Cloning and Characterization of GEF-H1, a Microtubule-associated Guanine Nucleotide Exchange Factor for Rac and Rho GTPases*

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The Rho-related small GTPases are critical elements involved in regulation of signal transduction cascades from extracellular stimuli to cell nucleus and cytoskeleton. The Dbllike guanine nucleotide exchange factors (GEF) have been implicated in direct activation of these GTPases. Here we have identified a new member of the Dbl family, GEF-H1, by screening a human HeLa cell cDNA library. GEF-H1 encodes a 100-kDa protein containing the conserved structural array of a Dbl homology domain in tandem with a pleckstrin homology domain and is most closely related to the lfc oncogene, but additionally it contains a unique coiled-coil domain at the carboxyl terminus. Biochemical analysis reveals that GEF-H1 is capable of stimulating guanine nucleotide exchange of Rac and Rho but is inactive toward Cdc42, TC10, or Ras. Moreover, GEF-H1 binds to Rac and Rho proteins in both the GDP- and guanosine 5′-3-O-(thio)triphosphate-bound states without detectable affinity for Cdc42 or Ras. Immunofluorescence reveals that GEF-H1 colocalizes with microtubules through the carboxyl-terminal coiled-coil domain. Overexpression of GEF-H1 in COS-7 cells results in induction of membrane ruffles. These results suggest that GEF-H1 may have a direct role in activation of Rac and/or Rho and in bringing the activated GTPase to specific target sites such as microtubules.

The Rho-related GTP-binding proteins of the Ras superfamily function as molecular switches in a variety of cellular signaling pathways, many of which influence the cell cytoskeletal organization and effect on physiological properties of cells such as cell polarity and motility (1–3). Rho family GTPases also play roles in transmission of growth factor and mitogen-initiated signals to the nucleus through kinase cascades (4–7). Members of this family of GTP-binding proteins include numerous Ras-like GTPases. Here we have identified a new member of the Dbl family, GEF-H1, by screening a human HeLa cell cDNA library. GEF-H1 encodes a 100-kDa protein containing the conserved structural array of a Dbl homology domain in tandem with a pleckstrin homology domain and is most closely related to the lfc oncogene, but additionally it contains a unique coiled-coil domain at the carboxyl terminus. Biochemical analysis reveals that GEF-H1 is capable of stimulating guanine nucleotide exchange of Rac and Rho but is inactive toward Cdc42, TC10, or Ras. Moreover, GEF-H1 binds to Rac and Rho proteins in both the GDP- and guanosine 5′-3-O-(thio)triphosphate-bound states without detectable affinity for Cdc42 or Ras. Immunofluorescence reveals that GEF-H1 colocalizes with microtubules through the carboxyl-terminal coiled-coil domain. Overexpression of GEF-H1 in COS-7 cells results in induction of membrane ruffles. These results suggest that GEF-H1 may have a direct role in activation of Rac and/or Rho and in bringing the activated GTPase to specific target sites such as microtubules.

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The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; GST, glutathione S-transferase; ORF, open reading frame; PH, pleckstrin homology; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinedithanesulfonic acid; GTPγS, guanosine 5′-3-O-(thio)triphosphate.

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H1, is a new member of Dbl family with a broad tissue distribution pattern. GEF-H1 functions as a GEF for Rac and Rho proteins in vitro and binds to Rac and Rho in both the GDP- and the GTP-bound states. Furthermore, GEF-H1 colocalizes with microtubules in cells mediated by its unique carboxy-terminal coiled-coil domain instead of its PH domain, and its overexpression in COS-7 cells results in induction of membrane ruffles. Thus, GEF-H1 represents a novel Dbl-like GEF that may have a direct role in activating Rac and/or Rho and serve to activate the activated GTPase to cellular microtubules.

EXPERIMENTAL PROCEDURES

Isolation of GEF-H1 cDNA—A HeLa cell cDNA library was generated using a kit from CLONTECH following standard protocols (35). A double-stranded adaptor was ligated to both ends of the cDNA library by using T4 DNA ligase, and the adaptor-ligated cDNAs were then used to selectively amplify 5' cDNA fragments by polymerase chain reaction with a combination of a p40-specific primer GSP4 (5' GCAGTGACCCCAGGACTCGTGTTAC3') and an adaptor-specific primer. The polymerase chain reaction products were cloned into pBluescript vector and were sequenced by the dyeodeoxy nucleotide method using Sequane 2.0 (U. S. Biochemical Corp.). Data bank searches for sequence homology were performed using the BLAST program (Genetics Computer Group, Madison, WI).

Northern Blot Analysis—A human multiple tissue Northern blot (CLONTECH) containing approximately 2 µg of poly(A)+ RNA per lane from eight different human tissues was hybridized with either 5' or 3' end (nucleotides 1–1624) or 3' end (nucleotides 2251–2573) sequences labeled with [32P]dATP. The hybridization and washing conditions were as described by the CLONTECH protocols.

Expression and Purification of Recombinant Proteins—The glutathione S-transferase (GST) fusion proteins of small GTP-binding proteins were expressed in Escherichia coli using the pGEX vectors as described (29). Purification of the GST-fused G-proteins from E. coli was also carried out as described (29). The GST-Lbc protein was synthesized in the baculovirus-infected Sf9 insect cells and purified by glutathione-agarose affinity chromatography (16).

The GEF-H1 baculovirus construct, pBacGEF-H1, was made by cloning an EcoRI/BglII fragment of GEF-H1 containing the complete ORF into the EcoRI/BglII sites of the transfer vector pVL1393 (PharMingen). Recombinant baculovirus was produced as described previously (33). The virus-infected Sf9 cells were washed once with ice-cold buffer A containing 100 mM KCl, 1 mM EDTA, and then mixed with the precleaned cell lysates (1:30 dilution) in phosphate-buffered saline for 1 h at room temperature. The cells were washed, the cells were incubated with anti-FLAG monoclonal antibody and permeabilized with 100% methanol at −20 °C for 10 min. After washing, the cells were incubated with anti-FLAG monoclonal antibody. The immune complexes were visualized by chemiluminescence reagents (Amersham Pharmacia Biotech).

Cell Culture—The cDNAs encoding full-length GEF-H1 and Lbc or the amino-terminal residues encompassing the DH and PH domains (residues 1–604) of GEF-H1 were subcloned into the mammalian expression vector pFLAG-CMV-2 (Eastman Kodak). COS-7 or NIH 3T3 cells were transfected by Lipofectin following the instructions from Life Technologies, Inc. Transfected COS-7 cells on chamber slides were fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.5% Triton X-100 in PEM buffer (100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM MgCl2), 5 µl of each GST-fused small G-proteins were immobilized on agarose-glutathione beads, washed three times in a buffer containing 20 mM Tris-HCl, pH 7.6, 60 mM NaCl, 2 mM EDTA, and then mixed with the precleaved cell lysates (~200 µg of total protein) for 1 h. The washed precipitates from the mixtures were subjected to 10% SDS-PAGE and transferred to nitrocellulose for Western-blot analysis using anti-GEF-H1 polyclonal antibody. The immune complexes were visualized by chemiluminescence reagents (Amersham Pharmacia Biotech).

RESULTS

Cloning of the cDNA Encoding the Complete Open Reading Frame of GEF-H1—Previously a partial cDNA clone termed p40 was isolated from a Hep-2 cDNA library, and the protein product was thought to play a role in regulation of cell proliferation (32). To obtain the complete ORF of this clone, a 5' rapid amplification of cDNA ends was performed by polymerase chain reaction in a HeLa cell cDNA library with the combination of a p40-specific primer GSP4 complementary to the p40 nucleotide residues 187–212 (32) and an adaptor-specific primer in the linker region of the library. A cDNA clone containing 3630 bases with a single ORF starting with an ATG codon at nucleotide 103 was obtained during the screening (Fig. 1).
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Fig. 2. Alignments of the homology domains of GEF-H1 protein with other Dbl-like GEFs. A, schematic representation of the GEF-H1 protein and analogous domains in Lfc, Lbc, Dbl, and RhoGEF, with relevant amino acid numbering indicated. DH, Dbl-homologous domain; PH, pleckstrin homology domain; COILED, coiled-coil region; L-rich, leucine-rich region; Zn, zinc finger-like motif. Percentages indicate relative identity in sequences of the domains in pairwise comparison with the corresponding domains in GEF-H1. B, comparison of the DH domains of GEF-H1, Lfc, Lbc, RhoGEF, and Dbl. Boxed residues represent sequence identities. C, comparison of the PH domains of GEF-H1, Lfc, Lbc, RhoGEF, and Dbl. D, comparison of the coiled-coil domains of GEF-H1, RhoGEF, Plectin, and myosin heavy chain.
The ATG codon is in a moderately good context of Kozak sequences for translation initiation with a purine (A) at +2 and a pyrimidine (T) at +4 (31). The ORF predicts a protein of 894 amino acids in length with a calculated molecular mass of 100 kDa. Comparison of the deduced amino acid sequences of this clone with protein sequences in the Swiss Protein Data Bank revealed no apparent identity, indicating that the cDNA is likely to represent a novel gene.

Subsequent protein homology searches revealed that the predicted amino acid sequences contain a DH domain followed by a PH domain from amino acids 238–572 (Fig. 2); these sequences are 23, 48, 51, and 88% identical to the homologous regions of Dbl (10), Lbc (16), RhoGEF (26), and Lfc (13), respectively. We therefore designated this protein as GEF-H1. In addition to the tandem DH and PH domains, GEF-H1 also contains a cysteine-rich zinc finger-like motif at its amino terminus, which is similar to that found in Lfc (13). Aside from the 12% differences over the amino-terminal 414 amino acids, GEF-H1 differs from Lfc by an extra region of 310 amino acid residues at the carboxyl-terminal half, which is weakly homologous to the cytoskeleton protein Plectin (21) and RhoGEF (26) (Fig. 2D). Further structural prediction by the COILS program suggests that this region of GEF-H1 may form an α-helical coiled-coil structure. These sequence analyses suggest that GEF-H1 is a novel member of the Dbl family GEFs.

Northern blot analysis using the 5′-half of GEF-H1 cDNA as a probe detected a single 4.4-kilobase pair mRNA band in the total RNA samples from a wide range of human tissues including spleen, thymus, prostate, testis, uterus, small intestine, colon, and leukocytes, with higher staining intensity in the normalized samples from thymus, testis, and leukocytes (Fig. 3). Northern blot using a probe derived from the 3′-half sequence (nucleotides 2251–2573; B) of GEF-H1 labeled with 32PdATP. As a control for the amount of RNA loaded, the blot was reprobed with a β-actin probe.

FIG. 3. Tissue distribution pattern of GEF-H1 mRNAs. Human multiple tissue Northern blot IV (CLONTECH) containing approximately 2 µg of poly(A)+ RNA per lane from eight different human tissues was hybridized with either a 5′-half (nucleotides 1–1624; A) or 3′-half sequence (nucleotides 2251–2573; B) of GEF-H1 labeled with 32PdATP. As a control for the amount of RNA loaded, the blot was reprobed with a β-actin probe.

GEF Activity of GEF-H1—Since GEF-H1 shares considerable sequence homology with Lbc and Lfc of the Dbl family small G-protein regulators which have been shown to be Rho-specific GEFs, we examined potential GEF activity of GEF-H1 toward RhoA. Fig. 4 shows that GEF-H1 is capable of stimulating the dissociation of [3H]GDP from RhoA when immunopurified from recombinant GEF-H1 baculovirus-infected Sf9 cells (lane 3).

FIG. 4. GEF-H1 functions as a guanine nucleotide exchange factor for RhoA in vitro. A, Coomassie Blue-stained SDS-PAGE (10%) showing protein standard (lane 1), purified GST-Lbc (lane 2), and immunoprecipitated GEF-H1 from infected Sf9 cells (lane 3). B, time courses of [3H]GDP release from recombinant RhoA (0.3 µg) in the presence of free CTP (0.5 mM) and GST (2 µg), GST-Lbc (0.1 µg), or GST-GEF-H1 (1 µg). □, Lbc %; ◦, GEF-H1 %; ■, Control %. Conditions of the [3H]GDP/GTP exchange were as described under “Experimental Procedures.”

FIG. 5. GEF-H1 stimulates GDP dissociation from Rac and Rho GTPases. GDP dissociation assays were carried out as in Fig. 4 using immunopurified GEF-H1 (1 µg) and 0.3 µg of recombinant GST fusion of Ras, Ran, RhoA, RhoB, RhoC, TC10, Rac1, or Cdc42. The reactions were terminated after 5 min incubation at 25 °C.
cells. In the absence of GEF-H1 protein, the half-time for dissociation of GDP from RhoA was relatively slow, i.e., >30 min at room temperature. However, GEF-H1 accelerated the rate of GDP dissociation by at least 10-fold; the half-time for GDP dissociation became 2–3 min. The purified GST-Lbc was used as a positive control, and the purified GST and anti-GEF-H1 immunoprecipitates of Dbl virus-infected Sf9 cell lysates were used as negative controls in similar assays (Fig. 4 and data not shown). When the activity of GEF-H1 to stimulate GDP/GTP exchange was examined for a panel of small G-proteins, it was found that GEF-H1 was active as a GEF for RhoA, RhoB, RhoC, and Rac1, but not for Ras, Cdc42, or TC10 under similar conditions (Fig. 5). Thus, GEF-H1 appears to be an effective GEF for Rho and Rac GTPases in vitro.

Interaction of GEF-H1 with Different Nucleotide Binding States of Rho Family GTPases—Certain members of the Dbl family, e.g. Ect-2, bind to Rho-related GTP-binding proteins without detectable GEF activity (15, 17). To determine whether GEF-H1 interacts solely with Rho and Rac and to examine the nucleotide dependence of the interaction, we carried out a complex formation assay using immobilized GST fusion RhoA, Rac1, Cdc42, or Ras and insect cell lysates overexpressing recombinant GEF-H1. The binding of GEF-H1 to the GTP-binding proteins was assayed when the G-proteins are in the guanine nucleotide-free state, GDP-bound state, or GTP\(\gamma\)S-bound state. As shown in Fig. 6, GEF-H1 bound to RhoA and Rac1 with relatively high affinity, and this binding occurred approximately equally well when RhoA and Rac1 were at any one of the three states. In contrast, GEF-H1 was unable to bind to Ras or Cdc42 in any of the nucleotide-binding states in the similar assay (Fig. 6 and data not shown). Therefore, GEF-H1 is a Rho- and Rac-specific GEF capable of direct interaction with the G-proteins in both the GDP- and the GTP-bound state.

Subcellular Distribution Pattern of GEF-H1—To investigate the potential role of GEF-H1 in vivo, an epitope-tagged variant of GEF-H1 was constructed in pFLAG-CMV-2 vector, and the cDNA was transfected into COS-7 and NIH 3T3 cells. The transfected cells were stained with either monoclonal anti-Flag antibody M2 or a polyclonal anti-GEF-H1 antibody raised against the carboxyl-terminal region of the molecule for visu-
alization of the expression pattern. As shown in Fig. 7A, staining with the M2 antibody revealed a filamentous distribution pattern of GEF-H1. This pattern does not correlate with that of actin structures of the cell, which was visualized by rhodamine-labeled phalloidin staining (data not shown). However, when the cells expressing GEF-H1 were stained with monoclonal anti-tubulin antibody, they displayed a similar filamentous fluorescent image as that by anti-GEF-H1 antibody staining (Fig. 8). To determine the possible colocalization relationship of GEF-H1 with tubulin, the effect of the microtubule-disrupting agent colcemid was examined. The GEF-H1-transfected cells were pretreated with 0.06 µg/ml colcemid for 30 min before GEF-H1, and cellular microtubules were labeled with immunofluorescent probes. Fig. 7B shows that colcemid caused an unwinding of the filamentous structure of GEF-H1. In some cases, the filamentous staining pattern of GEF-H1 was changed to a punctate form (Fig. 7B). Since colcemid specifically disrupts microtubule structures (19), these results indicate that GEF-H1 indeed colocalizes with microtubules of the cell.

To determine whether the amino-terminal Lfc homology half of GEF-H1 might have a role in influencing its subcellular localization pattern, a mutant form of GEF-H1 (residues 1–604) was generated with deletion of the carboxy-terminal coiled-coil domain. This truncation mutant was found to display a punctate distribution pattern throughout the cytoplasm and in dense irregular perinuclear aggregates (Fig. 7C), differing markedly from the filamentous pattern of the full-length protein (Fig. 7A). Thus, the carboxy-terminal coiled-coil domain appears to be essential for GEF-H1-microtubule colocalization. Moreover, this pattern of distribution seems unique for GEF-H1 since Lbc, which contains a few more amino acid sequences than the DH and PH domains, was found to display a punctate distribution throughout the cytoplasm when overexpressed in COS-7 cells (Fig. 7D).

**GEF-H1 Effects on Cell Morphology—**To see whether GEF-H1 may act as a GEF for Rac or Rho proteins in *vivo*, the effect of its overexpression on the morphology of COS-7 cells was examined. The expression of GEF-H1 was determined by fluorescence microscopy 36 h post-transfection by anti-GEF-H1 antibody staining, and cell morphology was visualized by phase contrast microscopy. In cells showing positive staining with GEF-H1 antibody (Fig. 9A), large membrane ruffles at the edges of cells were present (Fig. 9B). Further labeling of the cells with rhodamine-conjugated phalloidin revealed that these ruffling membranes contained dense actin patches (Fig. 9C), similar to the effect observed when a constitutively active form of Rac was present (15). On the other hand, little change in morphology was detected in mock-transfected cells (data not shown). Given the previous observation that membrane ruffling and lamellipodia formation are through a Rac-mediated pathway (15), these results are consistent with the possibility that GEF-H1 activates endogenous Rac in these cells resulting in actin reorganization.

**DISCUSSION**

In the present study, we have identified a complete ORF from a human HeLa cell cDNA library, which encodes a novel Dbl family protein GEF-H1. Like all known Dbl family members, GEF-H1 contains a DH domain in tandem with a PH domain, which is highly homologous to that of Lfc, an exchange factor specific for Rho (13, 30). Yet differing from Lfc, GEF-H1 contains an extended coiled-coil carboxyl terminus that shows a weak homology to Plectin, an intermediate filament binding protein (29). Further differing from Lfc, probing the mRNA of GEF-H1 revealed a single hybridization band of 4.4 kilobase pairs in size (comparing to 3.7-, 3.3-, and 4.5-kilobase pair mRNA bands seen for Lfc) which is broadly present in human tissues. Further biochemical and cell biological characterizations of GEF-H1 revealed a few additional unique properties of GEF-H1 as follows: 1) it shows guanine nucleotide exchange activity toward both Rho and Rac; 2) it binds to Rho and Rac in all three nucleotide-binding states; and 3) it colocalizes with microtubules in cells that are mediated through the carboxy-terminal coiled-coil sequences.

Although GEF-H1 is capable of stimulating GDP/GTP exchange on RhoA, this action does not appear to be catalytic since the stimulated GDP-release from RhoA can reach completion only when stoichiometric amounts of GEF-H1 (*versus* RhoA) were present (Fig. 4). This observation can be rationalized by the fact that GEF-H1 binds to Rho and Rac independently of their nucleotide binding status (Fig. 6) and therefore remains bound to RhoA-GTP even after the GDP/GTP exchange is accomplished. This nucleotide-independent binding to small GTPases is similar to the Dbl family members Ect2 and RhoGEF and to the Rho family G-protein effectors IQGAP2 (36) and PRK2 (22), suggesting that GEF-H1 recognizes the GTPase elements that do not make a major conformational change from the GDP- to GTP-bound state. If the classic GEF-G protein interaction mechanism in which the exchange of GDP by GTP has to undergo a nucleotide-depleted “transition state” can be applied to the GEF-H1-Rho interaction, it would be expected that the nucleotide-free state of Rho and Rac may have at least a slightly higher affinity toward GEF-H1. However, this effect of GEF-H1 binding to Rho and Rac was not detected, possibly due to the qualitative nature of the complex formation assay used to measure the binding interaction. Although recognition of the small G-proteins at the GTP-bound state may satisfy one criterion as a potential effector of G-protein function, a constitutively bound protein can also serve...
as a carrier to recruit binding partners to potential target sites in cells. In this sense, GEF-H1 may qualify both as a GEF activator and a shuttling/recruiting scaffold that brings the activated Rho proteins to microtubule structures.

The unique carboxyl-terminal coiled-coil region of GEF-H1 is weakly homologous to Plectin, an intermediate filament binding protein. Results from the carboxyl-terminal deletion mutant of GEF-H1 indicate that this section of GEF-H1 is essential for the microtubule-associated distribution pattern in cells (Fig. 7C). Interestingly, a fraction of Rac in cells was also found to colocalize with microtubules and to recognize tubulin in a GTP-dependent manner (25). Together with the biochemical results here showing that GEF-H1 is capable of activating Rac and interacts with Rac at both the GDP- and GTP-bound states, one possible mode of action by GEF-H1 may be that it recognizes Rac in the inactive GDP-bound state, stimulates its GDP/GTP exchange, and brings the GTP-bound, active form of Rac to one of the target sites at the microtubule locations. Consistent with the possibility that Rac is the preferred substrate for GEF-H1 in cells, GEF-H1 induces membrane-ruffling formation, which is the characteristic change controlled by Rac in many cell types. Alternatively, GEF-H1 may activate Rho in certain cells and further serves to recruit the GTP-bound form of Rho to microtubules to execute Rho function there, since there is also evidence that Rho may be an important regulator of microtubule structures (20).

Joining Dbl, Lbc, Lfc, Lsc, p115, Trio, and RhogEF of the Dbl family, GEF-H1 may provide another link to Rho activation. The reason that a surprisingly large number of Dbl family members can activate Rho may reside in their differences in tissue distribution, subcellular localization, substrate specificity, and mode of regulation by upstream and/or feedback signals. For example, localization of Lfc to the plasma membranes by the PH domain is important for its transforming function (13), targeting of Lbc and Dbl to cytoskeleton by the PH domains is essential to their oncogenic activity (11, 27), and membrane translocation mediated by an amino-terminal unique PH domain of Tiam1 is required for its transforming potential (18). The recent findings that the amino-terminal coiled-coil domain of p115 contains an RGS motif and is subjected to regulation by the heterotrimeric G-protein Go13 (23) further suggest that each Dbl-like GEF may employ a unique mode of regulation and links a subpopulation of Rho GTPases to specific upstream signals via a distinct mechanism.

Many Dbl family GEF members are oncogene products (8). Both the DH domain and PH domain are essential for their transforming activity, and in many cases an additional motif at the amino terminus or carboxyl terminus of the DH and PH module, particularly a coiled-coil domain, constitutes an inhibitory constraint on their oncogenic potentials (8). In NIH 3T3 cells, GEF-H1 displayed a very weak foci-forming activity when compared with Lbc. Whether GEF-H1 belongs to the oncogene superfamily and its carboxyl-terminal coiled-coil domain causes an inhibition in its transforming capability are currently under investigation. The important questions on which signaling pathway involves GEF-H1/Rac or GEF-H1/Rho combination and how the exchange factor is turned on in response to upstream signaling events will have to be addressed in future studies to gain a full understanding of the cellular functions of GEF-H1.

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