A domain containing the Cdc42/Rac interactive binding (CRIB) region of $p65^{PAK}$ inhibits transcriptional activation and cell transformation mediated by the Ras-Rac pathway

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Abstract The molecular bases of the versatile functions of Rho-like GTPases are still unknown. Using luciferase assays with rat 3Y1 cells, we found that Rac1 is integrated downstream of Ras in the TRE (TPA response element) activation pathway. Coexpression of a mutant of $p65^{PAK}$, PAK/RD, lacking the kinase domain but containing the Cdc42/Rac interactive binding (CRIB) region, suppressed the TRE activation and cell transformation caused by constitutively activated forms of Ras (RasV12) and Rac1 (Rac1V12). PAK/RD is a good tool to investigate the signaling pathways in which Rac and Cdc42 are involved.

Key words: Small GTP-binding protein; Transcriptional activation; Cell transformation; Signal transduction

1. Introduction

Small GTP-binding proteins or GTPases act as molecular switches in diverse cellular functions [1]. In mammalian cells, Rho (A, B, C isoforms), RhoE, RhoG, Rac (1, 2 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), and TC10, have been identified as members of the Rho family of GTP-binding proteins [1]. Microinjection experiments with Swiss 3T3 fibroblasts have revealed that Cdc42, Rac, and Rho control the formation of filopodia, membrane ruffles (lamellipodia), and actin stress fibers with focal adhesion complexes, respectively [2–6]. This dynamic rearrangement of the actin cytoskeleton is caused by the GTPase cascade reaction: Cdc42 activates Rac, which in turn activates Rho [6,7].

In addition to their effects on actin polymerization and cytoskeletal rearrangement, the Rho family of GTPases is involved in the regulation of cell growth. Rac and Rho are required for oncogenic Ras-induced transformation, cooperating with Raf [8–11]. Rac, Rho, and Cdc42 induce G1 cell cycle progression and subsequent DNA synthesis [12,13]. Knowledge about downstream signaling pathways stimulated by the Rho-like GTPases has been accumulating. Rac and Cdc42, but not Rho, have been shown to activate the JNK/SAPK and p38/Mpk2 signaling pathways, which are triggered by stress stimuli [13–18], and pp70S6K, which plays an important role in G1 cell cycle progression [19]. RhoA, Rac, and Cdc42 also stimulate a novel signaling pathway leading to transcriptional activation by SRF (serum response factor), which does not correlate with activation of known MAP kinase cascades [20].

In order to understand how the Rho family members execute these various functions, a number of candidate target proteins that interact with the Rho family of GTPases in a GTP-dependent manner have been described [21–28]. Among these candidate proteins, $p65^{PAK}$ seems to be a key molecule of the Rac/Cdc42 signaling pathway. $p65^{PAK}$ was originally isolated from rat brain as a serine/threonine kinase that was activated by the binding of GTP forms of Rac and Cdc42 [22]. A protein motif search using the GTPase-binding site of $p65^{PAK}$ has led to the identification of a minimum region of 16 amino acids required for Cdc42/Rac interactive binding (CRIB motif). More than 25 proteins containing a similar motif were identified from a wide range of eukaryotes, and several of these proteins have been shown to bind to Cdc42 and/or Rac in vitro in a GTP-dependent fashion [29].

It has been previously reported that Rac1 stimulates c-Jun transcriptional activity using GAL4-luciferase and GAL4-c-Jun fusion protein [15]. However, to date there is no report showing that Rac1 activates the TRE (TPA response element), which is an authentic c-Jun binding site. In this paper, using luciferase assays with rat 3Y1 cells, we demonstrate that a constitutively activated form of Rac1 potentiated transcriptional activation via a TRE, and Rac1 is integrated downstream of Ras in the Ras-mediated TRE activation. We also show that a mutant of $p65^{PAK}$, PAK/RD, lacking the kinase domain but containing the Cdc42/Rac interactive binding (CRIB) region, suppresses transcription via a TRE and cell transformation mediated by the Ras-Rac pathway. PAK/RD is thought to inhibit the activities of Rac/Cdc42 target proteins by titrating out endogenous Rac and Cdc42. Our studies suggest that PAK/RD and probably similar mutants for other CRIB motif-containing proteins may provide good tools to investigate the intracellular signaling pathways in which Rac and Cdc42 are involved.

2. Materials and methods

2.1. Isolation of $p65^{PAK}$ cDNA

We have recently identified a novel mammalian Ste20-related kinase, YSK1 [30]. In the process of the screening of a mouse brain cDNA library for YSK1, we isolated a cDNA clone 16L, related but not identical to that encoding YSK1. Screening of a rat 3Y1 cDNA library constructed in λ ZAPII using 16L as a probe permitted us to isolate full-length cDNA clone (16L-11) encoding rat $p65^{PAK}$ [31].

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA response element.

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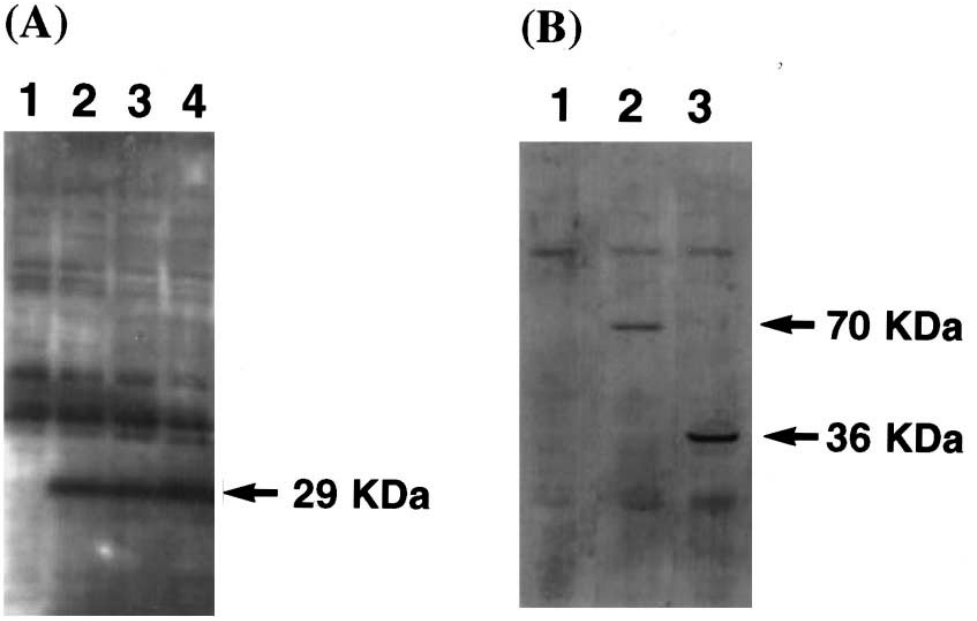

Fig. 1. Immunological detection of T7 epitope-tagged proteins. (A) COS1 cells were transfected with the empty vector (lane 1), the expression vector for T7-tagged wild-type Rac (lane 2), T7-tagged Rac1V12 (lane 3), and T7-tagged Rac1N17. (B) COS1 cells were transfected with the empty vector (lane 1), the expression vector for T7-tagged wild-type $p65^{PAK}$ (lane 2), and T7-tagged PAK/RD (lane 3). The same amount (100 μg) of each cell extract was reacted with the anti-T7 monoclonal antibody. The sizes of proteins are indicated in kDa.

2.2. Construction of expression plasmids

The SRHis expression vector was used for constructing all expression plasmids. The construction of the SRHis vector has been reported [32]. Expressed proteins contained 6 tandem histidine residues and the sequence, MASMTGGQQMGR, which is an epitope from the gene 10 leader peptide of T7 phage, at its N-terminus. An *Eco*RI site was introduced immediately upstream of the initiation codon by PCR to facilitate subsequent plasmid constructions. An *Eco*RI-*Eco*RI cDNA fragment of $p65^{PAK}$ containing the entire protein-coding sequence was blunt-ended and introduced into the *Pvu*II site of the SRHis expression vector. An *Eco*RI-*Bgl*II cDNA fragment (amino acid 1–251) corresponding to the regulatory domain (RD) of $p65^{PAK}$ was blunt-ended and introduced into the SRHis vector. A cDNA fragment containing the entire protein-coding region of Rac was obtained from rat brain cDNA by PCR amplification. Amplification was conducted with upstream primer (5′-GCGAATTCCCC-GAGATGCAGGCCATCAA-3′) and downstream primer (5′-GC-GAATTCGGCTCCGACATTTACAACAGC-3′), both of which have an *Eco*RI site at their 5′ end. The conditions for the PCR reactions were 25 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final step of 5 min at 72°C. The PCR fragment was digested with *Eco*RI and subcloned into the SRHis expression vector. Mutagenesis of Gly to Val at codon 12 or of Ser to Asn at codon 17 in Rac was carried out by overlapping PCR. The construction of expression plasmids for RasV12 and RasN17 has been previously reported [33].

2.3. Immunoblot analysis

COS1 cells were transfected with the expression plasmids for epitope-tagged Rac, Rac1V12, Rac1N17, $p65^{PAK}$, or PAK/RD by electroporation, and the following preparation of cell extracts were carried out as described [31]. The epitope-tagged proteins were reacted with anti T7-tag monoclonal antibody (Novagen), and then with horseradish peroxidase-conjugated sheep anti-mouse Ig (Amersham), and visualized using ECL detection reagent (Amersham). In some experiments alkaline phosphatase-conjugated goat anti-mouse Ig (TAGO) was used as a secondary antibody, and visualized using the alkaline phosphatase substrate kit I (Vector laboratories).

2.4. Luciferase assay

The TRE-luciferase reporter plasmid contains the luciferase structural gene positioned downstream of 6 tandem repeats of synthetic collagenase TRE (TRE×6) under the control of the SV40 promoter [34]. Rat 3Y1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The day before transfection cells were seeded at a density of 3×10^3 cells per 6 cm dish. Transfection was carried out using the calcium phosphate coprecipitation method. After 6 h, the medium was removed and the cells were washed twice with 3 ml of phosphate-buffered saline. The cells were then rendered quiescent by incubation in DMEM containing 0.5% FCS for 48 h. Harvesting the cells without stimulation, the preparation of cell extracts and the measurement of luciferase activity were performed as previously described [34].

2.5. Colony formation assay

NIH3T3 cells and a Ha-Ras-transformed cell line, 29-1, were grown in DMEM supplemented with 7% calf serum (CS). Cells were seeded at a density of 5×10^5 cells per 10 cm dish and cotransfected with 2 μg of pSV2-Neo and various combinations of expression vectors for RasV12, Rac1V12, Rac1N17, $p65^{PAK}$ and PAK/RD using the calcium phosphate coprecipitation method. The total amount of transfected DNA was adjusted to 20 μg per 10 cm dish with the SRHis vector. After 48 h culture in DMEM/7%CS, cells were grown in the presence of 300 μg/ml of G418 (geneticin, Gibco BRL) for 2 weeks. The selection medium was changed every 3 days. Tissue culture plates (6 cm) were coated with 3 ml of an agarose mixture (2.25×DMEM and 0.5% agarose were mixed at the ratio of 1:1). G418-resistant cells were collected, mixed and resuspended in 2 ml of DMEM. The cell suspension was combined with 4 ml of the agarose mixture (final 0.18% agarose), and 1.5 ml of this mixture was overlaid onto each plate. The cells were plated in triplicate at a density of 1×10^4 cells per dish and cultured for 3 weeks. The cells were fed with 1.5 ml of the 0.18% agarose mixture every 5 days. Colonies consisting of more than 10 cells were scored as positive. For morphological examination, a portion of G418-resistant cells were reseeded at a density of 5×10^5 cells per 10 cm dish and maintained in DMEM containing 200 μg/ml of G418 for a week.

3. Results

3.1. PAK/RD suppresses transcriptional activation via a TRE

Rac and Cdc42 have been implicated in activation of the JNK/SAPK [13–18]. Although using GAL4-luciferase and GAL4-c-Jun fusion protein it has been shown that c-Jun transcriptional activity is potentiated by Rac and Cdc42 [15], it is not clear that Rac1 activates the TRE, an authentic c-Jun

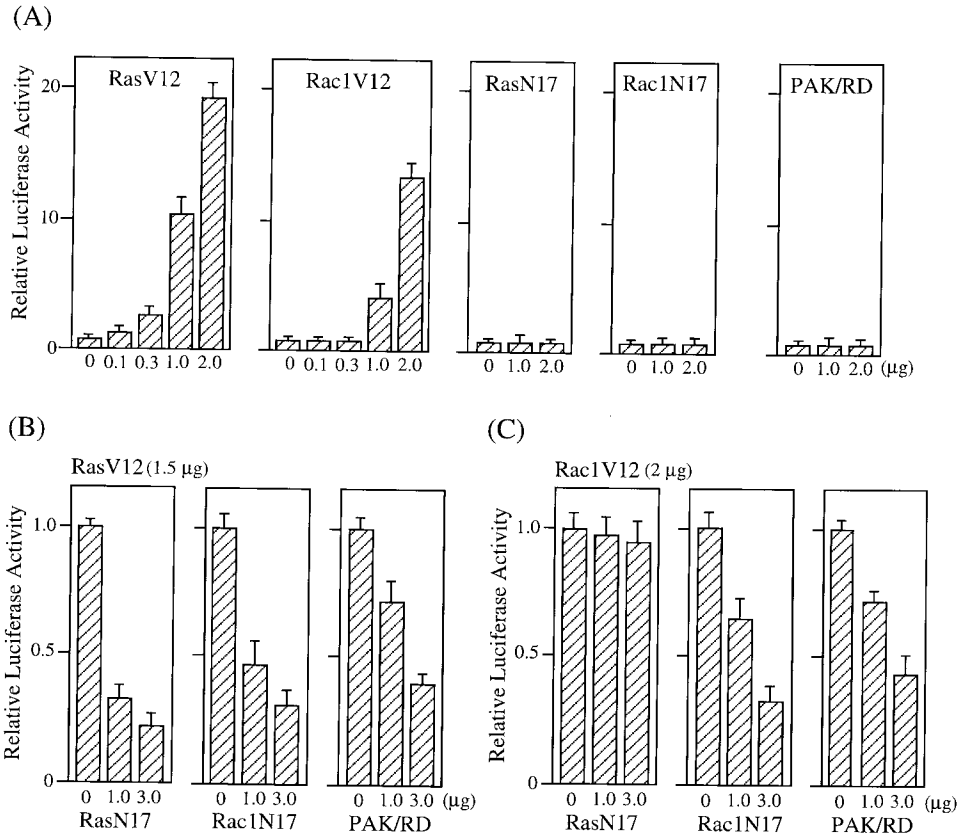

Fig. 2. Inhibitory effects of PAK/RD on the activity of TRE-luciferase by RasV12 and Rac1V12. (A) Rat 3Y1 cells were cotransfected with 1 μg of the TRE×6-luciferase reporter plasmid and 0–2 μg of the RasV12, Rac1V12, RasN17, Rac1N17, or PAK/RD expression vector as indicated. (B) 3Y1 cells were cotransfected with 1 μg of TRE-luc and 1.5 μg of the RasV12 expression vector together with RasN17, Rac1N17, or PAK/RD expression plasmid. (C) 3Y1 cells were co-transfected with 1 μg of TRE-luc and 1.5 μg of the Rac1V12 expression vector together with RasN17, Rac1N17, or PAK/RD expression plasmid. After serum starvation, cell extracts were prepared without stimulation.

binding site. We examined whether Rac is involved in transcriptional activation via a TRE using a constitutively activated and a dominant negative of Rac1, Rac1V12 and Rac1N17, respectively. The proteins of wild-type Rac1, Rac1V12, and Rac1N17 were expressed as T7 epitope-tagged proteins to facilitate immunological detection using the anti-T7 monoclonal antibody. As shown in Fig. 1A, T7 epitope-tagged Rac1, Rac1V12, and Rac1N17 were detected as 29-kDa proteins.

Transcriptional activities were measured in rat 3Y1 cells using TRE-luciferase as a reporter gene. RasV12, a constitutively activated form of Ras, activated the TRE-luciferase activity, consistent with the previous reports that Ras augments c-Jun activity (Fig. 2A). Rac1V12 activated TRE luciferase activity without any stimulation, in a dose-dependent manner, but Rac1N17 did not show any increase in luciferase activity (Fig. 2A). Rac1V12 did not activate luciferase from a mutated TRE (data not shown). We next examined whether Rac1 is integrated in the Ras-mediated TRE-luciferase activation. As shown in Fig. 2B, when dominant negative forms of Ras (RasN17) or Rac1 (Rac1N17) were cotransfected with RasV12, both RasN17 and Rac1N17 suppressed the activation of TRE-luciferase by RasV12 in a dose-dependent manner. However, when RasN17 or Rac1N17 were cotransfected with Rac1V12, Rac1N17 suppressed the TRE activation by Rac1V12, whereas RasN17 did not (Fig. 2C), suggesting that Rac functions downstream of Ras in the TRE activation.

We constructed a mutant, PAK/RD, which lacks the C-terminal kinase domain, but contains the N-terminal CRIB region. Rac and Cdc42 bind to the CRIB region of $p65^{PAK}$ and activate its kinase activity [22,29]. It is thought that PAK/RD could titrate out endogenous Rac and Cdc42 and inhibit the functions of molecules that rely on an interaction with Rac and/or Cdc42 for their activation, such as $p65^{PAK}$. Fig. 1B shows that T7-tagged $p65^{PAK}$ and PAK/RD were detected as 70- and 36-kDa proteins, respectively. PAK/RD itself did not lead to activation of TRE-luciferase (Fig. 2A) like RasV12 and Rac1V12. Coexpression of PAK/RD with RasV12 or Rac1V12 suppressed the activation of TRE-luciferase by RasV12 and Rac1V12 in a dose-dependent manner (Fig. 2B,C). Coexpression of wild-type p65PAK did not suppress the TRE activation by RasV12 or Rac1V12 (data not shown). These results suggest that molecules, which are activated by binding to Rac and/or Cdc42 and involved in transcriptional activation via a TRE through the Ras-Rac pathway, were blocked by PAK/RD.

3.2. PAK/RD inhibits cell transformation mediated by the Ras-Rac pathway

We next examined the effect of PAK/RD on cell transformation induced by overexpression of Rac1V12. NIH 3T3 fibroblasts stably transfected with the empty vector or with RasV12 were used as negative and positive controls of cell transformation, respectively (Fig. 3A,B). As previously re-

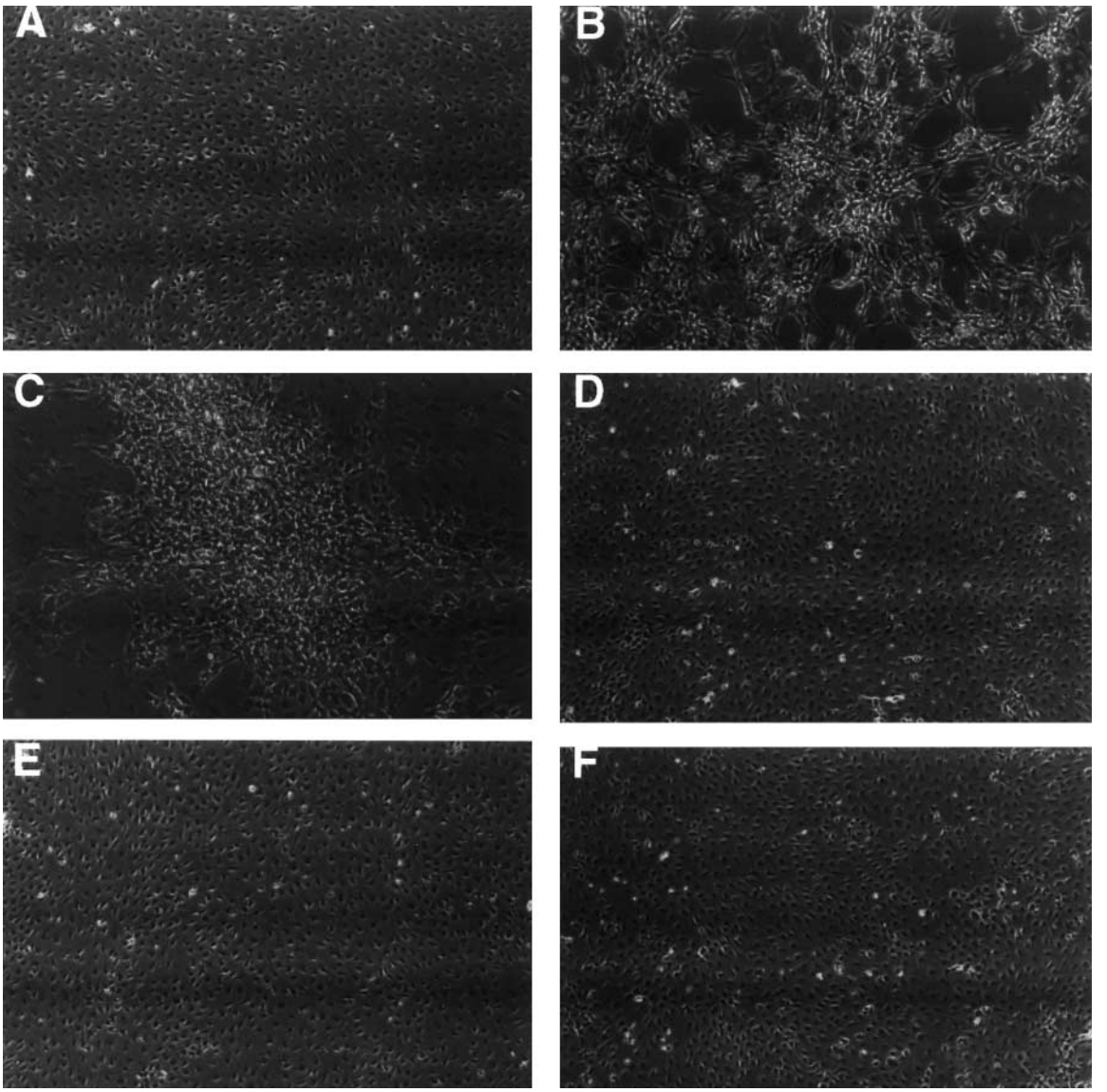

Fig. 3. Suppression of morphological transformation of Rac1V12 transfected NIH 3T3 cells by PAK/RD. Phase contrast photographs of NIH 3T3 cells stably transfected with (A) empty vector, (B) RasV12, (C) Rac1V12, (D) $p65^{PAK}$, (E) PAK/RD, and (F) Rac1V12 and PAK/RD. Magnification: ×40.

ported [8], overexpression of Rac1V12 promoted colony formation on soft agar, being comparable to the result with RasV12 (Table 1). Cells transfected with Rac1V12 showed an unorganized growth pattern, an increase in cell density, and piled up to make foci (Fig. 3C), indicating the loss of contact inhibition. In contrast, cells transfected with Rac1N17, wild type $p65^{PAK}$ or PAK/RD did not show an increase in colony forming activity (Table 1). These cells dem-

Table 1
Summary of the colony formation assay in NIH3T3 cells

	Ex 1	Ex 2	Ex 3
Vector	1.4 ± 0.6	2.2 ± 0.8	1.9 ± 0.2
RasV12	21.0 ± 1.1	9.2 ± 1.7	14.1 ± 2.4
Rac1V12	20.6 ± 2.7	16.9 ± 0.3	22.9 ± 3.0
Rac1N17	1.1 ± 0.4	1.8 ± 0.4	2.2 ± 0.7
PAK	1.0 ± 0.3	1.3 ± 0.9	ND
PAK/RD	0.7 ± 0.6	0.4 ± 0.4	2.3 ± 0.2
RasV12+Rac1N17	2.2 ± 0.3	4.3 ± 0.3	4.7 ± 0.2
RasV12+PAK/RD	1.1 ± 0.4	2.3 ± 0.3	6.2 ± 0.4
Rac1V12+PAK/RD	9.5 ± 1.6	3.2 ± 0.5	5.3 ± 1.5

ND, not determined. Each transfection was performed in triplicate. Colonies consisting of more than 10 cells were scored as positive. Values represent averages and standard deviations of colony formation normalized to total cell number within 5 randomly selected fields of view.

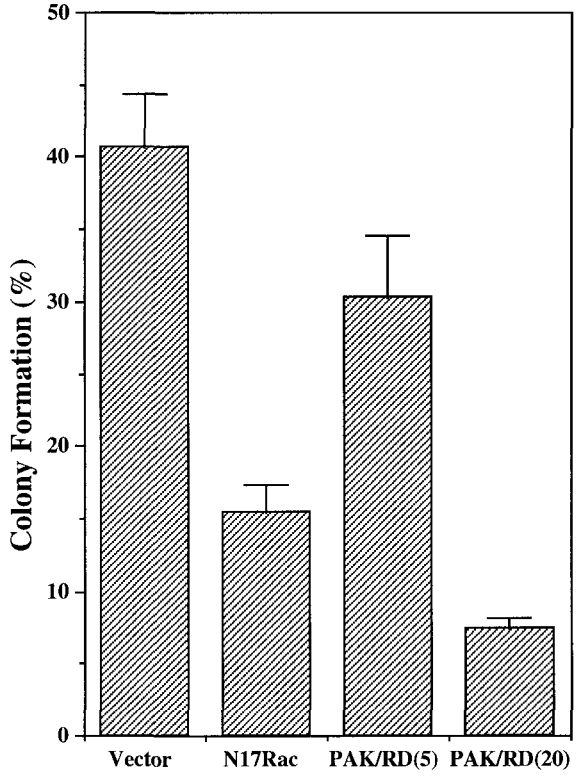

Fig. 4. Suppression of colony formation of a Ras-transformed cell line by PAK/RD. Ha-ras-transformed cell line, 29-1, was stably transfected with the empty vector (bar 1), Rac1N17 (bar 2), PAK/RD (5 μg per 10 cm dish) (bar 3), and PAK/RD (20 μg/dish, bar 4). The amount of transfected DNA adjusted to 20 μg per dish with the empty vector. Colonies consisting of more than 10 cells were scored as positive. Values represent averages and standard deviations of colony formation normalized to total cell number within 5 randomly selected fields of view. Bars indicate the averages and standard deviations of triplicate.

onstrated a flattened and cobblestone-like growth pattern like that of untransformed control cells (Fig. 3D,E). Coexpression of Rac1N17 with RasV12 inhibited oncogenic Ras-induced colony formation to the level of negative control as previously reported (Table 1) [8]. Coexpression of PAK/RD inhibited colony formation by RasV12, being comparable to Rac1N17, as well as colony formation by Rac1V12 (Table 1 and Fig. 3F). Similar results were observed in three independently performed experiments (Table 1) Coexpression of PAK/RD did not affect the expression level of RasV12 or Rac1V12 (data not shown).

In order to confirm the inhibitory effect of PAK/RD on transformation by RasV12 further, the 29-1 cell line, which was previously established by introducing a constitutively activated form of Ha-Ras into NIH3T3 cells [33], was stably transfected with PAK/RD. As shown in Figs. 4 and 5A, cells transfected with control selection vector alone showed significantly high colony formation activity. In contrast, introduction of PAK/RD suppressed colony formation of 29-1 cells in a dose-dependent manner, being comparable to the level seen in cells transfected with Rac1N17 (Fig. 4). The size of colonies detected in PAK/RD-transfected cells, if any, was much smaller than that of vector-transfected cells (Fig. 5B). These results suggest that PAK/RD suppressed the Ras-Rac-induced cell transformation by inhibiting the functions of Rac/Cdc42 interacting molecules.

4. Discussion

In this report, we have shown that expression of constitutively activated Rac (Rac1V12) in 3Y1 cells leads to transcriptional activation via a TRE. This activation is inhibited by Rac1N17, but not by RasN17, suggesting that Rac1 acts downstream of Ras in the c-Jun activation pathway. We also examined effects of a mutant of $p65^{PAK}$, PAK/RD, on the TRE activation and cell transformation caused by the Ras-Rac pathway. PAK/RD markedly antagonized cell transformation as well as TRE activation by RasV12 and Rac1V12. PAK/RD lacks the C-terminal catalytic domain, but has the N-terminal regulatory domain, which contains the CRIB motif, a minimal consensus sequence required for the binding of Rac and/or Cdc42. Therefore, PAK/RD is thought to inhibit the functions of cellular targets of Rac/Cdc42 by titrating out endogenous Rac and Cdc42.

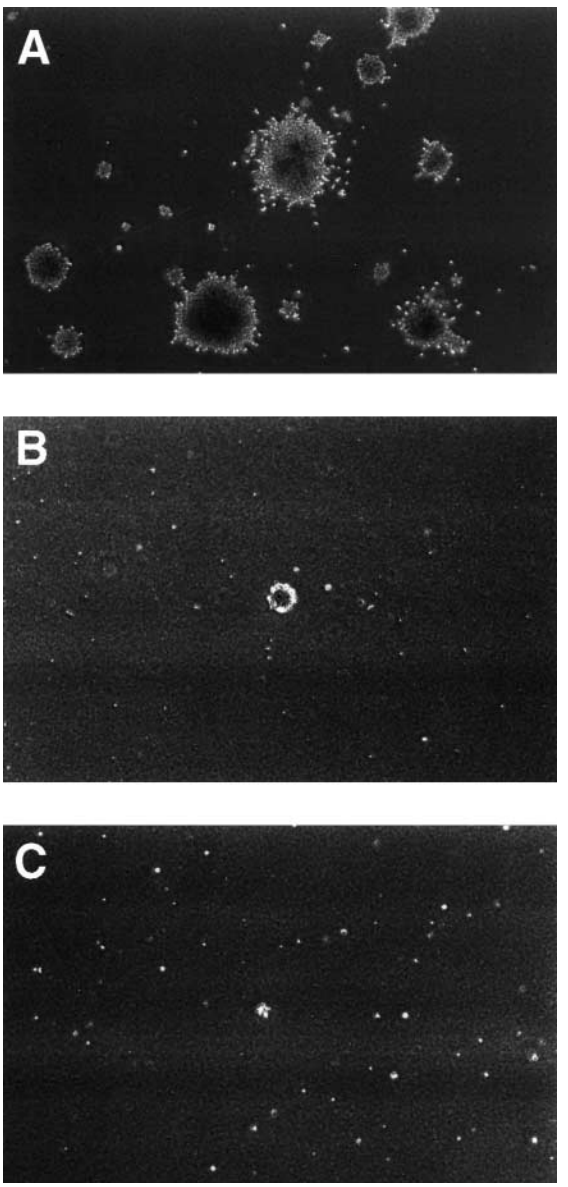

Fig. 5. Morphology of colonies in soft agar. Phase contrast photographs of cells from the Ha-ras-transformed NIH3T3 cell line 29-1: (A) stably expressed empty vector, (B) stably expressed Rac1N17, and (C) stably expressed PAK/RD. Magnification: ×40.

Inhibitory effects of the N-terminal regulatory domain of hPAK65 [35], one of the isozymes of $p65^{PAK}$, have been also reported [15]. The regulatory domain of hPAK65 (PAKR) inhibited JNK1 activation by constitutively activated Cdc42, and Dbl, which acts as an exchange factor for Cdc42 and RhoA [15]. As PAKR also contains the CRIB motif, the mechanism by which PAKR exerts its inhibitory effects seems to be similar to that of PAK/RD. More than 25 proteins containing the CRIB motif have been recently identified, including the growing members of the Ste20- and $p65^{PAK}$-related kinases [29]. Thus, the CRIB motif-containing domains from these proteins could act as good tools to block specifically the functions of Rac and Cdc42.

In order to understand the biochemical basis underlying a wide range of the biological effects of Rac and Cdc42, several cellular targets of Rac and Cdc42 have been identified [21–23,36,37]. Recently, using mutants of Rac and Cdc42 which harbor amino acid substitutions in the effector loop of the these GTPases, the roles of some of these target proteins have been analyzed [37]. A Y40C substitution in the effector loop of Rac and Cdc42 was not able to interact with $p65^{PAK}$, and could activate neither $p65^{PAK}$ nor the JNK/SAPK pathway. Despite this, Y40C-substituted Rac and Cdc42 were still able to induce actin polymerization and DNA synthesis. These results suggest that $p65^{PAK}$ activation by Rac and Cdc42 followed by the activation of the JNK/SAPK pathway are not required for cell transformation induced by Rac1V12 [37]. Consistent with this, to date there is no report that the components of the JNK/SAPK pathway, such as MEKK, JNKK/SEK1/MKK3, and JNK/SAPK could cause cell transformation. We also showed that overexpression of wild-type $p65^{PAK}$ in NIH 3T3 cells did not increase the colony forming activity. However, wild-type $p65^{PAK}$ is not necessarily in its active state. In the case of MAPK kinase (MEK), overexpression of constitutively activated forms of MEK, but not wild-type MEK, caused transformation in NIH3T3 cells [38,39] and differentiation in PC12 cells [38]. The transforming activity of a constitutively activated form of $p65^{PAK}$ will be needed to address this question.

Previous reports suggest that cell transformation by Ras bifurcates into two pathways, Rac/Rho-dependent pathway and Raf-dependent pathway [8–11]. The Raf-dependent pathway is linked to the ERK pathway, and the involvement of ERK in transformation of NIH3T3 has been reported [38,39]. On the other hand, the downstream components of the Rac/Rho-dependent pathway are still unknown. One of the possible candidates integrated into the pathway of Rac/Rho-dependent cell transformation is $p160^{ROCK}$. $p160^{ROCK}$, originally identified as a Ser/Thr kinase that interacts with Rho [23], can also bind to Rac in vitro in a GTP-dependent fashion [37]. The study using a mutant harboring a F37A substitution in the effector loop of Rac showed that this mutant was not able to interact with $p160^{ROCK}$ or induce DNA synthesis, suggesting that the interaction between Rac and $p160^{ROCK}$ is required for cell growth [37]. The Rho family of GTPases also activate PI3 kinase [40–42] and pp70S6K [19], both of which are key mediators of mitogenic stimuli. It will be of great interest to determine the interactions among these kinases in cell growth and transformation.

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