The yeast exchange assay, a new complementary method to screen for Dbl-like protein specificity: identification of a novel RhoA exchange factor

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Abstract The target Rho GTPases of many guanine nucleotide exchange factors (GEFs) of the Dbl family remain to be identified. Here we report a new method: the yeast exchange assay (YEA), a rapid qualitative test to perform a wide range screen for GEF specificity. In this assay based on the two-hybrid system, a wild type GTPase binds to its effector only after activation by a specific GEF. We validated the YEA by activating GTPases by previously reported GEFs. We further established that a novel GEF, GEF337, activates RhoA in the YEA. GEF337 promoted nucleotide exchange on RhoA in vitro and promoted F-actin stress fiber assembly in fibroblasts, characteristic of RhoA activation.

Key words: Rho GTPase; Exchange factor; Functional assay

1. Introduction

The Rho family of Ras-related GTPases comprises at least 15 distinct proteins that are key regulators of actin cytoskeleton dynamics also involved in a variety of cellular processes [1,2]. Most Rho GTPases function as molecular switches oscillating between a GDP-bound inactive and a GTP-bound active form [1]. The GTPase-activating proteins that stimulate its intrinsic nucleotide hydrolysis activity regulate inactivation of the GTPase. The release of GDP and binding of GTP are catalyzed by guanine nucleotide exchange factors (GEFs). A number of studies on Rho GTPase signaling have used constitutively active mutant GTPases. Nevertheless, recent studies have pointed out that the biological effects elicited by these non-cycling GTPases are not always consistent with those obtained by activation of endogenous GTPases with the corresponding exchange factor [3–5]. The identification of Rho GEFs and their target GTPases is an essential step in unraveling Rho-dependent signaling pathways.

The Dbl-like GEFs have very high transforming potential, as a consequence of the deregulated activation of Rho GTPases and their downstream signaling pathways [6]. GEFs also play an important role in the cross talk between the different Rho GTPase pathways [7–9] and in their connection with other signaling cascades [6]. Rho GEFs are characterized by the presence of a Dbl homology (DH) domain of 200 amino acids (aa) which harbors the guanine nucleotide exchange activity and in many cases a pleckstrin homology domain, implicated in intracellular membrane targeting of the protein [6]. The sequences of over 40 putative Dbl-related GEFs have been released in databases ranging from 400 to over 6500 aa long [10,11]. The specificity of about half of these GEFs was established. Some are specific of a single GTPase while others activate several ones [6]. Comparing the aa sequences of the DH domains of the GEFs studied so far did not lead to any obvious rule to deduce their GTPase specificity [6,12]. The specificity of a new exchange factor is usually established by in vitro guanine nucleotide exchange assays. One major limitation of this method is the purification of active exchange factors and GTPases, which in some instances cannot be achieved [13].

Here we present the YEA (yeast exchange assay), a new functional assay in yeast based on the two-hybrid interaction system, which allows a quick initial screen for the activity of Dbl-related GEFs on a wide range of Rho-related GTPases. With the YEA, we were able to reproduce the activation of Rac1, RhoG, Cdc42Hs and RhoA by Tiam-1 [14], Trio D1 [15], αPIX [16] and Trio D2 [17], respectively. We further studied GEF337, a putative Dbl-related exchange factor from the HUGE cDNA database [18]. In the YEA, we demonstrate that GEF337 activates RhoA. We confirmed this result by in vitro guanine nucleotide exchange assays. Finally, we establish that GEF337 induces the assembly of actin stress fibers when overexpressed in fibroblastic cells, demonstrating that it does activate RhoA in vivo.

2. Materials and methods

2.1. Plasmid construction

The LexA and GAL4 constructs have been described elsewhere [19]. pGEX-RhoA and pRK5-mycRhoA$_{V14}$ were gifts from A. Hall (London, UK) and pCEV-RhoG a gift from X. Bustelo (Stoney Brook, NY, USA). The catalytic regions of Trio (D1: aa 1232–1429 and D2: aa 1849–2450) [20], αPIX (aa 60–779) [16] and Tiam-C580 (aa 1011–1591) [14] were fused to the HA- (Trio D2) or myc- (others) tag and inserted into the yeast expression vector pRs426Met [21]. KIAA0337 is a cDNA from the HUGE database from the Kazusa sequencing project (accession number AB002335). The DH domain and full length open reading frame (respectively nucleotides 1802–3031 and nucleotides 534–6288) of KIAA0337 were fused to the myc-tag and inserted in pRs426Met or pRcCMV (Invitrogen). The DH domain was fused to GST in pGEX (Pharmacia).

2.2. Yeast techniques

Interactions were assayed in yeast strain TAT7 (Mata, trp1, his3,

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leu2, ura3, ade2, LYS::(LexAop)4-HIS3, URA3::(LexAop)8-lacZ), provided by J. Camonis (Paris, France). Filter assays were performed as described [19]. For histidine (HIS) auxotrophy assays, yeasts were spotted on the appropriate drop out (DO) plates lacking HIS and supplemented with the indicated amount of 3-aminotriazole (3-AT). Quantitative determinations of β-galactosidase activities were performed as described [15]. The basal β-galactosidase activity (A, nmol/min/mg) was calculated according to the formula $A = 20 \times 1.7\ OD_{405}/0.0045\ T$, where 1.7 corrects for the reaction volume and 20 for the amount of protein, 0.0045 is the optical density of a 1 nmol/ml solution of ONPG and T the time in minutes. Activation of β-galactosidase was then expressed as a fold increase of the basal activity. The yeast cdc24^{ts} strain 12.2 was obtained by mating TAT7 to YMP483, bearing the cdc24-5 ts (thermosensitive) mutation [22]. Diploids were selected, allowed to sporulate and individual spores were selected for the presence of both the lacZ reporter expression cassette and the cdc24^{ts} mutation. 12.2 strain was grown at 25°C and the β-galactosidase assays performed as in TAT7.

2.3. GDP release assay

GST fusion proteins were expressed in *Escherichia coli*, GTPases loaded with [^{3}H]GDP and exchange performed as described [15]. Briefly, 25 pmol of exchange factor and [^{3}H]GDP-loaded GTPase were mixed in 80 μl of reaction buffer (50 mM Tris pH 7.5, 1 mM GTP, 2 mM $MgCl_2$) and incubated at 20°C. The fraction of [^{3}H]GDP-bound GTPase was determined after 0, 1, 2, 5, 10 and 15 min of reaction.

2.4. Cell culture, transfection and immunofluorescence

Rat embryo fibroblasts (REF-52) were cultured, transfected and processed for immunofluorescence as described [15]. The myc-tagged proteins with 9E10 anti-myc monoclonal antibody followed by incubation in biotin-conjugated sheep anti-mouse IgG (1:200 dilution, Amersham) and then streptavidin FITC (1:200 dilution, Amersham).

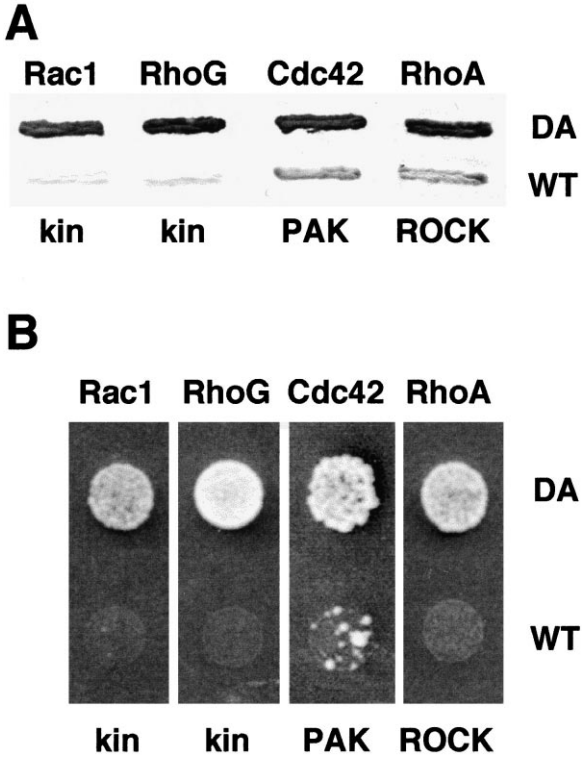


Fig. 1. Interaction of Rac1, RhoG, Cdc42Hs and RhoA GTPases with their effectors in the two-hybrid system. A: Filter assay for β-galactosidase activity in TAT7 yeasts expressing the constitutively active (DA) or wt (WT) GTPases fused to LexA DNA binding domain and their effector proteins fused GAD. B: The same yeasts were assayed for the transactivation of the HIS3 reporter gene by growth on DO plates lacking HIS and supplemented with 20 mM 3-AT in the case of Cdc42Hs.

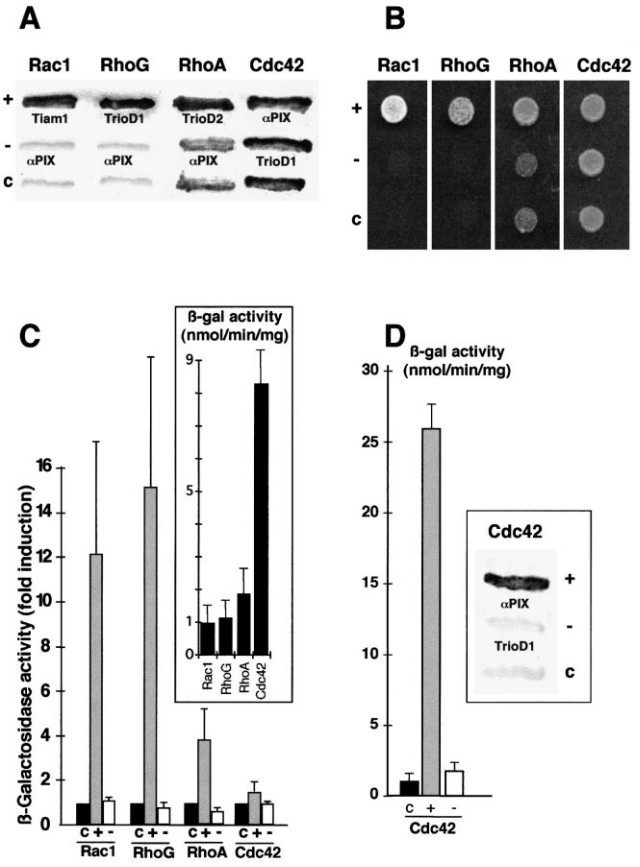


Fig. 2. Activation of the binding of wt GTPases to their effectors by expression of a positive exchange factor. A: β-Galactosidase activity in TAT7 yeasts expressing wt GTPases fused to LexA, their effectors fused to GAD and the indicated positive (+) or negative control (−) GEFs or the pRs vector alone (c). B: Transactivation of the HIS3 reporter gene by growth on DO plates lacking HIS supplemented with 5 mM 3-AT. C: The β-galactosidase activity was measured by ONPG liquid assay using 5 μg total protein extracts from yeasts expressing positive (+) or negative (−) GEFs, and expressed relative to the basal activity in yeasts expressing no exchange factor (c). Inset: basal β-galactosidase activity (nmol/min/mg) in 5 μg total protein extracts from yeast expressing no exchange factor (c). Reactions were performed for 3 h at 30°C. Mean and S.E.M. of three experiments are shown. D: ONPG liquid assay in cdc24^{ts} strain 12.1. Inset: β-galactosidase activity in 12.1 yeasts expressing wt Cdc42Hs, its effector PAK and αPIX positive (+) or Trio D1 negative control (−) GEFs or the pRs vector alone (c).

Actin was stained using rhodamine-conjugated phalloidin (0.5 U/ml, Sigma). Cells were observed under a DMR Leica microscope using a 63× planapochromat lens. Images were recorded using a micromax 1300Y/HS camera (Princeton Instruments Inc., USA) using the Metamorph acquisition software, transferred to Adobe Photoshop and printed on an Epson Color 750 printer. All transfections were repeated at least three times and at least 100 transfected cells were examined.

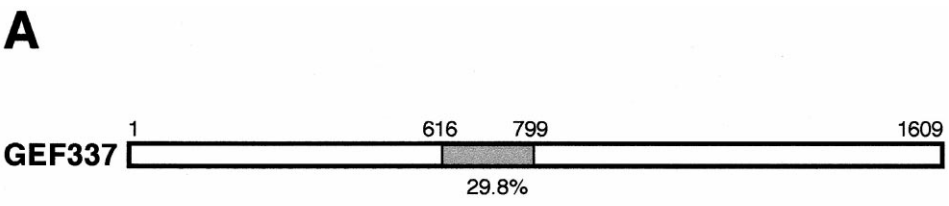
2.5. Bio-computing

Search for consensus protein domains in GEF337 protein sequence was performed with the Pfam program (http://pfam.wustl.edu/hmmsearch.shtml) [23] and DH domain of GEF337, ABR and BCR were aligned using MULTALIN [24].

3. Results

3.1. Establishment of YEA

We wanted to develop a functional assay in yeast, which



B

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          1                                                  50
      337 LNAKDAVRVA KEARPAFLKF LEQSMRENKE KQALSDLMIK PVQRIPRYEL
      abr ..VLSGFLAS EEIYINQLEA LLLPMKPLKA TATTSQPVLT .IQQIETIFY
      bcr ..VLSGILAS EETYLSHLEA LLLPMKPLKA AATTSQPVLT .SQQIETIFF
Consensus ..vlsg.las eE.y...Lea LllpMkplKa .attS#pvlt ..QqIetif.

          51                                                 100
      337 LVKDLLKHTP EDHPDHPLLL EAQRNIKQVA ERINKGVRSA EEAERHARVL
      abr KIQDIYEIHK EFYDNLCPKV QQWDSQVTMG HLFQK...LA SQLGVYKAFV
      bcr KVPELYEIHK EFYDGLFPRV QQWSHQQRVG DLFQK...LA SQLGVYRAFV
Consensus k!.#lyeihk Efyd.l.p.v #qw..q..vg .lf#K...lA s#lgvy.afv

          101                                                150
      337 QEIEAHIEGM EDLQAPLRRF LRQEMVIEVK AIGGKKDRSL FLFTDLIVCT
      abr DNYKVALETA EKCSQSNNQF QKISEELKVK GPKDSKDSHT SVTMEALLYK
      bcr DNYGVAMEMA EKCCQANAQF AEISENLRAR SNKDAKDPTT KNSLETLLYK
Consensus ##y.va.E.a Ekc.q.n.qF ..ise.l.vk ..kd.KD..t ....#.llyk

          151                                            195
      337 TLKRKSGS.L RRSSMSLYTA ASVIDTASKY KMLWK..... .....
      abr PIDRVTRSTL VLHDLLKHTP VDHPDYPLLQ DALRISQNFL SSINE
      bcr PVDRVTRSTL VLHDLLKHTP ASHPDHPLLQ DALRISQNFL SSINE
Consensus p.dRvtrStL vlhd$lkhTp ashpD.pllq daLrisqnfl ssine
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Fig. 3. A novel putative exchange factor from the Dbl family. A: Localization of the DH (gray box) domain in GEF337 as identified by the Pfam profile scanning server (St. Louis). Percent refers to the identity to the DH domain of Dbl. Further information on KIAA0337 is available at http://www.kazusa.or.jp. B: Alignment of the DH domains of GEF337, ABR and BCR. Sequences aligned using the MULTALIN program. !: I or V, $: L or M, %: F or Y and #: N, D, Q, E, B or Z.

would provide a quick test to screen for the specificity of newly identified GEFs. In the classical two-hybrid protein interaction assay, a dominant active (DA) mutant Rho GTPase fused to the LexA DNA binding domain (LexA) can interact with its effector fused to GAL4-activating domain (GAD). This interaction leads to the assembly of a transcriptionally active complex driving the expression of lacZ and HIS3 reporter genes. In the same assay, the wild type (wt) GTPase does not bind to its effector. In yeasts expressing a wt GTPase and its effector, we further introduced a third plasmid expressing the catalytic DH domain of an exchange factor. We postulated that a specific exchange factor should activate its target GTPase and allow its binding to its effector thereby leading to the transactivation of the reporter genes.

When we expressed LexA-Rac1$_{G12V}$, RhoG$_{G12V}$, Cdc42Hs$_{G12V}$ and RhoA$_{G14V}$ in TAT7 yeast strain, these DA mutant GTPases bound to their respective GAD-fused targets (Fig. 1): kinectin as a target of Rac1 and RhoG, PAK1 for Cdc42Hs and Rock for RhoA [19]. In the case of Rac1 and RhoG, the wt GTPases showed no binding to kinectin (Fig. 1) while wt RhoA, and to a larger extent wt Cdc42Hs, exhibited a higher basal binding activity to their effectors. Nevertheless, the DA RhoA$_{G14V}$ and Cdc42Hs$_{G12V}$ bound more efficiently to their effectors than the wt GTPases (Fig. 1).

We further transformed yeasts expressing the wt GTPases and their respective effectors with the previously characterized positive exchange factors: Tiam-1 for Rac1 [14], Trio D1 for RhoG [15], αPIX for Cdc42Hs [16] and Trio D2 for RhoA [17]. The catalytic domains of these proteins were expressed as myc-tagged (Tiam-1, Trio D1 and αPIX) or HA-tagged (Trio D2) proteins and the transactivation of HIS3 and lacZ reporter genes was assayed.

In the case of Rac1, RhoG and RhoA, we did observe increased β-galactosidase activity in filter assays and growth on HIS selection medium (Fig. 2A,B) in yeasts co-expressing a positive exchange factor (+ lanes): Rac1 and RhoG bound kinectin and RhoA bound Rock, upon Tiam-1, Trio-D1 and Trio D2 expression, respectively. Thus the wt GTPases Rac1, RhoG and RhoA were activated and now able to bind their effector proteins. In the case of Rac1 and RhoG, no activity was observed in the absence of an exchange factor (c lanes), or when a negative control exchange factor (αPIX) was expressed (− lanes). In the case of RhoA, the basal binding of the wt GTPase to Rock was higher than in the two-hybrid experiments (Fig. 1), due to the fact that the GTPase activation experiments needed to be run longer to obtain positive β-galactosidase activities and growth on HIS medium similar to the two-hybrid interaction assays. We then measured the increase in β-galactosidase activities after expression of a pos-

itive GEF using a quantitative colorimetric assay and confirmed the above results: the expression of a positive exchange factor markedly increased the basal β-galactosidase activity, while a negative control GEF had no effect (Fig. 2C).

In the case of Cdc42Hs, the basal β-galactosidase activity was very high as compared to the other GTPases, therefore we did not observe any clear increase in the binding of Cdc42Hs to PAK upon expression of the positive GEF αPIX (Fig. 2A–C). As the high basal β-galactosidase activity we observed in this assay was most likely due to the activation of Cdc42Hs by yeast Cdc24p [25], we performed the YEA on Cdc42Hs in the yeast strain 12.2 bearing a $cdc24^{ts}$ mutation. In this strain, we did observe β-galactosidase activity induction in filter assays (Fig. 2D, inset) in yeasts co-expressing the positive exchange factor αPIX (+ lane), while no activity was observed in the absence of an exchange factor (c lane), or when a negative control exchange factor (Trio D1) was expressed (− lane), which was confirmed by the quantitative colorimetric assay (Fig. 2D).

This new functional assay termed as YEA thus appears as a convenient qualitative test suitable to screen for the specificity of different Dbl-related exchange factors towards various Rho GTPases. We then further used the YEA method to assay for an exchange factor of unknown specificity.

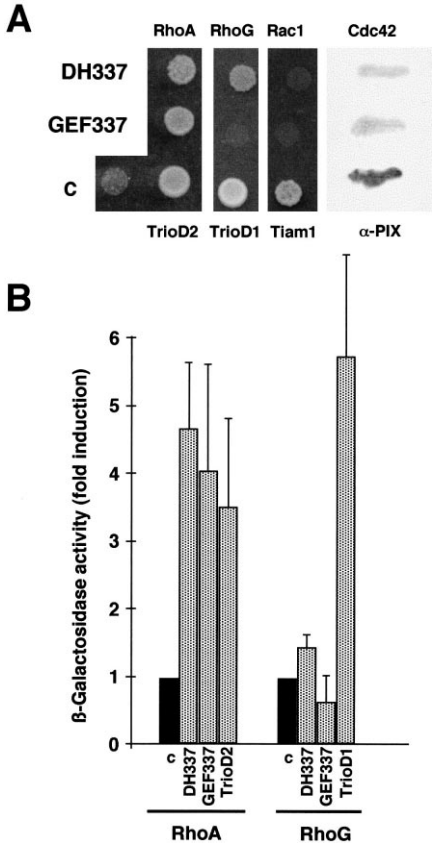

Fig. 4. Activation of wt GTPases upon expression of GEF337. A: The wt GTPase, their effectors and the DH domains (DH) or full length (GEF) GEF337 were expressed in TAT7 or 12.1 (Cdc42Hs). The transactivation of the HIS3 (TAT7) and of the β-galactosidase (12.1) reporter genes was assayed as in Fig. 2. B: The β-galactosidase activities determined with an ONPG liquid assay as in Fig. 2. Mean and S.E.M. of three experiments are shown.

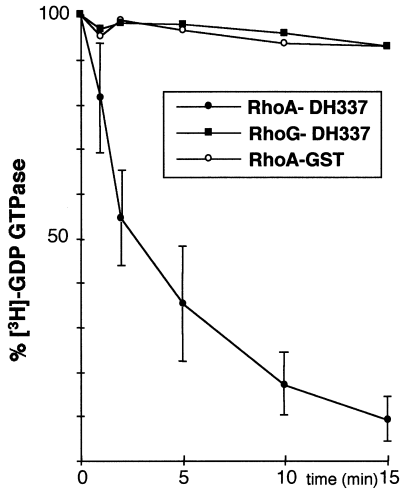

Fig. 5. GEF337 specifically promotes GDP/GTP exchange on RhoA. [^{3}H]GDP-loaded GST-fused RhoA and RhoG (300 nM) were incubated in the presence of GST-fused DH337 (300 nM) or GST (1 mM) and cold GTP (1 mM). Mean and S.E.M. from three experiments are shown.

3.2. GEF337 behaves as RhoA exchange factors in the yeast functional assay

KIAA0337 is a human cDNA from the HUGE cDNA database [18] that encodes a Dbl-like protein referred to as GEF337 (Fig. 3A). The DH domain of GEF337 is closer to that of Bcr (29 and 35% identity, respectively) and Abr (28 and 30%) (Fig. 3B), both reported to act as GEFs for RhoA, Rac1 and Cdc42Hs in vitro [26].

We used the YEA method to determine the GTPase specificity of this novel exchange factor. The DH domain (DH337) and full length GEF337 were fused to the myc-tag and transformed in yeasts TAT7 already expressing wt RhoA, RhoG or Rac1 and their respective effectors, as well as in 12.2 yeasts expressing wt Cdc42Hs and PAK. DH337 efficiently activated RhoA and to a lesser extent RhoG, but did not activate Rac1 or Cdc42Hs (Fig. 4A). The increase in β-galactosidase activity was over 4-fold in the case of RhoA while it was only 1.5-fold for RhoG (Fig. 4B). The expression of the full length GEF337 in yeast did not activate RhoG any longer, while maintaining the same level of activity on RhoA as DH337 (Fig. 4A,B). The use of the YEA method therefore suggests that GEF337 is an exchange factor specific for RhoA.

3.3. KIAA0337 can catalyze GDP exchange on RhoA in vitro

To confirm the results obtained in yeast, we performed in vitro exchange assays using purified proteins. DH337, RhoA and RhoG GST fusions were affinity-purified from bacteria. The GTPases were loaded with [^{3}H]GDP and incubated with an equimolar amount (300 nM) of DH337. The fraction of GDP-bound GTPase was determined after 0, 1, 5, 10 and 15 min of reaction. After 15 min in the presence of DH337, RhoA had released more than 90% of its bound [^{3}H]GDP (Fig. 5, black circles), while RhoG was still stably bound to GDP (Fig. 5, black squares). In similar experimental conditions, we obtained as much as 90% guanine nucleotide exchange on RhoG, using Trio D1 as an exchange factor (not shown, see [15]). As a control for the stability of the GDP-loaded RhoA GTPase, GST was used instead of the exchange

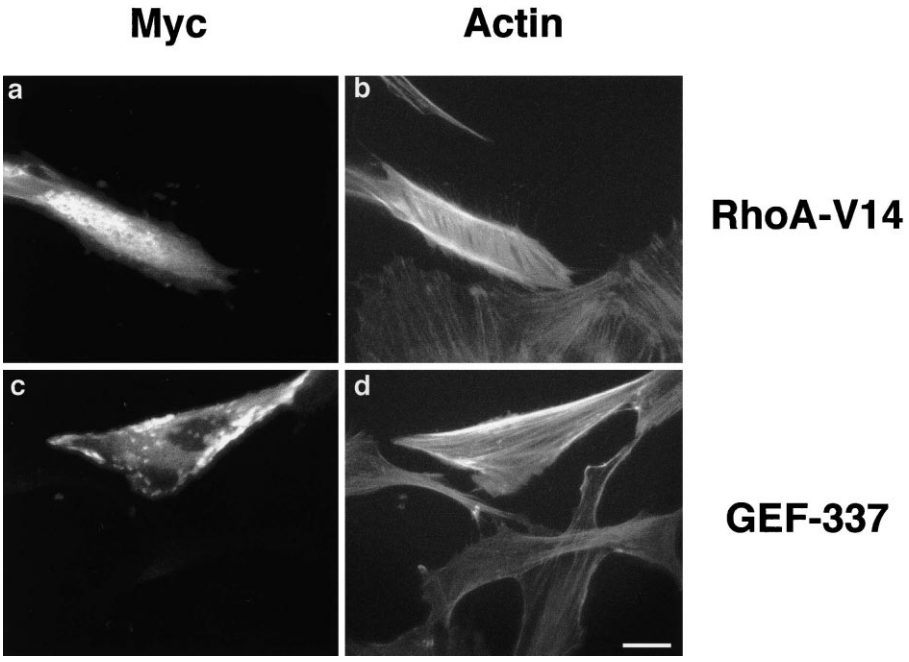

Fig. 6. GEF337 induces stress fiber assembly in fibroblasts. REF-52 cells were transfected with constructs expressing myc-tagged GEF337 or $RhoA_{G14V}$: myc epitope expression (a and c) and F-actin distribution (b and d). Bar: 10 μm. For each panel, cells shown are representative of more than 100 observed cells from at least three transfection experiments.

factor. In this case, RhoA remained stably bound to [^{3}H]GDP (Fig. 5, open circles).

As expected from the YEA results, DH337 efficiently promoted guanine nucleotide exchange on RhoA, but was not active on RhoG in vitro. To confirm the activity of GEF337 on RhoA, we further studied this putative RhoA activator in cultured cells.

3.4. GEF337 induces actin stress fiber assembly in fibroblasts

Activation of RhoA triggers actin stress fiber assembly in cultured fibroblastic cells [27]. To examine whether GEF337 could act as a RhoA exchange factor in vivo, we expressed this protein as myc-tagged fusion in exponentially growing rat embryo fibroblasts (REF-52). The resulting modifications in the actin containing structures were then examined with rhodamine-labeled phalloidin 24 h after transfection.

When myc-tagged full length GEF337 was expressed in REF-52 fibroblasts, 60–80% of the transfected cells exhibited a contracted morphology associated with an increase in actin stress fiber content (Fig. 6c,d) similar to what was observed upon $RhoA_{G14V}$ expression (Fig. 6a,b). They did not induce filopodia or microvilli formation, characteristic of Cdc42Hs and RhoG activation or the apparition of lamellipodia or ruffles, characteristic of Rac1 and RhoG activation [19]. In REF-52 cells, expression of GEF337 thus induced RhoA but not Cdc42Hs, Rac1 or RhoG activation. Similar results were obtained in Swiss 3T3 mouse fibroblastic cells (not shown). Combined with the results from YEA and in vitro exchange assays, these data confirm that GEF337 acts as a specific in vivo RhoA exchange factor.

4. Discussion

We report in this study a new functional assay termed YEA which provides a rapid test to perform wide range screens for GEFs specificity towards many GTPases. This assay, based on the classical two-hybrid system, has been designed to detect the activation of a wt GTPase by an exchange factor. The activation of the GTPase is revealed through its interaction with its effector, as in a classical two-hybrid system. The YEA proved successful to confirm the specificity of various GEFs previously established under different experimental conditions. We further studied a novel putative Dbl-related exchange factor: GEF337. We could demonstrate that GEF337 specifically activates RhoA using the YEA. Consistently, it catalyzes guanine nucleotide exchange on RhoA in vitro and when overexpressed in fibroblasts, GEF337 induced the assembly of actin stress fibers, characteristic of RhoA activation.

Dbl-related GEFs are key regulators of Rho-related GTPase signaling pathways, involved in many biological processes from oncogenic transformation to neuronal development [6]. Several complementary experimental approaches have been used to study the catalytic activity of exchange factors. In vitro guanine nucleotide exchange assays using purified proteins allow a biochemical analysis of the exchange reaction. In vivo, the ectopic expression of an exchange factor induces the activation of endogenous GTPases. The activation can be monitored by pulling down the activated GTPases using their effectors as a bait [28] or through the biological effects elicited by these GTPases, such as actin cytoskeleton reorganization or kinase activation. Nevertheless, both types of approaches suffer several limitations. In vitro exchange assays require prior purification of active proteins. This step proved critical for GTPases and GEFs, many of which have been reported to be poorly functional proteins, because of insolubility and non-proper folding [13]. Besides, although in vivo analysis of GEF activity might overcome this problem, final interpretation might be misleading due to cross talk between different Rho GTPases: for instance Cdc42Hs activates Rac1 [29] and RhoG activates Rac1 and Cdc42Hs [19], which

makes it difficult to readily establish GEF specificity in vivo. Finally, both methods are tedious to study a large number of GEFs and GTPases. As a consequence, most GEF specificities have been mainly assayed on Rac1, RhoA and Cdc42Hs, while the other Rho GTPases have never or scarcely been tested [1,6]. The YEA method we describe here provides a complementary approach based on cDNA cloning which allows large scale studies. Furthermore, this method is particularly suitable for site-directed mutagenesis analyses to identify residues critical for the activity of a GEF towards a given GTPase. Finally, the YEA can easily be adapted to assay for the activation of the other GTPases from the Ras superfamily.

The design of the YEA was motivated by many earlier studies where the two-hybrid system was successfully used to study the interaction of active mutant GTPases with their effectors. We validated the YEA using exchange factors of known specificity. A limitation of the YEA is the basal level of activation of some GTPases, probably due to endogenous yeast exchange factors. The mammalian GTPase RhoA is possibly activated by the yeast exchange factors Rom1p and Rom2p, which activate Rho1p, a yeast GTPase that is 70% similar to RhoA. Rom2p has indeed been shown to activate RhoA in vitro [30]. Notwithstanding the basal RhoA activity, ectopic expression of positive exchange factors led to a readily detected increase in RhoA activation. In the case of Cdc42Hs, the high basal activity due to the yeast exchange factor for Cdc42p [25] was suppressed by working in a $cdc24^{ts}$ strain, which allowed the detection of Cdc42Hs activation by exogenous GEFs.

Using the YEA, we found that the novel Rho exchange factor GEF337 displayed exchange specificity for RhoA. GEF337 can directly catalyze guanine nucleotide exchange in vitro and when expressed in REF-52 and Swiss 3T3 fibroblastic cells GEF337 indeed triggered actin stress fiber assembly, a characteristic of RhoA activation. The catalytic domain DH337, but not the full length GEF337, slightly activated RhoG in the YEA, which was not confirmed in vitro and in vivo. This suggests that the YEA might be in some instances either less stringent or more sensitive than the other methods. For instance, Trio D1 efficiently activates both RhoG and Rac1 in the YEA (our unpublished data), while it is 10-fold more active on RhoG than on Rac1 in vitro and specifically activates RhoG in vivo [15]. The above observations clearly stress out the necessity of using complementary approaches to investigate the catalytic specificity of any novel exchange factors.

The regulation of the different Rho pathways in vivo is still poorly understood. However, their cross talk must be finely regulated to ensure a correct control of cell morphology, adhesion and movement [31]. GEFs most likely play a crucial role in coordinating spatial and temporal activation of GTPase signaling cascades. The new challenge in Rho GTPase study is now to identify their cellular and physiological functions. This will require the ability to activate the endogenous proteins, rather than using DA mutants, which may not mimic the fine regulation of a cycling GTPase. A crucial step therefore resides in the study of Dbl-related exchange factors, the upstream regulators of Rho-related GTPase. They exhibit high tissue specificity and possess multiple regulatory domains, which allow a tight regulation of GTPase activation both in time and space as well as the cross talk of Rho pathways with many other signaling cascades.

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