Prenylation of RhoB Is Required for Its Cell Transforming Function but Not Its Ability to Activate Serum Response Element-Dependent Transcription*

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Rho regulates cytoskeletal actin structure and integrin-mediated cell adhesion. Rho also has a role in cell growth regulation and is required for cell transformation by oncogenic Ras. Recently, it has been demonstrated that Rho can activate transcription from the c-fos serum response element (SRE). This raised the possibility that functions required for Rho-mediated cell transformation might overlap with those involved in transcriptional regulation. Here we show that RhoB can activate the SRE and can synergize in cell transformation with constitutively activated Raf-CAAX. Significantly, unprenylated forms of RhoB that are biologically inert and unable to transform cells can still activate SRE-dependent transcription. This finding suggests that transcriptional activation by Rho may be separable from its cell transforming functions.

The Rho family GTP-binding proteins, whose members include Rho, Rac, and CDC42, have been shown to influence a number of cellular processes including motility, adhesion, and proliferation (1–5). The signaling pathways responsible for these diverse effects are currently an area of intense research (6). Early studies have established that a major function of Rho proteins is the regulation of cytoskeletal actin architecture with different Rho family members inducing distinct actin structures (reviewed in Ref. 6).

Recent work has indicated that Rho proteins can promote cell growth and transformation (reviewed in Ref. 7). One protein that is likely to have a physiological role is RhoB. Unlike most members of the Rho family, RhoB is a growth factor- and oncogene-induced immediate early gene product that is regulated during the cell cycle (8, 9). While weakly oncogenic on its own, RhoB is required for the initiation and maintenance of transformation by oncogenic Ras (10, 11). Additional support for a role in cell transformation is provided by evidence that RhoB is a critical target of farnesyltransferase inhibitors (10, 12, 13), a novel class of anti-tumor chemotherapeutics (14).

The mechanisms underlying the transformation-promoting effects of RhoB are unknown. Recently, work on the closely related RhoA protein showed that it can stimulate transcription through the serum response element (SRE)† (15), a regulatory sequence found in the c-fos promoter and many other mitogen-regulated promoters (16, 17). This finding raised the possibility that the transforming capabilities of RhoB might be mediated by its transcriptional effects. For this reason, we were interested in determining whether RhoB could also activate the SRE and, if so, whether this activity overlapped with its cell transforming functions.

Like other Ras superfamily proteins, Rho proteins are posttranslationally prenylated at their C terminus (18, 19). Prenylation involves the covalent thioether linkage of either a farnesyl or geranylgeranyl moiety to the C-terminal cysteine residue of Rho and is likely critical for proper membrane localization and some protein-protein interactions (18, 19). It is generally thought that Rho proteins, as well as other Ras superfamily small GTP-binding proteins, require prenylation to function.

In this study, we report that while prenylation is essential to Rho transformation potential, the modification is not required for activation of SRF-mediated transcription. Our work suggests a divergence in Rho pathways leading to transformation and SRE transcription.

MATERIALS AND METHODS

Plasmids—HA-RhoBV14 has been described previously (10, 11). Standard polymerase chain reaction mutagenesis techniques were used to generate the C193S mutant HA-RhoBV14, the T37A mutant HA-RhoBVA, and HA-RhoaV14. All constructs were expressed from the cytomegalovirus enhancer/promoter-containing vector pcDNA3 (Invitrogen). The SRE reporter construct, 3D.ACATC, with linker sequences are lowercase and SRE sites are bold (15). The Raf-CAAX construct was a gift of M. Symons (Onyx Pharmaceuticals).

Cell Culture—NIH3T3 cells were maintained in Dulbeco’s modified Eagle’s medium (Sigma) supplemented with 10% calf serum (Life Technologies, Inc.) and transfected using a modified calcium phosphate method (25). For foci assays, 5 × 103 cells were transfected with plasmids as indicated in the figure legends and passed into three dishes the day after transfection. Cell foci were scored 12–14 days later by fixing with methanol and staining with crystal violet. COS cells were maintained in Dulbeco’s modified Eagle’s medium supplemented with 10% fetal calf serum. COS cells were transfected by a modified DEAE-dextran method (26).

Western Analysis—Cells were washed in phosphate-buffered saline and harvested in Nonidet P-40 lysis buffer containing phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (25). Cell lysates were clarified by microcentrifugation and quantitated by Bradford assay. Total cellular protein (50 μg) was fractionated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and analyzed as described (27). The anti-HA antibody, 12CA5, was used at 2.5 μg/ml. A horseradish peroxidase-conjugated anti-mouse IgG antibody was used at a 1:5000 dilution to detect 12CA5 bound to Western blots (Boehringer Mannheim). Antibody complexes were detected by a chemiluminescence kit using reagents and a protocol provided by the vendor (Pierce).

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1 The abbreviations used are: SRE, serum response element; SRF, serum response factor; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase.

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RESULTS AND DISCUSSION

It has previously been shown that RhoA, Rac1, and CDC42 can activate the SRE of the c-fos promoter in a manner that depends upon SRF but not the associated ternary complex factor (15–17). Although RhoB is ∼85% identical to RhoA, it has a radically different subcellular localization; while RhoA is localized primarily in the plasma membrane and cytosol, RhoB is localized in internal vesicles and the nuclear membrane (9, 10, 20). Thus despite the similarity between RhoA and RhoB, it was not entirely clear whether RhoB would also be able to activate SRF-mediated transcription. To investigate this question, we examined the ability of an activated form of RhoB, HA-RhoBV14, to activate SRF in NIH3T3 cells. Rho-specific activation of SRF was monitored by employing the CAT reporter plasmid 3D.ACAT, which contains three SRE-binding sites but lacks the adjacent elements for ternary complex factor binding that are commonly found at SREs (15). In this assay, there was a >15-fold increase in transcriptional activation by HA-RhoBV14 compared with vector, an activity that was comparable with that of HA-RhoAV14 (see Fig. 1A). We, therefore, concluded that despite differences in cellular localization, both RhoB and RhoA communicate with the SRE.

To further analyze RhoB activation of SRF-mediated transcription, HA-RhoBV14 constructs were generated that included point mutations in either the prenylation acceptor site or the effector domain; both mutations were predicted to cause a loss of Rho activity. The CAAX motif mutation C193S abolishes prenylation of the protein and eliminates the normal localization of RhoB to cytoplasmic vesicles and the nuclear membrane (10, 20). Since prenylation is believed to be necessary for the physiological function of Ras superfamily proteins, the activated, unprenylated mutant, designated RhoBV14, would be predicted to be nonfunctional. The effector mutant, designated RhoBV14A, was engineered to contain a T37A muta-
tion in the putative effector domain of RhoB. An analogous effector mutation in RhoA has been demonstrated to eliminate its stress fiber-inducing activity (21); thus, this mutation would also be expected to destroy physiological RhoB functions. A dominant negative RhoB mutant, designated RhoB<sup>V14</sup>, has been described previously (11); this construct encodes a protein that preferentially binds GDP and has been shown to inhibit Rho function likely by competing for upstream activators (11). Western analysis demonstrated that each mutant protein accumulated to similar levels in transiently transfected COS cells (see Fig. 2).

NIH3T3 cells were transfected with 3D.ACAT and RhoB expression vectors to test the ability of the RhoB mutants to activate transcription via SRF. Interestingly, while the effector mutation in RhoB<sup>V14</sup> abolished SRF activation, the prenylation mutation in RhoB<sup>V8</sup> had no effect (see Fig. 1B). The RhoB<sup>V14</sup> result indicated that Rho effector functions are required for RhoB to activate SRF, similar to RhoA (15). Consistent with physiological activation, GTP binding was also required to activate SRF, because the dominant inhibitory RhoB mutant, RhoB<sup>N19</sup>, lacked activity. The RhoB<sup>V8</sup> result indicated that unprenylated forms of RhoB can also activate SRF. We concluded that effector functions, but not prenylation, are required for RhoB to induce SRE-mediated transcription.

The ability of RhoB<sup>V8</sup> to activate transcription raised the prospect that the cell transforming functions of RhoB might also be prenylation-independent. To test this possibility, we compared the transformation capabilities of HA-RhoB<sup>V8</sup>, HA-RhoB<sup>V14</sup>, and HA-RhoB<sup>V14</sup> in focus formation assays. RhoB<sup>V14</sup> potentiates focus formation by oncogenic Ras (11), but the −2-fold activity in this type of experiment was too weak for a significant comparison of RhoB mutant activities (data not shown). Therefore, we turned instead to a Rho-Raf cooperation assay, which has been used successfully to examine the transforming potential of activated RhoA (22, 23). This assay is based on a strong synergism which occurs when cells are cotransfected with activated RhoA and a Raf construct, Raf-CAAX, which are each quite weakly transforming on their own. Raf-CAAX includes a C-terminal CAAX motif that allows it to be prenylated and to associate constitutively with the plasma membrane; this modification confers a weak Ras-independent transforming ability (24). In this assay, we observed that RhoB<sup>V14</sup> synergistically transformed NIH3T3 cells with Raf-CAAX (see Fig. 3A). As expected, the effector mutation in RhoB<sup>V14</sup> abolished cooperation with Raf-CAAX (see Fig. 3B). In addition, the prenylation mutation in RhoB<sup>V8</sup> eliminated its ability to cooperate with Raf-CAAX, despite the fact that this mutant could activate SRF. We concluded that prenylation of RhoB is necessary for its transforming functions but not its transcriptional effects.

The finding of a prenylation-independent Rho function is notable given the widely held assumption that prenylation of Rho is necessary for its physiological functions. One possible interpretation is that SRF-mediated transcriptional activation is not a true physiological function of RhoB. However, since effector function and GTP-binding status were critical to RhoB activation of SRF-mediated transcription, another interpretation is that there exists a Rho-mediated effect on SRF, which is mechanistically novel.

In addition to demonstrating a prenylation-independent RhoB activity, our results indicate that Rho transcriptional activation of the SRE is clearly insufficient for transformation. The finding that RhoB<sup>V8</sup> can activate transcription but is unable to transform argues that other RhoB pathways must be required for transformation. A number of potential Rho-binding proteins and functions have been defined which might be critical to transformation ability; future study should define potential transforming roles soon (reviewed in Ref. 6). While we have shown that SRF-mediated transcriptional activation is not sufficient for transformation, it is not yet clear if it is necessary. Further mutational analysis is required to determine the importance of various Rho functions in transformation.

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