic GTPase activity of R-Ras containing a glycine-to-valine substitution at codon 38 (equivalent to codon 12 in Ras) is insensitive to stimulation by p120RasGAP or neurofibromin and this mutant protein is, therefore, constitutively active when introduced into cells (Rey et al., 1994). Using this activated version of R-Ras, it was first reported that, unlike Ras, it did not induce transformed foci in Rat1 fibroblasts (Lowe and Goeddel, 1987). However, others have shown that V38R-Ras can produce transformed foci in NIH3T3 cells, although at a much lower efficiency than V12Ras (Cox et al., 1994; Saez et al., 1994). Unlike cells derived from Ras foci, cells from the R-Ras foci do not appear morphologically transformed; however, they are able to proliferate in low serum, to form colonies in soft agar and to give tumours in nude mice (Cox et al., 1994). Furthermore, these cells have elevated levels of ERK1 and ERK2 MAP kinases, suggesting that R-Ras can activate similar signal transduction pathways to Ras.

R-Ras has also been shown to affect integrin function. Expression of activated R-Ras in 32D.3 mouse myeloid cells, which normally grow in suspension, causes the cells to become highly adherent. Introduction of dominant negative R-Ras into adherent Chinese hamster ovary cells, on the other hand, reduces their adhesiveness (Zhang et al., 1996). Zhang et al. further showed that R-Ras induces an increase in cell adhesion by activating $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins on the cell surface of CHO and 32D cells, respectively. Others, however, have suggested that the changes in adhesion of these cells are not due to R-Ras being a direct activator of integrins, but rather are a result of its ability to inhibit a Ras/Raf-mediated integrin suppression pathway (Sethi et al., 1999).

The major downstream target of Ras is c-Raf1 and in its active (GTP-bound) state, Ras induces recruitment of c-Raf1 to the plasma membrane and stimulation of its kinase activity (Leevers et al., 1994; Stokoe et al., 1994). This leads to activation of the ERK MAP kinase cascade and eventually to changes in gene transcription. Since R-Ras has also been shown to interact with c-Raf1 in vitro (Rey et al., 1994; Spaargaren et al., 1994; Marte et al., 1996), it is possible that it may also regulate the ERK cascade under some conditions and this might explain why it is able to induce foci in NIH3T3 cells. However, there are conflicting reports whether R-Ras can

in fact activate ERK. ERK is activated in foci derived from R-Ras transformed NIH3T3 cells (Cox et al., 1994), but others have suggested that this is not due to a direct effect of R-Ras but rather to other factors, which have been selected for during the transformation assay (Marte et al., 1996). A second important target of Ras is thought to be the p110 subunit of PI3-kinase (Rodriguez et al., 1994). R-Ras has also been reported to interact with this molecule to increase the levels of PIP_3 in cells (Marte et al., 1996). This lipid affects several important intracellular pathways associated with growth and survival, including activation of the kinase Akt/PKB (Franke et al., 1995) and activation of the Rac GTPase leading to membrane ruffling (Reif et al., 1996). Finally RalGDS has been shown to interact with both Ras and R-Ras (Albright et al., 1993; Hofer et al., 1994; Spaargaren and Bischoff, 1994; Urano et al., 1996). The interaction of Ras with RalGDS results in activation of its guanine nucleotide exchange activity towards Ral and this appears to signal a growth promoting activity (Urano et al., 1996; White et al., 1996).

Here we have analyzed the effects of expressing activated R-Ras in three distinct cell types: fibroblasts, PC12 cells and macrophages. Despite inducing G_1 cell cycle progression in fibroblasts, spreading in PC12 cells and integrin activation in macrophages, no detectable changes were observed in the levels of ERK activity or in membrane ruffling. Instead in the macrophage, we find that R-Ras-induced integrin activation is mediated by Rap1. This suggests that R-Ras is an important regulator of cell growth and adhesion, but that this is mediated through signals distinct from those used by Ras.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

V12Ras was expressed under the control of the tryptophan promoter in *E. coli* and proteins purified as previously described (Hall and Self, 1986). V38R-Ras and the three R-Ras effector mutants (provided by M. A. White) were expressed under the control of the T7 promoter and purified as for V12Ras. Amino acids 1-169 of c-Jun (provided by J. Ham), the C-terminal domain of p50RhoGAP residues (198-439) (Lancaster et al., 1994) and the Ras binding domains of human RalGDS (provided by P. Aspenström), human c-Raf (Warne et al., 1993), mouse Rlf and Rgl (provided by R. H. Cool), were all expressed as GST fusion proteins in *E. coli*. Fusion proteins were eluted from the beads with 5 mM reduced glutathione (Sigma) and concentrated by ultrafiltration using Centricon-10 (Amicon). Protein concentration was determined by BCA protein assay (Pierce) and the purity of protein preparations was visualized on Coomassie Blue-stained SDS-polyacrylamide gels.

Dot blot assay

The interaction of R-Ras with Ras effector proteins was carried out using a dot blot assay as previously described (Diekmann and Hall, 1995). Briefly, 10 μg of GST fusion protein, p50RhoGAP, c-Raf, RalGDS, Rlf or Rgl were spotted onto nitrocellulose membranes, the filter air-dried and then incubated with blocking buffer (1 M glycine, 5% milk powder, 1% ovalbumin and 5% foetal calf serum, FCS) for 2 hours at room temperature. The membrane was washed in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT) and incubated for 5 minutes at 4°C with 0.1 μg [γ-^{32}P]GTP-bound V12Ras, V38R-Ras, S61R-Ras, G63R-Ras or C66R-Ras in buffer A. The filters were washed quickly three times with cold buffer A containing 0.1% Tween-20, and interacting GTPases were visualized by autoradiography.

Swiss 3T3 cell culture, microinjection and DNA synthesis

Swiss 3T3 cells were maintained in DMEM containing 10% FCS at 37°C, 10% CO_2. Quiescent, serum-starved Swiss 3T3 cells were prepared for microinjection as follows: cells were seeded at a density of 5×10^4 cells/ml onto 13 mm glass coverslips. 7-10 days after seeding, cells were serum-starved for 16 hours in DMEM containing 2 g/l $NaHCO_3$. For the DNA synthesis assay, recombinant V12Ras and V38R-Ras proteins were microinjected at 1 mg/ml together with rat IgG (1 mg/ml) as an injection marker. After microinjection the cells were returned to the incubator in serum-free DMEM with 10 μg/ml bromodeoxyuridine (BrdU, Boehringer Mannheim) for 40-48 hours.

PC12 cell culture, microinjection and transfection

Rat pheochromocytoma (PC12) cells were seeded at a density of 5×10^5 onto Bio-Coat laminin-coated 60 mm culture dishes (Collaborative Biomedical Products) in RPMI medium 1640 containing 10% horse serum (HS) and 5% FCS and incubated at 37°C in 5% CO_2 for 24 hours prior to microinjection. PC12 cells were microinjected with V12Ras and V38R-Ras eukaryotic expression constructs (50 μg/ml), left for 48 hours and then fixed and stained for protein expression. PC12 cells grown in DMEM plus 10% HS and 5% FCS were transfected by electroporation using 10 μg of pMT, pMT-Myc-D12-Ras or pMT-Myc-V38R-Ras. Briefly, trypsinised cells were washed and resuspended in 250 μl HeBs buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose), transferred to a 0.4 cm^3 electroporation cuvette and electroporated in a BioRad Gene Pulser II device. Cells were resuspended in DMEM plus serum and replated. After 24 hours, transfected cells were serum-starved for 16 hours in DMEM containing 0.1% BSA and then harvested in S buffer (20 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$, 10 mM EGTA, 40 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 1% Triton X-100, 0.5% sodium deoxycholate, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 3 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane and activation of endogenous ERK1 and ERK2 determined by anti-active MAPK antibody (Promega). As a positive control, cells transfected with empty vector were stimulated with 100 ng/ml nerve growth factor (NGF, Boehringer Mannheim) 5 minutes before harvesting.

J774 cell culture, microinjection and phagocytosis assay

J774.A1 macrophages were seeded at a density of 1×10^5 cells/ml onto glass coverslips in DMEM plus 10% heat-inactivated FCS. Cells were transferred to serum-free DMEM supplemented with 10 mM Hepes immediately before microinjection with biotin-dextran (2.5 mg/ml), Myc-epitope-tagged Ras or R-Ras eukaryotic expression constructs (0.1 mg/ml) and incubated at 37°C in 10% CO_2 for 2.5 hours to allow for protein expression prior to the phagocytic assay. The macrophages were then challenged with complement-opsonized sheep red blood cells (RBCs) for 30 minutes at 37°C as described previously (Caron and Hall, 1998). The unbound RBCs were then washed off and the cells were fixed and stained for protein expression and the number of internalized red blood cells counted. For Rap1 inhibition, the Myc-tagged R-Ras construct was co-injected with an HA-tagged Rap1GAP construct (Rubinfeld et al., 1991).

Immunofluorescence

At the indicated times, microinjected Swiss 3T3, PC12 and J774 cells were washed in PBS and fixed for 10 minutes in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 for 10 minutes and free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 minutes. PC12 and J774 cells were then blocked for 30 minutes with 10% FCS and 0.5% BSA, respectively. For DNA synthesis experiments, Swiss 3T3 cells were stained for rat IgG to localize injected cells and BrdU incorporation was monitored

using an anti-BrdU antibody as reported earlier (Olson et al., 1995). In the PC12 microinjection experiments, R-Ras expressing cells were visualized using a rabbit anti-R-Ras polyclonal antibody (Lowe and Goeddel, 1987) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG. Ras expressing cells were visualised using the rat anti-Ras Y13-259 antibody (Furth et al., 1982) followed by incubation with FITC-conjugated anti-rat IgG. In microinjected J774 cells, protein expression of Myc-tagged constructs was monitored using mouse anti-Myc tag 9E10 antibody (Evan et al., 1985) followed by FITC-conjugated anti-mouse IgG and RBCs were visualised with rhodamine-conjugated anti-rabbit IgG. Where indicated HA-tagged constructs were detected using anti-HA 3F10 antibody (Boehringer). Coverslips were mounted by inverting them onto 5 μl moviol mountant containing p-phenylenediamine (1 mg/ml) as an antibleach agent. Swiss 3T3 cells were examined on a Zeiss Axiophot microscope using a Zeiss 40×1.3 oil immersion objective and fluorescent images recorded on Kodak T-MAX 400 ASA film. Confocal images of PC12 cells were obtained using an MRC 1024 laser scanner (BioRad) attached to a Nikon Optiphot 2 microscope. Hard copies of PC12 images were obtained on a Sony UP 5000 colour video printer. J774 cells were examined on a Zeiss Axioskop microscope (HBO 50) connected to a C5985 Hammamatsu CCD camera. J774 images were processed using Adobe Photoshop and printed on an Epson 600 colour printer.

Scrape-loading and 'in gel' kinase assay

For scrape-loading experiments, 8×10^5 Swiss 3T3 cells were seeded onto 10 cm dishes, grown to confluence for 72 hours in DMEM/10% FCS and then serum-starved overnight. Quiescent serum-starved cells were washed twice with PBSA and scrape-loaded as previously reported (Morris et al., 1989) using 200 μl buffer (10 mM Tris-HCl pH 7.0, 114 mM KCl, 15 mM NaCl, 5.5 mM $MgCl_2$) with or without recombinant V12Ras or V38R-Ras protein (3 mg/ml). Cells were resuspended in serum-free DMEM and incubated at 37°C, 10% CO_2. Cell samples were pelleted, lysed in S buffer and cell lysates resolved on a 12% polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP, Sigma). After electrophoresis, the gel was denatured in 6 M guanidine-HCl, washed as described (Kameshita and Fujisawa, 1989) and kinase activity assayed with 100 μCi [γ-^{32}P]ATP and 10 μM unlabelled ATP.

COS-1 cell transfections

COS-1 cells were transfected by the DEAE-dextran method as described (Olson et al., 1995). Plasmid amounts per 10 cm dish were as follows: 2 μg pEXV-Myc-ERK2 with or without 5 μg each of pMT, pMT-Myc-V38R-Ras, pEXV-MycD12Ras or pMT-Myc-RafCAAX; 5 μg pCMV-FLAG-JNK1 with or without 5 μg each of pMT, pMT-Myc-V38R-Ras, pEXV-Myc-D12Ras, pEXV-Myc-V12CDC42 or pMT-Myc-MEKK COOH-terminal domain and pRK5-Myc-p38/Mpk2 with or without 5 μg each of pMT or pMT-Myc-V38R-Ras. After 24 hours, transfected cells were serum-starved for 16 hours and then harvested.

Immunoprecipitation and kinase assays

For ERK2 assays, transfected COS-1 cells were harvested and lysed in S buffer. 50 μg of protein from each lysate were resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane and Myc-epitope-tagged proteins visualized with the anti-Myc monoclonal antibody 9E10. ERK2 was immunoprecipitated from lysates by incubation with 9E10 and kinase activity determined using 5 μg of myelin basic protein (MBP) as a substrate (Olson et al., 1995). For JNK assays, transfected cells were harvested in 25 mM Hepes, pH 7.6, containing 0.3 M NaCl, 5 mM EGTA, 40 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, 1% Triton X-100, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 3 mM PMSF. JNK1 expression was visualised using anti-FLAG M2 antibody (IBI). JNK1 kinase activity was measured after immunoprecipitation with M2 using 2 μg of GST-c-jun as a substrate (Olson et al., 1995). For p38/Mpk2 assays, transfected cells were harvested as described for JNK transfected cells. p38/Mpk2 kinase activity was measured after immunoprecipitation with 9E10 using 2 μg recombinant MAPKAP2 protein as a substrate (provided by A. Nebreda).

Immunocomplex kinase reactions were resolved by SDS-PAGE (15% gel) followed by blotting onto nitrocellulose membrane and autoradiography. The relative phosphorylation levels of c-jun, MBP or MAPKAP2 were determined by Phospho-Imager (BioRad) analysis. The amount of immunoprecipitated JNK1 was determined on western blots by M2 antibody and that of ERK2 and p38/Mpk2 by 9E10, as revealed by chemiluminescence detection using an ECL kit (Amersham).

RESULTS

V38R-Ras stimulates DNA synthesis in quiescent fibroblasts

The biological effects of R-Ras and its potential to activate the ERK cascade or PI3-kinase, were first examined in quiescent, serum-starved fibroblast cells. Activation of ERK is known to lead to the induction of G_1 progression and DNA synthesis, while activation of PI3-kinase induces Rac-dependent membrane ruffling in both NIH3T3 and Swiss 3T3 cells and activation of the kinase Akt/PKB (Leevers and Marshall, 1992; Morris et al., 1989; Reif et al., 1996; Franke et al., 1995).

To determine whether R-Ras can induce DNA synthesis, recombinant V38R-Ras protein, purified from *E. coli*, was microinjected into quiescent Swiss 3T3 cells. Cell cycle progression through G_1 and into S phase was assessed by the incorporation of bromodeoxyuridine (BrdU) into nascent DNA 40-48 hours later. Fig. 1A,B shows that microinjection of a rat IgG marker had no significant effect, but injection of V38R-Ras (1 mg/ml) very efficiently stimulated DNA synthesis, with 76% of injected cells positive for BrdU incorporation. V38R-Ras also stimulated DNA synthesis when microinjected into quiescent NIH3T3 cells, with 74% of cells positive for BrdU incorporation (data not shown). For comparison, V12Ras stimulated DNA synthesis in around 50% of injected cells in both Swiss 3T3 (Fig. 1A,B) and NIH3T3 (data not shown). V14Rho, on the other hand is unable to activate the ERK pathway, and does not induce DNA synthesis in NIH3T3 cells (data not shown), though it can in Swiss 3T3 cells (Olson et al., 1995). Analysis of Ras effector site mutants has shown that multiple pathways downstream of Ras contribute to cell transformation. Three effector site mutants of V38R-Ras S61, G63 and C66 (equivalent to codons 33, 35 and 40 in Ras, respectively) were constructed but none of these induced DNA synthesis when microinjected into quiescent Swiss 3T3 cells (data not shown).

It has previously been shown that the generation of PIP3 by addition of growth factors such as PDGF, or by expression of a constitutively activated form of PI3-kinase, leads to activation of Rac and membrane ruffling (Nobes and Hall, 1995; Reif et al., 1996). To determine whether R-Ras can activate PI3-kinase in fibroblasts, recombinant protein was microinjected into quiescent Swiss 3T3 cells and the actin cytoskeleton visualized in fixed cells 30 minutes later using rhodamine-phalloidin immunofluorescence (Reif et al., 1996). No increase in filamentous actin could be observed (data not shown). Furthermore, no phosphorylation of the PI3-kinase target Akt

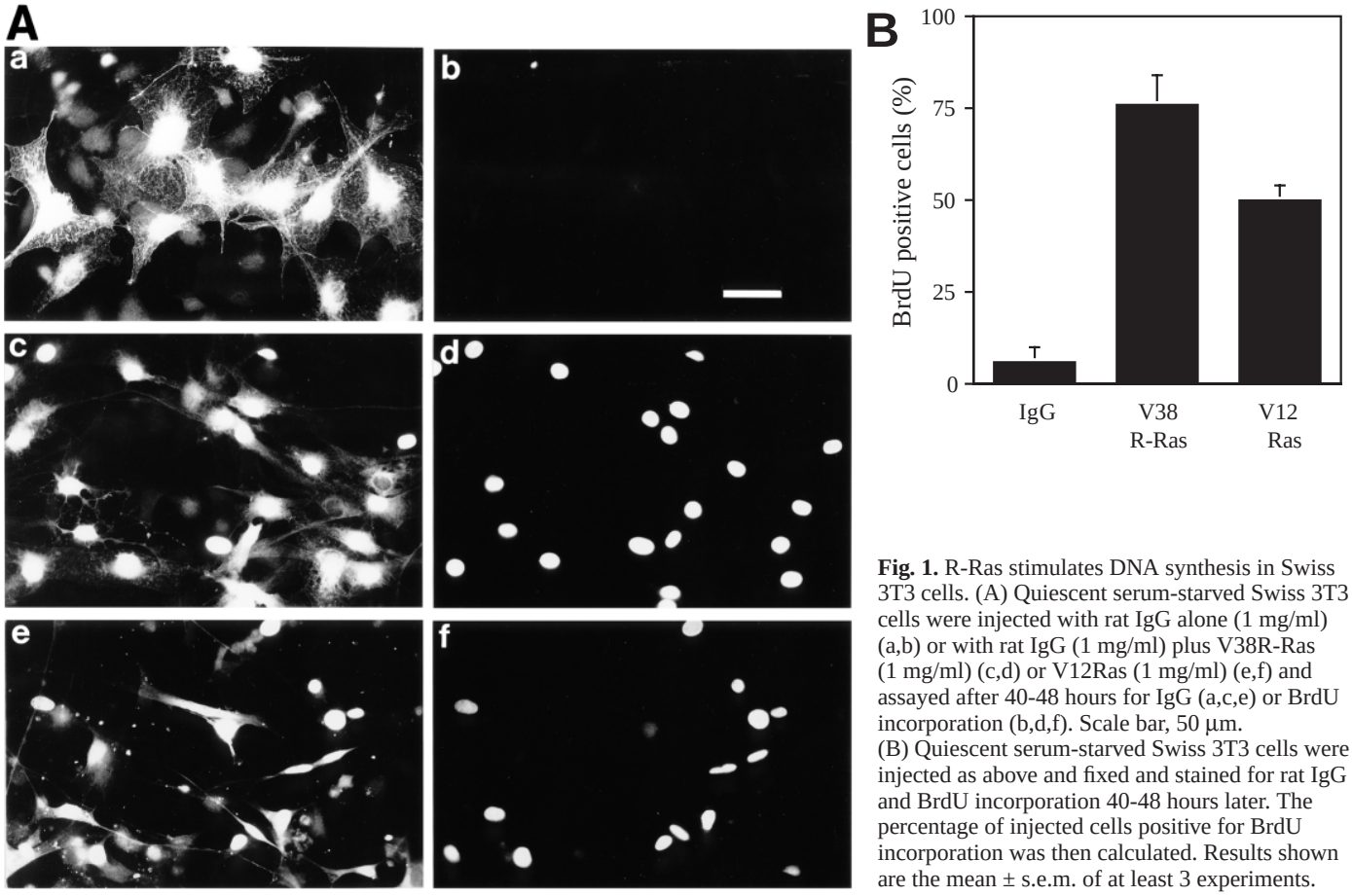

Fig. 1. R-Ras stimulates DNA synthesis in Swiss 3T3 cells. (A) Quiescent serum-starved Swiss 3T3 cells were injected with rat IgG alone (1 mg/ml) (a,b) or with rat IgG (1 mg/ml) plus V38R-Ras (1 mg/ml) (c,d) or V12Ras (1 mg/ml) (e,f) and assayed after 40-48 hours for IgG (a,c,e) or BrdU incorporation (b,d,f). Scale bar, 50 μm.
(B) Quiescent serum-starved Swiss 3T3 cells were injected as above and fixed and stained for rat IgG and BrdU incorporation 40-48 hours later. The percentage of injected cells positive for BrdU incorporation was then calculated. Results shown are the mean ± s.e.m. of at least 3 experiments.

could be detected by immunofluorescence, after microinjection of V38R-Ras, using an antibody specific to phosphorylated Akt (data not shown). We conclude that R-Ras is not able to activate PI3-kinase in these cells.

V38R-Ras does not activate ERK MAP kinases in quiescent fibroblasts

Since R-Ras can bind to c-Raf1 in vitro (Rey et al., 1994; Spaargaren et al., 1994) and since we have shown that R-Ras can induce DNA synthesis in both Swiss 3T3 and NIH3T3 cells, we next examined its ability to activate ERK. Quiescent serum-starved Swiss 3T3 cells are highly resistant to transfection protocols and so we have made use of the scrape-loading technique to introduce recombinant V38R-Ras protein into the cells (Morris et al., 1989). V38R-Ras (3 mg/ml protein), V12Ras (3 mg/ml protein) and a buffer control were scrape-loaded into quiescent Swiss 3T3 cells in serum-free medium. After 30 and 60 minute time points, the cells were harvested and lysates examined using an 'in gel' kinase assay with myelin basic protein (MBP) as substrate. This technique has been shown previously to detect changes in ERK1 and ERK2 (Leevers and Marshall, 1992) and theoretically it might detect other kinases activated under these conditions. As shown in Fig. 2A, after 60 minutes V12Ras increased the activity of both ERK1 and ERK2, elevenfold and fourfold, respectively. V38R-Ras, however, failed to activate either kinase significantly above background levels. This approach provided no indication for any other kinase being activated by R-Ras. We conclude that under conditions where R-Ras efficiently induces DNA synthesis, it does not lead to activation of ERK1 or ERK2.

V38R-Ras does not activate the ERK, JNK or p38/Mpk2 MAP kinases in COS-1 cells

To determine whether R-Ras can activate ERK when it is overexpressed along with ERK, Myc-epitope-tagged expression constructs for V38R-Ras and for ERK2 were cotransfected into COS-1 cells. Expression from the transfected constructs was confirmed by immunoblotting with the monoclonal anti-Myc 9E10 antibody (Evan et al., 1985). Whereas D12Ras and RafCAAX (a constitutively activated Raf construct) stimulated ERK2 kinase activity 39-fold and 35-fold, respectively, V38R-Ras did not induce any increase (Fig. 2B).

Other MAP kinase pathways, particularly JNK, have been reported to play a role in cell cycle progression (Robinson and Cobb, 1997). To test whether V38R-Ras can activate either of the two other well-characterized MAPK cascades, JNK (the stress-activated c-Jun NH_2 kinase) and p38/Mpk2, COS-1 cells were first cotransfected with NH_2-terminal FLAG-epitope-tagged JNK1 along with Myc-epitope-tagged V38R-Ras, D12Ras, V12Cdc42 or the C-terminus of MEKK (a constitutively activated upstream regulator of JNK). Whereas D12Ras, V12Cdc42, C-MEKK and UV irradiation stimulated the activity of immunoprecipitated JNK1 to phosphorylate recombinant c-jun protein 16-, 37-, 67- and 23-fold,

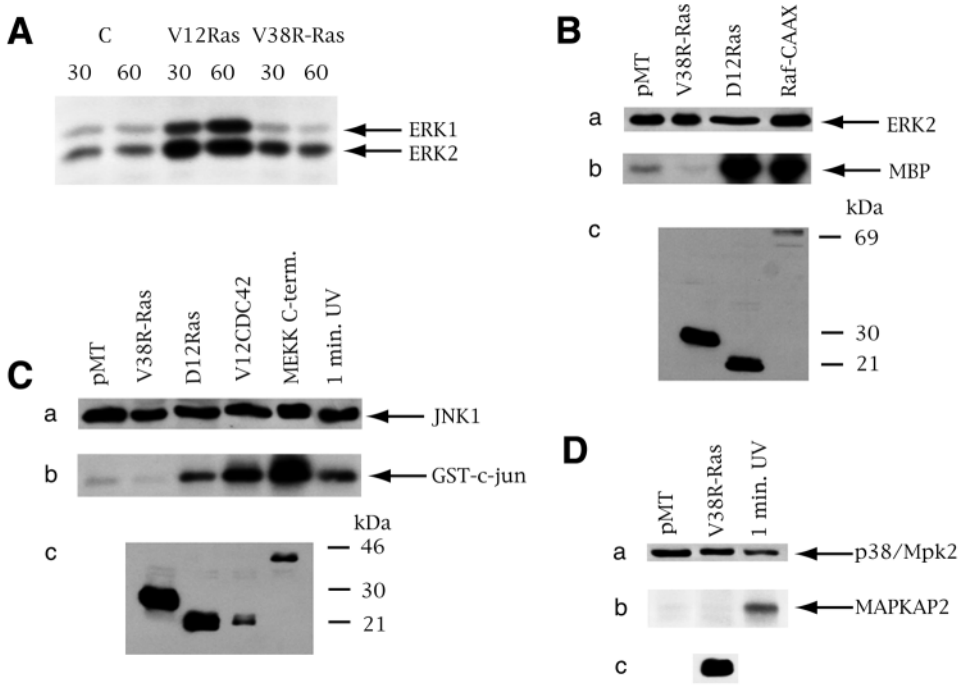

Fig. 2. R-Ras does not activate ERK, JNK or p38/Mpk2 MAP kinases. (A) Quiescent serum-starved Swiss 3T3 cells were scrape-loaded with V12Ras (3 mg/ml) or V38R-Ras (3 mg/ml) protein or buffer alone (C), resuspended in serum-free medium and incubated for 30-60 minutes before being lysed. 25 μg of each cell lysate was then analysed for ERK1 and ERK2 kinase activity as described in Materials and Methods. (B) COS-1 cells were transfected with pEXV-Myc-ERK2 together with the indicated constructs. (a) Immunoblot with antibody to the Myc-epitope showing expression of ERK2 in cell lysates of transfected COS-1 cells, (b) myelin basic protein phosphorylation by immunoprecipitated ERK2 and (c) immunoblot with antibody to the Myc-epitope showing expression of V38R-Ras, D12Ras and RafCAAX. (C) COS-1 cells were transfected with pCMV-FLAG-JNK1 together with the indicated constructs, or subjected to 1 minute of UV irradiation. (a) Immunoblot with antibody to the FLAG-epitope showing expression of JNK1 in cell lysates, (b) GST-c-jun phosphorylation by immunoprecipitated JNK1 and (c) immunoblot with antibody to the Myc- epitope showing expression of V38R-Ras, D12Ras, V12CDC42 and MEKK C terminus. (D) COS-1 cells were transfected with pRK5-Myc-p38/Mpk2 together with the indicated constructs, or subjected to 1 minute of UV irradiation. (a) Immunoblot with antibody to the Myc-epitope showing expression of p38/Mpk2 in cell lysates, (b) MAPKAP2 phosphorylation by immunoprecipitated p38/Mpk2 and (c) immunoblot with antibody to the Myc-epitope showing expression of V38R-Ras.

respectively, V38R-Ras failed to do so above background levels (Fig. 2C). Secondly, COS-1 cells were cotransfected with Myc-tagged p38/Mpk2 and V38R-Ras constructs and p38/Mpk2 immunoprecipitated and assayed using recombinant MAPKAP2 protein. UV light stimulated the activity of p38/Mpk2 to phosphorylate MAPKAP2 24-fold, whereas V38R-Ras had no effect (Fig. 2D). We conclude that R-Ras is unable to activate any of the three MAP kinase pathways in these cells.

V38R-Ras induces a spreading phenotype in PC12 cells

The introduction of activated versions of Ras into PC12 cells leads to activation of ERK, which in turn, induces their terminal differentiation into neuronal-like cells (Marshall, 1995). Although PC12 cells express both c-Raf1 and B-Raf, it has been reported that Ras signals preferentially through B-raf to activate MEK and the MAP kinase cascade (Jaiswal et al., 1994). We therefore looked to see if V38R-Ras could activate endogenous ERK1 or ERK2 in PC12 cells. Myc-epitope-tagged V38R-Ras, Myc-epitope-tagged D12Ras or empty vector were transfected into PC12 cells by electroporation and activation of endogenous ERK1 and ERK2 detected by western blot analysis using an antibody specific for dually phosphorylated ERKs. Whereas D12Ras and NGF activated both endogenous ERK1 and ERK2, V38R-Ras failed to do so (Fig. 3A). V38 R-Ras, therefore, does not activate ERK1/2 MAP kinases in PC12 cells.

To see whether R-Ras could nevertheless induce differentiation, plasmids encoding V38R-Ras or D12Ras were microinjected into PC12 cells. As shown in Fig. 3B, D12Ras induced differentiation with neurite extensions appearing after 18 hours. V38R-Ras on the other hand did not induce any neurites; instead a dramatic shape change occurred, with the cells undergoing flattening and substantial spreading approximately 6 hours after injection (see Fig. 3B). When the three R-Ras effector mutants described earlier were injected, only the G63 mutant induced the PC12 cells to spread to the same extent as V38R-Ras (data not shown). These effects are consistent with functional activation of integrins, similar to what has been reported in some other cell types (Zhang et al., 1996; Keely et al., 1999), though the identity of the integrins involved in PC12 cells is not clear.

V38R-Ras activates the integrin $\alpha_M\beta_2$ in J774 macrophages

To investigate the mechanism by which R-Ras activates integrins more closely, we examined its effects on the $\alpha_M\beta_2$ integrin in the J774 macrophage cell line. $\alpha_M\beta_2$ is the complement receptor (CR3) that mediates phagocytosis of C3bi-opsonized particles in macrophages and neutrophils. The integrin is functionally inactive in resting cells but can be activated experimentally with the phorbol ester PMA, for example, which leads to phagocytosis (Wright and Griffin, 1985). J774 macrophage cells were microinjected with a V38R-Ras expression construct and the ability of the cells to phagocytose complement-opsonized sheep red blood cells was assessed 2.5 hours later. As shown in Fig. 4A,B, V12Ras was unable to activate the integrin under these conditions, but R-Ras was as efficient at inducing phagocytosis as PMA treatment.

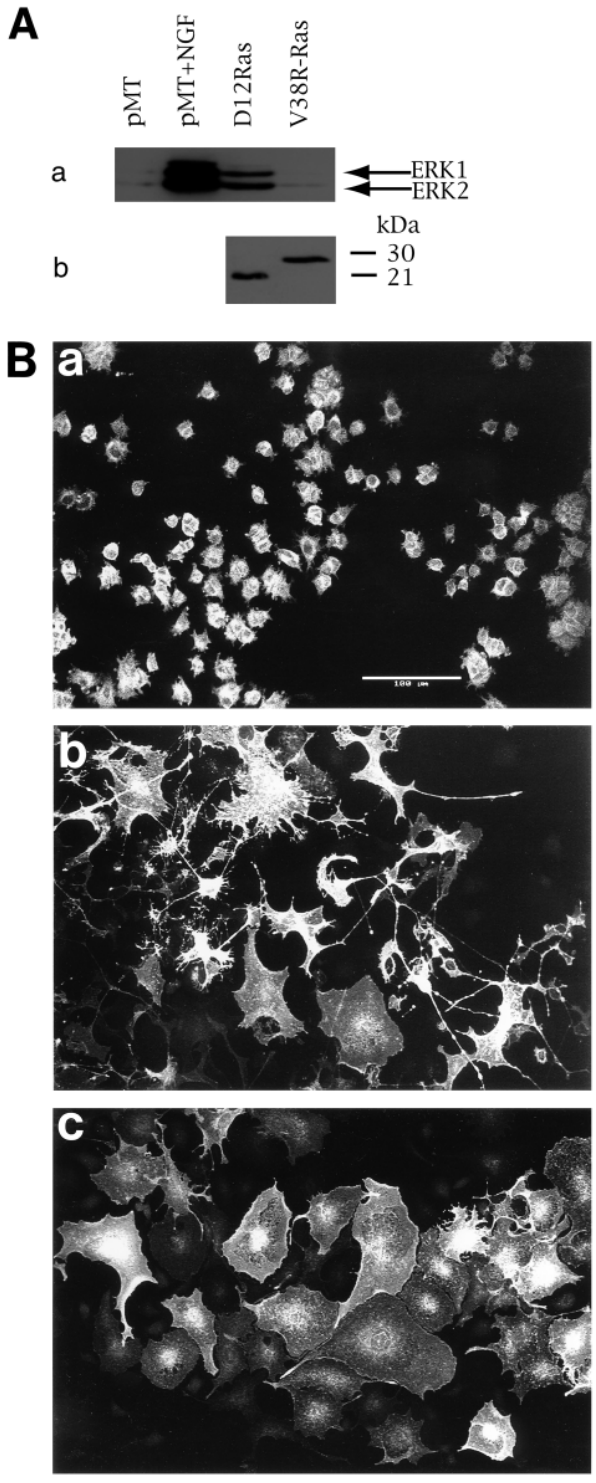

Fig. 3. R-Ras does not activate ERK MAP kinase in PC12 cells but does cause extensive cell spreading. (A) PC12 cells were transfected with pMT, pMT-Myc-D12-Ras, pMT-Myc-V38R-Ras or pMT+100 ng/ml NGF for 5 minutes. (a) Immunoblot with anti-active MAP kinase antibody showing activated ERK1 and ERK2 in transfected PC12 cell lysates and (b) immunoblot with antibody to the Myc-epitope showing expression of V38R-Ras and D12Ras. (B) PC12 cells were (a) uninjected or microinjected with 50 μg/ml pEXV-Myc-D12Ras (b) or pEXVV38R-Ras (c) and left for 48 hours before fixing and staining for protein expression. Uninjected cells were stained for actin and tubulin. Scale bar, 100 μm.

To determine whether R-Ras overexpression leads to activation of ERK in macrophages under these conditions, injected cells were analyzed by immunofluorescence using an antibody specific for dually phosphorylated ERKs. V38R-Ras did not lead to activation of ERK above background levels (data not shown). Furthermore, inhibition of MEK using the inhibitor PD98059 (Dudley et al., 1995) had no effect on R-Ras induced phagocytosis (data not shown), nor did the PI3-kinase inhibitor wortmannin (data not shown).

To explore the mechanism of $\alpha_M\beta_2$ activation further, the three R-Ras effector mutants S61, G63 and C66 were analyzed for their ability to induce phagocytosis. As shown in Fig. 5A, only the G63 mutant was as competent as V38R-Ras at internalising complement-opsonized red blood cells (Fig. 5A). In Ras, the corresponding G37 mutation has been shown to still interact with members of the RalGDS family of exchange factors. We therefore tested the ability of the R-Ras mutants to interact with the three known members of this family, RalGDS, Rlf and Rgl, using dot blot assays. As shown in Fig. 5B, the G63 mutant interacted strongly with these molecules but S61 and C66, which were unable to induce phagocytosis, interacted very weakly or not at all. The interaction studies suggested that RalGDS family proteins might be mediating the activation of integrins by R-Ras. To test this, Rlf-CAAX, which has previously been shown to act as a constitutively active Rlf (Wolthuis et al., 1997), was microinjected into J774 cells. However, as shown in Fig. 5A, Rlf-CAAX failed to induce phagocytosis in J774 macrophages.

We have recently shown that the Ras-related protein Rap1 regulates activation of the $\alpha_M\beta_2$ integrin in response to a wide variety of inflammatory mediators (Caron et al., 2000). To determine whether R-Ras induced integrin activation is dependent on Rap1, we microinjected V38R-Ras along with an expression vector for the Rap-specific GTPase activating protein, Rap1GAP. As shown in Fig. 5A, coexpression of Rap1GAP completely blocked phagocytosis induced by R-Ras, indicating that R-Ras acts upstream of Rap1 in the activation of $\alpha_M\beta_2$.

DISCUSSION

R-Ras is closely related to Ras and appears to be widely expressed (Lowe et al., 1987; Saez et al., 1994). In vitro experiments have shown that it shares several biochemical activities with Ras, including its ability to interact with two RasGAP proteins, p120RasGAP and neurofibromin and with three downstream effectors, the serine/threonine kinase c-Raf1, the p110 subunit of PI3-kinase and members of the RalGDS family (Kikuchi et al., 1994; Rey et al., 1994; Spaargaren and Bischoff, 1994; Spaargaren et al., 1994; Marte et al., 1996; Wolthuis et al., 1996). When introduced into NIH3T3 cells R-Ras, like Ras, can induce foci, though this is much less efficient and the ‘transformed cells’ are morphologically similar to parental cells (Cox et al., 1994; Saez et al., 1994). Stable cell lines expressing activated R-Ras, however, give tumours in nude mice and contain elevated levels of ERK MAP kinases (Cox et al., 1994). These results suggest that R-Ras, like Ras, has an essential role in growth control and may activate some elements of the Ras signalling pathway.

Previous reports have shown that microinjection of Ras

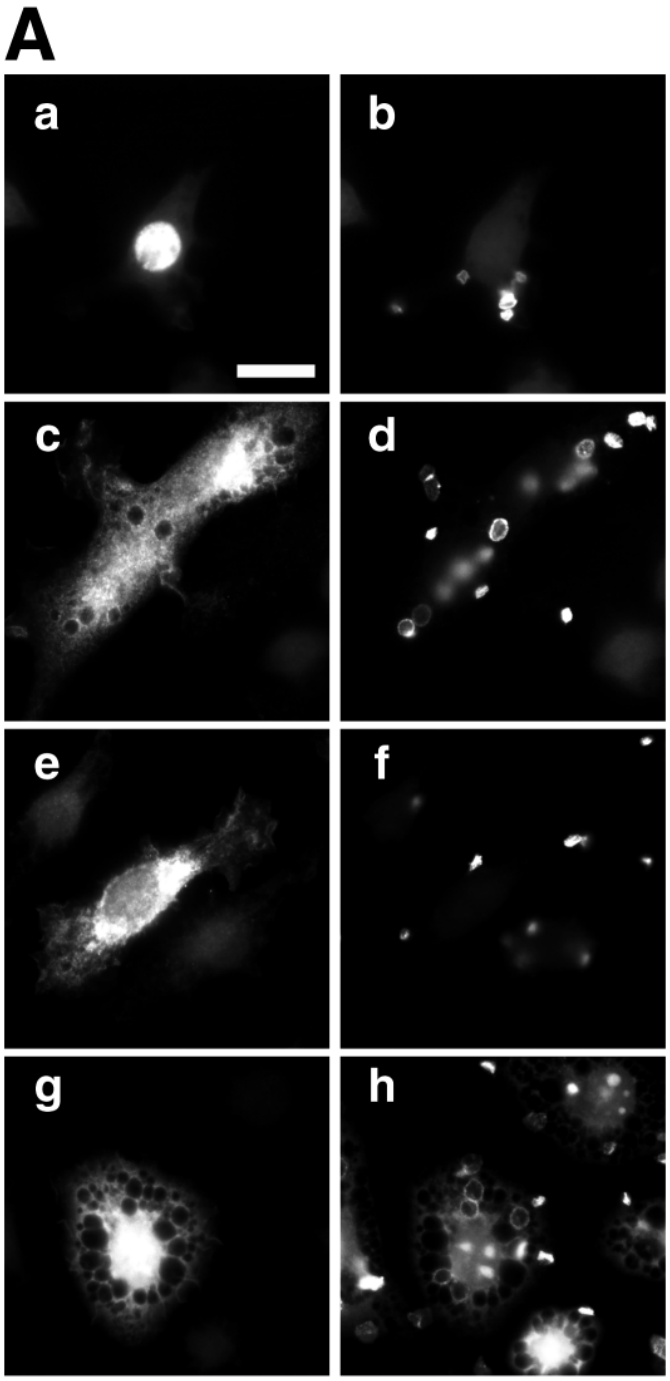

Fig. 4. R-Ras, but not Ras, activates the $\alpha_M\beta_2$ integrin in J774 macrophages. (A) J774 macrophages were microinjected with biotin-dextran (2.5 mg/ml) (a,b,g,h) or 100 μg/ml pRK5-Myc-V38R-Ras (c,d) or pRK5-Myc-V12Ras (e,f). Injected cells were left for 2.5 hours to allow for protein expression prior to the phagocytic assay. Cells injected with biotin-dextran were visualised using AMCA-coupled streptavidine (a,g), protein expression was assayed using antibody to the Myc-epitope (c,e) and red blood cells were visualised using rhodamine-conjugated anti-rabbit IgG (b,d,f,h). As a positive control, cells injected with biotin-dextran were activated for 15 minutes with 150 ng/ml PMA prior to the phagocytic challenge (g,h). Scale bar, 10 μm. (B) J774 macrophages were microinjected as above. Phagocytosis was assessed only in the retrieved Myc-positive cells and is expressed using the phagocytic index, which is defined as the number of internalised red blood cells per 100 macrophages. Results shown are the mean ± s.e.m. of at least three experiments where at least 50 cells were expressing the Myc-epitope.

protein into fibroblasts stimulates DNA synthesis (Feramisco et al., 1984; Stacey and Kung, 1984; Trahey et al., 1987). We previously reported that microinjection of R-Ras protein into quiescent Swiss 3T3 cells was unable to induce DNA synthesis, but have subsequently found that the V38R-Ras protein used was inactive due to proteolytic cleavage (Rey et al., 1994). Using full-length protein we show here that R-Ras induces DNA synthesis in both Swiss 3T3 and NIH3T3 as efficiently as does Ras. Treatment of quiescent, serum-starved Swiss 3T3 with PDGF leads to activation of Rac and membrane ruffling via a pathway involving PI3-kinase (Nobes and Hall, 1995; Reif et al., 1996). Microinjection of the V38R-Ras into Swiss 3T3 cells did not, however, produce any membrane ruffling or any other actin changes. Furthermore, V38R-Ras did not lead to phosphorylation of the PI3-kinase target Akt. We therefore conclude that R-Ras is unable to activate PI3-kinase in fibroblasts.

Since R-Ras induces G_1 progression in Swiss 3T3 cells and can bind to c-Raf1 in vitro, we investigated whether R-Ras could activate the ERK MAP kinase pathway using the scrape-loading technique under identical conditions to those used in the DNA synthesis assay. Ras protein strongly activated both ERK1 and ERK2, whereas R-Ras protein did not. This result contrasts with a previous report that cells overexpressing V38R-Ras had elevated levels of ERK MAP kinases (Cox et al., 1994). We therefore further investigated whether overexpression of both V38R-Ras and ERK could activate ERK using COS cell transfections and found that, in contrast to Ras, R-Ras was unable to do so. Since the experiments carried out by Cox and co-workers used stable cell lines overexpressing V38R-Ras (Cox et al., 1994), it is very possible that the increased levels of MAP kinase observed were not a direct effect of R-Ras, but rather a consequence of an autocrine loop in the transformed cells. We also found that R-Ras does not activate either the Jun or p38/Mpk2 MAP kinase signalling cascades.

Microinjection of oncogenic Ras into PC12 cells leads to activation of ERK, but in these cells this induces terminal differentiation into neuronal-like cells. It has been shown that although PC12 cells contain both c-Raf1 and B-Raf, Ras signals preferentially through B-Raf to activate MEK and the MAP kinase cascade (Jaiswal et al., 1994). Since R-Ras can also bind to B-Raf (Spaargaren et al., 1994; Marte et al., 1996), we looked at its effects in PC12 cells and found that it was

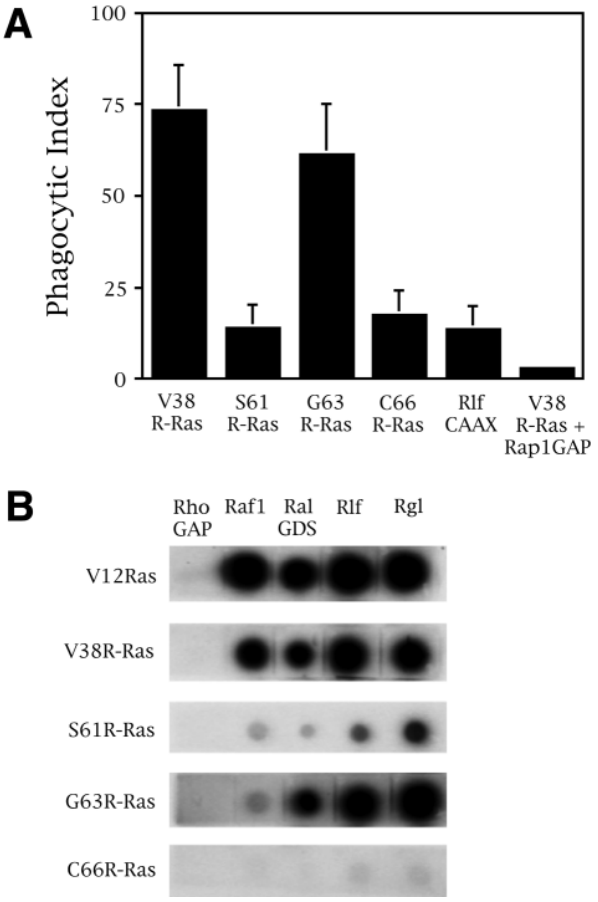

Fig. 5. Activation of the $\alpha_M\beta_2$ integrin by R-Ras is not mediated by Ras effector proteins. (A) J774 macrophages were injected with pRK5-Myc-tagged V38, S61, G63 or C66R-Ras or RlfCAAX or pRK5-Myc-tagged V38R-Ras plus pMT-HA-tagged Rap1GAP as before, and then activation of $\alpha_M\beta_2$ determined as measured by the phagocytic index of expressing cells. Results shown are the mean ± s.e.m. of at least three experiments. (B) Interaction of R-Ras effector mutants with Ras effector proteins in dot blot assays. 10 μg of the Ras binding domains of GST-RhoGAP, GST-c-Raf1, GST-RalGDS, GST-Rlf and GST-Rgl were spotted onto a nitrocellulose filter in a volume of 10 μl and incubated with 0.1 μg of [γ-^{32}P]GTP-loaded V12Ras, V38R-Ras, S61R-Ras, G63R-Ras or C66R-Ras. GTPase interactions were visualized by autoradiography.

unable to activate endogenous ERK1 and ERK2 and it did not induce differentiation. Interestingly, however, R-Ras did induce a dramatic change in cell shape with the cells undergoing flattening and substantial spreading. These observations are consistent with R-Ras having a role in the activation of integrins, which has been previously reported in some cell types (Zhang et al., 1996; Keely et al., 1999). However, due to the lack of suitable reagents to look at PC12 integrins, we moved to a better defined integrin activation system, the macrophage.

In macrophages, CR3-mediated phagocytosis of opsonized particles is dependent upon activation of the $\alpha_M\beta_2$ integrin. This integrin is functionally inactive in nonactivated macrophages but can be activated experimentally by phorbol esters or physiologically by inflammatory mediators such as lipopolysaccharide, platelet-activating factor or TNFα (Wright and Griffin, 1985). Our results show that R-Ras, but not Ras, can induce activation of $\alpha_M\beta_2$ and phagocytosis. Since this is not accompanied by activation of ERK and is not inhibited by the MEK inhibitor or by wortmannin, we conclude that R-Ras induced activation is not mediated by Raf or PI3-kinase. R-Ras has been reported to lead to activation of some integrins by antagonizing a Ras/Raf-initiated integrin suppression pathway (Sethi et al., 1999). Since expression of dominant negative (N17) Ras in J774 macrophages does not lead to activation of $\alpha_M\beta_2$ (Caron et al., 2000), we also conclude that R-Ras integrin activation in macrophages does not function by antagonizing Ras.

To further analyze the mechanism of R-Ras induced integrin activation we have made use of three R-Ras effector site mutants. Two of these, S61 and C66, were unable to induce phagocytosis but a G63 mutant was as efficient as V38R-Ras. Dot blot interaction assays revealed that G63 R-Ras does not bind to c-Raf1, but does strongly interact with the exchange factors RalGDS, Rlf and Rgl, which specifically activate Ral. S61 and C66 R-Ras on the other hand do not interact with RalGDS proteins. Since these three RalGDS members have been implicated in pathways downstream of Ras (White et al., 1996; Murai et al., 1997; Wolthuis et al., 1997), we reasoned that they might also be involved in the R-Ras integrin pathway. To test this, constitutively activated Rlf (Rlf-CAAX) was microinjected into macrophages but it did not induce CR3-mediated phagocytosis.

We have recently shown that the Ras related GTPase Rap1 controls a signal transduction pathway leading to the activation of the $\alpha_M\beta_2$ integrin. Micronjection of the Rap-specific Rap1GAP protein completely blocked phagocytosis induced by V38R-Ras, indicating that R-Ras is inducing phagocytosis via activation of Rap1. Recently many exchange factors that activate Rap1 have been identified (Zwarkruis and Bos, 1999), but it has not been reported whether any of these are activated by R-Ras. We conclude that R-Ras can activate the $\alpha_M\beta_2$ integrin in macrophages via a Rap1 dependent pathway that does not involve the ERK MAP kinase, PI3-kinase or the Ral exchange factors. Whether the effects of R-Ras on integrin activation reported previously by others in different cell types are also mediated through Rap1 remains to be seen (Zhang et al., 1996; Keely et al., 1999).

In conclusion, we have analyzed the function of R-Ras in three distinct cell types and have found that it induces G_1 progression in fibroblasts and integrin activation in PC12 cells and macrophages. R-Ras does not activate ERK or cause membrane ruffling in fibroblasts and activation of integrins in macrophages does not involve any of the three Ras targets Raf, PI3-kinase or RalGDS. Interestingly, R-Ras activation of the $\alpha_M\beta_2$ integrin proceeds via a pathway involving Rap1. Whether other putative targets of R-Ras, such as AF6 (Linnemann et al., 1999), have a role in R-Ras signalling has yet to be established. Therefore, whilst R-Ras clearly plays an important role in cell cycle control and integrin activation, the downstream effectors involved remain to be identified.

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