Potent transforming activity of the small GTP-binding protein Rit in NIH 3T3 cells: evidence for a role of a p38γ-dependent signaling pathway

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Abstract A novel branch of the Ras family, Rit, was recently identified. Rit exhibits a distinct C-terminus and effector domain, and does not activate mitogen-activated protein kinase (MAPK) but can cooperate with Raf to transform fibroblasts. Here, we found that when overexpressed, activated mutants of Rit transform NIH 3T3 cells efficiently, and stimulate p38γ but not MAPK, p38α, p38β, p38δ, or ERK5. Furthermore, we provide evidence that p38γ activation is required for the ability of Rit to stimulate gene expression and cellular transformation. These findings suggest that this unique GTPase stimulates proliferative pathways distinct from those regulated by other Ras family members. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Rit; Mitogen-activated protein kinase; p38γ; Focus formation

1. Introduction

The number of proteins in the Ras superfamily has expanded dramatically over the last decade. Two *ras* genes, v-H-*ras* and v-K-*ras*, were originally identified as the transforming sequences in the Harvey and Kirsten sarcoma viruses, respectively. Presently, more than 50 distinct Ras-like proteins controlling many aspects of cell growth and cellular behavior have been identified in mammals and other organisms. Based on their protein structure, the Ras superfamily can be divided into five subfamilies (see Fig. 1). The Ras family includes H-Ras, K-Ras, and N-Ras; the Rap subfamily, including Rap 1A, 1B, 2A and 2B; the R-Ras subfamily; the Ral subfamily, which includes Ral A and Ral B; and TC21 [1].

More recently, a novel branch of the Ras family, Rit (Ras-like protein in tissues) has been described. Rit was cloned as a homolog of Ric (calmodulin-binding Ras-like GTPase) in *Drosophila* [2]. This GTPase is highly expressed in most embryonic and adult tissues, and exhibits an effector (G2) domain distinct from that of Ras and lacks a C-terminal prenylation motif. Although the function of Rit is still unknown, it

was recently shown that Rit can cooperate with Raf to transform NIH 3T3 cells, in spite of its inability to stimulate mitogen-activated protein kinase (MAPK) pathways [3]. This observation prompted us to explore whether Rit may stimulate novel proliferative pathways distinct from those regulated by other members of the Ras superfamily. In this study, we show that, when overexpressed, Rit is alone sufficient to transform NIH 3T3 cells. Furthermore, we found that Rit can potently activate expression from the c-*jun* promoter. In search for the underlying mechanisms, we found that transforming Rit mutants did not stimulate MAPK, p38α, p38β, p38δ, or ERK5. In contrast, we observed that Rit can potently promote the activation of p38γ, a recently identified member of the p38 family of MAPKs, and present evidence that the MKK3/MKK6-p38γ pathway may regulate the transforming ability of this unique Ras-like GTPase.

2. Materials and methods

2.1. Cell lines and transfection
NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% calf serum, and transfected by the calcium-phosphate precipitation technique or the Lipofectamine PLUS (Life Technologies) technique following the manufacturer's instructions, adjusting the total amount of DNA to 2–5 µg/plate with vector alone.

2.2. DNA constructs
Expressing plasmids for epitope-tagged MAPKs and GTPases, including pCEFL HA-MAPK, pCEFL HA-JNK, pCEFL HA-p38α, pCEFL HA-p38γ, pCEFL HA-p38δ, pCEFL HA-ERK5, pCEFL AU5-c-H-Ras V12 and pCEFL AU5-RhoA Q63L, were described previously [4,5]. pCEFL HA-p38β and pCEFL AU5-Rit WT were obtained upon amplification of the corresponding coding sequence by the polymerase chain reaction technique, and subcloned into pCEFL. pCEFL AU5-Rit 30V and pCEFL AU5-Rit Q79L were obtained by site-directed mutagenesis (Quick Change kit; Stratagene), replacing glycine-30 and glutamine-79 by valine and leucine, respectively.

2.3. Western blots
Lysates containing approximately 50 µg of total cellular protein, anti-HA (12CA5, Covance) immunoprecipitates, or proteins bound to glutathione-Sepharose beads, (Pharmacia) were analyzed by Western blotting after SDS–polyacrylamide gel electrophoresis. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Corp.) using goat anti-mouse or goat anti-rabbit IgGs coupled to horseradish peroxidase as a secondary antibody (Cappel).

2.4. Kinase assays
MAPK activity in NIH 3T3 cells transfected with an epitope-tagged MAPK (HA-ERK2, referred in here as HA-MAPK), p38α, p38β,

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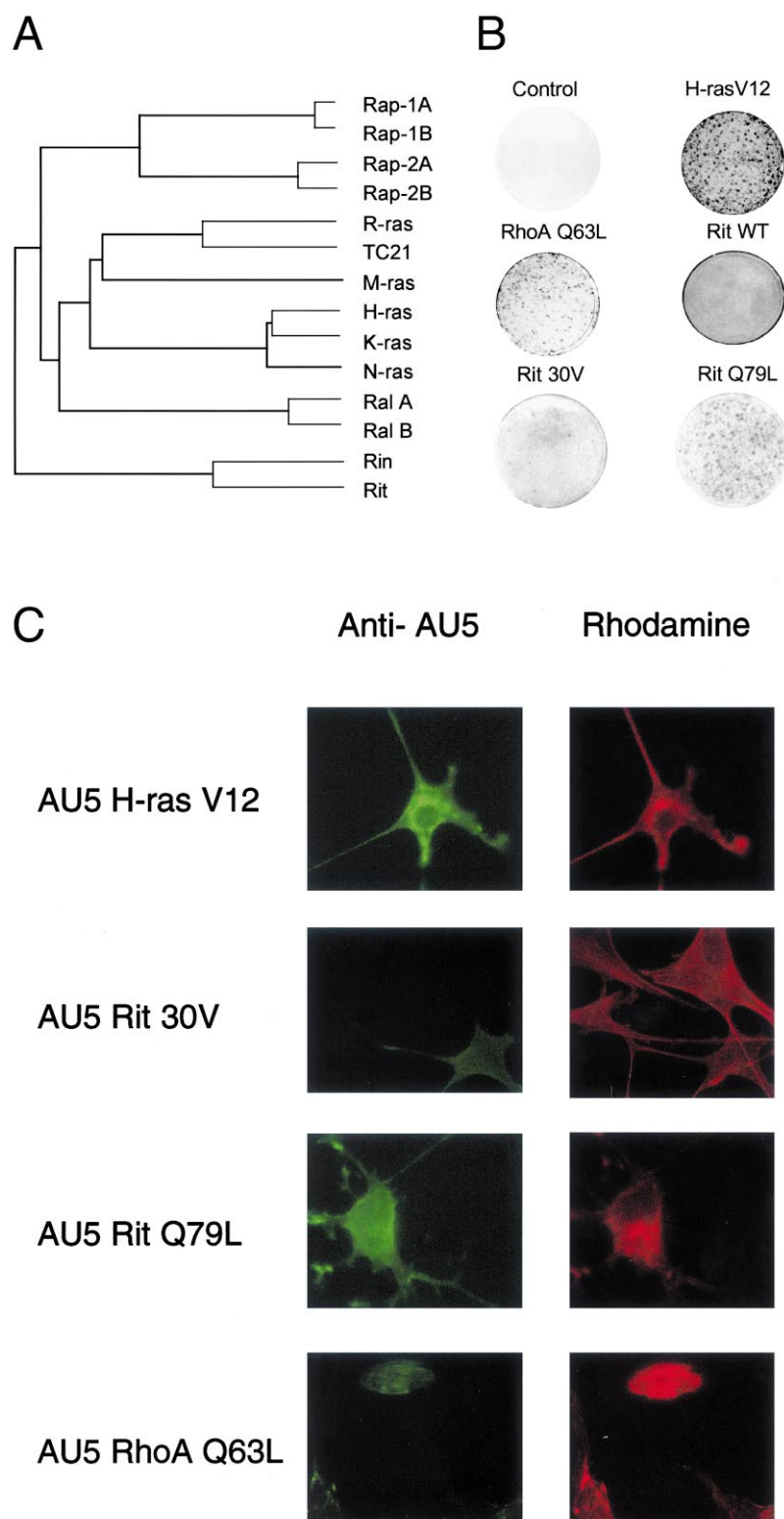[2] Present address: Bio-Technology General, Kiryat Weizmann, 76326 Rehovot, Israel.

Fig. 1. Phylogenic family tree of the Ras small GTPase superfamily members (A). Sequence alignment was performed using the Pileup program from the Genetics Computer Group (GCG) package using the following human protein sequences obtained from the NCBI and the Swiss-Prot data bank: Rin (NP 008843), Rit (NP 002921), Rap-1A (p10113), Rap-1B (p09526), Rap-2A (p10114), Rap-2B (p17964), H-Ras (p01112), N-Ras (p01111), K-Ras (p01118), R-Ras (p10301), TC21 (p17082), M-Ras (o14807), Ral A (p11233), Ral B (p11234). B: Focus formation assay in NIH 3T3 cells transfected with small G proteins. NIH 3T3 cells were transfected by the calcium phosphate technique with pCEFL vector (control) or with the same expression vector carrying cDNAs for H-Ras V12 (0.05 μg per plate), RhoA Q63L (0.5 μg per plate), Rit 30V (0.5 μg per plate) and Rit Q79L (0.5 μg per plate). Cells were cultured for 2 weeks in 5% calf serum, fixed, and then stained. C: Actin reorganization induced by the small GTPases. NIH 3T3 cells were transfected with expression plasmids encoding the small GTP-binding proteins Ras V12 (1 μg per plate), Rit 30V (1 μg per plate), Rit Q79L (1 μg per plate), and RhoA Q63L (1 μg per plate). Cells were fixed and stained after 48 h with rhodamine-labeled phalloidin to evaluate actin microfilament formation. Expression of each small G protein was confirmed by immunostaining using an anti-AU5 monoclonal antibody. Similar results were obtained for each experimental plasmid in three to five independent experiments.

p38δ, p38γ, JNK, and ERK5 were carried out as previously described [6,7], using myelin basic protein (Sigma), bacterially expressed GST-ATF2 (96) or GST-MEF2C fusion proteins as substrate. Samples were analyzed by SDS–gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen.

2.5. Reporter gene assays

NIH 3T3 cells were transfected with 1 μg of each of the different expression plasmids together with 0.1 μg of pJun-Luc reporter plasmid. After overnight incubation, the cells were washed with serum-free DMEM, and kept for 24 h in DMEM supplemented with 20 mM HEPES. Cells were then lysed using reporter lysis buffer (Promega).

2.6. Indirect immunofluorescence

NIH 3T3 cells were transfected by the Lipofectamine PLUS technique. Serum-starved transfected NIH 3T3 cells were washed twice with PBS then fixed with 4% formaldehyde with 5% sucrose in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. The cells were incubated with anti-AU5 mAb (Covance) at 10 μg/ml for 1 h, washed three times with PBS and then with a 1:100 dilution of fluorescein-conjugated goat F(ab')₂ IgG anti-mouse (Bio-

source International) and rhodamine-conjugated phalloidin (Molecular Probes). Coverslips were mounted in Gel-mount (Biomeda Corp.) containing p-phenylenediamine (ICN) at 1 mg/ml to inhibit photobleaching and viewed using an Axioplan2 fluorescence microscope (Zeiss). When required, pictures were taken at 40× magnification using a SPOT digital camera and software (Diagnostic Instruments) and analyzed using Adobe Photoshop.

2.7. Focus formation of NIH 3T3 cells

Plasmid DNA transfection of NIH 3T3 cells was performed by the calcium phosphate precipitation technique, and transformed foci were scored after 2–3 weeks as described [7].

3. Results and discussion

3.1. Focus formation in NIH 3T3 cells by Rit

A dendrogram of the Ras family (Fig. 1A) shows that Rit defines a distinct subfamily of Ras-related GTPases. Rit possesses C-terminal sequences not seen in any other Ras family proteins, which are modified by farnesyl or geranylgeranyl
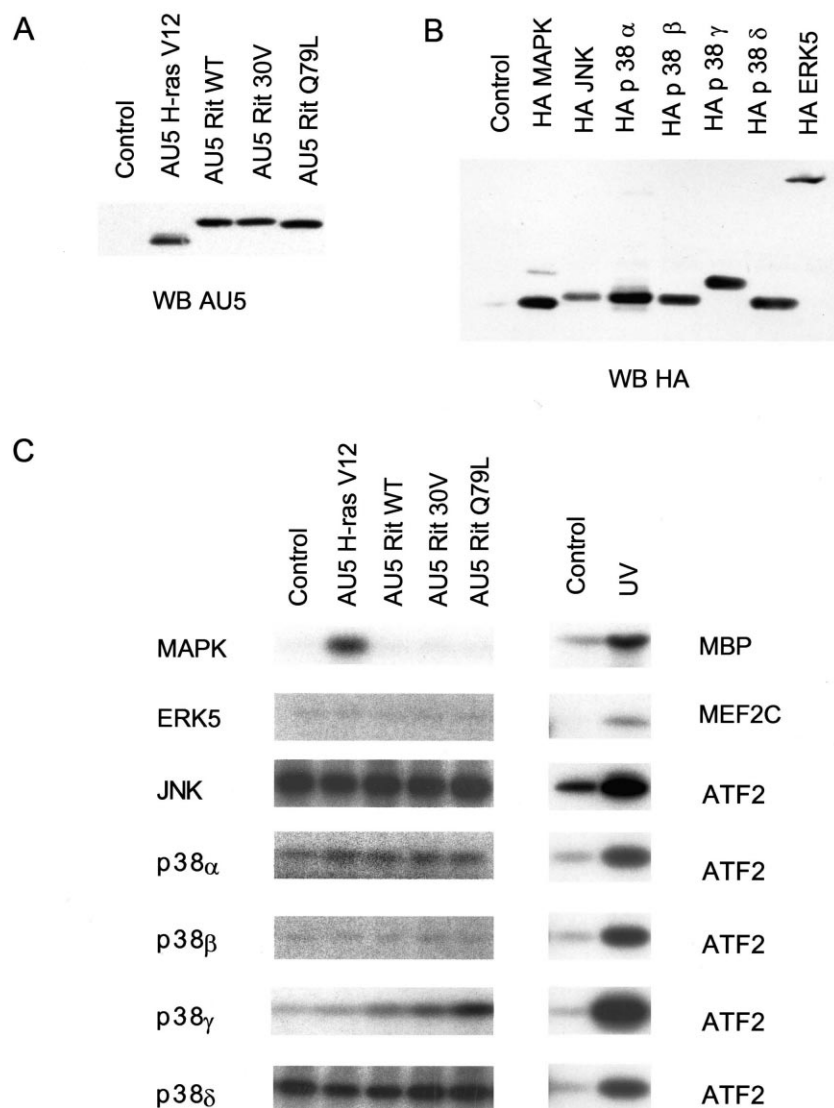

Fig. 2. Activation of MAPK pathways by UV and small GTPases. NIH 3T3 cells were transfected by the Lipofectamine PLUS technique with pCEFL vector (control) or with the same expression vector carrying cDNAs for the designated small GTPases (A) and MAPKs (B) (1 μg per plate in each case). Expression of the indicated epitope-tagged molecules was confirmed by Western blot analyses. C: In vitro kinase assays on anti-HA immunoprecipitates were performed in transfected cells, with or without UV stimulation (120 mJ, UV-Stratalinker 1800, Stratagene), as indicated.
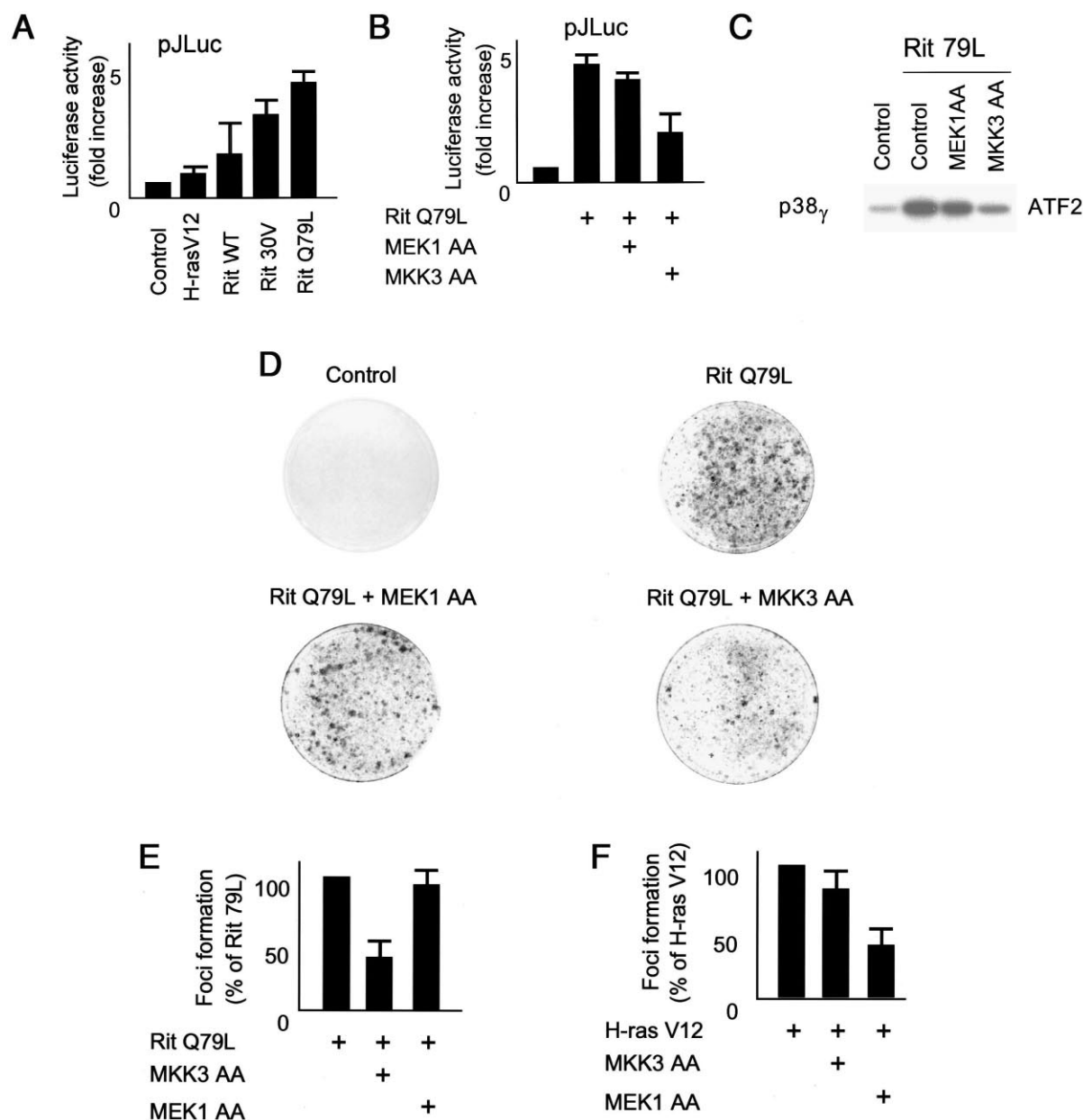
Fig. 3. Stimulation of the c-*jun* promoter activity by small GTPases. A: NIH 3T3 cells were cotransfected with pJLuc (0.1 μg per plate) and pcDNA3β-gal (1 μg per plate) plasmid DNAs together with expression plasmid containing the indicated small G protein (1 μg per plate). B: NIH 3T3 cells were cotransfected with pJLuc (0.1 μg per plate) and pcDNA3β-gal (1 μg per plate) plasmid DNAs together with expression plasmid for Rit Q79L (1 μg per plate) alone or in combination with MEK1 AA or MKK3 AA (1 μg per plate). Data represent luciferase activity normalized by the β-galactosidase activity present in each cellular lysate, expressed as fold induction with respect to control cells, and are the average ± S.E.M. of triplicate samples from a typical experiment which was repeated four to six times. C: NIH 3T3 cells were transfected with pCEFL vector (control) (1 μg per plate) or with vectors for MEK1 AA and MKK3 AA together with Rit Q79L and HA-p38γ, as indicated (1 μg per plate in each case) and subjected to in vitro kinase assays. D: NIH 3T3 cells were transfected with pCEFL vector (control) (1 μg per plate) or with the same expression vectors for Rit Q79L (0.5 μg per plate) (D,E) or Ras V12 (0.05 μg per plate) (F) alone or in combination with MKK3 AA or MEK1 AA (1 μg per plate in each case) for focus formation assays.

isoprenoids [2], and exhibits poor sequence conservation in the Ras effector domain, thus suggesting that this Ras family member may perform distinct cellular functions. Although its normal function is still unknown, recent evidence indicates that Rit can cooperate with other oncogenes to transform cells [3]. However, this approach did not reveal whether Rit could control growth-promoting pathways, nor the nature of the biochemical routes regulated by this GTPase. To explore whether Rit can itself transform NIH 3T3 cells, we used an

EF-1 (elongation factor 1) promoter driven expression plasmid (pCEFL), whose transcription activity is reported to be stronger than that of the CMV promoter [14].

As shown in Fig. 1B and Table 1, when used as controls H-Ras V12 and RhoA Q63L were highly transforming, each eliciting the formation of foci with a characteristically distinct morphology, being Ras V12 foci larger and diffuse, and Rho Q63L punctuate and clearly defined [4]. Rit WT did not transform NIH 3T3 cells effectively, but two activated mutants, Rit

30V and Rit Q79L produced the formation of foci, albeit with a clearly different efficiency (Table 1). Rit 30V produced few scattered foci, but Rit Q79L was nearly as transforming as Rho Q63L, although the morphology of Rit Q79L foci was more similar to that of H-Ras V12. Thus, this observation allowed us to begin exploring how this intriguing Ras-related protein promotes uncontrolled cell growth.

3.2. Rit induces a Ras-like change in the cytoskeleton

To investigate the effects of Rit in inducing actin rearrangement, NIH 3T3 cells were transfected with the AU5-tagged small GTP-binding proteins H-Ras V12, Rit 30V, Rit Q79L and RhoA Q63L, and studied by fluorescence microscopy employing an anti-AU5 antibody to detect the GTPases and with rhodamine-conjugated phalloidin to directly identify the filamentous actin containing structures. As shown in Fig. 1C, serum-starved NIH 3T3 cells exhibited very low levels of stress fibers. In contrast, RhoA Q63L overexpression induced the formation of multiple actin stress fibers and enhanced overall staining for F-actin associated with a contracted cellular morphology, clearly distinguishing transfected cells from adjacent cells. H-Ras, Rit 30V and Rit Q79L expression, on the other hand, did not induce stress fibers, but provoked changes in the cellular morphology, which became detached from the adjacent cells and exhibited membrane extensions in a stellate shape, with some ruffle-like structures at their end that were more remarkable for Rit Q79L.

3.3. Regulation of MAPK, JNK, p38 (α, β, δ, γ) and ERK5 kinase by Rit in NIH 3T3 cells

Emerging evidence indicates that many of the biological functions of small GTP-binding proteins are dependent on their ability to activate intracellular signaling pathways resulting in the stimulation of members of the MAPK superfamily of serine-threonine kinases [8]. Ras and certain Ras-related proteins activate ERK1 and ERK2, referred herein as MAPKs, while Rac and Cdc42 activate the stress activated protein kinases JNK and p38 [9]. As activated Rit had a profound biological activity in NIH 3T3 cells, we next examined whether this GTPase could activate MAPK family members in these cells. For these experiments, Ras V12, Rit WT, Rit 30V and Rit Q79L (Fig. 2A) were co-expressed with epitope-tagged forms of MAPK, JNK p38α, p38β, p38γ, p38δ and ERK5 in NIH 3T3 cells (Fig. 2B). As a general control, we used UV stimulation, which resulted in the activation of all MAPKs (Fig. 2C). As expected, Ras stimulated MAPK po-

Table 1
Focus-forming activity of wild-type and mutated Rit

DNA construct	Focus-forming activity (foci per μg of DNA)
pCEF AU5	< 1
pCEF AU5 H-Ras V12	1030 ± 76
pCEF AU5 RhoA Q63L	102 ± 18
pCEF AU5 Rit WT	10 ± 2
pCEF AU5 Rit 30V	22 ± 3
pCEF AU5 Rit Q79L	253 ± 24

Wild-type or mutated Rit cDNAs were subcloned in an expression vector, pCEF AU5, and 0.05–1 μg of plasmid DNA was transfected into NIH 3T3 cells. Cultures were scored for focus formation 2–3 weeks after transfection. Data shown represent mean values of the number of foci formed per μg of transfected DNA ± S.E.M. of triplicate plates from three independent experiments.

tently, but did not activate any of the other members of the MAPK superfamily in these cells. In contrast, Rit did not stimulate MAPK, as reported [3], nor it activated ERK5, JNK and the best-studied p38s, p38α and p38β (Fig. 2C). Rit did not activate p38δ, but surprisingly stimulated p38γ potently. Even wild-type Rit induced a nearly two-fold activation, which was slightly higher for Rit 30V. Furthermore, Rit Q79L provoked a remarkable activation of p38γ, which, of note, correlated well with its enhanced biological activity.

3.4. Rit stimulates c-jun transcription

As p38γ can regulate c-jun expression [7], we next examined the ability of Ras and Rit to stimulate the activity of the c-jun promoter (pJLuc) in this cellular system. As shown in Fig. 3A, Rit activated c-jun promoter potently, while Ras did not enhance the expression from this promoter. MKK3 and MKK6 function as upstream activators of the p38 MAPK family [10]. Thus, we asked whether these kinases participate in the activation of p38γ by Rit, using the expression of a dominant-negative form of MKK3, MKK3 AA [11], as an approach. As shown in Fig. 3C, c-jun transcriptional activity was blocked potently by the dominant-negative MKK3. Similarly, a kinase deficient mutant of MKK6, MKK6 KR [12] was also able to diminish the activation of the c-jun promoter by Rit (not shown). In contrast, MEK1 AA, a dominant-negative form of the upstream activator of the MAPK pathway [13], had no effect on the expression from the c-jun promoter.

3.5. Inhibition of Rit-induced transformation by a dominant-negative MKK3

We next examined whether inhibition of p38γ activation can affect the ability of Rit to transform cells. First, we confirmed that MKK3 AA but not MEK1 AA could prevent p38γ activation by Rit Q79L in NIH 3T3 cells (Fig. 3C). Furthermore, focus formation assays revealed that interfering with MKK3 function could diminish dramatically the transforming activity of Rit (Fig. 3D,E), although under identical conditions the inactive MKK3 did not affect Ras-induced focus formation (Fig. 3F). Conversely, a dominant-negative mutant of MEK1 effectively inhibited the transforming Ras pathway, but had a marginal effect on Rit (Fig. 3E,F).

4. Conclusion

The emerging picture from this study is that Rit can initiate cytoskeletal changes, c-jun transcription activity, and cellular transformation when expressed in NIH 3T3, utilizing biochemical routes distinct from those activated by other Ras family members. Specifically, available evidence suggests that the p38γ but not the MAPK pathway is required for the stimulation of the c-jun promoter and cellular transformation by Rit. Why Rit activates only p38γ is not known, as MKK3 and MKK6 can act upstream of all p38 family members. Additional factors, such as the presence of specific kinase-kinase recognition sequences [15], the expression of p38-specific phosphatases, or the existence of scaffolding molecules favoring the activation of specific kinase modules may help explain the specific activation of p38γ by Rit. These, as well as additional possibilities are under current investigation. On the other hand, prolonged activation of p38γ and other p38 isoforms by overexpression of MKK3 and MKK6 did not result in focus formation or cytoskeletal changes, nor they potenti-

ated these effects when elicited by Rit (data not shown). Thus, we can speculate that Rit may utilize additional proliferative pathways to transform NIH 3T3 cells. Further work will be required to elucidate fully the nature of the signaling events regulated by Rit, as well as to unravel the complexity of the molecular mechanisms whereby this GTPase may promote aberrant cell growth.

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