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Role of Phosphatidylinositol 4,5-Bisphosphate in Ras/Rac-Induced Disruption of the Cortactin-Actomyosin II Complex and Malignant Transformation

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Oncogenic Ras mutants such as v-Ha-Ras cause a rapid rearrangement of actin cytoskeleton during malignant transformation of fibroblasts or epithelial cells. Both PI-3 kinase and Rac are required for Ras-induced malignant transformation and membrane ruffling. However, the signal transduction pathway(s) downstream of Rac that leads to membrane ruffling and other cytoskeletal change(s) as well as the exact biochemical nature of the cytoskeletal change remain unknown. Cortactin/EMS1 is the first identified molecule that is dissociated in a Rac–phosphatidylinositol 4,5-biphosphate (PIP_2)-dependent manner from the actin-myosin II complex during Ras-induced malignant transformation; either the PIP_2 binder HS1 or the Rac blocker SCH51344 restores the ability of EMS1 to bind the complex and suppresses the oncogenicity of Ras. Furthermore, while PIP_2 inhibits the actin-EMS1 interaction, HS1 reverses the PIP_2 effect. Thus, we propose that PIP_2, an end-product of the oncogenic Ras/PI-3 kinase/Rac pathway, serves as a second messenger in the Ras/Rac-induced disruption of the actin cytoskeleton and discuss the anticancer drug potential of PIP_2-binding molecules.

The nonmuscle actin cytoskeleton plays the major role in both maintenance of cell shape and variety of cell motility (32). More than 2 decades ago it was demonstrated that the actin cytoskeleton is rearranged rapidly when normal fibroblasts or epithelial cells are transformed with simian virus 40 (SV40) (48). A variety of oncoproteins (e.g., Src and Ras) also cause changes in the actin cytoskeleton during malignant transformation, typically disruption of actin stress fibers followed by induction of membrane ruffling (1). The actin cytoskeleton microfilament is an actomyosin (a complex of actin filament and myosin ATPases)-based contractile apparatus which is associated with a number of other actin- or myosin-binding proteins. These actin- or myosin-binding proteins form a variety of cytoskeleton structures, such as actin stress fibers, membrane ruffles, contractile ring, and microspikes. The transition from the stress fibers to membrane ruffles involves both the dissociation of stress-fiber-specific proteins from and the association of membrane-ruffle-specific proteins with actin filament. For example, single-headed myosin ATPases (myosins I) are associated with membrane ruffles but not with stress fibers or contractile ring, whereas double-headed myosin ATPases (myosins II) are mainly associated with stress fibers and contractile ring (10).

Interestingly, the expression of several genes encoding actin-binding proteins (ABPs), including alpha-actinin, vinculin, gelsolin, and tropomyosin, is suppressed during malignant transformation caused by SV40 or Ras (45). Furthermore, overexpression of alpha-actinin, vinculin, gelsolin, and tropomyosin genes suppresses SV40/Ras-induced malignant transformation of NIH 3T3 normal murine embryonic fibroblasts (6, 12, 13, 19, 30, 33). However, the molecular mechanism underlying the antioncogenic action of these ABPs remains to be determined. In addition to F-actin, at least the first three proteins of these multifunctional ABPs bind phosphatidylinositol 4,5-bisphosphate (PIP_2). Production of PIP_2 is induced by oncogenic Ras through PI-3 kinase and the G protein Rac, both of which are essential for Ras-induced malignant transformation (14, 34, 35). Ras activates PI-3 kinase, which in turn activates Rac (35) through an as-yet-uncharacterized Rac GDP dissociation stimulator. Rac then activates PI-4 and PI-5 kinases that produce PIP_2 (14). This unique acidic phosphoinositide then binds F-actin plus-end capping proteins, such as tensin, thereby causing the uncapping of actin filament and inducing a rapid actin polymerization at this end (14). Since cytochalasins, which cap the plus-end of actin filament and block membrane ruffling (50), and the drug SCH51344, which blocks Ras/Rac-induced membrane ruffling (46), suppress Ras/Rac-induced malignant transformation (21, 27, 46), it is likely that the Rac-induced PIP_2 production, uncapping, and membrane ruffling play a critical role in Ras/Rac-induced malignancy.

HS1 is closely related to an F-actin cross-linking protein called cortactin/EMS1, both structurally and functionally, particularly in the N-terminal F-actin binding and the C-terminal SH3 domains (15, 17, 20, 25, 26, 39, 49). However, unlike EMS1, which is expressed ubiquitously, HS1 is normally ex-

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pressed only in hematopoietic cells (20, 39) and is required for B-cell surface antigen receptor-mediated apoptosis (15, 52). Using two distinct anti-Ras tumor suppressors, i.e., the cytoplasmic protein HS1, which binds PIP_2, and the drug SCH51344, which blocks downstream of Rac, we have identified the first biochemically defined pathway in which Ras causes the disruption of an actomyosin complex through Rac and PIP_2, without suppressing the expression levels of its protein components, during malignant transformation, suggesting the anticancer potential of various PIP_2-binding molecules.

MATERIALS AND METHODS

Detection of the EMS1-actomyosin complex. Cells (10^7) cultured on a petri dish (150 mm in diameter) were disrupted with 1 ml of radioimmunoprecipitation assay buffer (47). The cell lysates were cleared by spinning at 13,000 rpm for 10 min. The resultant supernatants were incubated with the 1:500 antiserum RA23 against EMS1 (39), and the immunocomplex was precipitated by protein A beads with or without 5 mM ATP. The pellets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) followed by staining with 0.1% Coomassie blue. In control experiments with protein A beads alone, nonimmune serum, or antiserum against HS1 (51), no actomyosin complex was detected in the pellets by SDS-PAGE.

SCH51344 treatment of Ras-transformed cells. To examine the effect of SCH51344, an anti-Ras cancer drug (21), v-Ha-Ras-transformed cells (10^6) were cultured for 60 h in the presence of 50 μM SCH51344 or 0.08% dimethyl sulfoxide alone, the solvent for dissolving the drug. This drug has no effect on the growth rate of these cells in a liquid culture but flattens the cells and blocks their focus formation as previously described (21). The confluent cells were then harvested and subjected to the analysis of the EMS1-actomyosin complex formation as described above.

Identification of the 200-kDa protein as a nonmuscle myosin IIA heavy chain by microsequencing. The SDS-PAGE-purified 200-kDa protein (1.5 μg) immunoprecipitated with an EMS1-actin complex from the NIH 3T3 cells (see Fig. 1A) was digested by trypsin (29), and the resulting peptides were separated by high-performance liquid chromatography (28) and identified as the fragments of murine nonmuscle myosin IIA heavy chain by the microsequencing procedures previously described (2, 5, 28, 29) and comparison with the amino acid sequences of the rat and human nonmuscle myosin IIA heavy chains that were determined previously (3, 36, 40, 44) (Table 1).

Preparation of muscle actin and HS1 or EMS1 fragments. Actin was purified from acetone powder of rabbit skeletal muscle (Sigma Chemicals) as described previously (41). Full-length human HS1 and the actin-binding domain (residues 82 to 192) were produced in bacteria as thrombin-cleavable glutathione *S*-transferase (GST) fusion proteins by subcloning the corresponding PCR DNAs (*Eco*RI fragments) into the vector pGEX-2TH. Full-length human EMS1-GST fusion protein (17) and other GST fusion proteins were affinity purified by glutathione beads (24).

F-actin cross-linking assay. F-actin cross-linking activity of EMS1 or HS1 was measured by the superprecipitation procedure (17) with a slight modification in the presence or absence of PIP_2. Purified GST-fusion proteins of EMS1 or HS1 were preincubated with PIP_2 on ice for 30 min. F-actin was then added into the above-mentioned mixtures and incubated for another 30 min on ice. The mixture was then centrifuged at 15,000 × *g* for 10 min at 4°C, and the resultant pellets were subjected to SDS-PAGE. The Coomassie blue-stained gels were then subjected to densitometry for quantitation of actin bands. Under these conditions, the control GST alone caused no cross-linking of F-actin.

PIP_2-binding assay. Purified GST fusion proteins of EMS1 or HS1 were fixed onto a nitrocellulose membrane with a dot blot apparatus. The membrane was then blocked with 10% skim milk and washed (47). The membrane was then incubated with 10 mg of PIP_2 per ml in TSBT (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% bovine serum albumin, and 0.02% Tween 20) at 25°C for 30 min. After being washed, the membrane was incubated with the 1:1,000 rabbit antiserum against PIP_2 (7) at 25°C for 60 min, washed three times, and immunoblotted with 1:10,000 goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad, Richmond, Calif.) at 25°C for 60 min. The membrane was incubated with enhanced chemiluminescence detection solution (Amersham, Little Chalfont, United Kingdom) at 25°C for 1 min and exposed to XAR5 film (Kodak, Rochester, N.Y.) for 2 min. The density of each dot was quantitated by a densitometer. Under these conditions, the control GST alone did not bind PIP_2.

Construction of plasmids expressing human HS1 (wild type and mutants) in mammalian cells. For expression of wild-type HS1 and its mutants in mammalian cells, *Eco*RI DNA fragments containing the *Hin*dIII site followed by a Kozak consensus sequence (AAGCTTGCCGCCACCATG) at the 5′ end and encoding full-length human HS1 (HS1F) or its SH3-negative (HS1-SH3) actin-binding domain-negative mutants (HS1ad) and HS1-EMS1 chimera (HEaH) were prepared by PCR and subcloned into the retroviral vector pMV7 as described previously (24). The orientation of the inserts was determined by *Hin*dIII digestion (a second *Hin*dIII site is located 100 bp downstream of the *Eco*RI site in the vector). The resultant plasmids were purified by a Plasmid Maxi Kit Qiagen (Hilden, Germany) according to the manufacturer's instructions and used for transfection. Each plasmid expresses a neomycin G418 resistance selectable marker in addition to HS1 (wild type or mutants).

Effect of wild-type HS1 or its mutants on the colony-forming ability of v-Ha-Ras transformants in soft agar. v-Ha-Ras-transformed NIH 3T3 cells were transfected with HS1F or other plasmids as complexes with liposomes (31). A parallel transfection with the vector alone was also carried out as a negative control. The resultant transfectants were cloned in the presence of 400 μg of G418 per ml (24). The whole-cell lysate of each G418-resistant clone was then subjected to immunoblotting with the rabbit antiserum against a human HS1 peptide (residues 306 to 320) (51) for selecting out the clones that overexpress the wild-type HS1 or its mutants. The colony formation in a soft agar (anchorage-independent growth) of these HS1 transfectants in comparison with that for the clones with the control vector alone was examined by incubating 1,000 cells/plate at 37°C for 18 days under standard culture conditions (24). The colonies were stained with 0.005% crystal violet and counted.

Autokinase assay for v-Ha-Ras GTPase. Unlike normal Ras GTPases, v-Ha-Ras GTPase, which uniquely contains Thr at position 59, is autophosphorylated at this position during the hydrolysis of GTP (11). According to the previously described autokinase assay procedures (4), the cell lysate containing 100 μg of protein prepared from the parental v-Ha-Ras transformants or HS1 transfectants was immunoprecipitated with the anti-Ras antibody Y13-259, and then each immunoprecipitate resuspended in the kinase buffer (4) was subjected to the

TABLE 1. The 200-kDa protein immunoprecipitated with an EMS1-F-actin complex is murine myosin IIA heavy chain[a]

Source and fragment no.	Sequence
15	
Murine	NFINNPLAQADWAAK
Rat	NFINNPLAQADCGAK
Human	NFINNPLAQADWAAK
290	
Murine	TDLLLEPYNK
Rat	TDLLLEPYNK
Human	TDLLLEPYNK
843	
Murine	HEDELLAK
Rat	HEDELLAK
Human	QEEEMMAK
1325	
Murine	LSLSTK
Rat	LSLSTK
Human	LSLSTK
1394	
Murine	DLEGLSQR
Rat	DLEGLSQR
Human	DLEGLSQR
1479	
Murine	ALSLAR
Rat	ALSLAR
Human	ALSLAR
1756	
Murine	ANLQIDQINTDLNLER
Rat	ANLQIDQINTDLNLER
Human	ANLQIDQINADLNLER
1817	
Murine	IAQLEEQLDNETK
Rat	IAQLEEQLDNETK
Human	IAQLEEQLDNETK

[a] The primary sequences of tryptic fragments derived from the murine 200-kDa protein are compared with those of rat and human myosin IIA heavy chains (3, 36, 40, 44). The number of each fragment indicates the first residue of the corresponding fragment from the human nonmuscle myosin IIA heavy chain (36, 40, 44).

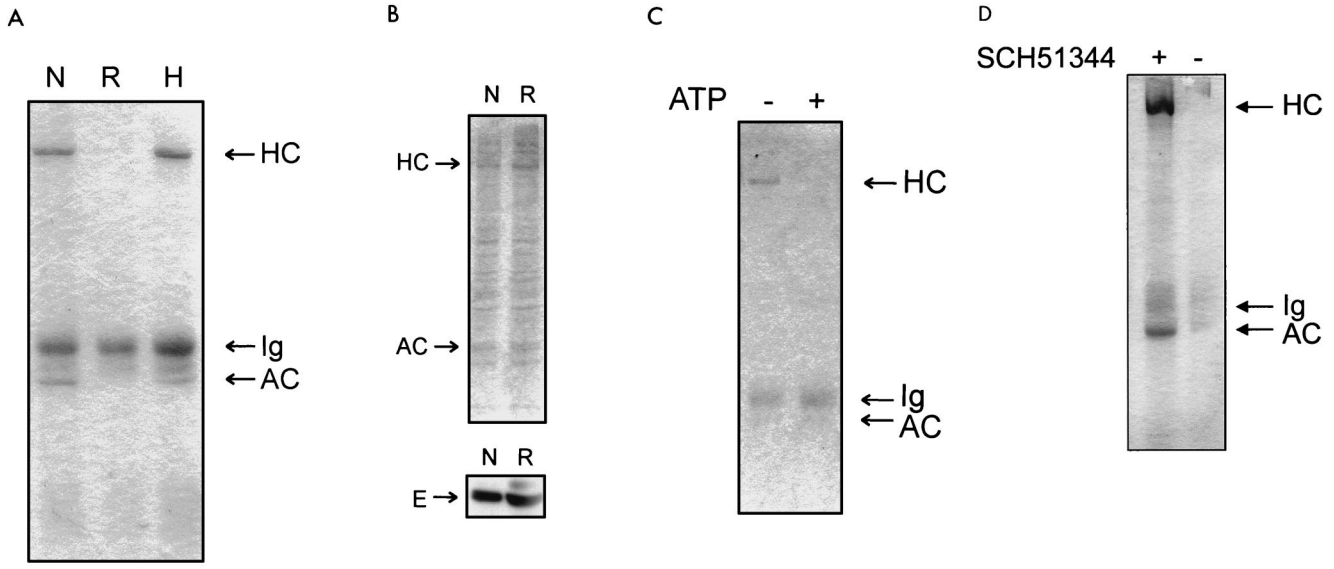


FIG. 1. Ras induces the disruption of an EMS1-actomyosin complex. (A) Proteins immunoprecipitated with EMS1. Normal (N), Ras-transformed (R), and HS1-transfected Ras-transformed (H) NIH 3T3 cells stained with Coomassie blue. HC, a protein of 200 kDa comigrating with myosin II heavy chain; AC, actin of 42 kDa. (B) No effect of Ras transformation on the expression levels of myosin II heavy chain (HC), actin (AC), and EMS1 (E). (Top panel), Coomassie-blue stained gel of the lysates from normal (N) and Ras-transformed (R) NIH 3T3 cells. (Bottom panel) Immunoblot showing the level of EMS1 in normal (N) and Ras-transformed (R) NIH 3T3 cells. EMS1 had a size of 85 kDa. Fifty micrograms of protein was loaded in each lane. (C) Effect of ATP. Dissociation of HC from the actin-EMS1 complex in the presence of 5 mM ATP (+). (D) Effect of SCH51344. Both myosin IIA heavy chain (HC) and actin (AC) were immunoprecipitated with EMS1 from Ras-transformed cells treated with SCH51344 (+) but not with cells without (−) drug treatments. Ig, immunoglobulin.

autokinase assay with 10 nM [γ-^{32}P]GTP at 37°C for 30 min and to SDS–15% PAGE. The autophosphorylated v-Ha-Ras band was visualized by autoradiography.

F-actin binding assay. The assay was performed according to the F-actin cosedimentation procedure (49). Briefly, F-actin (4 μM) was mixed with the fibroblast lysate containing the native full-length HS1 or HS1-EMS1 chimera. The mixture (100 μl) was kept on ice for 60 min and then centrifuged at 100,000 × *g* for 30 min at 4°C in a Beckman model TL-100 ultracentrifuge. The resultant supernatants and pellets were subjected to SDS-PAGE, followed by immunoblotting with the antibody against HS1.

RESULTS AND DISCUSSION

Ras-induced disruption of an EMS1-actomyosin complex. Microinjection of oncogenic Ras mutants into normal fibroblasts causes the disruption of actin stress fibers, followed by the induction of membrane ruffles in half an hour (1). To understand how Ras causes such a rapid change in the actomyosin complexes, presumably without any change in the levels of its protein components, we have examined the interaction of EMS1 with F-actin in normal and v-Ha-Ras-transformed NIH 3T3 cells. EMS1 is an F-actin cross-linking SH3 protein with 550 amino acids (17). With an antibody specific for EMS1, proteins associated with EMS1 were immunoprecipitated from the cytosol of both normal and Ras-transformed cells and separated by SDS-PAGE. In normal cells, EMS1 forms a stable complex with F-actin and an additional protein of 200 kDa, whereas in Ras-transformed cells, such a complex fails to coprecipitate with EMS1 (Fig. 1A). Furthermore, overexpression of HS1 in the Ras transformants restores the complex with EMS1 and suppresses Ras-induced malignancy as described later. Under these conditions, either F-actin or the 200-kDa protein is not coprecipitated with protein A beads alone, nonimmune serum, or an antibody specific for HS1 (data not shown), confirming that these two proteins are coprecipitated only when they form a complex with EMS1. Since EMS1 is an F-actin cross-linker, even a few EMS1 molecules per actin filament consisting of an average of 1,000 actin monomers are sufficient for the anti-EMS1 to coprecipitate the F-actin 200-kDa protein complex. Ras has no effect on the expression level of either EMS1 or actin (Fig. 1B), indicating that Ras causes a disassembly of the EMS1-actin filament complex.

We suspected that the 200-kDa protein is a heavy chain of a nonmuscle myosin II isozyme, because it is one of the major cytoplasmic proteins in the fibroblasts and comigrates with the heavy chain of skeletal muscle myosin (SDS-PAGE). In support of this notion, 5 mM ATP, which dissociates any myosin from actin filament (23), causes a dissociation of the 200-kDa protein from the EMS1-actin complex (Fig. 1C). Finally, the microsequencing of several tryptic peptides derived from the 200-kDa protein confirmed that it is the heavy chain of a nonmuscle myosin II type A (Table 1). The cytoplasmic myosin II level is not affected by Ras transformation (Fig. 1B). This notion was further confirmed by immunoblotting with an antibody specific for myosin IIA heavy chain (data not shown). Thus, it is clear that Ras transformation is associated with the disruption of an EMS1-actomyosin II complex, and the reversion of Ras transformation by HS1 overexpression restores the ability of EMS1 to bind actomyosin II. This is the first biochemical demonstration of the disruption by Ras of the specific interaction of actomyosin with ubiquitous cellular proteins such as EMS1. However, so far, no clear-cut change has been detected in the overall intracellular localization pattern of EMS1 upon Ras transformation by fluorescent EMS1 staining (44a).

SCH51344, which blocks Rac-induced membrane ruffling suppresses Ras-induced disruption of the EMS1-actomyosin II complex. We have shown previously that the drug SCH51344 blocks both Ras/Rac-induced membrane ruffling and malignant transformation and stimulates stress fiber formation (21, 46). However, this drug has no effect on either Raf-induced activation of MEK/mitogen-activated protein kinase (extracellular signal-regulated protein kinases) or Rac-induced activation of JNK kinase pathways which are not essential for

A

			* * *			
HS1-1	82-118	GYGG	*RFGVERDR*	MDKSAVGH	EYVA EVEKHS	SQTDAAK
HS1-2	119-155	GFGG	*KYGVERDR*	ADKSAVGF	DYKGEVEKHT	SQKDYSR
HS1-3	156-192	GFGG	*RYGVERDK*	WDKAALGY	DYKGETEKHE	SQRDYAK
			* * *			
EMS1-1	83-119	GYGG	KFGVEQDR	MDKSAVGH	EYQS KLSKHC	SQVDSVR
EMS1-2	120-156	GFGG	KFGVQMDR	VDQSAVGF	EYQGKTEKHA	SQKDYSS
EMS1-3	157-193	GFGG	KYGVQADR	VDKSAVGF	DYQGKTEKHE	SQRDYSK
EMS1-4	194-230	GFGG	*KYG I D KDK*	VDKSAVGF	EYQGKTEKHE	SQKDYVK
EMS1-5	231-267	GFGG	KFGVQTDR	QDKCALGW	DHQEKLQLHE	SQKDYKT
EMS1-6	268-304	GFGG	KFGVQ SER	QDSAAVGF	DYKEKLALHE	SQQDYSK

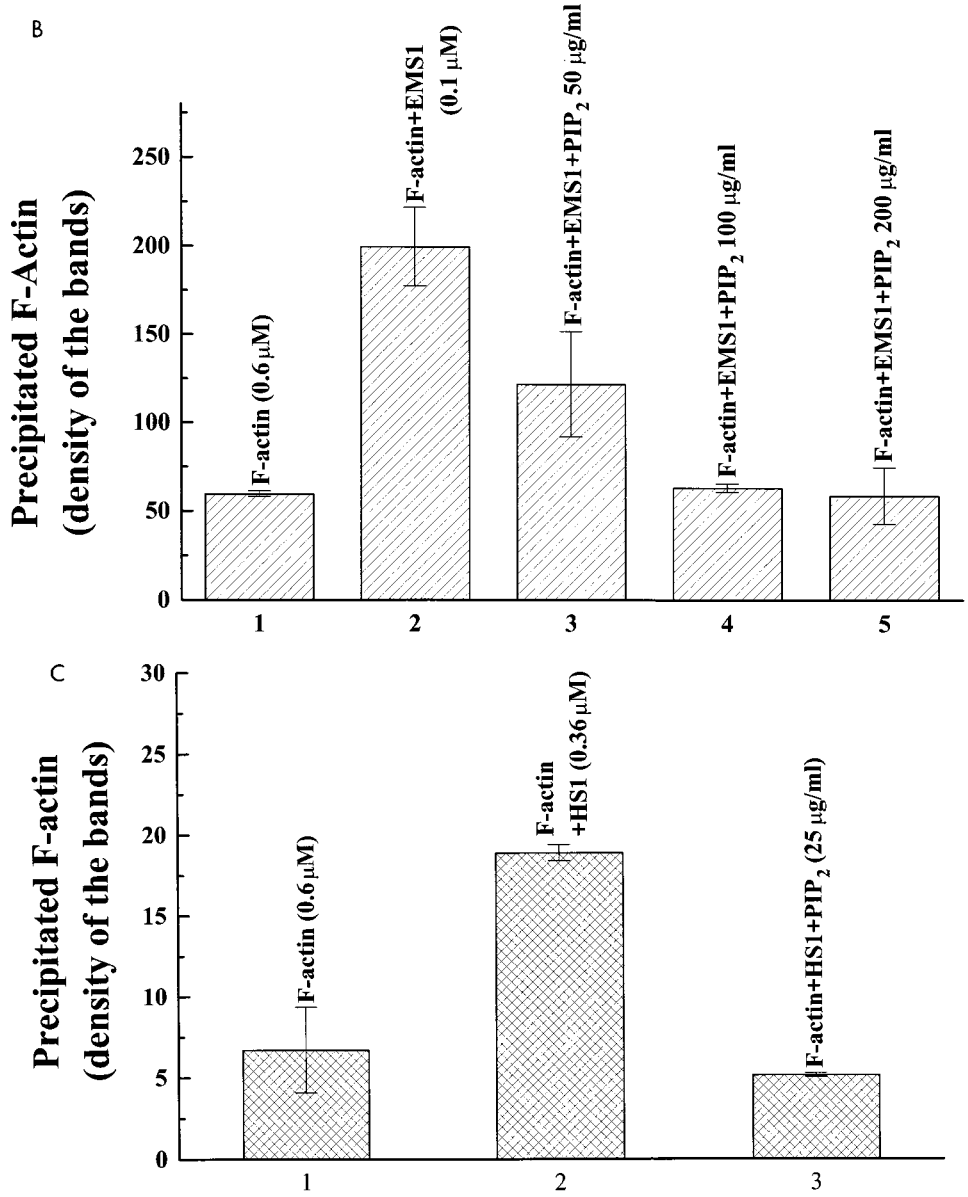


FIG. 2. PIP_2 inhibits F-actin cross-linking activity of both EMS1 and HS1. (A) The putative PIP_2-binding motifs (in boxes) within the actin-binding repeats (underlined) of EMS1 and HS1. Asterisks indicate the critical basic residues for PIP_2 binding. (B) Inhibition by PIP_2 of EMS1-induced F-actin cross-linking. F-actin alone or a mixture of F-actin and EMS1 with or without PIP_2 was centrifuged under conditions in which only the cross-linked F-actin was significantly precipitated. PIP_2, at 100 μg/ml, completely abolished the F-actin cross-linking activity of EMS1. The data presented are the averages of three duplicated samples. (C) Inhibition by PIP_2 of HS1-induced F-actin cross-linking. Much less PIP_2 (25 μg/ml) was sufficient to inhibit the F-actin cross-linking activity of HS1. Under these conditions, GST alone did not cross-link F-actin. The data are the averages of two duplicated samples.

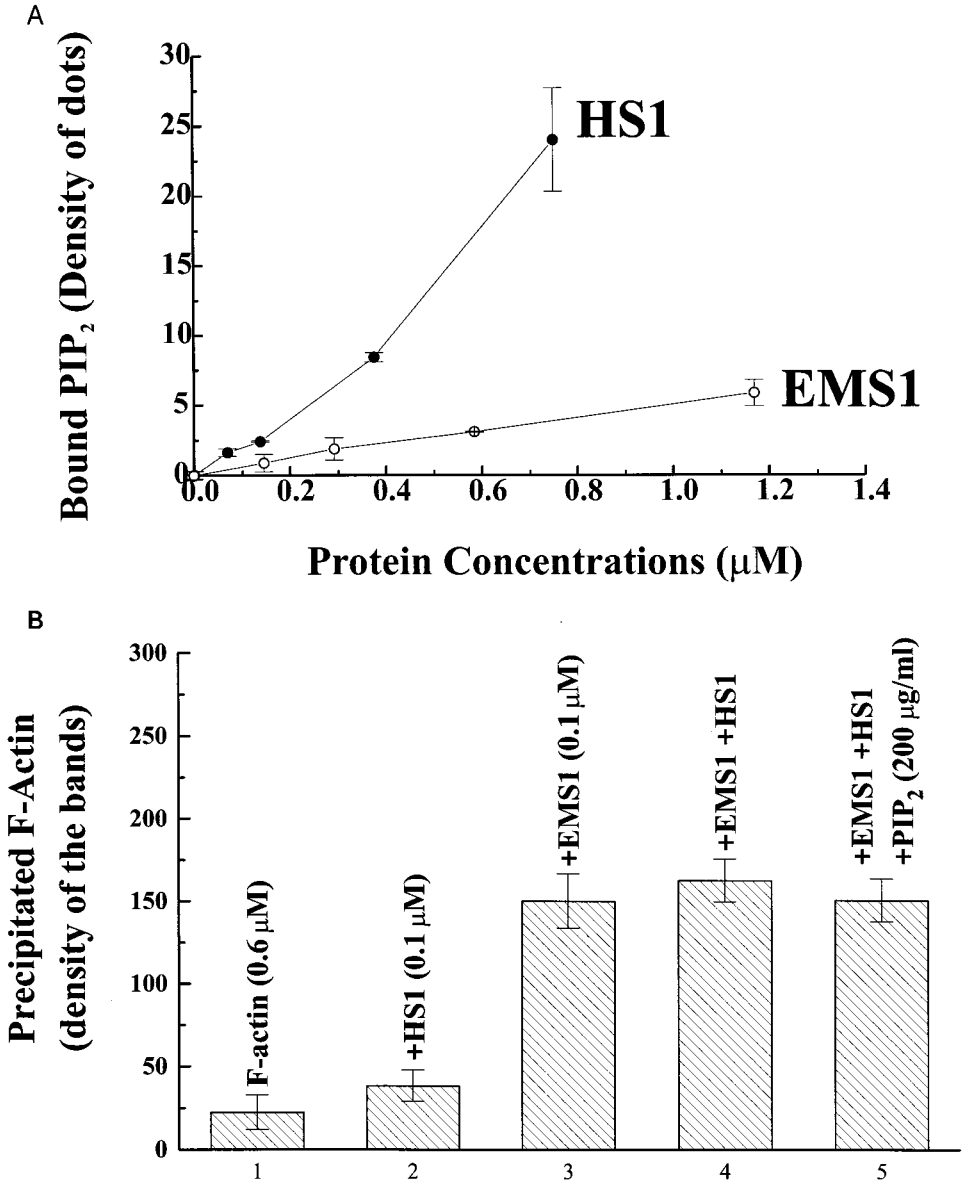


FIG. 3. (A) HS1 has a much higher affinity for PIP_2 than EMS1. PIP_2-binding assay was performed with GST fusion proteins of full-length HS1 or EMS1 fixed on a nitrocellulose membrane. Under these conditions, GST alone did not bind PIP_2. The data are the averages of three duplicated samples. (B) HS1 reverses the effect of PIP_2 that inhibits the F-actin cross-linking activity of EMS1. HS1 (0.1 μM) that does not cause F-actin cross-linking abolished the inhibition by PIP_2 (200 μg/ml) of EMS1-induced F-actin cross-linking, as the assay was performed under the essentially same conditions as those described in the legend for Fig. 2B. The data presented are the averages of two duplicated samples.

membrane ruffling (21, 46). Interestingly, the treatment of v-Ha-Ras-transformed cells with 50 μM SCH51344 completely restores the EMS1-actomyosin II complex (Fig. 1D), clearly indicating that Ras requires a component(s) acting downstream of Rac to both disrupt the EMS1-actomyosin II complex and induce membrane ruffling.

PIP_2 inhibits the F-actin cross-linking activity of either EMS1 or HS1 in vitro. The G protein Rac that is activated by Ras through PI-3 kinase (35) induces overproduction of PIP_2 by activating PI-4 and PI-5 kinases (14). Interestingly, as shown in Fig. 2A, we found that both EMS1 and HS1 contain a putative PIP_2-binding motif (R/K YG V/I E/D R/K D R/K) in their N-terminal 37-amino-acid repeats (20, 39, 54) as do other F-actin cross-linking proteins, such as alpha-actinin and vinculin (8, 9), and F-actin-severing proteins, such as gelsolin and cofilin (18, 53). In fact, PIP_2 clearly inhibits the F-actin cross-linking activity of EMS1 in a dose-dependent manner in vitro (Fig. 2B). While the F-actin cross-linking activity of HS1 requires higher concentrations, the cross-linking is inhibited more effectively by PIP_2 (Fig. 2C). As expected, HS1's apparent affinity for PIP_2 appears to be at least several fold higher than that of EMS1 (Fig. 3A). EMS1 contains only one putative PIP_2-binding motif, whereas HS1 contains three (Fig. 2A).

HS1 rescues EMS1 from the PIP_2 effect that blocks its F-actin cross-linking. Since the F-actin cross-linking activity of HS1 is much weaker than that of EMS1, HS1 (0.1 μM) does not significantly cross-link F-actin (0.6 μM), whereas EMS1 at the same concentration is a strong actin cross-linker (Fig. 3B).

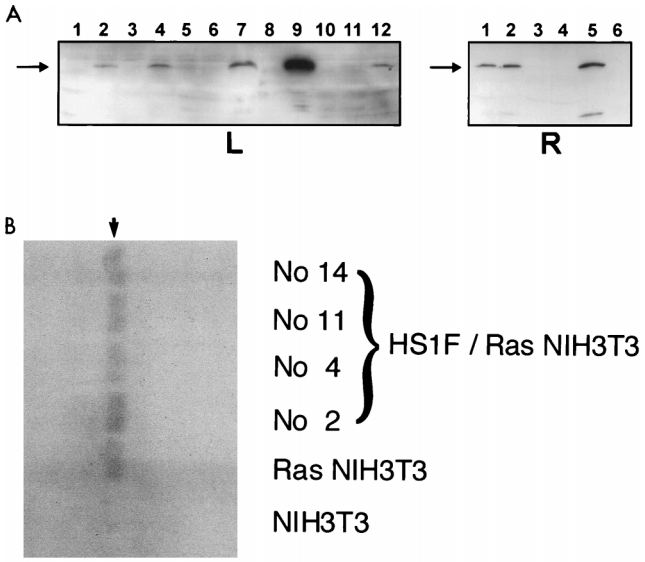


FIG. 4. Characterization of HS1 overexpressors derived from v-Ha-Ras-transformed NIH 3T3 cells. (A) Screening of HS1 overexpressors by immunoblot analysis. The supernatants (50 μl each) of cell lysates prepared from G418-resistant transfectants were subjected to SDS-PAGE and blotted by the antiserum against the human HS1 fragment. L, blotted with 1:1,000 antiserum; lanes 2, 4, 7, 9, and 12 correspond to the full-length HS1 overexpressors no. 2, 4, 7, 11, and 14, respectively. R, blotted with 1:2,000 antiserum; lanes 1, 2, and 5 correspond to the full-length HS1 overexpressors no. 15, 16, and 11. Arrow, HS1 of 75 kDa. (B) Autophosphorylation of v-Ha-Ras GTPase in HS1 revertants. Arrowhead, v-Ha-Ras GTPase of 21 kDa. There was no significant reduction in the v-Ha-Ras GTPase level of Ras-transformed cells transfected with HS1F (full-length HS1).

In the presence of both HS1 and EMS1 (0.1 μM), PIP_2, even at 200 μg/ml, fails to inhibit F-actin cross-linking by EMS1 (Fig. 3B). These observations clearly indicate that the high-affinity PIP_2 binder HS1 is able to abolish completely the action of PIP_2 that inhibits the F-actin cross-linking activity of EMS1 at least in vitro. These in vivo and in vitro effects of the PIP_2-binding protein HS1 and the Rac blocker SCH51344 on the EMS1-actin interaction support the notion that PIP_2 mediates Ras-induced disruption of the EMS1-actomyosin II complex, at least in part, as a factor acting downstream of Rac.

HS1 reverses Ras-induced malignant phenotype. Two distinct PIP_2-binding or F-actin cross-linking proteins, alpha-actinin and vinculin, suppress SV40-induced malignant transformation of NIH 3T3 cells when they are overexpressed (6, 13).

TABLE 2. Full-length HS1 suppresses anchorage-independent growth of v-Ha-RAS transformation

Clone	No. of colonies[a]				Suppression (%)
	Large	Medium	Small	Total	
Vector alone	31	334	357	721	0
HS1 transfectants					
Clone 14	2	25	94	119	83
Clone 04	2	47	89	134	81
Clone 02	1	25	126	152	79
Clone 11	3	47	138	188	74

[a] A total of 10^3 cells from each clone were plated in soft agar. After 18 days, the number of colonies formed in each dish was counted. Colony size: large, more than 100 cells; medium, 30 cells; small, fewer than 10 cells. Each value is the average of data from triplicate assay, and the standard deviation of each was less than 5%.

TABLE 3. SH3 and actin-binding domains are required for HS1 to suppress Ras-induced malignancy

v-Ha-Ras-transformed NIH 3T3 cells[a]	Colonies formed in soft agar/10^3 cells[b]
Vector alone	648 ± 122
HS1 (full length)	148 ± 30
Vector alone	600 ± 43
HS1 (SH3 negative)	565 ± 103
Vector alone	865 ± 143
HS1 (ACT negative)	645 ± 97
Vector alone	728 ± 19
HS1-EMS1 chimera (ACT negative)	678 ± 25

[a] HS1 (SH3 negative), lacking SH3 domain; HS1 (ACT negative), lacking the actin-binding domain; HS1-EMS1, non-actin-binding chimera.

[b] Each value is the average of data from at least four individual clones in duplicate assays ± standard deviation.

Furthermore, a mutant of gelsolin, the PIP_2-binding or F-actin-severing or -capping protein, also suppresses v-Ha-Ras-induced malignancy of the same fibroblasts (30). In addition, we have shown that NF2/Merlin, which is closely related to the Ezrin-Radixin-Moesin (ERM) family of PIP_2-binding and F-actin-capping proteins (16, 38), reverses v-Ha-Ras-induced malignancy (43). These previous observations prompted us to examine the anticancer potential of the newly identified PIP_2-binding and F-actin cross-linking protein HS1.

v-Ha-Ras-transformed fibroblasts were transfected with a DNA encoding full-length human HS1 with a retroviral vector (24). Several neomycin-resistant HS1-overexpressing clones were screened by immunoblot analysis with an antibody specific for the HS1 epitope which is not present in EMS1 (Fig. 4A). Unlike the parental Ras transformants, none of HS1 overexpressors forms any focus at their confluence (data not shown), suggesting that HS1 restores contact inhibition of growth. The colony-forming ability in soft agar (anchorage-independent growth) of these HS1-overexpressing clones is greatly reduced (by 70 to 80%) compared with that of the parental or vector-alone-transfected v-Ha-Ras transformants (Table 2). This reduction was not due to any loss of v-Ha-Ras gene expression, because all HS1 overexpressors show the basically same autophosphorylating activity of v-Ha-Ras as that of the parental cells (Fig. 4B). These observations clearly indicate that overexpression of HS1 suppresses Ras transformation.

Both the SH3 domain and the F-actin- and PIP_2-binding motifs of HS1 are required for the anti-Ras action. Full-length HS1 contains several distinct functional domains (Fig. 5): (i) the N-terminal three repeats of 37 amino acids, each of which binds F-actin (25, 26) and contains a putative PIP_2-binding motif, (ii) the Pro-rich domain (PLLP) that binds the SH3 domain of the Tyr-kinase Lck (42), (iii) two critical Tyr residues at positions 378 and 397, whose phosphorylation by the kinase Syk is required for the apoptosis of B lymphocytes (52), (iv) a nuclear localization sequence (residues 263 to 274), and (v) the C-terminal SH3 domain, deletion of which causes the nuclear localization of HS1 (15, 26). As summarized in Table 3, we found that deletion of either the SH3 domain or the entire F-actin- and PIP_2-binding motifs abolishes the ability of HS1 to suppress Ras-induced malignancy, although both deletion mutants are expressed as much as the full-length HS1 (data not shown). Here we describe the biological properties of only the following two distinct mutants of HS1 lacking all three

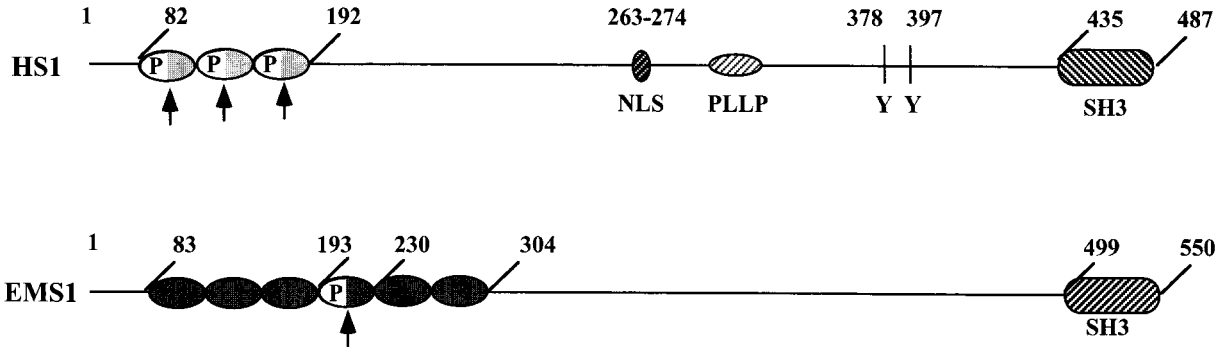


FIG. 5. Schematic structures of HS1 and EMS1. Numbers indicate amino acid residues. Arrowheads indicate only the 37-amino-acid motifs that bind F-actin. P, putative PIP$_2$-binding motif; NLS, nuclear localization sequence; PLLP, Pro-rich domain; Y, critical Tyr residue.

actin-binding 37-amino-acid repeats of HS1 (ACT−) that no longer bind either F-actin or PIP$_2$.

The N-terminal F-actin-binding domain of HS1 (residues 82 to 192) contains three unique tandem repeats, and each repeat comprises 37 amino acids and binds F-actin (25, 26). Although the corresponding domain of EMS1 (residues 83 to 193) also contains three similar tandem repeats of 37 amino acids possessing 60 to 70% sequence identity with the HS1 repeats, this EMS1 domain does not bind F-actin. Instead, only the fourth 37-amino-acid repeat of EMS1 (residue 194 to 230) binds the F-actin (25, 26). EMS1 appears to form a homodimer for cross-linking F-actin (17). Only these three F-actin-binding 37-amino-acid motifs of HS1 and the single F-actin-binding motif of EMS1 contain a putative PIP$_2$-binding motif (Fig. 2A).

To examine whether the three F-actin binding motifs of HS1 are involved in the anti-Ras action of HS1, we have generated an HS1-EMS1 chimera in which the whole F-actin-binding domain (residues 82 to 192) of HS1 is replaced by the corresponding domain of EMS1 (residues 83 to 193), which contains the highly homologous three 37-amino-acid motifs but does not bind F-actin. Unlike the wild-type HS1, the HS1-EMS1

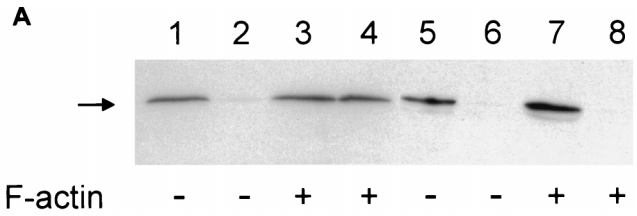
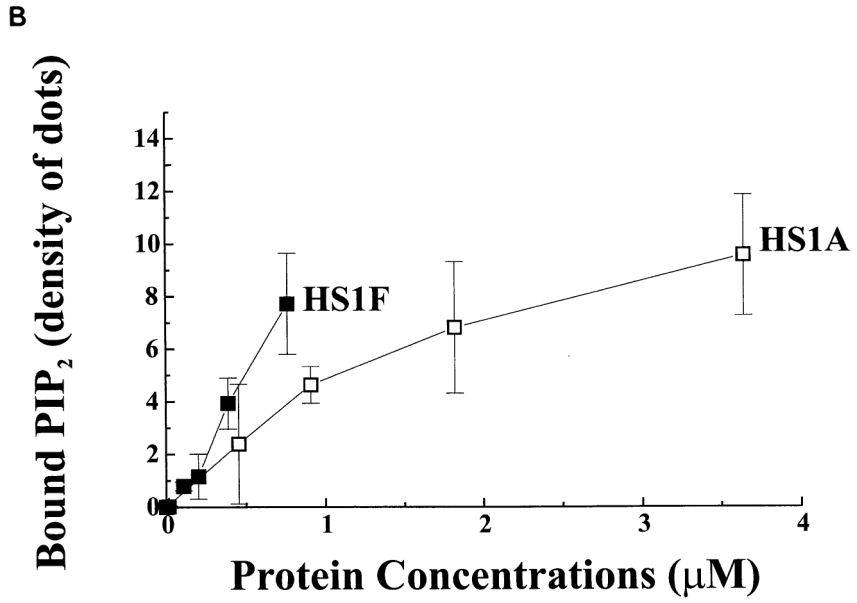


FIG. 6. Characterization of HS1 mutant (HS1ad) lacking the actin-binding domain and HS1-EMS1 chimera (HEaH). (A) F-actin binds the full-length HS1 but not the HS1-EMS1 chimera. v-Ha-Ras-transformed NIH 3T3 transfectants (10^6 cells) that overexpress the wild-type HS1 (lane 1 to 4) or chimera (lane 5 to 8) were disrupted, and the cleared cell lysate was incubated with (+) or without (−) F-actin and then centrifuged at 100,000 × *g*. The immunoblot of resultant supernatants (lanes 1, 3, 5, and 7) and pellets (lanes 2, 4, 6, and 8) indicates that the wild-type HS1 coprecipitates with F-actin (lane 4), whereas the chimera does not coprecipitate with F-actin (lane 8). (B) Actin-binding domain of HS1 binds PIP$_2$. A GST fusion protein of actin-binding domain (residues 82 to 192) of HS1 fixed on a nitrocellulose membrane was subjected to a PIP$_2$-binding assay as described in the legend for Fig. 3A. The actin-binding domain alone (HS1A) is sufficient to bind PIP$_2$, as is the full-length HS1 (HS1F). The data presented are the averages of two duplicated samples.



chimera overexpressed in v-Ha-Ras-transformants fails to bind F-actin (Fig. 6A). The HS1-EMS1 chimera does not suppress the colony-forming ability of Ras transformants in soft agar (Table 3). Similarly, an internal deletion mutant of HS1 that lacks all three actin-binding motifs (ACT−) no longer suppresses Ras transformation (Table 3). These observations clearly indicate that the actin-binding domain of HS1 is also essential for the anti-Ras action. A reverse HS1 mutant alone, consisting of only three actin-binding 37-amino-acid motifs, binds PIP_2 (Fig. 6B), strongly supporting the notion that the putative PIP_2-binding motifs within these actin-binding repeats are responsible for the PIP_2 binding. Thus, it remains to be clarified whether F-actin-binding or PIP_2-binding activity (or both) is required for the anti-Ras action of HS1.

Interestingly, like HS1, all other actin-binding tumor suppressors, such as alpha-actinin, vinculin, NF2, tensin, and gelsolin or cofilin mutants, bind PIP_2 (26, 27). However, a mutant of cofilin (Gln112 Gln114) that no longer binds F-actin but still binds PIP_2 is able to suppress Ras-induced malignancy (26, 27). In addition, we have previously demonstrated that microinjection of the antibody specific for PIP_2 into Ras-transformed NIH 3T3 cells inhibits their proliferation and reverts their morphology to the normal flat phenotype, although these effects are both partial and transient (7), suggesting an essential role for PIP_2 in Ras transformation. It would be of great interest to examine whether other PIP_2-binding proteins, such as alpha-actinin and vinculin, also restore the EMS1-actomyosin II complex in Ras-transformed cells and suppress Ras transformation.

To test more directly the notion that the PIP_2 binding alone is sufficient for both restoring the EMS1-actomyosin II complex and suppressing the malignancy, we are currently examining the effects of PIP_2-binding antibiotics such as neomycin, which inhibits thrombin-induced actin polymerization in vivo (22), presumably by blocking the PIP_2-induced uncapping of actin filament plus-ends (14). If some PIP_2-binding drugs or peptides prove to have potent anticancer activities, the screening for various other PIP_2-binding compounds or PI-4 and PI-5 kinase inhibitors such as ribofuranosyl derivatives of echiguanine analogs (37) could lead to the development of novel anticancer therapeutics useful for the treatment of Ras-associated tumors, which represent more than 30% of all human cancers.

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