Unfarnesylated transforming Ras mutant inhibits the Ras-signaling pathway by forming a stable Ras·Raf complex in the cytosol

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Abstract **Farnesyltransferase inhibitors cause the growth arrest of *ras*-transformed cells, but not that of normal cells. To elucidate the mechanism of this differential effect, we examined the effect of accumulation of unfarnesylated Ras in the cytosol by using $Ras^{G12V,C186S}$ and Ras^{C186S}, which mimic unfarnesylated form of the oncogenic and the normal Ras, respectively. We found that $Ras^{G12,C186S}$ inhibited activation and membrane translocation of Raf by forming a stable complex with Raf in the cytosol. In contrast, Ras^{C186S} showed inhibitory effect on neither Raf activation nor Raf translocation. These results indicate that unfarnesylated oncogenic Ras interacts with Raf in the cytosol and inhibits its membrane translocation, a crucial step for the Raf activation, while unfarnesylated normal Ras does not.**

Key words: Ras; Farnesylation; Dominant-negative mutant; Raf

1. Introduction

Ras belongs to a family of low-molecular-weight GTP-binding proteins and functions in the regulation of cellular proliferation, differentiation, and transformation [1–3]. Ras cycles between two forms, an active GTP-bound form (Ras·GTP) and an inactive GDP-bound form (Ras·GDP). Ras is localized to the cytoplasmic membrane and this is facilitated by a series of posttranslational modification occurring at the C-terminus [4,5]. In the case of Ha-Ras, the modification is triggered by farnesylation of Cys-186, followed by proteolytic removal of the C-terminal three amino acids, carboxyl methylation of Cys-186 and palmitoylation of Cys-181 and Cys-184. Substitution of Cys-186 with other amino acids results in a mutant protein which is completely blocked in processing, localized in the cytosol, and devoid of the transforming activity [6].

The importance of the farnesylation of Ras to its transforming function has raised a possibility that farnesyltransferase inhibitors may act as anticancer drugs [7,8], since oncogenic mutations of ras gene are found in a wide variety of human tumors [1,9]. Recently, several groups have succeeded in the isolation of farnesyltransferase inhibitors which induce growth arrest of *ras*-transformed cells [10,11]. Interestingly, these inhibitors showed a differential effect on the growth of normal and transforming cells. At the concentration which causes the growth arrest of *ras*-transformed cells, the inhibitors had no effect on the growth of normal cells [11,12]. Moreover, detailed analyses have revealed that the reversion of the *ras*-transformed cells occurs prior to the complete inhibition of processing of Ras [12,13]. Thus, it is unlikely that the observed growth arrest was caused by the depletion of the farnesylated Ras. Previous reports indicated that mutants of oncogenic Ras defective in targeting to membrane, function as dominant-negative mutants [14–17], suggesting that unfarnesylated oncogenic Ras may inhibit the Ras-signaling pathways.

It has been shown that Ras·GTP interacts directly with Raf [18–22], which is a cytosolic serine/threonine kinase involved in the activation of the mitogen-activated protein kinases (MAPKs) [23,24]. After interaction with Ras·GTP, Raf is activated by a still unknown mechanism [25]. The activated Raf then phosphorylates and activates MAPK kinase (MEK), which in turn activates MAPK by phosphorylation on tyrosine and serine/threonine residues [26,27]. Though the precise role of Ras in Raf activation remains still ambiguous, recent reports have revealed that Ras-mediated Raf translocation from cytosol to membrane is a prerequisite for the activation of Raf [28,29].

We assume that treatment of *ras*-transformed cells with farnesyltransferase inhibitors may result in accumulation of unfarnesylated form of oncogenic Ras, which sequesters Raf in the cytosolic fraction. On the other hand, unfarnesylated wild-type Ras which accumulates in the GDP-bound form does not interact with Raf. To assess this hypothesis, we examined the effect of accumulation of unfarnesylated oncogenic Ras on Ras-mediated Raf activation. In the present study, we constructed two Ha-Ras mutants, $Ras^{G12V,C186S}$ (V12Ras-SVLS) and Ras^{C186S} (G12Ras-SVLS), which fail to undergo farnesylation because of the C186S substitution and thus mimic unfarnesylated form of oncogenic and normal Ras, respectively. Using these two mutants, we have shown that V12Ras-SVLS functions as a dominant-negative mutant to inhibit Ras-induced activation of Raf as well as that of MAPK. Expression of V12Ras-SVLS resulted in the inhibition of Ras-induced membrane translocation of Raf. In the cytosol, V12Ras-SVLS accumulated in the GTP-bound form to form a stable complex with Raf. In contrast, G12Ras-SVLS which accumulates in the GDP-bound form failed to interact with Raf and did not affect the activation and translocation of Raf. These results indicate that unfarnesylated oncogenic Ras inhibits Ras-induced Raf activation by preventing the translocation of Raf to membrane, while unfarnesylated normal Ras does not.

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Abbreviations: G12Ras-SVLS, Ha-Ras^{C186S}; V12Ras-CVLS, Ha-Ras^{G12V}; V12Ras-SVLS, Ha-$Ras^{G12V,C186S}$; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; Raf-FH6, Raf-1 with flag-epitope tag and six histidine residues in its C-terminus.

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2. Materials and methods

2.1. Materials

Antibodies against Raf-1 (C-20) and Ha-Ras (C-20) were obtained from Santa Cruz Biotechnology. Antibodies against MAPK (#05–157), flag epitope (M2), and Ha-Ras (LA069) were purchased from Upstate Biotechnology, Eastman Kodak and Quality Biotech, respectively. Recombinant histidine-tagged *Xenopus* MEK [30] and glutathione-*S*-transferase (GST)-fused kinase-deficient *Xenopus* MAPK [31] were produced using bacterial expression systems (kindly given by E. Nishida and Y. Gotoh, Kyoto University, Japan) and purified as described in [30,31]. Complementary DNAs of V12Ras-CVLS and V12Ras-SVLS were kindly provided by P. Kirshmeier (Schering-Plough Research Institute, NJ).

2.2. Plasmid DNAs

To make G12Ras-SVLS, we substituted Val^{12} of V12Ras-SVLS with Gly by oligonucleotide-directed mutagenesis. The mutation was confirmed by dideoxynucleotide sequencing. The cDNAs of Ras^{G12V}(V12Ras-CVLS), V12Ras-CVLS, G12Ras-SVLS and V12Ras-SVLS were subcloned into the *Eco*RI-*Hin*dIII site of pCMV5 vector [32] to generate pCMV-V12RasCVLS, pCMV-G12RasSVLS and pCMV-V12RasSVLS, respectively. For expression of Raf, we used pLNC-Raf-1:FH6 (a kind gift from M. McMahon, DNAX Research Institute, Palo Alto, CA) encoding human c-Raf-1 with flag-epitope tag and six histidine residues in its C-terminus (Raf-FH6).

2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 10% CO_2/90% air. DNA transfection was performed by the calcium phosphate coprecipitation method. HEK293 cells were exposed for 1 day to DNA precipitates in DMEM containing 10% FBS and then starved for 1 day in DMEM containing 1 mg/ml bovine serum albumin before being used in subsequent experiments.

2.4. MAP kinase mobility shift

Two days after transfection, the cells were harvested, washed twice with cold phosphate-buffered saline and lysed in buffer A (20 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) containing 150 mM KCl, 10% glycerol and 0.5% Triton X-100. The lysate was boiled in the sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The boiled sample was fractionated by SDS-PAGE (10%) and transferred to a nitrocellulose filter. Western blot analysis was performed with an anti- MAPK antibody (#05–157) using the ECL detection system (Amersham).

2.5. Raf kinase assay

Raf kinase assay was performed as described in [33]. Briefly, transfected cells were lysed with buffer A containing 150 mM KCl, 10% glycerol and 0.5% Triton X-100 and then endogenous c-Raf-1 in the lysate was immunoprecipitated with anti-c-Raf-1 antibody (C-20). After washing, the precipitate was incubated at 30°C for 20 min with 0.3 μg of recombinant histidine-tagged MEK, 3.4 μg of recombinant GST-fused kinase-deficient MAPK, 0.28 mM ATP and 150 kBq [γ-^{32}P]ATP. The reaction was stopped by boiling in the sample buffer for SDS-PAGE and the samples were resolved by SDS-PAGE (10%). The radioactivity incorporated into the kinase-deficient GST-MAPK was detected by autoradiography and measured by an image analyzer (FUJI BAS2000).

2.6. Cell fractionation

Transfected HEK293 cells were harvested and homogenized in ice-cold buffer A containing 250 mM sucrose. The postnuclear supernatant was centrifuged for 60 min at 100,000 × *g* yielding the cytoplasmic fraction and the membrane pellet. The latter was washed, resuspended in buffer A containing 250 mM sucrose and 1% Triton X-100, and centrifuged for 10 min at 10,000 × *g*. The supernatant was saved as the membrane fraction.

3. Results

3.1. Inhibition of Ras-mediated signaling pathway by V12Ras-SVLS

First, we examined whether V12Ras-SVLS inhibits the Ras-mediated signaling pathway. Transfection of HEK293 cells with the V12Ras-CVLS expression vector induced a shift in the mobility of MAPK, indicating MAPK activation (Fig. 1A). This shift was inhibited by co-expression of V12Ras-SVLS (Fig. 1A). In contrast, G12Ras-SVLS showed a negligible effect on the Ras-induced MAPK activation. In these experiments, we confirmed by Western blotting with an anti-Ras antibody (C-20) that both V12Ras-SVLS and G12Ras-SVLS were expressed in a dose-dependent manner and existed in the soluble, but not in the particulate fraction (data not shown). Next, we examined the effect of these mutants on Raf. Measurement of the Ras-induced activation of Raf with the in vitro kinase assay revealed that V12Ras-SVLS inhibited the activation of Raf, while G12Ras-SVLS did not show any effect (Fig. 1B).

3.2. V12Ras-SVLS inhibits Ras-induced membrane translocation of Raf

Upon activation, Ras induces translocation of Raf to the

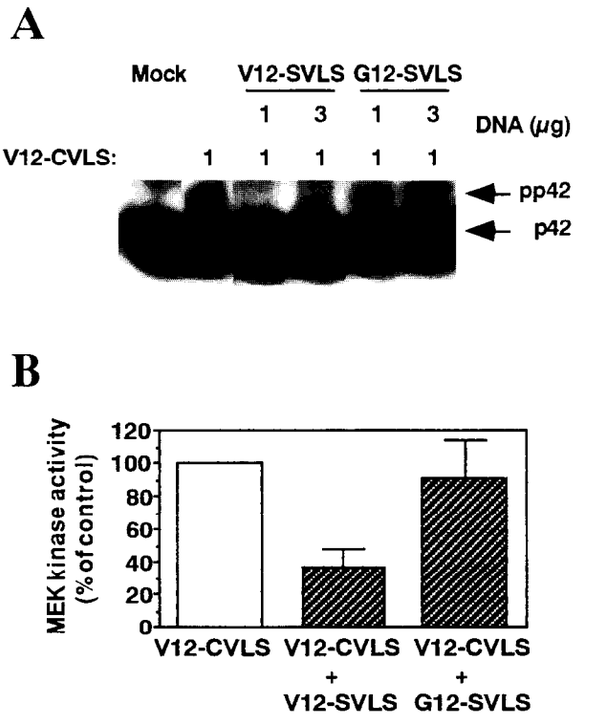

Fig. 1. Inhibition of the Ras-induced activation of MAP kinase (A) and Raf (B) by V12Ras-SVLS. (A) HEK293 cells were transfected with the indicated amount of expression vectors. Detergent lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with an anti-MAPK antibody. The data are representative of three separate experiments. (B) Cells were transfected with 1 μg of pCMV-V12RasCVLS and 5 μg of either pCMV-V12RasSVLS or pCMV-G12RasSVLS. From the cell lysate, endogenous c-Raf-1 was immunoprecipitated and incubated at 30°C for 20 min with purified recombinant MEK and kinase-deficient MAPK in the presence of [γ-^{32}P]ATP. The data are expressed as percentage of the ^{32}P incorporated into MAPK with V12Ras-CVLS alone and are shown as the mean±S.D. from three independent experiments.

cytoplasmic membrane. As shown in Fig. 2, expression of V12Ras-CVLS together with Raf-FH6 resulted in the accumulation of Raf-FH6 in the membrane fraction, which was reduced by co-expression of V12Ras-SVLS, but not G12Ras-SVLS. The result indicates that Ras-mediated translocation of Raf to the cytoplasmic membrane was inhibited by V12Ras-SVLS.

3.3. *V12Ras-SVLS forms a stable complex with Raf in the cytosol*

V12Ras-SVLS is deficient in the intrinsic GTPase activity and stays in the GTP-bound form. Therefore, it is expected that Raf is trapped in the cytosolic fraction by forming a stable complex with V12Ras-SVLS. We tested the possibility by immunoprecipitation method. The cytosol prepared from the cells expressing V12Ras-SVLS and Raf-FH6 was treated with an anti-Ras antibody (LA069). After Western blot analysis, we detected Raf-FH6 in the immunoprecipitate (Fig. 3), suggesting that V12Ras-SVLS formed a stable complex with Raf-FH6 in the cytosol. On the other hand, in the case of G12Ras-SVLS, we did not detect significant amount of Raf-FH6 in the precipitate (Fig. 3), although the amount of Ras in the immunoprecipitate was comparable to that obtained with V12Ras-SVLS (data not shown).

4. Discussion

In this paper, we demonstrate that: (1) V12Ras-SVLS, but not G12Ras-SVLS, inhibits the Ras-mediated signaling pathway; (2) expression of V12Ras-SVLS inhibits Ras-induced translocation of Raf to the cytoplasmic membrane, while expression of G12Ras-SVLS has no effect; and (3) V12Ras-SVLS, but not G12Ras-SVLS, forms a complex with Raf in the cytosol. From these results, we conclude that V12Ras-SVLS inhibits Ras-induced Raf activation by sequestering Raf in the cytosols. This is supported by the observation that overexpression of Raf overcomes the inhibitory effect of V12Ras-SVLS (data not shown). The mechanism of activation of Raf in the cytoplasmic membrane has not yet been fully understood. It has been shown that Src, a membrane-bound tyrosine kinase, activates Raf synergistically with Ras [34], suggesting that Src may be involved in the Ras-induced Raf activation. In consistent with this idea, we observed that V12Ras-SVLS also inhibits v-Src-induced Raf activation (data not shown).

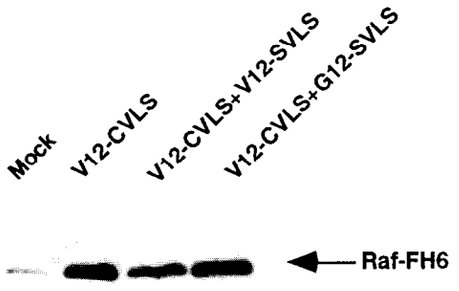

Fig. 2. Inhibition of the Ras-induced translocation of Raf to the cytoplasmic membrane by V12Ras-SVLS. Cells were transfected with 1 μg of pCMV-V12RasCVLS, 5 μg of pLNC-Raf-1:FH6, and 5 μg of either pCMV-V12RasSVLS or pCMV-G12RasSVLS. After cell fractionation, Raf-FH6 was immunoprecipitated from the membrane fraction with antiflag antibody. The precipitate was probed with antiflag antibody to detect Raf-FH6. An arrow indicates the Raf-FH6 band. The results shown are the representative of three separate experiments.

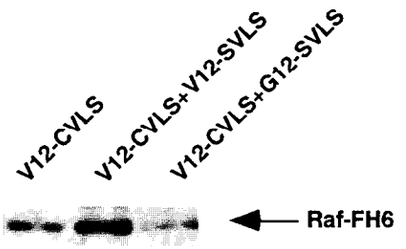

Fig. 3. Accumulation of the Ras·Raf complex formation in the cytosol induced by RasV12-SVLS. Cells were transfected with 1 μg of pCMV-V12RasCVLS, 10 μg of pLNC-Raf-1:FH6 and 5 μg of either pCMV-V12RasSVLS or pCMV-G12RasSVLS. From the cytosolic fraction, Ras was immunoprecipitated with an anti-Ras antibody (LA069) and Raf-FH6 in the immunoprecipitate was detected by Western blot analysis with an antiflag antibody. An arrow indicates the Raf-FH6 band. The data are representative of three independent experiments.

The present study demonstrates clearly that V12Ras-SVLS, which mimics unfarnesylated oncogenic Ras, blocks the Ras-induced Raf activation, while G12Ras-SVLS, which mimics unfarnesylated normal Ras, showed negligible effect on the Raf activation. This corresponds well to the result that the farnesyltransferase inhibitors arrest the growth of *ras*-transformed cells, but not that of normal cells. We consider that the differential sensitivity to the farnesyltransferase inhibitors of *ras*-transformed and normal cells is due to the fact that the oncogenic Ras accumulates in the cytosols in the GTP-bound conformation, while the normal Ras in the GDP-bound conformation.

Although V12Ras-SVLS inhibits the signal transmission from Ras to Raf, it is not known whether this mutant can block also the interaction of Ras with other effectors such as RalGDS and PI3-kinase [35–38]. Recently, Ras has been shown to transmit the signal, in addition to the Raf-MEK-MAPK pathway, to Rac-Rho pathway [39–41]. The Rac-Rho pathway is shown to be involved in morphological change of transformed cells [39–42]. Therefore, it would be interesting to see whether V12Ras-SVLS blocks also this pathway. The isolation and characterization of the complex involving Ras and Raf may lead to the identification of other molecule(s) contained in the complex. The possible candidates are the other Ras-binding molecules such as RalGDS and PI3-kinase, and Raf-binding proteins such as 14–3–3 [43] and Hsp90 [44]. We hope that this kind of analysis may help the further understanding of the mechanism of Ras-mediated signal transduction.

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