Biochemical Clustering of Monomeric GTPases of the Ras Superfamily


To date phylogeny has been used to compare entire families of proteins based on their nucleotide or amino acid sequence. Here we developed a novel analytical platform allowing a systematic comparison of protein families based on their biochemical properties. This approach was validated on the Rho subfamily of GTPases. We used two high throughput methods, referred to as AlphaScreen™ and FlashPlate®, to measure nucleotide binding capacity, exchange, and hydrolysis activities of small monomeric GTPases. These two technologies have the characteristics to be very sensitive and to allow homogenous and high throughput assays. To analyze and integrate the data obtained, we developed an algorithm that allows the classification of GTPases according to their enzymatic activities. Integration and hierarchical clustering of these results revealed unexpected features of the small Rho GTPases when compared with primary sequence-based trees. Hence we propose a novel phylobicbiochemical classification of the Ras superfamily of GTPases. Molecular & Cellular Proteomics 4:936–944, 2005.

Ras GTPases are small GTP-binding proteins involved in diverse cellular processes such as apoptosis, cell proliferation/differentiation, cytoskeleton reorganization, and membrane trafficking. These proteins cycle between an inactive GDP-bound form and an active GTP-bound form. Under their GTP-bound form their active conformation allows them to interact with effector molecules and to generate specific biological responses (1). Two intrinsic enzymatic activities regulate the balance between GTP-bound and GDP-bound conformations: 1) GDP/GTP exchange and 2) GTP hydrolysis activities (2). Whereas the intrinsic enzymatic activities have been characterized for a few of these proteins, such studies were carried out in non-systematic manners and by different research groups (3–6).

The analysis of full genome sequences has allowed large scale biology experimental approaches consisting of systematic characterization of entire families of proteins instead of investigating them individually. Developing such “functional proteomic” approaches requires the design of high throughput and versatile experimental procedures (7, 8). For the study of small G proteins, conventional filtration assays, although powerful at small scale, are not appropriate for systematic studies. Indeed this methodology has a low to very low throughput, is not versatile, and requires large amounts of reagents. To overcome these issues and characterize GTP-binding proteins of the Ras superfamily in a systematic manner, we developed a robust assay platform using two well adopted high throughput technologies, AlphaScreen™ (9) and FlashPlate® (10).

AlphaScreen is a bead-based non-radioactive and homogeneous proximity assay used to measure interaction between biological binding partners. The principle of this technology relies on the use of a Donor bead and an Acceptor bead that generate a light signal when brought into proximity (<200 nm). Upon laser excitation at 680 nm, the Donor beads, containing a photosensitizer, will generate short lived singlet oxygen that can diffuse only a short distance before returning to the ground state. The Acceptor beads, containing chemiluminescers and fluorophores, will react with this singlet oxygen and will emit an amplified light signal measurable at ~600 nm. AlphaScreen (PerkinElmer) provides highly versatile, sensitive, and homogeneous assays that allow us to perform studies at a higher throughput and at a lower cost.

FlashPlates are white polystyrene microplates in which the interior of wells is coated with a thin layer of polystyrene-based scintillation reagent. The principle of this homogeneous radiometric technology is based on proximity between a radioligand and the scintillation reagent. A target protein, such as antibodies or GSH, is also labeled on the wall of the plate and will bring the protein of interest close to the scintillation reagent. The interaction between the radioisotopes and the proteins of interest will activate the scintillation reagent and emit a luminescent signal. FlashPlate (PerkinElmer) technology reduces considerably the amount of reagent to use and eliminates the time-consuming washes normally needed to separate bound from unbound radioligands.
In this study we took advantage of these two technologies to characterize in a systematic manner four biochemical properties of the Ras family of small G proteins and we reclassified them according to their activities instead of their primary amino acid sequences. Due to their high level of interspecies conservation, we selected the Rho GTPases as prototype proteins to develop and validate our assays.

**EXPERIMENTAL PROCEDURES**

**Materials**

[\(\gamma^{35}\text{S}]\text{GTP} \gamma \text{S} \text{ and } [\gamma^{32}\text{P}]\text{GTP}] \text{ were purchased from PerkinElmer Life Sciences. ATP, GDP, GTP, and GTP} \gamma S \text{were purchased form Sigma. Goat anti-GST antibodies were from Amersham Biosciences.}

**Plasmids and Constructs**

*Caenorhabditis elegans* CDC-42 (RO7G3.1), RAC-2 (KO3D3.10b), CED-10 (C09G12.8b), MIG-2 (C35C5.4), and RHO-1 (YS1H4A.3) plus one putative uncharacterized GTPase, CRP-1 (Y514A3.3) were obtained from the *C. elegans* ORFeome (11). The mRAC-1 and mRAC-1 \text{N}17 were described previously (12). The above ORFs were recombined in a bacterial expression vector (pGEX-2TK) to produce N-terminally tagged GST fusion proteins using the Gateway technology (Invitrogen).

**Nucleotide Biotinylation**

GTP\gamma S and GDP were biotinylated, respectively, using biotin-PEG-maleinate (Pierce) dissolved in MES, pH 6, and biotin-PEG-COO-NHS (Pierce) dissolved in carbonate buffer, pH 8.5, according to the manufacturer’s instructions. For both nucleotides, reaction mixtures were incubated for 2 h at 37 °C and purified using HPLC. Concentrations were measured by optical density at 260 nm. Biotinylated nucleotides were validated for their biological properties toward GTPases (data not shown).

**Expression and Purification of Recombinant Proteins**

Bacterial recombinant GST-GTPases were expressed and purified as described previously (13).

**AlphaScreen Assays**

AlphaScreen assays were performed in Costar 384-well microplates in a final reaction volume of 25 \(\mu\)l Streptavidin Donor beads and protein G Acceptor beads (PerkinElmer BioSignal) were used at a final concentration of 0.02 mg/ml per well. The assays were performed in AlphaScreen buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, and 1 mg/ml BSA). All incubations were performed at 23 °C. Laser excitations were carried out at 680 nm, and readings were performed at 520–620 nm using an AlphaQuest reader (Packard).

**Competition Assays**—During purification, GST-GTPases were unloaded from their intrinsic nucleotide by adding 5 mM EDTA in lysate before purification. Different concentrations of unlabeled GDP (ranging from 0.4 nM to 3 \(\mu\)M) were then added to microplate wells with different concentration of unlabeled GDP (ranging from 0.4 nM to 3 \(\mu\)M). The plate was incubated for 20 min and 25 \(\mu\)l goat anti-GST (Amersham Biosciences) was added and incubated for 20 min. Donor and Acceptor beads were added simultaneously and incubated for 1 h before reading.

**Exchange Activity**—Purified GST-GTPases were preloaded with a biotinylated derivative of GDP (b-GDP) as follows. 150 \(\mu\)M proteins were incubated with 100 \(\mu\)M b-GDP in loading buffer (25 mM Tris, pH 7.4, 100 mM NaCl, 0.1 mM DTT, and 5 mM EDTA) for 30 min at 30 °C. Reactions were then placed on ice, and 10 \(\mu\)M MgCl\(_2\) was added. 5 \(\mu\)l of preloaded GST-GTPases (final concentration, 30 \(\mu\)M) were then added in microplate wells with different concentration of unlabeled GDP (ranging from 0.4 nM to 3 \(\mu\)M). The plate was incubated for 20 min, and 25 \(\mu\)l goat anti-GST (Amersham Biosciences) was added. After 20 min of incubation, Donor and Acceptor beads were added simultaneously, and the plate was analyzed after 1 h of incubation at 23 °C.

**FlashPlate Assays**

These assays were performed in glutathione-coated 96-wells FlashPlates (PerkinElmer). Bacterial lysates containing GST-GTPases were diluted with PBS (50 \(\mu\)l) and incubated in the FlashPlate for 45 min at 4 °C in the presence of 5 \(\mu\)M EDTA to allow the binding of GST-GTPases on the GSH plate to and to unblock the protein from its intrinsic nucleotide.

**Binding Assay**—The lysate was removed, and the wells were washed twice with loading buffer (20 mM Tris, pH 7.4, 100 mM NaCl, and 0.1 mM DTT). Then 50 \(\mu\)l of loading buffer containing different concentrations of [\(\gamma^{35}\text{S}]\text{GTP} \gamma \text{S} (20–1000 \text{nCi}) were added. The signal produced was monitored on a \(\beta\) scintillation counter (Packard) every 5 min until the plateau was reached. The plate was placed back on ice, and 10 \(\mu\)M MgCl\(_2\) was added. The radioactive reaction mixture was then removed from the plate, and 50 \(\mu\)l of stopping buffer (20 mM Tris, pH 7.4, 0.1 mM DTT, 5 mM MgCl\(_2\), 1 mg/ml BSA, and 100 \(\mu\)M GTP\gamma S) were added. Signal was monitored on a \(\beta\) scintillation counter (Packard) every 5 min for another 30 min to measure nucleotide dissociation.

**Hydrolysis Activity**—Bacterial lysate containing EDTA was replaced by 50 \(\mu\)l of loading buffer (20 mM Tris, pH 7.4, 100 mM NaCl, and 0.1 mM DTT). Then either 500 nCi of [\(\gamma^{32}\text{P}]\text{GTP} \gamma \text{S} or 500 nCi of [\(\gamma^{32}\text{P}]\text{GTP} \gamma \text{S} were added. The interaction between GTPases and these radionucleotides was carried out for 30 min after which 10 \(\mu\)M MgCl\(_2\) was added. The radioactive reaction mixture was then replaced by 50 \(\mu\)l of hydrolysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, 0.01% BSA, and 10 \(\mu\)M GTP\gamma S), and the radioactivity was monitored for another 60 min using a \(\beta\) scintillation counter (Packard).

**Data Clustering, Tree Representation, and Comparison**

* C. elegans GTPase amino acid sequences were aligned using ClustalW (Blosum30 [Henikoff] matrix). Matrices were computed from the alignment using PHYLIP ProtDist according to the Jones-Taylor-Thornton model. The hierarchical clustering tree was then computed using the PHYLIP Neighbor command based on the UPGMA method and visualized using TreeView. Biochemical values obtained for each GTPase were normalized (between 0 and 1) and used to generate distance matrices based on activity(ies) similarities (alone or in combination). These matrices were calculated based on Euclidian distances, and hierarchical clustering trees were then computed using the PHYLIP Neighbor command based on the UPGMA method and visualized using TreeView. Tree comparison was carried out using PHYLIP Treedist.

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1 The abbreviations used are: GTP\gamma S, guanosine 5’-3-O-(thio)triphosphate; b-GDP, biotinylated GDP; SB, sequence-based; b-GTP\gamma S, biotinylated GTP\gamma S; PEG, polyethylene glycol; NHS, N-hydroxysuccinimide; UPGMA, unweighted pair-group method arithmetic averaged; mRAC-1, mouse RAC-1.

2 See [www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/) EMBL-EBI, ClustalW submission form.
RESULTS

Rho GTPases Identified in the C. elegans Genome—The ORFeome projects, which consist of the individual cloning of nearly all protein-encoding ORFs from the ATG to the stop codon predicted from genome sequences, are the cornerstone of systems biology approaches. The C. elegans ORFeome project is one of the most advanced and had reached more than 84% completion in 2003 (11). In C. elegans, six genes have been predicted to encode potential Rho GTPases, and the corresponding amino acid sequences were aligned and compared with the mouse RAC-1 protein sequence as shown in Fig. 1A. The corresponding ORFs were retrieved from the C. elegans ORFeome (14, 15) and expressed as N-terminally tagged GST fusion proteins in Escherichia coli (DH5α). The GST fusion proteins were expressed at high levels and purified to homogeneity (Fig. 1B). Using these small G proteins, we developed four distinct assays to measure their affinity for guanine nucleotides as well as their exchange and hydrolysis activities.

Small G Protein Affinity for Nucleotides—The first two assays, developed using both FlashPlates and AlphaScreen, aimed at measuring the affinity of small G proteins for GTP and other nucleotides. GSH-coated FlashPlates were used to perform saturation binding assays where increasing concentrations of non-hydrolyzable [γ-35S]GTPγS were incubated in the presence of a constant amount of nucleotide-free GST-GTPase. GST alone was also used as a negative control (see “Experimental Procedures”; Fig. 2A). When [γ-35S]GTPγS interacts with the proteins associated with the plate, β particles emitted from 35S activate the scintillation reagent embedded in the walls of the microplate. A luminescent signal is then emitted and measured. As shown in Fig. 2B, the signal intensity increased proportionally to the radionucleotide concentration and reached a plateau. An apparent $K_d$ ($K_{d(app)}$) corre-
sponding to the concentration of $\gamma^{35S}$GTPγS necessary to reach 50% of the maximum signal was measured (Fig. 2B) in a prototypical experiment using CDC-42 ($K_{d(app)} = 7.2$ nM). $K_{d(app)}$ values obtained with the six C. elegans putative Rho GTPases (CDC-42, RAC-1, CED-10, RHO-1, MIG-2, and CRP-1) were then compared together to set a rank order of GTPase GTP binding potency. In addition, the well characterized mRAC-1 (12, 16) and the GTP binding-deficient mutant mRAC-1N17 were used as controls. Our results show that these proteins clustered in two groups according to their affinity toward $\gamma^{35S}$GTPγS: on one hand CDC-42, RAC-2, CED-10, RHO-1, and mRAC-1 showed a high $K_{d(app)}$ binding with $K_{d(app)}$ values ranging from 2.8 to 7.4 nM, and on the other hand MIG-2, CRP-1, and mRAC-1N17 showed a low affinity for $\gamma^{35S}$GTPγS with $K_{d(app)}$ ranging from 11.7 to 14.5 nM (Fig. 2C). The results obtained with mRAC-1N17 were consistent with previous reports showing that this mutant displays low GTP affinity (17).

Our next objective was to develop competition assays to determine specific nucleotide affinities. These assays were carried out using both AlphaScreen and FlashPlate. Both platforms gave comparable results for CDC-42 affinities for GTP or GDP (Supplemental Fig. 1). To compare the six potential C. elegans Rho GTPases, we used AlphaScreen because of its higher throughput potential and its non-radioactive applications. In this assay, b-GTPγS was produced and used as a tracer binding to GTP-unloaded GST-GTPases as described under “Experimental Procedures.” In our system, the binding of b-GTPγS to GST-GTPases brings anti-GST Acceptor beads into proximity of streptavidin-coated Donor beads thus allowing the generation of an AlphaScreen signal (Fig. 3A). Competition isotherms are generated by adding...
GTPases were incubated with a constant concentration of b-GTP and increasing concentrations of unlabeled competitors. The streptavidin Donor bead ([A]) recognizes b-GTP and the protein G Acceptor bead ([D]) binds to the GST-GTPase via an anti-GST antibody. When the b-GTP binds to the GST-GTPase, a light signal is detectable upon laser excitation of the Donor bead. The concentration of competitor required for the loss of 50% of the maximum signal (obtained in the absence of competitor) corresponds to the competitor concentration required for the loss of 50% of the maximum signal (obtained in the absence of competitor). The IC50 values correspond to the competitor concentration required for the loss of 50% of the maximum signal (obtained in the absence of competitor). When the IC50 values were compared together, Rho GTPases clustered in the same two groups previously reported with saturation binding studies using the FlashPlate. High affinity Rho GTPases showed IC50 values ranging from 11.8 to 33.5 nM, whereas the lowest affinity Rho GTPases generated IC50 values ranging from 97.6 to 294.5 nM (Fig. 3C). ATP was used as a nonspecific nucleotide control. As expected, ATP was not able to compete the binding of b-GTP to the GTPases. In all the assays, GST was included as another negative control to measure background level.

**Small G Protein Exchange Activity**—A third assay aiming to measure the GDP/GTP exchange activity was developed using AlphaScreen. GST-GTPases were loaded with b-GDP and incubated with increasing concentrations of GTP. Donor and Acceptor beads were added, and the exchange of b-GDP to GTP was measured as described under “Experimental Procedures” (Fig. 4A). As described above for the b-GTP binding assay, exchange of b-GDP to GTP led to a signal decrease. The exchange activity was also characterized by an IC50 value obtained when increasing concentrations of GTP were used to compete the binding of b-GDP to a Rho GTPase such as CDC-42 (Fig. 4B). The IC50 value corresponds to the concentration of GTP necessary for dissociating 50% of the GDP initially bound. To compare exchange activities for various GTPases, the specific activity of the GTPases was measured, and a similar amount of GTPase-GDP complexes was used in the assay. Parallel analyses of the exchange activities displayed by the Rho GTPases showed that CDC-42, RAC-2, CED-10, RHO-1, and mRAC-1 have high exchange activities with IC50 values ranging from 0.65 to 13.3 nM, whereas MIG-2 and mRAC-1N17 showed lower exchange activities (IC50 values of 49 and 2000 nM, respectively) consistent with their lower affinity for the GTP than CDC-42 (Fig. 4B). In all the assays, GST was included as another negative control to measure background level.

**Small G Protein GDP Hydrolysis Activity**—A fourth assay was developed with GSH-coated FlashPlates to measure GTP hydrolysis activity. GTPases were loaded with [γ-33P]GTP, and the level of radionucleotide associated with increasing concentrations of unlabeled nucleotides, which progressively prevent b-GTP binding to GST-GTPase. A concentration-dependent signal decrease is then observed (Fig. 3B). Competition curves with GTP were used to generate IC50 values, which are apparent affinity constants: an IC50 value corresponds to the GTP concentration inducing 50% of signal loss. As shown in Fig. 3B, GTP competed the binding of b-GTP to CDC-42 with an IC50 value of 45 nM. IC50 values specific for each GTPases were then measured except for mRAC-1N17, for which an outranged signal was detected due to its expected low affinity for b-GTP. When the IC50 values were compared together, Rho GTPases clustered in the same two groups previously reported with saturation binding studies using the FlashPlate. High affinity Rho GTPases showed IC50 values ranging from 11.8 to 33.5 nM, whereas the lowest affinity Rho GTPases generated IC50 values ranging from 97.6 to 294.5 nM (Fig. 3C). ATP was used as a nonspecific nucleotide control. As expected, ATP was not able to compete the binding of b-GTP to the GTPases. In all the assays, GST was included as another negative control to measure background level.

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**Small G Protein GDP Hydrolysis Activity**—A fourth assay was developed with GSH-coated FlashPlates to measure GTP hydrolysis activity. GTPases were loaded with [γ-33P]GTP, and the level of radionucleotide associated with
the GTPases was measured as a function of time (Fig. 5A). The signal decrease observed is produced from a combination of hydrolysis of the [γ-33P]GTP and dissociation of the nucleotide. To account for the latter, GTP dissociation from the GTPase was measured independently after loading with a non-hydrolyzable GTP analog, [γ-33P]GTP. The pharmacodynamic parameter measured is the half-life of radionucleotide binding to the Rho GTPases. This parameter corresponds to the time required to achieve 50% hydrolysis after signal correction to account for nucleotide dissociation. A prototypical experiment using CDC-42 revealed that this protein has a high hydrolysis activity (half-life 10.4 min; Fig. 5B). In our assay, CDC-42, RAC-2, CED-10, MIG-2, and CRP-1 showed comparable hydrolysis activities with half-lives ranging from 12.0 to 14.1 min, whereas RHO-1 and CRP-1 clustered in another group with half-lives of 41.4 and 95.8 min, respectively (Fig. 5C). Our results were consistent with those reported in the literature for several Rho GTPases using conventional filtration assays (Supplemental Table 1 and Ref. 21).

Computation of Hierarchical Trees Based on Biochemical Properties of Small G Proteins—To compare and integrate our data, we computed hierarchical clustering trees based on the biochemical characteristic of the proteins tested (Fig. 6). As predicted from our results, the enzymatic tree based on GTP binding and GTP-Y-S competition underlined the lower affinity of MIG-2 and CRP-1 for GTP-Y-S. These two GTPases clustered in a separate group from the rest of the RHO GTPases tested (Fig. 6, A and B). Similarly the exchange activity-based tree (Fig. 6C) demonstrated that MIG-2 segregated from the others GTPases, and the hydrolysis activity-based tree showed the partitioning of CRP-1 and RHO-1 from the rest of
Biochemical Clustering of Ras GTPases

Fig. 5. [$\gamma$-35P]GTP hydrolysis activity assay. A, FlashPlate experimental design for the [$\gamma$-35P]GTP hydrolysis activity. GST-GTPases were fixed on a GSH FlashPlate and were loaded with either 500 nCi of [$\gamma$-35S]GTP [$\gamma$S ($) or 500 nCi of [$\gamma$-35P]GTP ($\bullet$). When the GTPase hydrolyzes the $\gamma$ phosphate of [$\gamma$-35P]GTP, the radioactivity is liberated in the reaction mixture, and $\beta$ particles are quenched by the aqueous solution leading to the inactivation of the scintillation reaction. A decrease of signal is then measured. [$\gamma$-35S]GTP [$\gamma$S, a non-hydrolyzable analog of the GTP, is used as a control to measure the nucleotide dissociation from the GST fusion protein. B, the half-life value is the time needed for a GTPase to hydrolyze 50% of the initially bound [$\gamma$-35P]GTP with the [$\gamma$-35S]GTP [$\gamma$S dissociation parameter excluded. The graph shows the hydrolysis and dissociation curves obtained for CDC-42 in a single experiment done in triplicate. C, comparison of the half-life values obtained for six C. elegans GST-GTPases (CDC-42, RAC-1, CED-10, RHO-1, MIG-2, and CRP-1) and two mouse GST-GTPases (mRAC-1 and the GTP binding-deficient mRAC-1N17) using the hydrolysis activity assay. The half-life values represent the average of at least three independent experiments performed in triplicate.

We then hypothesized that integrative trees combining different biochemical characteristics should be representative of the amino acid sequence-based tree in the context of conserved families of proteins, and the sequence-based trees should be proportional to the number of biochemical characteristics included in the integrative trees. When the hierarchical clustering tree combining the four biochemical properties of Rho GTPases (Fig. 6E) was compared with the amino acid sequence-based (SB) tree (Fig. 6F), we found that, as expected, the distance was smaller than when the latter was compared with the trees based on individual biochemical characteristics (Supplemental Fig. 3). In all the analyses, GST was used as a control for tree calculation. For each activity, GST clustered out of the Rho GTPases family validating the process of tree calculation. We then calculated the distance between the SB tree and various combinatorial trees based on biochemical properties of the Rho GTPases (combining one, two, three, or four activities; Fig. 6G). Unexpectedly the smallest distances observed between the SB and biochemical trees were when only two biochemical characteristics were combined (Fig. 6G, B + D and A + D). More specifically, the minimal distance to the SB tree was obtained when the affinity for GTP was combined with the hydrolysis activity, thus reflecting the large number of amino acid residues involved in those two processes. In addition, the fact that the exchange activity parameter led to an increase in distance to the SB tree indicates that the small number of residues involved in this process may not be conserved within the Rho family. Together our results indicate that sequence-based trees only provide a partial classification of the Rho GTPases.

DISCUSSION

In this study, we developed four different high throughput enzymatic assays to characterize the biochemical properties of small G proteins. To fulfill the needs created by ORFeome scale studies, we used two well accepted high throughput detection technologies, and we developed the algorithms allowing the integrated analysis of the combined data. The first technology, AlphaScreen, allowed us to measure GTP affinity and exchange activity for the small G proteins with a very high reproducibility, with homogeneity, and at low cost. In addition to allowing the characterization of the affinity of GTPases for different nucleotides, this technology represents a powerful tool to characterize GTPases, such as CRP-1, that have unusual properties toward GTP. However, the AlphaScreen platform was not best suited to measure GTP hydrolysis activity. Therefore, we opted for the FlashPlate technology to measure [$\gamma$-35S]GTP [$\gamma$S binding as well as the [$\gamma$-35P]GTP hydrolysis activity. When compared with more traditional filtration methods, FlashPlate provided a high throughput tool to follow in a homogenous manner the hydrolysis activity of small G proteins.

We used AlphaScreen and FlashPlate in combination to cover the entire spectrum of small G protein enzymatic activities. Significant results were obtained for six C. elegans and
two mouse GTPases of the Rho family. In addition, comparing the biochemical activities of CED-10 and its mouse ortholog mRAC-1 revealed that these two proteins displayed similar affinity for GTP as well as GDP/GTP exchange and GTP hydrolysis activities (Figs. 2–5) even if they share only 83% identity in their amino acid sequence (Fig. 1B). Finally activities obtained for mRAC-1, CED-10, and CDC-42 were comparable to those described in the literature (5, 16, 18–20). These observations taken together confirmed that (i) our novel approach provides results comparable in significance to the data previously reported from other studies, (ii) these results are comparable between orthologous proteins, (iii) our experimental platform allows a high coverage of the enzymatic activities of the small G protein, and (iv) our assays are sensitive and reproducible enough to identify subtle enzymatic differences between members of the Ras superfamily of proteins.

This analytical platform would have been incomplete without the tools enabling an integrated analysis of our experimental data. We computed hierarchical trees reporting amino acid sequence-based trees (F). The bar graph (G) shows the distance, calculated using Euclidean matrices, between the SB tree and different hierarchical trees integrating one, two, three, or four activities (A, B, C, and D). The smaller the distance is, the closer the biochemical tree is from the SB tree.

**Fig. 6.** Comparison and integration of the enzymatic trees of Rho GTPases. Hierarchical clustering trees based on GTPase biochemical activities are shown. The trees show the distance between each GTPase according to their GTP binding (A), their b-GTP competition (B), their kinetic of exchange (C), their speed of GTP hydrolysis (D), and according to the integration of these four activities (E). These trees were then compared with the amino acid sequence-based tree (F). The bar graph (G) shows the distance, calculated using Euclidean matrices, between the SB tree and different hierarchical trees integrating one, two, three, or four activities (A, B, C, and D). The smaller the distance is, the closer the biochemical tree is from the SB tree.
Biochemical Clustering of Ras GTPases

frequency-based trees reflect mostly the combination of two biochemical characteristics, namely the affinity for GTP and the GTP hydrolysis activity. Thus, our analytical platform allowed us to propose an alternative classification of the Rho GTPases based on their enzymatic characteristics.

In this study, we established and designed a platform combining AlphaScreen and FlashPlate technologies to assess the enzymatic characteristics of small GTPases of the Ras superfamily. The proof of concept validation of these assays was made possible by using the subfamily of Rho GTPases from both murine and C. elegans origins. This platform may present several advantages applicable to academic research, for example to study the relationships between phylogeny and functional characteristics by a parallel study of many different proteins from the same phyla. On the other hand this type of platform may be profitable to the industrial/pharmaceutical sector for the screening of GTPase inhibitors/activators. The platform may be suitable to the industrial/pharmaceutical sector for the screening of GTPase inhibitors/activators. The targeting of specific characteristics such as exchange or hydrolysis activities may therefore represent a specific application of this platform.

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