



The CDC42-specific inhibitor derived from ACK-1 blocks v-Ha-Ras-induced transformation

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Based on the previous experiments with the N17 mutant of CDC42, it has been speculated, but not proved as yet, that CDC42 is required for Ras-induced malignant transformation of fibroblasts. However, since this inhibitor could sequester many GDP-dissociation stimulators (GDSs), such as DBL, OST and Tiam-1 which activate not only CDC42, but also Rho or Rac, in fact it is not a specific inhibitor that inactivates only CDC42. Thus, we have taken the minimum CDC42-binding domain (residues 504–545, called ACK42) of the Tyr-kinase ACK-1 that binds only CDC42 in the GTP-bound form, and thereby blocking the interactions of CDC42-GTP with its downstream effectors such as ACKs, PAKs and N-WASP. First of all, using the ACK42-GST fusion protein as a specific ligand for the GTP-CDC42 complex, we have revealed that CDC42 is activated by oncogenic Ras mutants such as v-Ha-Ras in NIH3T3 fibroblasts, and similarly in PC12 cells by both NGF (Nerve Growth Factor) and EGF (Epidermal Growth Factor) which activate the endogenous normal Ras, providing the first direct evidence that CDC42 acts downstream of Ras and NGF/EGF. Furthermore, over-expression of ACK42 completely reversed Ras-induced malignant phenotypes such as focus formation and anchorage/serum-independent growth of the fibroblasts, and a cell-permeable derivative of ACK42 called WR-ACK42 strongly inhibited the growth of Ras transformants, with little effect on the parental normal cell growth, and also abolished Ras-induced filopodium/microspike formation of the fibroblasts which is CDC42-dependent. These observations unambiguously proved for the first time that the RAS-induced activation of CDC42 is indeed essential for Ras to transform the fibroblasts, and furthermore suggest that ACK42 or its peptidomimetics are potentially useful for genotherapy or chemotherapy of Ras-associated cancer.

Keywords: CDC42; ACK; NGF; neurite outgrowth; Ras transformation; tumor suppressor

Introduction

Rho family GTPases including Rho, Rac and CDC42 play the major role in controlling actin cytoskeleton organization (Hall, 1998). Rho is required for the

formation of stress fibers, focal adhesion plaques and contractile ring. Rac is essential for membrane ruffling. CDC42 is required for microspike/filopodium formation. Ras is involved in the activation of some of these Rho family GTPases. For instance, Rac is activated by a Rac GDP-dissociation stimulator (GDS) called Vav that is activated by the Tyr-kinase Lck and an end-product of PI-3 kinase (Crespo *et al.*, 1997; Han *et al.*, 1998), the latter kinase being activated by Ras (Rodriguez-Viciano *et al.*, 1997). CDC42 is activated through bradykinin B2 receptor (BB2R), and the BB2R gene is activated by Ras through an as yet uncharacterized pathway (Kozma *et al.*, 1995; Downward *et al.*, 1988). These findings together lead us to a speculation that Ras is involved in the activation of CDC42. Interestingly, the N17 mutant of CDC42 inhibits both Ras/Raf-induced focus formation of normal fibroblasts (Qui *et al.*, 1997), suggesting that (i) CDC42 might be essential for both Ras/Raf-transformation, and that (ii) Raf might be involved in Ras-induced activation of CDC42.

However, strictly speaking, the N17 mutant of CDC42 blocks not only the activation of CDC42 by sequestering a CDC42-specific GDP-dissociation stimulator (GDS) called FGD1, but also could block at least in part the activation of other Rho family G proteins such as Rac and Rho by sequestering DBL, OST and Tiam-1, GDSs that activate Rho or Rac, in addition to CDC42 (Maruta, 1998; Feig, 1999). Thus, it still remains to be clarified whether the observed effect of the N17 mutant of CDC42 is due solely to the inactivation of CDC42, or also to the inactivation of both Rac and Rho. The N17 mutant of Rac also inhibits Ras-induced malignant transformation (Qui *et al.*, 1995). Rho GTPases, in particular RhoB, also appear to be essential for Ras transformation, as a farnesyltransferase inhibitor that blocks the farnesylation of Rho B reverses Ras transformation (Lebowitz *et al.*, 1995), and the bacterial exotoxin C3 that specifically inactivates Rho GTPases by ADP ribosylation selectively inhibits the growth of Ras transformants, but not the parental normal fibroblasts (He *et al.*, manuscript in preparation). It has recently been suggested that Rho is activated by Ras through the SH3 domain of a Ras GAP of 120 kDa (Leblanc *et al.*, 1998). Thus, to determine unambiguously whether CDC42 is essential for Ras transformation, it is critical to create a new inhibitor which is highly-specific for CDC42, and has no effect on any other related GTPases. Interestingly, the Tyr-kinases ACK-1 and ACK-2 bind only CDC42 in the GTP-bound form (Manser *et al.*, 1993; Yang and Cerione, 1997).

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In this study, using ACK42 which is the minimum CDC42-binding fragment of ACK-1 (residues 504–545) (Mott *et al.*, 1999), we provide the direct evidence for the Ras-induced activation of CDC42. Furthermore, by either the conventional DNA transfection or a novel delivery through a cell-permeable peptide vector of 16 amino acids called penetratin or WR (Derossi *et al.*, 1998), we demonstrate that v-Ha-Ras-induced malignant transformation and is completely suppressed by this truly CDC42-specific inhibitor, ACK42, clearly proving for the first time that CDC42 is essential for Ras transformation. Our findings indicate that the ACK42-WR conjugate is the first cell-permeable tool for blocking selectively the CDC42-dependent signaling pathways of the intact cells, and suggest that ACK42 mimetics are potentially useful for the treatment of Ras-associated cancers.

Results

Ras and NGF induce the activation of CDC42

Like full-length ACK-1 and ACK-2, ACK42 binds only the CDC42-GTP complex, and not any other GTPases (E Manser, personal communication). Thus, to monitor whether Ras transformation causes any activation of CDC42 (conversion from the GDP-bound form to the GTP-bound form), we have used ACK42-GST fusion protein as a specific ligand for the active GTP-CDC42 complex. We found that ACK42-GST beads bind much more CDC42 in the lysate from Ras transformed than the parental normal NIH3T3 cells, although the total levels of CDC42 are not changed by Ras transformation (see Figure 1a), clearly indicating that Ras induces the activation of CDC42. Similarly, NGF which induces the activation of Ras also induces the activation of CDC42 in PC12 cells (see Figure 1b).

Over-expression of ACK42 suppresses v-Ha-Ras-induced transformation of NIH3T3 cells

To determine unambiguously whether CDC42 is essential for Ras transformation, we have over-expressed ACK42, the CDC42-specific inhibitor, in v-Ha-Ras-transformed NIH3T3 cells. ACK42 selectively blocks the interactions of CDC42 with any of its effectors including ACK family kinases, PAK family kinases and N-WASP. Approximately 30 neomycin resistant transfectants were selected at random, and their morphology was analysed (see Figure 2). Like normal NIH3T3 fibroblasts, the ACK42 transfectants formed a thin monolayer in a liquid culture, and no longer formed foci by piling up on top of each other, indicating that contact inhibition of growth is restored in these transfectants.

Changes in growth behavior of v-Ha-Ras transformed cells by blocking CDC42 signals

Unlike the parental v-Ha-Ras transformants which grow in soft agar in an anchorage-independent manner, the ACK42 over-expressors are no longer able to grow in soft agar (see Table 1), clearly indicating that CDC42 is required for the anchorage-independent

growth. The saturation density and contact inhibition of the ACK42 over-expressors were compared with those of parental v-Ha-Ras transformants. The ACK42 over-expressors stop growing at much lower density of cells, compared with the parental v-Ha-Ras transformants (see Figure 3a). Furthermore, the parental v-Ha-Ras transformants grew at much lower serum concentrations, compared with the ACK42 over-expressors which require much higher serum concentrations as do normal fibroblasts (Figure 3b). Northern blot analysis confirmed that all ACK42 transfectants

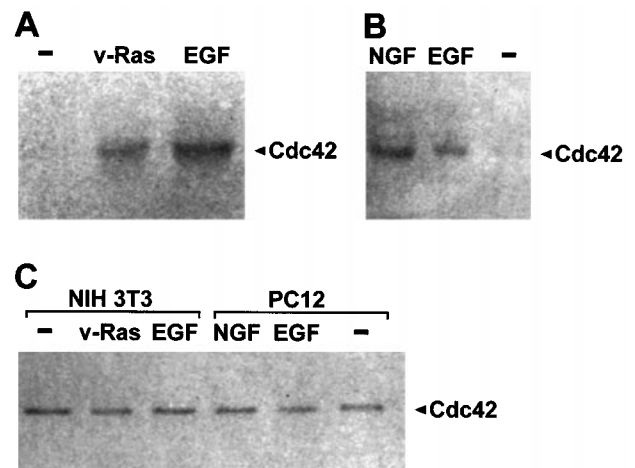


Figure 1 Ras/NGF-induced activation of CDC42 in NIH3T3/PC12 cells. (a) v-Ha-Ras/EGF-induce activation of CDC42 in fibroblasts. The GTP-CDC42 complex (indicated by the arrow-head), that binds the ACK42-GST fusion protein on GSH beads, in lysates derived from either normal (minus), v-Ha-Ras-transformed (v-Ras) or EGF-treated NIH3T3 cells (150 μ g protein each) was immuno-blotted with the anti-CDC42 antibody after the separation by SDS-PAGE as described under the 'Materials and methods'. (b) NGF/EGF-induced activation of CDC42 in PC 12 cells. The GTP-CDC42 complex (indicated by the arrow-head), that binds the ACK42-GST on beads, in lysates derived from NGF-treated, EGF-treated or non-treated (minus) PC12 cells (150 μ g protein each) was immuno-blotted as described in (a). (c) No change in the total CDC42 levels by v-Ha-Ras-transformation or NGF/EGF-treatment of cells. The total CDC42 (GDP/GTP-bound forms, indicated by the arrow-head) levels in lysates (30 μ g protein each) derived from NIH3T3 or PC12 cells (normal, Ras-transformed or NGF/EGF-treated) was directly immuno-blotted as described in (a) or (b) (without ACK42-GST beads)

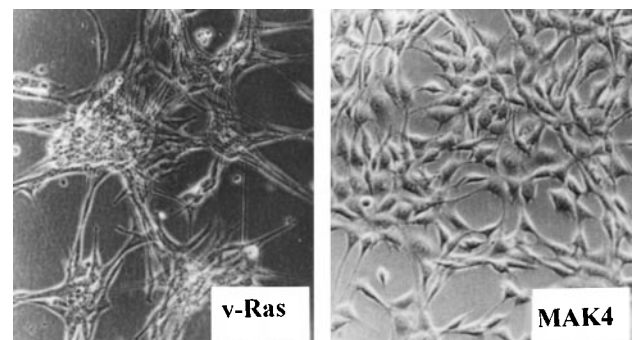


Figure 2 Morphological changes of v-Ha-Ras transformants by ACK42. The parental v-Ha-Ras transformants (v-Ras) and one of the typical ACK42 transfectants (MAK4) were grown to near confluency. Unlike the transformants, the ACK42 transfectants no longer formed a focus, and showed a flat morphology

express ACK42 mRNA (Figure 4a). Furthermore, v-Ha-Ras protein levels in the parental, pMV7 transfected and ACK42 over-expressing cells were quite similar (Figure 4b), confirming that ACK42 does not

Table 1 Suppressed colony formation of v-Ha-Ras transformants in soft agar. 10^3 cells (Parental, pMV7 alone transfected or ACK42 over-expressing v-Ha-Ras transformants) were plated in soft agar. After 20 days of incubation the number of cell colonies were counted under a microscope

| Clone number | Number of colonies | | | Suppression (%) |
|--------------------------|--------------------|-------|-------|-----------------|
| | Small | Large | Total | |
| Parental v-Ha-Ras cells | 650 | 320 | 970 | 0 |
| pMV7 alone transfectants | 720 | 270 | 990 | 0 |
| ACK42 transfectants: | | | | |
| Clone 1 | 50 | 0 | 50 | 95 |
| Clone 2 | 10 | 0 | 10 | 99 |
| Clone 3 | 80 | 0 | 80 | 92 |
| Clone 4 | 0 | 0 | 0 | 100 |
| Clone 5 | 5 | 0 | 5 | 99.5 |
| Clone 6 | 0 | 0 | 0 | 100 |
| Clone 7 | 20 | 0 | 20 | 98 |

Size of colonies: Large, more than 30 cells per colony; Small, less than 30 cells per colony

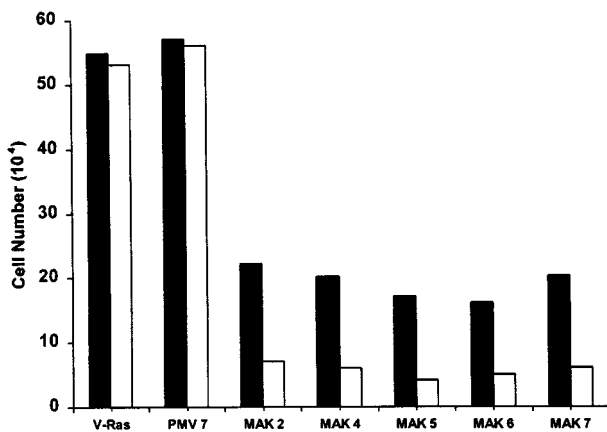
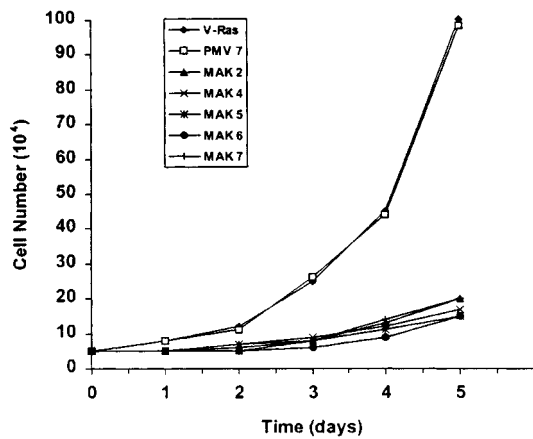


Figure 3 Effects of ACK42 on the growth behavior of Ras transformants. Equal number (5×10^4 cells) of either the parental v-Ha-Ras transformants (v-Ras), the vector alone transfectants (pMV7) or ACK42 transfectants (MAK2-7) were seeded and their growth was monitored as described under the 'Materials and methods'. (Top panel), their growth rate and saturation density were compared in the presence of 10% FCS. (Bottom panel), their serum dependency for the growth was compared by culturing in the presence of 10% FCS (closed bar) and 0.5% FCS (open bar). Data presented are representative of three independent experiments

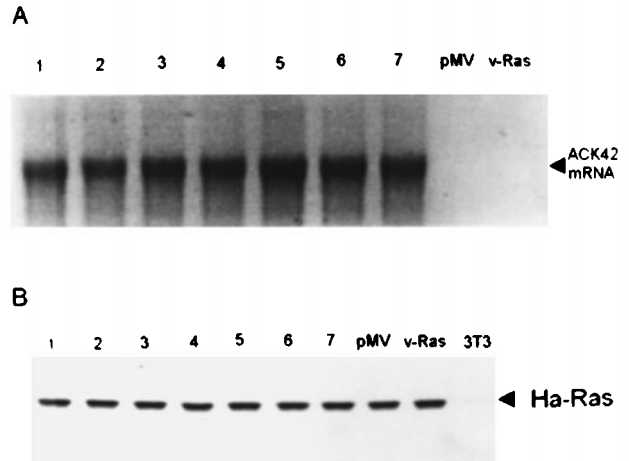


Figure 4 ACK42 mRNA and v-Ha-Ras protein levels in ACK42 transfectants. (a) Northern blot analysis of ACK42 mRNA levels in $2 \mu\text{g}$ of poly(A)⁺ RNAs isolated from the parental (v-Ras), the vector alone transfectants (pMV) or ACK42 transfectants (lanes 1–7). (b) Immunoblot analysis of v-Ha-Ras protein levels in $20 \mu\text{g}$ of proteins isolated from the total lysate of each clone with an anti Ha-Ras antibody as described under the 'Materials and methods'. The cells are the same as those indicated in (a), except for the lane 7, i.e. the negative control (the lysate from normal NIH3T3 fibroblasts that express normal Ras proteins, but not v-Ha-Ras) in (b)

affect v-Ha-Ras expression levels. These observations clearly indicate that over-expression of ACK42 suppresses v-Ha-Ras-induced malignant transformation, and confirm that CDC42 is indeed required for Ras-induced malignant phenotypes including the anchorage-dependent growth, loss of contact inhibition and serum-independency for growth.

The cell-permeable ACK42 derivative blocks the growth of Ras transformants and filopodium formation

To deliver a variety of 'cargo' peptides of less than 100 amino acids such as ACK42 into the cytoplasm or nucleoplasm of target cells, a series of novel peptide vectors/vehicles of 16 amino acids called penetratins have been recently developed (for a review see Derossi *et al.*, 1998). Penetratins are potentially quite useful tools for comparing the anti-cancer potential of the given 'cargo' peptide with its mutants quantitatively to screen for its higher-affinity (and therefore more potent) mutants. The simplest version of penetratins, called WR, consists of only ten Arg residues and six Trp residues (Williams *et al.*, 1997). To determine a dose-response effect of the anti-Ras peptide ACK42 on the growth of Ras transformants, we first generated in *E. coli* a recombinant thrombin-cleavable fusion protein called GST-HA-WR-ACK42 in which the HA, a hemagglutinin epitope/flag of 12 amino acids, is flanked by GST (at the N-terminus) and WR-ACK42 (at the C-terminus), and then affinity-purified it on GSH-beads. Since GST is too large as a cargo for the WR-mediated penetration across the plasma membranes, GST was then separated from the HA-WR-ACK42 by thrombin-cleavage.

The resulting 'free' HA-WR-ACK42 (and a trace amount of thrombin) at various concentrations was added to the culture of Ras transformants or the parental normal fibroblasts, and its effect was

monitored for 48 h. The HA-WR-ACK42 is indeed cell-permeable as judged by a fluorescent staining of the treated cells with an anti-HA polyclonal antibody (see Figure 5). Furthermore, HA-WR-ACK42 significantly inhibits the growth of Ras transformants in a dose-dependent manner (see Table 2) and their filopodium/microspike formation (see Figure 6). Under the same conditions, HA-WR-ACK42 has no

effect on the growth of the normal cells (data not shown). These observations indicate that the cell-permeable ACK42 is a very useful tool for selectively inhibiting the CDC42-mediated pathways, and suggest the anti-cancer drug potential of its peptidomimetics for the treatment of Ras-associated cancers.

Discussion

Here, using a specific peptide which binds only CDC42 in the GTP-bound form, we have clearly demonstrated that (i) Ras/NGF induces the activation of CDC42, and that (ii) CDC42 is required for Ras-induced malignant transformation. Using a similar ACK42-based approach, we have found that the NGF-induced neurite outgrowth (NOG) of PC12 cells also requires CDC42 (Nur-E-Kamal *et al.*, submitted). The CDC42/GTP complex binds several distinct proteins including CDC42 GAPs such as n-chimaerin and p190-A as well as its effectors such as the Tyr kinase family ACKs and the Ser/Thr kinase family PAKs. The next question is what is an effector(s) of CDC42 that is responsible for NOG or Ras transformation. One of the ubiquitous CDC42-binding proteins called N-WASP, which is an F-actin severing protein rich in brain, has been recently shown to be an effector of CDC42 which is essential for the microspike/filopodium formation in fibroblasts (Miki *et al.*, 1998). More recently, a dominant negative mutant of N-WASP, which no longer binds actin but still binds CDC42, blocks NOG of PC12 cells, supporting the notion that N-WASP is essential for the NOG as well (Miki *et al.*, personal communication). These findings suggest the possibility that N-WASP acts as an effector of CDC42 for Ras transformation of fibroblasts as well. We are currently examining this possibility more directly by over-expressing an anti-sense N-WASP RNA.

Our finding that over-expression of ACK42 suppresses Ras transformation suggests a new avenue to the development of anti-Ras cancer drugs based on ACK42: a conjugate of ACK42 with the cell-permeable peptide vector ('penetratin' or its derivative WR) could be used for the peptidotherapy of Ras-associated cancers. In fact we have shown that addition of the ACK42-WR conjugate to the culture medium is sufficient to inhibit strongly the growth of Ras-transformed cells, but not the parental normal fibroblasts. Thus, ACK42 mimetics, chemical compounds that functionally mimic ACK42, also it could be potentially useful for the cancer chemotherapy. Previously we have shown that the minimal Ras-binding fragment (Raf81, residues 51–131) of the Ser/Thr kinase c-Raf-1 suppresses Ras transformation (Fridman *et al.*, 1994), and furthermore, to potentiate its anti-Ras action, we have created a few mutants of Raf81 which have much higher affinity for the Ras/GTP complex by replacing Gln64, Val70, Ala85 or Val 88 with basic amino acids (Arg or Lys) (Maruta, 1996). Similarly, by a PCR-based site-directed mutagenesis, we are currently screening for the mutants of ACK42 that show a much higher affinity for the CDC42/GTP complex than the wild-type ACK42, to increase the anti-cancer therapeutic potential of ACK42.

We have found that at least two basic residues of ACK42, His17 and Arg/Lys 34, are absolutely essential

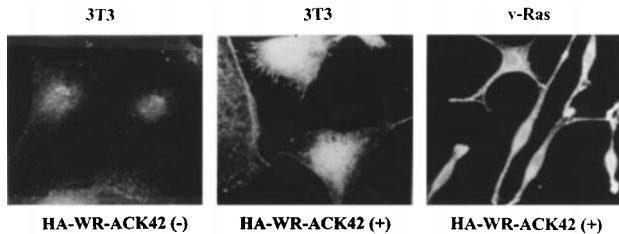


Figure 5 Penetration of HA-WR-ACK42 into normal and v-Ha-Ras transformed NIH3T3 cells. Cells cultured in the presence (+) or absence (–) of HA-WR-ACK42 (2.5 $\mu\text{g}/\text{ml}$) were fixed and stained with the fluorescent anti-HA antibody to visualize the penetration of HA-WR-ACK42 into the cells as described under the 'Materials and methods'. Both normal and Ras-transformed cells uptake HA-WR-ACK42

Table 2 Effect of a cell-permeable derivative of ACK42 on the growth of Ras transformants. Ras transformants were incubated in the presence of indicated concentrations of thrombin-cleaved GST-HA-WR-ACK42 (or GST or thrombin alone as controls) for 48 h, and cells were counted as described under the Materials and methods

| Added | Cell number (10^4) |
|--|---------------------------|
| GST-HA-WR-ACK42 ($\mu\text{g}/\text{ml}$): | |
| 0 (no thrombin) | 50 |
| 0.5 (thrombin 0.5 $\mu\text{g}/\text{ml}$) | 30 |
| 1.0 (thrombin 1.0 $\mu\text{g}/\text{ml}$) | 25 |
| 2.0 (thrombin 2.0 $\mu\text{g}/\text{ml}$) | 25 |
| Thrombin alone ($\mu\text{g}/\text{ml}$): | |
| 0 | 55 |
| 0.5 | 53 |
| 1.0 | 55 |
| 2.0 | 55 |
| GST alone ($\mu\text{g}/\text{ml}$): | |
| 0 | 53 |
| 1.5 | 55 |
| 3.0 | 54 |
| 6.0 | 55 |

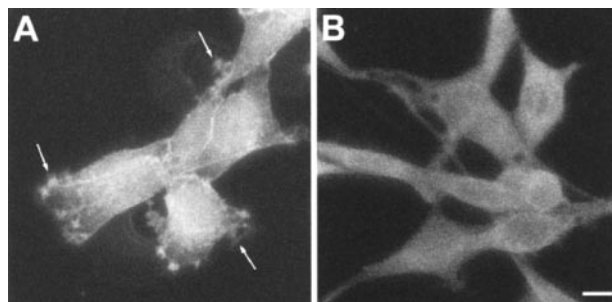


Figure 6 HA-WR-ACK42 blocks filopodium formation of Ras transformants. Phalloidin-staining of the control cells (a), and HA-WR-ACK42-treated cells (b). The arrows indicate filopodia/microspikes. HA – wr – ACK42, but not GST alone (the control), strongly inhibits the filopodium formation. The scale bar indicates 10 micron

for the ACK-CDC42 interaction (Vuong Nheu *et al.*, manuscript in preparation). Interestingly, His17 appears to bind Asp38 of CDC42 directly (Mott *et al.*, 1999). To identify the residue(s) in the switch II domain of CDC42 that binds Arg/Lys 34, we are currently determining the three-dimensional (3D) structure of ACK42 bound to the CDC42/GTP complex by X-ray crystallography (Rittinger *et al.*, 1997). Furthermore, based on the 3D structure of the highest-affinity mutant of ACK42, we intend to design through a computer-based molecular modeling an ACK42 mimetic, the chemical compound that functionally mimics the ACK42 mutant. A similar approach could be taken with the as yet unidentified minimal high-affinity CDC42-binding fragment of N-WASP, PAKs or other CDC42/GTP-binding proteins.

We have shown that CDC42 acts downstream of Ras. How does Ras activate CDC42? Since Ras up-regulates BB2R gene expression, we are currently determining whether BB2R antagonists such as Hoe 140 and FR173657 (Aramori *et al.*, 1997) suppress Ras transformation, in order to explore the possible role of BB2R in Ras-mediated activation of CDC42.

Materials and methods

Monitoring the activation of CDC42 by ACK42-GST fusion protein

The ACK42 binds selectively CDC42 only in the active GTP-bound form, and not in the inactive GDP-bound form (Manser *et al.*, 1993; Mott *et al.*, 1999), as Raf 81/RBD (residues 51–131) of c-Raf-1 binds the GTP-Ras, but not the GDP-Ras (Fridman *et al.*, 1994; de Rooji and Bos, 1997). Thus, the GTP-CDC42 levels in the Triton X-100 lysates derived from normal or v-Ha-Ras-transformed NIH3T3 cells and the control or NGF/EGF-treated PC12 cells were measured by using ACK42-GST beads as the activation-specific probe for CDC42, through the essentially same procedures previously described for monitoring the Ras activation (de Rooji and Bos, 1997).

The following are the specific conditions used for this particular experiment: after overnight starvation of PC12 cells or normal NIH3T3 cells, they were incubated in the presence of either NGF or EGF (50 ng/ml) for 6 h, and these cells or v-Ha-Ras transformed NIH3T3 cells (5×10^7) were then disrupted in the lysis buffer containing 1% Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 0.2 mM sodium 0-vanadate, 50 mM NaF, 0.5 mg/ml PMSF, and 0.5 µg/ml aprotinin. 500 µg protein of each cell lysate was mixed with 50 µl of ACK42-GST on GSH-beads, and incubated at 4°C for 2 h. After washing the beads three times with the lysis buffer, proteins bound to the beads were boiled in 50 µl of an SDS sample buffer, and 15 µl of each sample was subjected to SDS-PAGE followed by immuno-blotting with a rabbit antibody against CDC42 (Santa Cruz, CA, USA).

Immunoblotting

10^6 cells of parental cell lines or each transfectant were suspended in 200 µl of SDS sample buffer and boiled for 15 min. Proteins in each sample were separated by 12% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Millipore) and immunoblotting was carried out according to the procedure described previously (Wang *et al.*, 1997) using antibodies against Ha-Ras (Oncogene Science) or CDC42 (Santa Cruz).

Construction of the plasmid that expresses the CDC42 binding domain of ACK-1

To express the minimal CDC42 binding domain (residues 504–545, ACK42) of human ACK-1 (Manser *et al.*, 1993; Mott *et al.*, 1999), a pMV7-derived plasmid called pMAK was constructed as previously described (Wang *et al.*, 1997). Briefly, an *EcoRI/HindIII* DNA fragment encoding the ACK42 was prepared by polymerase chain reaction (PCR) using a pair of primers (sense, AA GAA TTC GCC ACC ATG GGC CTG TCG GCC CAG GAC A TC; anti-sense, CC AAG CTT TTA GTT TCC CAG ATA CAG TTC GTC). In this DNA the consensus Kozak sequence is followed by the initiation codon. The DNA was subcloned into the vector pMV7 (Maruta *et al.*, 1991), and both orientation and nucleotide sequence of the insert were confirmed by sequencing. The resultant plasmid was purified by CsCl density gradient centrifugation and used for subsequent transfection experiments.

Construction of a plasmid that expresses GST-HA-WR-ACK42 fusion protein

To prepare a gene encoding a GST fusion protein containing a hemagglutinin epitope (HA), a penetratin of 16 amino acids called WR, and ACK42, first a template DNA for HA-WR was generated by an end-filling reaction using a pair of primers, BHRS (5'-G GAA GGA TCC ATG AAG AAC GGA AAC ATG CGG GCT ACT ATT GCA ATA AGA AGA-3') and RHA (5'-TCT TCT CCA CCA TCT TCT CCA CCA TCT TCT CCA TCT TCT TAT TGC AAT AG-3'). Second, a DNA encoding ACK42 was generated by PCR using a pair of primers, WAS (5'-AGA TGG TGG AGA AGA TGG AGA AGA GGC CTG TCG GCC CAG-3') and EAA (5'-TT GAA TTC CTA GTT TCC CAG ATA CAG T-3'). The template DNAs from end-filling and PCR reactions were separated by agarose gel electrophoresis (AGE) followed by purification using 'Sephaglass Band Prep Kit' (Pharmacia) according to the manufacturer's instruction. These two DNA pieces were ligated and amplified by PCR using the primers BHRS and EAA. The PCR product was digested with *Bam*HI and *Eco*RI, and subcloned into the *Bam*HI/*Eco*RI sites of the vector pGEX-2TH (Maruta *et al.*, 1991). The insert was confirmed by nucleotide sequencing.

Affinity-purification of GST-HA-WR-ACK42 fusion protein and its thrombin cleavage

The GST fusion protein of HA-WR-ACK42 was induced in *E. coli* by 20 µM of isopropyl-beta-galactopyranoside (IPTG) at 28°C for 7 h, and affinity-purified as described previously (Maruta *et al.*, 1991). The cell-permeable ACK42 derivative, HA-WR-ACK42, was then separated from GST by thrombin (Sigma Chemicals)-cleavage according to the manufacturer's instruction.

Assay for pharmacological effect of the cell-permeable ACK42 derivative

2×10^5 v-Ha-Ras transformed NIH3T3 cells were seeded in 2 ml of the culture medium RPMI containing 1% fetal calf serum (FCS), and incubated overnight as described previously (Maruta *et al.*, 1991). The thrombin-digested GST-HA-WR-ACK42 (or GST and thrombin alone as a control) at various concentrations was added to the culture, and cells were further incubated for another 48 h. Cells were then trypsinized and counted in a hemocytometer.

Expression of ACK42 in v-Ha-Ras transformed NIH3T3 cells

v-Ha-Ras-transformed NIH3T3 cells (Maruta *et al.*, 1991), which are sensitive to neomycin, were cultured to 60% confluency in RPMI medium containing 10% FCS under the

standard culture conditions. The cells were then transfected with either the plasmid pMAK or the vector pMV7 alone, 2 µg each as complexes with 20 µl of transfectin (GIBCO-BRL), as described previously (Nur-E-Kamal *et al.*, 1992). The transfected cells were allowed to grow in the presence of 400 µg/ml G418 (a neomycin analog). After 18 days, neomycin resistant transfected colonies (clones) were isolated under microscope, and each of these clones was cultured separately for further analysis.

Soft agar colony (SAC) formation assay

10³ cells of either the parental v-Ha-Ras transformants, the vector alone or pMAK (ACK42) transfectants were inoculated per plate in soft agar as described previously (Maruta *et al.*, 1991) in tetraplicate. The cells were incubated for 20 days under standard culture conditions. After staining, the number of colonies were determined under microscope.

Determination of contact inhibition and serum requirement

v-Ha-Ras transformed 3T3 cells and their clones derived by transfection with either the pMAK or the vector alone were cultured in RPMI containing 10% FCS, and G418 (stable transfectants only). To investigate the contact inhibition (or saturation density) of growth, the same number of cells were seeded, and at various intervals cells were trypsinized and counted in a hemocytometer. To study their serum requirement, the same number of cells were inoculated in the medium containing either 0.5 or 10% FCS. At various intervals cells were trypsinized and counted. Each experiment was performed in triplicate and the average values are shown.

Northern blotting

Poly(A)⁺RNA was isolated from parental cells as well as from the stable transfectants as described previously (Nur-E-Kamal *et al.*, 1992). Two µg of poly(A)⁺RNA from each cell line was applied to 1.2% agarose gel electrophoresis (AGE) to separate poly(A)⁺RNAs. Poly(A)⁺RNAs from the gel were transferred onto nylon membrane and probed with a radiolabeled ACK24 cDNA. Membranes were washed as described previously (Nur-E-Kamal *et al.*, 1992) and scanned by phosphorimager (Bio Rad Model, Molecular Imager System GS-525).

Immunostaining of HA-WR-ACK42 treated cells

Fibroblasts were cultured in RPMI medium containing 1% FCS in the presence or absence of HA-WR-ACK42 (2.5 µg/ml) for 2 h. The cells were fixed with 3.7% formaldehyde in

phosphate buffered saline (PBS) for 20 min. They were then treated with 0.5% Triton X-100 in PBS for 5 min, and washed three times in PBS. Cells were treated with rabbit antibodies against HA tag. The samples were then treated with TRITC conjugated anti-rabbit IgG antibodies (Sigma Chemicals). After mounting the coverslip with immunofluor mounting medium (ICN), cells were analysed under a fluorescent microscope (Zeiss Axivert 405M).

Effect of ACK42 on filopodium/microspike formation

Ras-transformants were cultured on coverslips under the standard conditions, until reaching around 60% confluency. The wounds were made by scraping cells through the middle of coverslips with sterile yellow tips (Nobes and Hall, 1999). Cells were then cultured in a fresh RPMI (plus 0.5% FCS) containing HA-WR-ACK42 (3 µg/ml) or GST alone as the control for 6 h, until 25% of the gap was filled. Cells were fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, and stained with TRITC-conjugated phalloidin (Sigma Chemicals). Cells were then visualized under fluorescent light and images were taken using a confocal microscope (Axiovert 100 TV, Zeiss LSM).

Abbreviations

ACK42, CDC42-binding fragment of ACK-1; AGE, agarose gel electrophoresis; BB2R, bradykinin B2 receptor; FCS, fetal calf serum; GAP, GTPase activating protein; GDSs, GDP-dissociation stimulators; GST, glutathione S-transferase; IPTG, isopropyl-beta-galactopyranoside; NGF, nerve growth factor; NOG, neurite outgrowth; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; WR-ACK42, cell-permeable ACK42 derivative.

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