Differential properties of D4/LyGDI versus RhoGDI: phosphorylation and rho GTPase selectivity

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Abstract RhoA/B/C and CDC42/Rac, which form two subgroups of the rho guanosine triphosphatase (GTPase) family, regulate various aspects of actin cytoskeleton organisation. In cytosol, guanosine diphosphate (GDP) dissociation inhibitor (GDI) interacts with and maintains rho GTPases in their inactive GDP-bound form. RhoGDI is a ubiquitously expressed GDI, whereas D4/LyGDI is hematopoietic cell-specific and 10-fold less potent than RhoGDI in binding to and regulating rho GTPases. We have combined microanalytical liquid chromatography with the use of specific antibodies in order to separate D4/LyGDI and RhoDGI-complexes from the cytosol of U937 cells and to demonstrate that the two GDIs associate with different rho protein partners. RhoGDI can form a complex with CDC42Hs, RhoA, Rac1 and Rac2, while none of these GTPases was found to interact with D4/LyGDI. In addition, we found that stimulation of U937 cells with phorbol ester leads to phosphorylation of D4/LyGDI. Our results suggest that LyGDI forms complexes with specific rho GTPases expressed in hematopoietic cells where it may regulate specific pathways.

Key words: Rho guanosine triphosphatase; Guanosine diphosphate dissociation inhibitor; Hematopoietic cell; Phosphorylation

1. Introduction

Rho guanosine triphosphatases (GTPases), which form a distinct branch of the Ras-like low molecular weight GTP-binding protein superfamily, are involved in actin cytoskeleton organization [1]. Even though the precise biochemical mechanisms of their function remain largely elusive, several effector molecules that bind to the GTP-bound form of Rho GTPases and mediate their regulatory activity on the actinomyosin system have been recently identified (for review see [2]). Indeed, and as for the other members of the Ras superfamily, rho GTPases switch between an inactive guanosine diphosphate (GDP)-bound state and an active GTP-bound state. Much effort has been put into identifying and characterizing regulators of this GDP/GTP cycle. Guanine nucleotide exchange factors promote the exchange of GTP for GDP. GTPase-activating proteins (GAP) catalyze GTP hydrolysis, which returns the protein to the inactive conformation. Another class of regulators appears to prevent GDP dissociation. RhoGDI (GDP Dissociation Inhibitor) was first identified in bovine brain cytosol on the basis of its ability to inhibit GDP dissociation from RhoA [3]. Later on it was found that RhoGDI could also complex other Rho family members including CDC42Hs [4] and Rac1/2 [5–7] and that geranylgeranylation of the C-terminal CAAX motif of rho partners was required for these interactions [8]. In addition, RhoGDI solubilizes the GDP-bound form of rho proteins from membranes [9] and may also control their translocation from the cytosol to the target membrane [10].

Recently a second GDI has been discovered that shares 67% amino acid identity with RhoGDI [11–13]. In contrast to RhoGDI which is ubiquitous, this novel protein is almost exclusively expressed in the hematopoietic lineages and has been called LyGDI [11] or D4 [12]. Although recombinant D4/LyGDI (the nomenclature adopted throughout this paper) binds to and inhibits GDP dissociation from purified CDC42Hs, RhoA and Rac in vitro, it appears at least 10-fold less potent than RhoGDI in doing so [11,14]. Cerione's group has provided an explanation for this reduced efficiency: the affinity of D4/LyGDI for CDC42Hs is 15-fold lower than the affinity of RhoGDI for CDC42Hs [15]. Therefore the question remains whether D4/LyGDI is an actual regulator of these rho proteins in cells of the hematopoietic lineage where both inhibitors should coexist.

In order to assess the substrate specificity of RhoGDI and D4/LyGDI in U937, a human myelomonocytic cell line, we developed a chromatographic fractionation procedure allowing the separation of complexes containing either RhoGDI or D4/LyGDI. The distribution of RhoA, CDC42Hs, Rac1 and Rac2 in the different fractions enriched for RhoGDI or D4/LyGDI was determined using specific antibodies. In addition, the effect of activation of U937 cells by phorbol 12-myristate 13-acetate (PMA) on the phosphorylation of D4/LyGDI and RhoGDI was determined.

2. Materials and methods

2.1. Cells, antibodies and reagents

U937 cells (ATCC CRL 1593) were maintained in RPMI-1640 (Gibco BRL) supplemented with 10% fetal calf serum (FCS). Activation with phorbol 12-myristate 13-acetate (PMA, Sigma) was performed at a concentration of 100 ng/ml. The polyclonal serum against D4/LyGDI was raised against the N-terminal peptide (MTEKAPEPHVEEDDDDELDSK, position 1–21 in the human D4/LyGDI protein [11,12]) conjugated to keyhole-limpet haemocyanin (Calbiochem) with glutaraldehyde (Sigma). A sample of 1 mg of the conjugated peptide was homogenized in Freund's complete adjuvant (DIFCO Laboratories) by vortexing and injected subcutaneously into rabbits. The rabbits were boosted at 2-week intervals with 0.5 mg of conjugated peptide in Freund's incomplete adjuvant (DIFCO Laboratories). The polyclonal antibody raised against CDC42Hs internal peptide has been described [16]. The anti-RhoGDI and anti-Rac2 antibodies were purchased from Santa Cruz. Mouse mAbs specific

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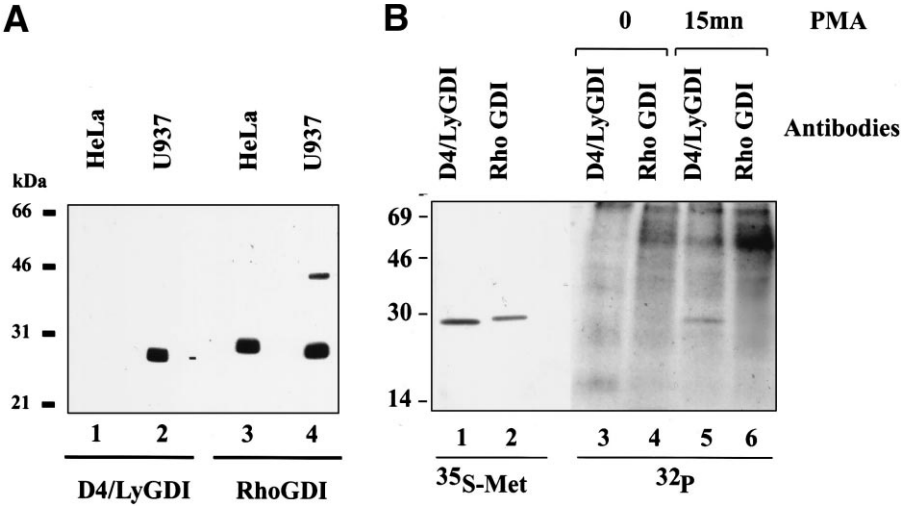

Fig. 1. PMA activation of myelomonocytic U937 cells induces the phosphorylation of D4/LyGDI. A: The D4/LyGDI N-terminal antiserum is specific, as shown by Western blotting analysis of HeLa (lanes 1 and 3) or U937 (lanes 2 and 4) cell extracts. Lanes 1, 2, D4/LyGDI N-terminal antiserum; lanes 3, 4, anti-RhoGDI antibodies. B: Immunoprecipitation from detergent extracts prepared from ^{35}S-labeled U937 cells (lanes 1, 2) or [^{32}P]orthophosphate labeled U937 cells (lanes 3–6). After labeling as described in Section 2, cells were incubated for 15 min in the absence (lanes 3, 4) or in the presence of PMA (100 ng/ml) (lanes 5, 6). Immunoprecipitations were performed with D4/LyGDI N-terminal antiserum (lanes 1, 3 and 5) or anti-RhoGDI antibodies (lanes 2, 4 and 6). Molecular weight markers are indicated (kDa).

for RhoA or Rac1 have been raised against the recombinant proteins (T. Azuma, unpublished). [^{35}S]Methionine/cysteine (TRAN^{35}S-LABEL™) was obtained from ICN Pharmaceuticals Inc. [^{32}P]Orthophosphate was purchased from Amersham. Unless indicated, chemicals were purchased from Sigma.

2.2. Immunoprecipitation of metabolically labeled proteins

For metabolic labeling of phosphorylated proteins, cells were first incubated for 2 h in phosphate-free RPMI-1640 (Gibco BRL) with 10% FCS which had been dialyzed for 15 h against Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). Then cells were labeled for 3 h in 0.5 mCi/ml of [^{32}P]orthophosphate in phosphate-free medium (10^7 cells/ml). To activate the cells, PMA was added to a concentration of 100 ng/ml and cells were further incubated for 15 min. For [^{35}S]methionine/cysteine labeling, cells were incubated for 2 h in methionine/cysteine-free RPMI-1640 (Gibco BRL) supplemented with 10% dialyzed FCS and then labeled for 3 h in 0.25 mCi/ml TRAN^{35}S-LABEL™ in methionine/cysteine-free medium (10^7 cells/ml). After labeling, cells were solubilized in 0.5 ml of 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, containing 1 mM PMSF, 0.1 mM VO_3^- and 10 mM KF. Solubilized samples were added to antibodies (0.1 ml antiserum) which had been pre-coupled to Protein A–Sepharose beads (0.05 ml, Pharmacia) and mixed at 4°C for 2 h. Immunoprecipitates were washed 3 times in solubilization buffer.

2.3. Cytosol preparation

Cytosol was prepared from 10^9 cells grown in a spinner as already described [17]. Briefly, cells were homogenized in 250 mM sucrose, 3 mM imidazole, pH 6.8, 10 μg/ml antipain, 1 μg/ml leupeptin, 1 mM PMSF. The homogenate was centrifuged for 10 min at $1000\times g$ and the post-nuclear supernatant (PNS) was collected. The PNS was centrifuged at $100\,000\times g$ for 30 min. The supernatant (20 mg/ml of protein) corresponding to the cytosolic extract, was aliquoted and stored in liquid nitrogen. Cytosol from PMA-differentiated U937 cells was prepared from 10^8 cells cultured in the presence of 100 ng/ml PMA for 5 days in 175 cm^2 plastic flasks. U937 cells that adhered to plastic upon differentiation were scraped with a rubber policeman and cytosol was prepared as described above except that the homogenization buffer was supplemented with 0.1 mM VO_3^- and 10 mM KF.

2.4. Chromatographic separation of complexed RhoGDI and D4/LyGDI

Separation of complexes was performed by a combination of ion exchange chromatography (Mono Q PC 1.6/5, Pharmacia, Uppsala, Sweden) followed by size exclusion chromatography (Superdex 75 PC 3.2/30, Pharmacia, Uppsala, Sweden) using the microanalytical liquid chromatography SMART system (Pharmacia, Uppsala, Sweden). Cytosol was prepared as described above. Four mg of protein in 500 μl of 250 mM sucrose, 3 mM imidazole, pH 6.8, was loaded onto a Mono Q column. The flow rate was 50 μl/min. Elution of proteins from the column was induced by applying a NaCl gradient in 250 mM sucrose, 3 mM imidazole, pH 6.8, starting from 0 up to 0.4 M NaCl in 42 min. The fraction size corresponded to 100 μl. After chromatography, 50 μl of fractions 8 or 11 were adjusted to 0.3 M NaCl in 250 mM sucrose, 3 mM imidazole, pH 6.8 and loaded onto a Superdex 75 column equilibrated in the same buffer. The flow rate was 40 μl/min and the fraction size 50 μl. Calibration of the Superdex 75 column was performed with chicken albumin (45 kDa, peak fraction: 16) and chymotrypsin (25 kDa, peak fraction: 20). After separation, fractions were analyzed by SDS-gel electrophoresis and Western blotting.

2.5. Electrophoresis and Western blotting

SDS-PAGE was carried out according to the system of Laemmli [18] using 12% gels. Gels with ^{35}S-labeled proteins were treated with Enhance (Dupont-NEN). For two-dimensional gel electrophoresis, samples (50 μg cytosolic protein) were run on IEF tube gels, with a linear pH gradient between pH 4.5 and 7.4 and then on 12% acrylamide second dimension resolving gels as described [19]. The resolved proteins were electroblotted onto nitrocellulose and detected with specific antibodies. Detection of bound antibodies was performed using goat anti-rabbit or anti-mouse peroxidase-conjugated antibodies (Sigma) with the enhanced chemiluminescence system (ECL, Amersham).

3. Results and discussion

The comparison of the amino acid sequence of RhoGDI [20] and D4/LyGDI [11,12] revealed that the two proteins are highly homologous except for the N-terminal region (not shown). Therefore, we raised a D4/LyGDI N-terminal antiserum directed against a synthetic peptide corresponding to residues 1 to 21 of human D4/LyGDI. D4/LyGDI could be specifically detected as a ~28-kDa species in cytosolic extracts from human U937 myelomonocytic cells by our N-terminal antiserum, while it was absent in HeLa cell cytosol (Fig. 1A). In contrast, the ~29-kDa RhoGDI was present in both cell lines. These results are in agreement with previous reports

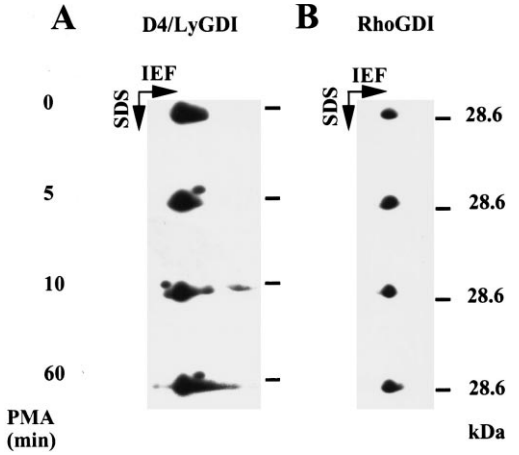

Fig. 2. Kinetics of phosphorylation of D4/LyGDI in U937 cells in response to PMA. Cytosolic extracts were prepared from U937 cells after 0, 5, 10 or 60 min of incubation in the presence of PMA and were resolved by two-dimensional gel electrophoresis. After electrotransfer D4/LyGDI (panel A) or RhoGDI (panel B) were identified by Western blotting with specific antibodies. Only the sections of 2D gels corresponding to the area where the GDIs migrate are shown. The position of a 28.6-kDa molecular weight marker is indicated.

showing that RhoGDI is ubiquitously expressed whereas D4/LyGDI expression is restricted to hematopoietic cells [11,12] in which both factors coexist (this study).

D4/LyGDI is phosphorylated upon T cell activation by phorbol ester [11] and RhoGDI appears to be constitutively phosphorylated in bovine neutrophils [21]. We thus investigated the phosphorylation of RhoGDI and D4/LyGDI in PMA-stimulated U937 cells. Both GDIs could be immunoprecipitated from ^{35}S metabolically labelled U937 cells using specific antibodies (Fig. 1B, lanes 1–2), but only D4/LyGDI was immunoprecipitated as a phosphorylated protein from ^{32}P-labelled U937 cell extracts in response to PMA treatment (Fig. 1B, lanes 3–6). Next, we characterized the kinetics of D4/LyGDI phosphorylation in PMA-stimulated U937 cells. U937 cells were incubated for various periods of time in the presence of PMA, cytosolic extracts were prepared and analyzed by two-dimensional gel electrophoresis followed by Western blotting with anti-D4/LyGDI or anti-RhoGDI antibodies. Prior to stimulation, a single spot was detected using anti-D4/LyGDI antibodies (Fig. 2A). Several spots of lower p*I* values were detected in response to PMA and very likely corresponded to different phosphorylated forms (Fig. 2A). Phosphorylation of D4/LyGDI was detected after 5 min of PMA treatment and persisted for at least 1 h in the presence of the phorbol ester. In the same conditions, RhoGDI was detected as a unique spot (Fig. 2B). Since phosphorylated RhoGDI has already been reported as multiple spots that could be resolved by IEF [21], our data show that RhoGDI is not phosphorylated in U937 cells upon phorbol ester activation in contrast to D4/LyGDI.

We next addressed the substrate specificity of these two GDIs vs. different Rho GTPases. Conditions were first set up in order to separate cytosolic complexes containing RhoGDI or D4/LyGDI. Cytosols prepared from U937 cells were applied onto a Mono Q column and after extensive washing, bound proteins were eluted by increasing salt concentration. Analysis of the column fractions by SDS-PAGE followed by immunoblotting with specific antibodies showed that both GDIs could be separated into fractions enriched for RhoGDI (peaking between fractions 6–11, ~120–150 mM NaCl, Fig. 3A) or D4/LyGDI (peaking between fractions 10–13, ~150–200 mM NaCl, Fig. 3B).

In order to identify the GTPases complexed with RhoGDI or D4/LyGDI in U937 cell cytosol, the fractions eluted from the Mono Q column were analyzed for the presence of various rho proteins by immunoblotting using specific antibodies. As shown in Fig. 3C, CDC42Hs peaked in fractions 7–8 eluted from the Mono Q column. In addition, anti-CDC42Hs antibodies gave a weak but reproducible signal in fractions 16 and 17 of the eluate. Rac1 and RhoA were detected using specific mouse mAbs. Rac1 was most abundant in fractions 6–8 of the eluate (Fig. 3D), while RhoA was present in fractions 8 and 9 (Fig. 3E). Finally, since the hematopoietic-specific Rac2 GTPase is expressed at very low level in resting U937 cells but can be induced upon differentiation in the presence of PMA ([22] and data not shown), cytosol from differentiated U937 cells that had been incubated for five days in the presence of PMA were applied onto the Mono Q column. As shown in Fig. 3F, specific antibodies detected the Rac2 GTPase in fractions 8–9 of the eluate (the positions of RhoGDI and D4/LyGDI in the Mono Q fractions from PMA-differentiated cell extracts were unchanged as compared to untreated cells, data not shown). Several observations support the conclusion that RhoGDI and Rho proteins that coeluted from the MonoQ column were in stochiometric complexes. First and as shown in Fig. 4, when Mono Q fractions 8 (peak of RhoGDI) or 11 (peak of D4/LyGDI) were applied on a Superdex 75 gel filtration column, the GDIs eluted as a single peak in fraction 16 of the sizing column with an apparent molecular mass of 45–50 kDa (see Section 2). In contrast, recombinant D4/LyGDI, N-terminally tagged with six histidine residues, eluted with an apparent mass of 20–25 kDa from the Superdex 75 column (data not shown). These results

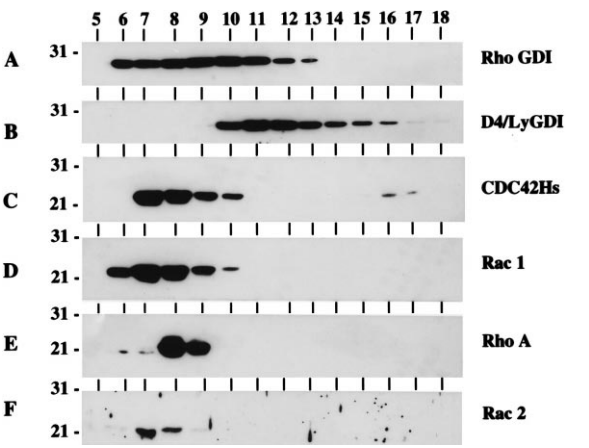

Fig. 3. CDC42Hs, RhoA, Rac1 and Rac2 are complexed with RhoGDI in U937 cell cytosol. U937 cell cytosol was chromatographed on a Mono Q anion-exchange column as in Section 2. Fractions were analyzed by SDS-PAGE and Western blotting with specific antibodies against RhoGDI (A), D4/LyGDI (B), CDC42Hs (C), Rac1 (D), RhoA (E) or Rac2 (F). Rac2 distribution was analyzed from cytosol extracts prepared from PMA-differentiated U937 cells. The position of RhoGDI and D4/LyGDI eluted from the Mono Q column was identical in cytosol from untreated or PMA-differentiated cells (not shown).

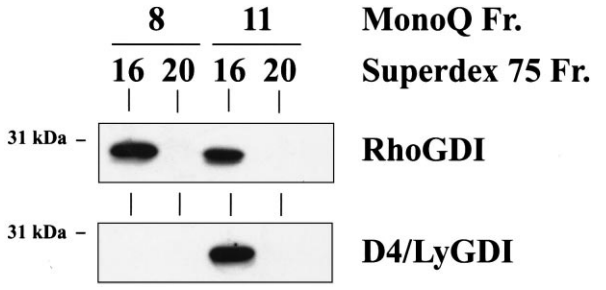

Fig. 4. GDIs eluted from the Mono Q column are complexed with Rho GTPases. Peak fractions eluted from the Mono Q column containing RhoGDI (fraction 8) or D4/LyGDI (fraction 11) were chromatographed on Superdex 75 column and fractions were analyzed for RhoGDI or D4/LyGDI by Western blotting. Both GDIs were eluted in fraction 16 with an apparent molecular mass of 45–50 kDa. Fraction 20 corresponds to proteins eluted with a molecular mass of 25–30 kDa.

indicate that the GDIs that were separated on the MonoQ column were associated with Rho proteins in heterodimeric complexes with an apparent mass of about 45–50 kDa. In addition, when U937 cytosol was directly applied onto the Superdex 75 column, both RhoGDI and D4/LyGDI were exclusively eluted with an apparent molecular mass of 45–50 kDa, indicating that the GDIs were entirely complexed with rho GTPases in U937 cytosol (data not shown). Finally, the differences in the predicted isoelectric points of RhoA (pI = 5.9), CDC42Hs (pI = 6.5), Rac1 (pI = 8.53), Rac2 (pI = 7.56) and RhoGDI (pI = 4.96) or D4/LyGDI (pI = 4.85) [13,23] make it very unlikely that RhoGDI and these four distinct rho proteins could be eluted from the Mono Q column at similar salt concentrations unless they were separated as heterodimeric complexes.

Altogether, these results demonstrate that CDC42Hs, Rac1, RhoA and Rac2 are specifically associated with the ubiquitous RhoGDI in U937 cell cytosol, while D4/LyGDI is complexed with distinct rho GTPase(s).

An important conclusion drawn from this study is that two related GDIs which are coexpressed in U937 cells, the ubiquitous RhoGDI and the hematopoietic-specific D4/LyGDI, are found in the cytosol as heterodimeric complexes involving different rho GTPases. Since its discovery by Takai's group, various studies have reported the association of RhoGDI with CDC42Hs, RhoA, Rac1 and Rac2 [3–7]. D4/LyGDI was initially identified as a hematopoietic cell-specific gene product, homologous to RhoGDI and acting as an inhibitor of GDP dissociation from RhoA [11]. Later on, it was found that D4/LyGDI is less efficient by a factor of 10–20 than RhoGDI in its ability to inhibit GDP/GTP exchange or to promote membrane release of CDC42Hs (as well as RhoA and Rac1) [14]. The regions of RhoGDI and D4/LyGDI that are responsible for the difference in substrate specificity have been mapped to the carboxyl terminal residues 172–180 of RhoGDI and particularly Ile-177 (Asn-174 of D4/LyGDI) [24]. Recently, Nomanbhoy and Cerione using a fluorescence spectroscopic assay have determined the binding affinity of the RhoGDI/CDC42Hs complex ($K_d \sim 30$ nM) which appears to be 15-fold higher than the binding of D4/LyGDI to CDC42Hs ($K_d \sim 440$ nM) [15]. Therefore, although it is possible to observe a weak affinity and inhibitory activity of D4/LyGDI for RhoA, CDC42Hs or Rac1/2 in vitro, our study clearly demonstrates that D4/LyGDI is not able to form stable complexes with these rho GTPases in vivo and is associated with as yet uncharacterized rho proteins.

Two new GTPases belonging to the Rho subgroup have recently been identified: TTF [25] and RhoE [26]. They share unusual structural features as compared to the classical Rho GTPases we found complexed with RhoGDI. In the TTF protein, the conserved Gly-12 is replaced by a serine residue (Ser-13 of TTF), while RhoE contains three amino acid differences at position 17 (Gly to Ser), 64 (Ala to Ser) and 66 (Gln to Ser). Mutations at the corresponding positions (12, 59 and 61) confer oncogenicity to Ras [27] and, as expected, totally abolish both intrinsic and GAP-stimulated GTP hydrolysis by RhoE which is maintained in the GTP-bound form [26]. Whether TTF and/or RhoE form complexes with a GDI, and more specifically D4/LyGDI, could not be addressed due to the lack of specific antibodies. The observation that RhoGDI binds equally well to the GDP- and GTP-bound form of CDC42Hs [15] suggests that TTF and RhoE, although they are probably locked in the active conformation, should also be able to interact with GDI. Interestingly, TTF is expressed in a restricted manner in hematopoietic cells [25] and represents a potential interacting partner of D4/LyGDI (we have observed that D4/LyGDI can solubilize overexpressed TTF from membranes; Dallery, E. and Chavrier, P., unpublished data).

Another observation concerns the rapid and specific phosphorylation of D4/LyGDI in response to PMA treatment of U937 cells, in contrast to RhoGDI which remains unphosphorylated. In bovine neutrophils, phosphorylation/dephosphorylation events have been implicated in the regulation of the dissociation of the RhoA/RhoGDI complex [21]. In addition, phosphorylation has also been shown to regulate p55RabGDI interaction with its rab protein partners [28]. So far, we have not been able to correlate the PMA-induced phosphorylation of D4/LyGDI with a modulation of its association with rho proteins. By gel filtration analysis, phosphorylated D4/LyGDI is eluted with an apparent molecular mass of 45–50 kDa and so behaves as the unphosphorylated molecule (data not shown). It has been postulated that GDI may serve as a chaperone-like molecule to transport rho proteins through the cytosol to their site of function at a target membrane [29]. Phosphorylation of D4/LyGDI could be involved in the regulation of this chaperone function by favoring the recruitment of D4/LyGDI complexes to the membrane. Concomitant to Rho translocation, free D4/LyGDI would be released (as shown for the Rac/RhoGDI complex [10]) and rapidly dephosphorylated, returning to the cytosol to interact with inactivated Rho. Interestingly, exposure of U937 cells to PMA induces their differentiation from non-adherent myelomonocytic cells to adherent macrophage-like cells. Rho GTPases have been implicated in the regulation of adhesion in several systems including U937 cells [30–33]. The rapid and sustained phosphorylation of D4/LyGDI upon PMA treatment may be related to the increased adhesion of U937 cells upon differentiation.

Altogether, these results suggest that LyGDI may be involved in the regulation of hematopoietic-specific rho GTPases and are possibly engaged in biochemical pathways restricted to the hematopoietic lineage. The chromatographic procedure developed in the present study should facilitate the purification of D4/LyGDI-containing complexes and we hope it will lead to the identification of its rho GTPase partners.

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