XPLN, a Guanine Nucleotide Exchange Factor for RhoA and RhoB, But Not RhoC*

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Rho proteins cycle between an inactive, GDP-bound state and an active, GTP-bound state. Activation of these GTPases is mediated by guanine nucleotide exchange factors (GEFs), which promote GDP to GTP exchange. In this study we have characterized XPLN, a Rho family GEF. Like other Rho GEFs, XPLN contains a tandem Dbl homology and pleckstrin homology domain topography, but lacks homology with other known functional domains or motifs. XPLN protein is expressed in the brain, skeletal muscle, heart, kidney, platelets, and macrophage and neuronal cell lines. In vitro, XPLN stimulates guanine nucleotide exchange on RhoA and RhoB, but not RhoC, Rac1, or Cdc42. Consistent with these data, XPLN preferentially associates with RhoA and RhoB. The specificity of XPLN for RhoA and RhoB, but not RhoC, is surprising given that they share over 85% sequence identity. We determined that the inability of XPLN to exchange RhoC is mediated by isoleucine 43 in RhoC, a position occupied by valine in RhoA and RhoB. When expressed in cells, XPLN activates RhoA and RhoB, but not RhoC, and stimulates the assembly of stress fibers and focal adhesions in a Rho kinase-dependent manner. We also found that XPLN possesses transforming activity, as determined by focus formation assays. In conclusion, here we describe a Rho family GEF that can discriminate between the closely related RhoA, RhoB, and RhoC, possibly giving insight to the divergent functions of these three proteins.

The Ras superfamily of small GTPases serve as molecular switches, relaying biochemical signals from extracellular stimuli to elicit intracellular responses that ultimately give rise to changes in cell behavior. Within the Ras superfamily, the members of the Rho family regulate an assortment of downstream effectors that contribute to their distinct cellular functions. Among the best characterized Rho effectors are mDia and the Rho kinases, ROCK1 and ROCK2. The specificity of XPLN for RhoA and RhoB, but not RhoC, is surprising given that they share over 85% sequence identity. We determined that the inability of XPLN to exchange RhoC is mediated by isoleucine 43 in RhoC, a position occupied by valine in RhoA and RhoB. When expressed in cells, XPLN activates RhoA and RhoB, but not RhoC, and stimulates the assembly of stress fibers and focal adhesions in a Rho kinase-dependent manner. We also found that XPLN possesses transforming activity, as determined by focus formation assays. In conclusion, here we describe a Rho family GEF that can discriminate between the closely related RhoA, RhoB, and RhoC, possibly giving insight to the divergent functions of these three proteins.

The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; GST, glutathione S-transferase; mant, N-methylanthraniloyl; RBD, Rho-binding domain of Rhotekin; XPLN, exchange factor found in platelets and in leukemic and neuronal libraries; ITSN, intersectin; HA, hemagglutinin; GFP, green fluorescent protein; GTP,S, guanosine 5′-3′-(thio)triphosphate.

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GTases (3). Consequently, because each Rho GTase serves distinct roles in controlling cellular processes, elucidating the precise Rho GTase specificity of a particular Rho GEF is crucial to understand its function in the regulation of cell behavior.

Although RhoA has been the most intensively studied, RhoB and RhoC are highly related proteins that share over 85% amino acid identity with RhoA. Therefore, it is not surprising that these proteins share related functions and regulation. However, there is evidence that they also facilitate distinct cellular functions. For example, RhoB has been shown to exhibit a growth inhibitory function that is not seen with RhoA (12). Interestingly, RhoC, but not RhoA or RhoB, overexpression has been observed in a variety of human cancers and was shown to enhance tumor cell invasion and metastasis (13–15). When tested, Rho GEFs that activate RhoA have also activated RhoB and RhoC. Rho GEFs that selectively activate RhoA, RhoB, or RhoC have as yet not been described.

While the spectrum and diversity of extracellular signals that cause activation of Rho GTases continue to grow at a remarkable pace, the identity of the Rho GEFs that link these signals to Rho GTases is amazingly quite limited. To better understand how Rho GTase activity is regulated by extracellular signals, we have characterized the product of the ARH-GEF3 gene, a Rho family GEF henceforth called XPLN. XPLN is expressed in brain, skeletal muscle, heart, kidney, platelets, and macrophages. We demonstrate by several criteria that in vitro and in vivo XPLN stimulates guanine nucleotide exchange on RhoA and RhoB, but not RhoC or other Rho family GTPases. We show that the inability of XPLN to exchange RhoC is mediated by isoleucine 43, a position occupied by valine in RhoA and RhoB.

**EXPERIMENTAL PROCEDURES**

**Cloning and Generation of Expression Vectors—**XPLN/ARHGEF3 cDNA was cloned of a human acute myeloid leukemia cDNA library (kindly provided by Dr. G. Reuther, University of North Carolina, Chapel Hill) and a human brain cDNA library (Marathon, Clontech) by PCR but could not be detected in two human cDNA libraries of a breast cancer cDNA library. The DNA sequences obtained from both libraries (1581 bp) were identical to the coding sequence of GenBankTM accession number NM_019555. The full-length cDNA, encoding amino acids 1–526, a cDNA fragment coding for the DH-PH tandem repeat (amino acids 117–457), as well as fragments corresponding to NH2- and COOH- terminal proteins (amino acids 117–296 (DN) and 1–457 (DC), respectively) were subcloned into the pCMV-Myc (Clontech) and pEGFP-C1 (Clontech) mammalian expression vectors as well as the pGEX 4T-1 glutathione S-transferase (GST)-tagged bacterial expression vectors (Amersham Biosciences). To obtain pCMV-Myc-Q63L RhoA, the cDNA of human RhoA containing a Q63L mutation was subcloned into pCMV-Myc. Bacterial expression vectors for GST-tagged RhoB or RhoC were made by subcloning rat RhoB and human RhoC coding sequences into pGEX 4T-1. The expression vectors for bacterial GST-tagged nucleotide-free RhoA, Rac1, and Cdc42, wild-type RhoA, and GTase-deficient RhoA have been described previously (16). Mammalian expression vectors for constitutively activated RafY340D), amino-terminal truncated and activated ΔN186-Vav1, and intersectin DH-PH have been described previously by our laboratories (17–19). The mammalian expression vector for Myc epitope-tagged Tiam1 C1199, pAC 90M-1 (20), was kindly provided by Gideon Bollag (Onyx Pharmaceuticals).

**Detection of XPLN Expression—**Antiserum against XPLN was produced by Covance Research Products Inc. by inoculating rabbits with full-length recombinant XPLN. The antiserum was then affinity purified with 5 mg of recombinant Tiam1 C1199, an amino-terminal truncated and activated Sepharose-agarose column as described by the manufacturer (Sigma). Immunoblot analysis with the XPLN antibody was performed with human tissue membranes from IMGENEX or with 40 μg of whole cell lysates from the indicated cells. Membranes were also stained with Ponceau S (Sigma) prior to immunoblot analysis and indicated that comparable amounts of total protein in each extract sample were used.

**In Vitro Guanine Nucleotide Exchange Factor Assays—**XPLN was prepared by incubating glutathione-Sepharose (Amersham Biosciences)-bound GST-XPLN with 5 units of bovine thrombin (Sigma) overnight. Released XPLN protein was then cleaned of free thrombin with benzamidine-agarose (Sigma). Fluorescence spectroscopic analysis of ethymylthiuranilloyl (mant)-GTP incorporation into GDP-prefolded GST-XPLN proteins using a FLUOstar fluorescence microplate reader at 25 °C similar to as described previously (21). Exchange reaction assay mixtures containing 20 μM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 50 μM/mg bovine serum albumin, 1% glycerol, 500 mM mann-GTP (Biomol), and 2 μM GST-Pase were prepared and allowed to equilibrate. For Fig. 3, Vav2 DH/PH/CRD (100 nM full-length XPLN (457 C) were subcloned into the indicated vector and the relative mant fluorescence (excitation = 360 nm, emission = 460 nm) was monitored. Experiments were performed in duplicate for every condition. For Table I, Vav2 DH/PH/CRD (100 nM), LARG DH/PH (150 nM), or full-length XPLN (650 nM) were added and the linear velocity of exchange was determined as follows. Baseline nucleotide exchange rates were calculated by dividing the change in emission at 460 nm by change in time. Values were averaged and standard deviations were calculated. Velocity was considered linear if the regression value of the exchange slope was greater than 0.97. After GEF addition, reactions were allowed to equilibrate and rates were calculated as above, and fold-induction was calculated by dividing GEF-induced exchange rates by the basal exchange rates.

**Precipitation of GEFs with Recombinant Rho Proteins—**For pull-down experiments with nucleotide-free mutants, cells expressing epitope-tagged GEFs were lysed in a buffer consisting of 1% Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl2, and protease inhibitors. Lysates were cleared by centrifugation at 16,000 × g for 10 min. Samples (250 μg) were incubated at 4 °C for 30 min with 10 μg of GST or GST fusion proteins containing nucleotide-free Rho family proteins (G15A Cdc42, G15A Rac1, and G17A RhoA) bound to glutathione-Sepharose. In some experiments lysates were also incubated with GST fusion proteins of constitutively active (Q63L) and wild type RhoA. After 1 h of incubation, the beads were washed 4 times with lysis buffer. Samples were then subjected to immunoblot analysis for the relevant epitope tag with monoclonal antibodies against Myc (Sigma), HA (Covance), or GFP (Clontech). Membranes were also stained with Ponceau S prior to immunoblot analysis and indicated that similar amounts of total proteins were used. For experiments using wild type Rho proteins stripped of nucleotide were performed using the same technique except that the cells were lysed in a buffer containing 5 mM EDTA and no MgCl2.

**Rho Family Activity Assays—**The formation of activated, GTP-bound RhoA subfamily proteins were measured using a technique similar to the method described by Ren and colleagues (22). Briefly, cells were lysed in 1% of 50 mM Tris, pH 7.4, 10 mM MgCl2, 0.5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, and protease inhibitors. 500–750 μg of lysates were cleared at 16,000 × g for 5 min, and the supernatant was rotated for 30 min with 30 μg of GST-RBD (GST fusion protein containing the Rho-binding domain (RBD, amino acids 7–89 of Rhotekin) bound to glutathione-Sepharose beads. Samples were then washed in 50 mM Tris, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. GST-RBD pull-downs and lysates were then immunoblotted with an antibody (clone 55, BD Transduction Laboratories) followed by incubation with Texas Red-donkey anti-mouse (Jackson ImmunoResearch Laboratories). Images were obtained and analyzed using ImageJ software (available at http://www.nih.gov.nih.gov/).
Sequence alignment of XPLN with Net1 and intersectin. The amino acid sequences of the DH (black) and PH domains (gray) of XPLN, and its two closest homologs, Net1 and intersectin (ITSN), were aligned. Residues that are identical (*), conserved (.), semiconserved (‘), or divergent (empty space) among the three proteins are indicated.

Expression of XPLN in Tissues and Cells—Initially, we examined XPLN expression in various human tissues and cell types. XPLN protein expression was detected by immunoblot analysis using an affinity purified rabbit antibody raised against the full-length GEF. Immunobots of human tissue extracts revealed that XPLN is expressed prominently in adult brain and skeletal muscle and to a lesser level in the heart and kidney (Fig. 2a). XPLN was not found in tissue extracts from the small intestine, liver, lung, testis, spleen, or pancreas. We also examined XPLN expression in several primary cells and common cell lines. XPLN expression was detected in human platelets, the murine macrophage cell line RAW264, and PC12 pheochromocytoma cells, but not in primary human monocytes or other hematopoietic cells, such as HL-60 promyelocytic, HuT 78 T-cell lymphoma, Namalwa Burkitt’s lymphoma, or K-562 chronic myelogenous cells (Fig. 2b).

Guanine Nucleotide Exchange by XPLN in Vitro—We investigated XPLN function by measuring its ability to stimulate guanine nucleotide exchange on members of the Rho family in vitro (Fig. 3). For these analyses, we evaluated the activity of bacterially expressed full-length XPLN. An assay was used that measures the incorporation of mant-GTP into recombinant Rho proteins. Nucleotide loading was detected by an increase in fluorescent emission upon insertion of mant-GTP into the hydrophobic nucleotide-binding pocket of the Rho proteins (21). We found that XPLN stimulated guanine nucleotide exchange on RhoA and RhoB, but had no effect on RhoC, Rac1, Cdc42, or RhoG at the concentrations of XPLN tested (350 μM). The VAV2 catalytic domain promoted nucleotide loading of all GTPases tested, indicating that the Rho proteins were functional. Similar to VAV2, the exchange factor LARG exchanged RhoA, RhoB, and RhoC (see Table 1). Collectively, these data demonstrate that in vitro XPLN stimulates nucleotide exchange specifically on RhoA and RhoB.

Molecular Basis for the Differential Activation of RhoA, RhoB, and RhoC by XPLN—The RhoA subfamily of GTPases (RhoA, RhoB, and RhoC) share over 85% amino acid sequence identity, with RhoA and RhoC being the most closely related...
For this reason, it was surprising that only a subset of the RhoA subfamily were substrates of XPLN whereas other GEFs such as VAV2 and LARG activated all three of these proteins. We aligned the amino acid sequences of RhoA, RhoB, and RhoC to identify residues that might mediate this selective regulation. We found four amino acid positions that were identical in RhoA and RhoB, but different in RhoC (corresponding to residues 43, 178, 184, and 192 in RhoA). Significantly, recent studies by Snyder and colleagues (28) have shown that valine 43 in RhoA loses extensive solvent accessible surface area upon binding of the GEF Dbs, highlighting the role of this residue in the interaction with at least one exchange factor. The amino acid found at position 43 is a valine in RhoA and RhoB, but is a bulkier isoleucine residue in RhoC.

We hypothesized that position 43 in the RhoA subfamily is involved in determining the substrate specificity of XPLN. To test this idea, we performed in vitro nucleotide exchange assays with XPLN, LARG, and VAV2 using wild type and position 43 mutants of RhoA, RhoB, and RhoC (Table I). Valine 43 in RhoA and RhoB was replaced with an isoleucine, as is found in RhoC. Although a decrease in nucleotide incorporation by XPLN was observed for the V43I RhoA mutant, exchange by VAV2 and LARG was also reduced. The decline in fold-induction of exchange activity was largely because of an enhanced basal incorporation of nucleotide by this mutant relative to wild type

![Image of graphs showing nucleotide exchange](http://www.jbc.org/)

**Fig. 3.** XPLN stimulates guanine nucleotide exchange in vitro on RhoA and RhoB. In vitro exchange assays were performed by measuring the increase in fluorescence emitted with time upon incorporation of mant-GTP into the indicated Rho family proteins in the absence of GEFs (Buffer) or in the presence of XPLN or VAV2. The data shown are representative of at least four individual experiments.
G15A mutant of H-Ras that fails to stably associate with gua-
in RhoA and in the analogous position, 15, in Cdc42 and Rac1
small GTPases. Thus, we generated nucleotide-free mutants of
nism, GEFs bind preferentially to the nucleotide-free form of
ing is thought to stabilize the nucleotide-free or empty pocket
by other GEFs, such as LARG and VAV2.

Nucleotide exchange by XPLN, yet it is permissive for exchange
position 43 in RhoC plays a determining role in preventing
stimulated nucleotide incorporation into the I43V RhoC mu-
whereas wild type RhoC is not a XPLN substrate, this GEF
were more active against I43V RhoC than wild type RhoC.

GEF binds to nucleotide-free RhoA and RhoB and leads us to pos-
tulate that this GEF is specific for these two members of the
Rho family and then used to precipitate expressed exchange factors out of
cells transfected with pCMV-Myc-XPLN or pAX-HA-oncoVAV1. c. GEF or GST
fusion proteins of wild type RhoA, RhoB, or RhoC were stripped of
nucleotide then used to pull GEFs out of cells transfected with pCMV-
Myco-XPLN or pAX-HA-oncoVAV1. The pull downs and lysates were then processed for immunoblot
analysis with antibodies against the relevant epitope. The data shown
are representative of at least three individual experiments.

Table I
Effect of residue 43 mutations in the RhoA subfamily on exchange

| Condition | Linear velocity of exchange (units Em \(^{-460}\) s \(^{-1}\)) |
|-----------|--------------------------------------------------|
| Buffer    | GST-wild type RhoA: 0.21 ± 0.05 (S.D.) |
|           | GST-V43I RhoA: 0.62 ± 0.05 (S.D.) |
| Vav2      | GST-wild type RhoB: 1.86 ± 0.16 (8.9) |
|           | GST-V43I RhoB: 2.47 ± 0.14 (4.0) |
| LARG      | GST-wild type RhoC: 2.79 ± 0.18 (13.3) |
|           | GST-143V RhoC: 3.07 ± 0.12 (5.0) |
| XPLN      | GST-wild type RhoB: 1.78 ± 0.13 (8.5) |
|           | GST-V43I RhoB: 1.79 ± 0.24 (2.9) |

RhoA. Both LARG and VAV2 stimulate nucleotide exchange of
wild type and V43I RhoB proteins to similar extents. However,
the V43I mutation of RhoB strongly attenuated nucleotide
exchange by XPLN, indicating impairments in nucleotide binding. We ex-
amined the ability of GST fusion proteins of the nucleotide-free
Rho proteins stripped of nucleotide to specifically associate with GEFs transiently
expressed in NIH 3T3 cells. We found that GST-G17A RhoA
precipitated Myc-XPLN and Myc-DH/PH XPLN from cells lys-
es (Fig. 4a). In contrast, GST, GST-G15A Cdc42, and GST-
G15A Rac1 failed to pull down Myc-XPLN or Myc-DH/PH
XPLN (Fig. 4a). However, we detected specific interactions of
GST-G15A Cdc42 with GFP-DH/PH intersectin and GST-G15A
Rac1 with Myc-Tiam1 C1199 (Fig. 4a), findings that are con-
sistent with previous studies which demonstrated that intersectin and Tiam1 are specific for Cdc42 and Rac1, respectively
(27, 30).

Given that our in vitro exchange assay data showed that
XPLN is active toward RhoA and RhoB but not RhoC, we
analyzed the association of XPLN with RhoA subfamily mem-
bers. For these experiments, GST or GST fusion proteins of
wild type RhoA, RhoB, and RhoC were stripped of nucleotide
and then used to precipitate expressed exchange factors out of
cell lysates. We found that nucleotide-free GST-RhoA and GST-
RhoB precipitated substantially more Myc-XPLN out of lysates
did GST or nucleotide-free GST-RhoC (Fig. 4b). However,
comparable levels of HA-oncoVAV1 were pulled down by GST-
RhoA, GST-RhoB, and GST-RhoC (Fig. 4b). Collectively, our
GEF pull-down assays using nucleotide-free Rho mutants or
Rho proteins stripped of nucleotide demonstrate that XPLN
binds to nucleotide-free RhoA and RhoB and leads us to postu-
late that this GEF is specific for these two members of the
Rho family.

To further explore the interaction between RhoA and XPLN,
we examined the nucleotide dependence of the binding of this
GEF to different RhoA mutants. We employed GST fusion
proteins containing wild type RhoA (GDP-bound), G17A RhoA
(nucleotide free), or Q63L RhoA (GTP-bound) and tested their ability to bind to XPLN and other transiently expressed RhoA subfamily GEFs. Myc-XPLN was pulled down by GST-Q63L RhoA, in addition to GST-G17A RhoA, but not by GST or GST-wild type RhoA (Fig. 4c). Similarly, HA-oncoAVAV1 (Fig. 4c) and endogenous Lsc and Lfc (data not shown) were precipitated by both GST-G17A RhoA and GST-Q63L RhoA, but not by GST or GST-wild type RhoA. These data are consistent with previous reports which demonstrated that the GEFs p114RhoGEF (31) and PDZ-RhoGEF/KIAA0380 (32) associate with both GTPγS-bound RhoA and RhoA that has been stripped of nucleotide, but not by GDP-bound RhoA. We could not detect any binding between GTPγS-bound RhoC and XPLN (data not shown), in agreement with the lack of XPLN binding to nucleotide-free RhoC.

**Induction of Changes in Cell Morphology by XPLN**—The different members of the Rho family rearrange the architecture of the actin cytoskeleton into specific structures such as filopodia, lamellipodia, membrane ruffles, podosomes, and stress fibers. Rho proteins also regulate the assembly of two types of cellular proteins (rRhoA, rRhoB, and rRhoC, data not shown). However, focal complexes were resistant to disrupted stress fibers and focal adhesions in untransfected cells and cells expressing GFP, Myc-XPLN, or GFP-Q63L RhoA (data not shown). However, focal complexes were resistant to Y-27632 and were found around the circumference of both transfected and untransfected cells (Fig. 5b). These data provide evidence that in vivo XPLN activates members of the RhoA subfamily, and subsequently Rho kinase, to stimulate stress fiber and focal adhesion formation.

**Activation of RhoA Subfamily by XPLN in Vivo**—The changes in cell morphology that arose as a result of XPLN expression suggested that in vivo this exchange factor activates members of the RhoA subfamily. To determine whether this GEF activates all, or only a subset, of these proteins in vivo, we measured the activity of RhoA, RhoB, and RhoC in response to transient expression of XPLN. The activities of these proteins were determined by precipitating the active, GTP-bound Rho proteins from cell lysates with GST fusion proteins containing the RBD of the downstream effector Rhotekin (22). The GST-RBD pull downs (Fig. 6, a–d, left panels) and lysates (Fig. 6, a–d, center panels) were then immunoblotted with an antibody that recognizes all three members of the RhoA subfamily (pan-Rho) or with antibodies specific for RhoA (b), RhoB (c), or RhoC (d) as determined by immunoblots of recombinant RhoA subfamily proteins (rRhoA, rRhoB, and rRhoC, right panels). The data shown are representative of at least four individual experiments.
XPLN Activates RhoA and RhoB

Transforming Activity of XPLN—In addition to triggering changes in cell morphology, Rho family GTPases and Rho GEFs have been implicated in oncogenesis and cell transformation (2). Given that XPLN activates specific members of the RhoA subfamily, we examined the ability of this GEF to transform cells. NIH 3T3 mouse fibroblasts were transfected with expression vectors encoding Myc-XPLN, Myc-ΔN XPLN, Myc-ΔC XPLN, Myc-DH/PH XPLN, Myc-Q63L RhoA, or an empty vector. The cells were then cultured for 2 weeks, at which time the number of foci that had formed was scored (23). When expressed alone, Myc-XPLN and Myc-Q63L RhoA both had very weak transforming activities as judged by the number of foci formed (between 1 and 4 foci per plate in both cases, data not shown). On the other hand, when cotransfected with a transformation-cooperating mutant of Raf(Y340D) (17) the transforming potential by both proteins was strongly enhanced, as described before with both RhoA and other Rho GEFs (17). In this case, we could determine that XPLN had a transforming potential comparable with activated RhoA (31 ± 3 and 41 ± 4 foci per plate, respectively, Fig. 7). Parallel experiments were performed with truncated mutants of XPLN. Relative to the full-length GEF, NH₂-terminal (1 ± 1 foci per plate) and COOH-terminal (15 ± 3) truncated XPLN and the isolated DH/PH domain (2 ± 2) had reduced transforming activity (Fig. 7). Taken together, these data suggest that XPLN also at stable overexpression levels is a functional Rho GEF capable of rodent fibroblast transformation, thus, making it into a potential oncogene.

DISCUSSION

Understanding the specificity of Rho family GEFs is crucial for appreciating the biological roles of these proteins. In the present study, we have characterized the Dbl family Rho exchange factor XPLN. Our data indicate that XPLN protein expression is limited to the brain, skeletal muscle, heart, kidney, platelets, and macrophages. We show that in vitro and in vivo this GEF specifically associates with and activates the RhoA subfamily GTPases RhoA and RhoB, but not RhoC. Importantly, XPLN is the first described Rho regulatory protein that discriminates between members of the RhoA subfamily. In agreement with its regulation of RhoA and RhoB, XPLN stimulates the formation of stress fibers and focal adhesions in a Rho kinase-dependent manner. Like other Rho GEFs and activated mutants of Rho proteins (2), XPLN expression is sufficient to cause transformation of NIH 3T3 fibroblasts.

Given that the members of the RhoA subfamily share 85% overall sequence identity, with RhoA and RhoC being 92% identical, a surprising result of this study was that XPLN activates RhoA and RhoB but not RhoC. Based on amino acid sequence alignments, we focused on the amino acid at position 43 in the RhoA subfamily as a possible determinant in the differential activation of RhoA, RhoB, and RhoC. This position is an isoleucine in RhoC, but is occupied by a less bulky valine in RhoA and RhoB. In agreement with this position serving an important function in exchange by XPLN, a I43V mutation in RhoB and RhoA did not abolish XPLN-stimulated nucleotide exchange. However, the V43I mutation in RhoB and RhoC did not restore XPLN-induced nucleotide incorporation to the levels observed with wild type RhoA and RhoB, indicating that there are other residues in the Rho proteins and in XPLN that participate in substrate specificity.

One candidate residue in XPLN that may confer specificity is aspartic acid 257. The corresponding residues in the GEFs intersectin (leucine 1376), Tiam1 (isoleucine 1187), and Dbs (leucine 766) are thought to play an important role in governing the specificity of exchange (28). In LARG and VAV2, which possess exchange activity for RhoA, RhoB, and RhoC (this study), the positions analogous to aspartic acid 257 in XPLN are held by uncharged residues (prolines, leucines, isoleucines, or valines) in nearly all GEFs with activity toward RhoA, such as Lbc, Lfc,
Lsc, Trio, and Tim (28). The presence of a charged aspartic acid in this position may be incompatible for XPLN association and exchange on RhoC, but not RhoA or RhoB. Only one other characterized RhoA GEF, NET1, has an aspartic acid at the equivalent position (residue 255, see Fig. 1). In future studies, it will be interesting to determine whether NET1, like XPLN, stimulates GTP incorporation into RhoA and RhoB, but fails to exchange RhoC.

The members of the RhoA subfamily all stimulate the formation of stress fibres and focal adhesion and are assumed to couple to a similar set of downstream effectors. Of the members of the RhoA subfamily, RhoB appears to be the most divergent with respect to primary structure, subcellular localization, and function (34). RhoB predominantly localizes to intracellular membranes, whereas RhoA and RhoC are largely cytoplasmic and plasma membrane-bound (35, 36). Although all members of the RhoA subfamily are capable of transforming cells (2), RhoB can also inhibit tumor growth in some cases (12). With regards to RhoC, recent studies have suggested that it possesses some unique characteristics. Increased RhoC expression has been observed in metastatic melanoma cells and transformed mammary epithelial cells, and overexpression is sufficient for induction of metastasis (13–15). In agreement with these findings mark a distinction from RhoA, which antagonizes cell migration and invasion in tissue culture cells (13). These findings argue that RhoC, which antagonizes migration in several cell types (37–41). Sahai and Marshall (42) demonstrated that RhoC is also more effective than RhoA at disrupting adherens junctions, a hallmark of epithelial cell transformation, through Rho kinase-mediated contractility.

In the light of these described functional differences between the RhoA subfamily members, it is interesting that only a few differences in regulation and downstream signaling have been proposed. The only previously described difference is that RhoC is suggested to bind Rho kinase more efficiently than RhoA (42). With most RhoA subfamily regulators and effectors, however, the binding and effects on the three family members have not been studied. Our finding that XPLN activates RhoA and RhoB but not RhoC is interesting given the oncogenic and differential morphogenic properties of RhoC. It is plausible to think that there are cellular events where an activation of RhoA and/or RhoB is required but where the activation of RhoC could be disadvantageous. Furthermore, the relatively weak transforming activity of XPLN, compared with several other RhoA subfamily GEFs (17), might be because of lack of RhoC activation. Future studies will address this, and determine whether there are other RhoA subfamily GEFs with differential preferences among the three RhoA subfamily members.

The catalytic activity of many GEFs is negatively regulated by sequences NH₂-terminal or COOH-terminal to the DH domain (3). Consequently, truncation mutants of GEFs are often constitutively active. However, whereas NH₂- and COOH-terminal truncations in XPLN do not have any major effects on its ability to activate RhoA in transiently overexpressed conditions (data not shown), the truncations strongly reduce XPLN transforming activity in stable overexpression conditions. In contrast, truncations of NET1, the closest homolog of XPLN, have enhanced transforming activity (25). Our findings suggest that the intrinsic activity of XPLN is not negatively regulated by intramolecular interactions, but instead that XPLN is regulated by expression or by sequestration into specific subcellular compartments. Expression of the related protein NET1 has been found to be regulated at the level of gene transcription (43). Unlike NET1, XPLN/Arhgef3 is not regulated by nucleocytoplasmic shuttling (44). Clearly, elucidation of the mechanisms of XPLN regulation awaits further characterization. Our observation that XPLN is expressed in only a limited number of tissues and specialized cell types could serve to clarify the signaling pathways and biological functions mediated by this exchange factor. For example, XPLN protein was detected in a macrophage cell line and could possibly participate in RhoA-dependent phagocytosis by these cells (45). XPLN is also expressed in platelets. An essential role for RhoC has recently been described in the ability of platelet integrin αIIbβ3 to maintain adhesion under shear stress (46). We are currently examining the role of XPLN in regulating the integrity of cell-cell and cell-matrix adhesion in platelets and other cell types, as well as its regulation in vivo.

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