Contribution of guanine exchange factor H1 in phorbol ester-induced apoptosis

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Abstract

Phorbol-12-myristate-13-acetate (PMA) treatment induces erythroleukemia D2 cells kept in suspension to undergo RhoA-dependent contraction and to become proapoptotic, while attached cells are induced to differentiate accompanied by the reduction of RhoA activity. In this study, we found that guanine exchange factor H1 (GEF-H1) is highly expressed in D2 cells. Depletion of GEF-H1 expression in D2 cells decreased RhoA activity and prevented PMA-induced contraction and apoptosis. Upon PMA stimulation, GEF-H1 became associated with microtubules in cells that were induced to differentiate. As a contrast, in the proapoptotic population of cells GEF-H1 stayed in the cytoplasm without showing PMA-responsive microtubule translocation. Given that GEF-H1 is inactivated when associated with microtubules and its release into cytosol due to depolymerization of microtubules activates RhoA, our results demonstrated that nonmicrotubule-associated GEF-H1 in D2 cells contributes to the sustained activation of RhoA/ROCK signaling in suspension cells, making cells susceptible to PMA-induced apoptosis.

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Abbreviations: GEF-H1, guanine exchange factor H1; PMA, phorbol-12-myristate-13-acetate; LPA, lysophosphatidic acid; RNAi, RNA interference; shRNA, short hairpin RNA

Introduction

Exposure of cells to phorbol ester induces differentiation in a number of myeloid leukemia cell lines. However, the same treatment can also lead to apoptosis in other cell lines. Presumably, the different response is highly dependent on the

cellular context. Our laboratory has previously used D2, an erythroleukemia cell line, to investigate the molecular mechanism underlying the different outcomes induced by PMA treatment. We have shown that adhesion signaling can prevent PMA-induced apoptosis in D2 cells, and the presence of serum or lysophosphatidic acid (LPA) in culture medium interferes with PMA-induced adhesion, thus promoting PMA-induced apoptosis via the G_{12/13}/RhoA/ROCK signaling pathway.^{1,2} As a result, ROCK-mediated phosphorylation of myosin light chain then generates contraction force, triggering death receptor-mediated apoptosis.³ In this study, we addressed the question of how RhoA is sustained activated in PMA-induced proapoptotic but not in PMA-induced differentiating D2 cells.

RhoA is a small GTPase that regulates a wide spectrum of cellular processes.^{4,5} An immediate target of RhoA, Rho-associated kinase/ROCK, has been shown to be involved in the formation of stress fibers and focal adhesion complexes and in various contractile processes.^{6,7} Although a basal level of RhoA activity is required for fibroblast adhesion, various lines of evidence have shown that too much RhoA/ROCK signaling indeed makes cell rounding and poor spreading. For example, it has been shown that an elevated level of RhoA activity is required for maintaining the round shape of undifferentiated embryonic mesenchymal cells.⁸ In culture condition, hematopoietic cells are round in shape. Following phorbol ester treatment, many myeloid and lymphoma cell lines become adherent and differentiated in the culture condition.^{9–12} Treatment of human blood monocytes, U937 and THP-1 cells, with C3 which inactivates RhoA by ADP-ribosylation significantly enhances PMA-induced monocyte adhesion,¹³ indicating that Rho is a negative regulator in human monocyte adhesion and spreading.

RhoA activity is regulated by three groups of proteins: Rho GTPase-activating proteins (RhoGAPs) facilitate the inactivation of active GTP-bound Rho by increasing the intrinsic GTPase activity of Rho.¹⁴ Rho guanine nucleotide dissociation inhibitors (RhoGDIs) sequester some Rho family GTPases from binding to membranes and prevent nucleotide dissociation and activation,¹⁵ and Rho guanine nucleotide exchange factors (RhoGEFs) facilitate the activation of Rho by promoting the dissociation of GDP and subsequent binding of GTP.¹⁶ Currently, RhoGEF proteins are regarded as the main regulators of Rho activation. It has been well established that several Rho-specific exchange factors, including LARG,^{17,18} PDZ-RhoGEF,¹⁹ p115RhoGEF,²⁰ and AKAP-Lbc,²¹ relay signals from heterotrimeric G proteins to Rho, by which extracellular signals can control RhoA activation across the plasma membrane through G protein-coupled receptors. In addition, several GEFs, including murine Lfc and its human homologue GEF-H1, and p190RhoGEF, that interact with microtubules were microtubule-regulated RhoA activator.^{22–25} In HeLa cells, GEF-H1, originally known as Rho/Rac nucleotide exchange factor 2,²³ associates with microtubules where GEF-H1 loses its RhoA activation

function.²² During microtubule depolymerization in mitosis or nocodazole-treated cells, GEF-H1 is released to the cytoplasm and activates RhoA, which plays a role in generating the contractile force during mitosis.^{22,24,26} Since RhoA is also an F-actin regulator, alteration of RhoA activity through the action of GEF-H1 regulated by microtubule reorganization represents a coordinated change between microtubule and F-actin structures during cytoskeletal rearrangement. A recent study has shown that the released GEF-H1 during microtubule disassembly induced by thrombin stimulation in lung endothelial cells (EC) increases RhoA-mediated actomyosin contraction and EC permeability,²⁷ further supporting the physiological importance of the microtubule-association status of GEF-H1 in regulation of RhoA signaling.

Our previous study has shown that when adhesion is prevented, PMA treatment could induce D2 and TF-1 cell apoptosis in a RhoA/ROCK-dependent manner under the serum- or LPA-free condition,²⁸ indicating that intracellular signals other than LPA-mediated stimulation via G protein-coupled receptors can contribute to RhoA activation. In an attempt to address the question of how RhoA activity is elevated in D2 cells in a sustained manner, we used the affinity capture method to identify protein(s) interacting with RhoA in these cells. This led us to find that GEF-H1 is abundantly present in the cytosolic soluble fraction of D2 cells. Here, we demonstrated the role of GEF-H1 in determining RhoA activity in D2 cells and in affecting susceptibility to PMA-induced apoptosis.

Results

RhoA/ROCK signaling in PMA-induced apoptosis

When D2 cells were incubated in serum-free medium condition, all cells adhered to the plates and displayed extensive spreading upon PMA treatment. By plating cells onto hydrogel-coated dishes in serum-free medium to prevent cell adhesion, following PMA treatment these suspension cells exhibited contraction followed by apoptosis (Figure 1a and b), indicating that lack of adhesion signal prompts PMA-induced apoptosis. Pretreatment of cells with ROCK inhibitor Y27632 abrogated PMA-induced apoptosis, demonstrating the essential role of ROCK activation in PMA-induced apoptosis in suspension cells. Since ROCK is a direct downstream effector of RhoA, we then examined endogenous RhoA activity in response to adhesion and PMA treatment. Endogenous RhoA activity was decreased in D2 cells after adhering to regular dishes in serum-free medium, followed by a further down-regulation upon the PMA treatment (Figure 1c). In contrast, high levels of RhoA activity were observed in cells kept in suspension condition irrespective of PMA treatment (Figure 1c), indicating that PMA-induced downregulation of RhoA activity requires the adhesion signal. These results also showed that high level of RhoA activity is sustained in D2 cells kept in suspension even in serum-free condition.

GEF-H1 is highly expressed in D2 cells

To search for factors involved in maintaining RhoA activation in suspension D2 cells, we incubated D2 cell lysates with

Figure 1 Effects of cell adhesion and PMA treatment on cell viability and RhoA activity. (a) D2 cells pretreated with or without Y27632 (20 μ M) for 1 h in serum-free RPMI-1640 medium in hydrogel-coated plates were then plated on regular or hydrogel-coated dishes following PMA (32 nM) treatment. After 2 h, cells were photographed by a phase-contrast microscope. (b) D2 cells (5×10^5 /ml) were treated as described above. Following PMA treatment for 8 h, cell viability was analyzed by trypan blue exclusion method. The results are means $\pm$ S.D. from two independent experiments. (c) D2 cells were washed twice with PBS and were either maintained in suspension in serum-containing medium (NT) or plated on a hydrogel-coated or regular plate in serum-free medium for 30 min in the presence or absence of PMA as indicated. Cell lysates were harvested for RhoA activity assay as described in 'Materials and methods'. RhoA in GTP-bound form and in total lysates were analyzed by Western blotting with anti-RhoA antibody. By densitometric scanning, the intensity ratio of RhoA in GTP-bound form to total RhoA in lysate of non-treated cells (NT) was set to 1, and relative ratio of that in cell lysate of each condition was shown below from two individual experiments

GST-RhoA-coupled beads. One of the major proteins pulled-down by this method was identified to be GEF-H1 (Figure 2a), which has been previously shown to be a GEF specific to RhoA.^{22,23} According to an earlier study, GEF-H1 is ubiquitously expressed in a broad range of human tissues, with a higher level in thymus, testis, and leukocytes.²³ Here, we examined GEF-H1 expression in different cell lines and found high expression level of GEF-H1 in myeloid leukemia cell line TF-1 and its cytokine-independent variant D2 cells (Figure 2b), both of which have been shown to undergo PMA-induced apoptosis.²

Depletion of GEF-H1 expression prevents PMA-induced apoptosis

To directly assess the physiological function of endogenous GEF-H1 in regulating RhoA activation in D2 cells, we used lentiviral-mediated RNA interference (RNAi) to confer stable knockdown of GEF-H1 expression. Western blot analysis of cells pooled from various stable clones expressing short hairpin RNA (shRNA) of GEF-H1 or LacZ as a control showed that lentiviral-mediated shRNA for GEF-H1 specifically depleted GEF-H1 expression in D2 cells (Figure 3a). We then measured endogenous RhoA activity assay in the stable

cell clones to examine the effect of GEF-H1 knockdown. RhoA activity was significantly decreased in cells depleted of GEF-H1 in serum-containing medium. When these cells were incubated in serum-free medium, depletion of GEF-H1 expression abolished endogenous RhoA activity (Figure 3b). Upon PMA treatment in serum-free medium, the LacZ control cells exhibited contraction and cell death as a contrast to no effect seen in cells depleted of GEF-H1 (Figure 3c and d), indicating that GEF-H1 expression is necessary for PMA-induced contraction and death in D2 cells. Since PMA-induced apoptosis is dependent on the contraction force generated from RhoA/ROCK signaling, it is clear that depletion of GEF-H1 abrogates RhoA activation, thus preventing PMA-induced apoptosis.

Difference in subcellular localization of GEF-H1 in PMA-induced differentiating and pro-apoptotic cells

In HeLa cells, it has been shown that the activity of GEF-H1 in RhoA activation relies on whether it dissociates from microtubules.²² We, therefore, determined the subcellular distribution of GEF-H1 in D2 cells by subcellular fractionation using the NP40 extraction method, which enriches cytoskeletal microtubules in the pellet fraction. We treated D2 cells with PMA in serum-containing medium, resulting in one population of cells attached and the other remaining in suspension. As shown in Figure 4a, a great proportion of GEF-H1 was extracted into the soluble fraction in nontreated cells. In PMA-induced adherent cells, GEF-H1 became shifted to the pellet fraction, whereas in PMA-induced proapoptotic cells the distribution of GEF-H1 was similar to the nontreated cells.

Using immunofluorescent staining with confocal microscope observation, we found that bundling microtubules were well organized, with a clear GEF-H1 colocalization signal in the PMA-treated, well-spread adherent D2 cells (Figure 4b). This is consistent with the results observed by the NP40 extraction method. Since we also found that GEF-H1 still stayed in the soluble cytosolic fraction in D2 cells adherent to fibronectin-coated dishes (data not shown), it seems that a cellular context induced by a combinatorial effect of adhesion and PKC-activating signals is required for the microtubule translocation of GEF-H1 in D2 cells. In contrast, in the PMA-treated proapoptotic cells, microtubules were found to be mainly distributed in the nuclear peripheral area, while GEF-H1 was evenly detected in the cytosol. In parallel, we compared endogenous RhoA activity in suspension and attached cells after PMA treatment. Consistently, reduction of RhoA activity was observed in the PMA-induced attached cells, but not in PMA-induced suspension cells (Figure 4c). Thus, the extent of GEF-H1 associated with bundling microtubules was well correlated with the downregulation of RhoA activity in PMA-induced differentiating cells. Accordingly, we proposed that the elevated level of GEF-H1 present in the cytosolic soluble fraction of these cells contributes to RhoA activation, by which PMA-induced signal triggers contraction and apoptosis.

We also treated PMA-induced adherent cells with nocodazole to verify whether depolymerization of bundling

Figure 2 Proteins specifically interacting with GST-RhoA in D2 lysates. (a) Cell lysates each containing 10 mg of protein were incubated with the control GST and GST-RhoA^{WT} beads, separately, for 2 h at 4°C. Binding proteins were separated by SDS-PAGE, followed by LC/MS/MS analysis of proteins. Mass spectrometric identification of proteins was listed as below: 1, myosin heavy chain 9; 3, GEF-H1 (Rho/Rac guanine nucleotide exchange factor 2); 5, albumin; 6, SmgGDS (RAP1, GTP-GDP dissociation stimulator 1); 7, vimentin; 2 and 4, keratin contamination. (b) Total cell lysates containing 50 µg of protein in RIPA buffer from the indicated cell line were separated by 10% SDS-PAGE and analyzed by Western blot analysis with antibodies against GEF-H1 and β-tubulin

Figure 3 Effect of knockdown of GEF-H1 by RNAi on RhoA activity and PMA-induced apoptosis. (a) Lentivirus expressing shRNA that targets the position at 217 nt downstream of the start codon of GEF-H1 was transduced to D2 cells. Lentivirus expressing LacZ shRNA was used as a control. Stable shRNA-expressing cells were further selected in medium containing Blasticidin ($2 \mu\text{g/ml}$) for 2 weeks and harvested for Western blot analysis. Abbreviations: NT, nontransduction; LacZ^{shRNA}, LacZ shRNA expressing lentivirus transduction; GEF-H1^{shRNA}, GEF-H1 shRNA expressing lentivirus transduction. (b) LacZ shRNA (LacZ^{shRNA})- and GEF-H1 shRNA (GEF-H1^{shRNA})-expressed D2 cells (4×10^6) were incubated in serum-containing or serum-free condition for 1 h before lysis for RhoA activity assay. By densitometric scanning, the intensity ratio of RhoA in GTP-bound form to total RhoA in lysate of LacZ control cells in serum-containing medium was set to 1, and relative ratio of that in each cell lysate was shown below from two individual experiments. (c) Two stably transduced cells treated with PMA (32 nM) were plated on a hydrogel-coated plate in serum-free medium for 2 h and were photographed by phase-contrast microscopy. (d) After PMA treatment for 8 h, cell viability was determined by the trypan blue exclusion method and results are means $\pm$ S.D. from two independent experiments

microtubules in PMA-induced adherent cells would result in RhoA activation and PMA-induced apoptosis. As shown in Figure 5a and b, treatment of PMA-induced adherent cells with nocodazole caused cells blebbing, a characteristic of apoptosis, and a concurrent five-fold increase of RhoA activity with GEF-H1 released from microtubules. To ascertain that GEF-H1 is responsible for increase of RhoA activity induced by microtubule depolymerization, we further tested the effect of GEF-H1 depletion on endogenous RhoA activity in PMA-treated adherent cells after microtubule depolymerization. As a contrast to the control cells, in GEF-H1 knockdown cells RhoA activity remained undetectable regardless of nocodazole treatment (Figure 5c), indicating that the increase of RhoA activity induced by nocodazole treatment in PMA-induced adherent cells is mainly due to the release of active GEF-H1 during depolymerization of microtubules.

Since PMA-induced adherent cells displayed a spreading and bundling structure of microtubules, we then raised the question whether microtubule stabilization could affect GEF-H1 distribution and PMA-induced apoptosis. By subcellular fractionation, we found that taxol treatment caused β -tubulin shifted into the pellet fraction completely, but had no significant effect on distribution of GEF-H1 and the level of endogenous RhoA activity in cells kept in suspension (Figure 6). Consistently, this treatment did not change PMA-induced apoptosis in suspended cells (data not shown). These results suggest that microtubule stabilization is

insufficient to induce a cellular context that can prevent GEF-H1-mediated RhoA activation.

Expression of GEF-H1 mutant defective in microtubule binding alleviates PMA-induced adhesion via the RhoA-mediated pathway

Next, we tested the effect of expressing a deleted form of GEF-H1(1-572), which has been characterized to be deficient in microtubule binding,²² on PMA-induced adhesion. For comparison, cells were transfected with expression vector of control GFP, active GFP-RhoAV14, GFP-GEF-H1(1-572), and inactive GFP-GEF-H1(Y393A) individually. GEF-H1(Y393A) is a catalytic inactive form of GEF-H1, which exhibits weak RhoA-GDP binding. We found that overexpression of either GEF-H1(1-572) or RhoAV14 significantly reduced PMA-induced adhesion in the serum-free medium (Figure 7a). Furthermore, transfection of cells with increasing amounts of GEF-H1(1-572) expression vector decreased PMA-induced adhesion in a dosage-responsive manner (Supplementary Figure 1). Since all D2 cells underwent apoptosis as long as they were still nonadherent after PMA stimulation,³ these results demonstrated that elevated expression of nonmicrotubule-associated GEF-H1 promotes PMA-induced apoptosis. In contrast, expression of GEF-H1(Y393A) had a much lesser effect to decrease PMA-induced attachment. Treatment of cells with Tat-C3

Figure 4 Subcellular localization of GEF-H1 and RhoA activity in PMA-induced apoptotic and differentiating cells. **(a)** D2 cells were treated with or without PMA (32 nM) for 40 min. Suspended nontreated (NT), PMA-induced suspended (sus.) and attached (att.) cells were collected for cytoskeletal microtubule-enriched (pellet, P) and cytosolic (supernatant, S) fractionation as described in 'Materials and Methods' from D2 cells and subjected to Western blot analysis with antibody against GEF-H1, β-tubulin and vimentin. **(b)** Suspended non-treated (NT), PMA-induced suspended (sus) and attached (att) D2 cells were fixed and stained with Rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG antibodies to label GEF-H1 and β-tubulin, respectively. Bar, 4 μm. **(c)** D2 cells treated with (PMA-induced suspended and attached cells, S and A) or without (nontreated cells, NT) PMA were harvested at the indicated time point for endogenous RhoA activity assay

recombinant protein reversed GEF-H1(1-572)-mediated inhibition of PMA-induced adhesion (Figure 7b), supporting the notion that active GEF-H1 in cytosol promotes proapoptotic pathway through RhoA activation in suspended D2 cells.

Discussion

Exposure of cells to phorbol ester induces apoptosis or differentiation depending on cell type. We have previously

Figure 5 GEF-H1 mediates microtubule-regulated RhoA in PMA-induced differentiating cells. D2 cells plated on regular dish were treated with PMA for 2 h and then treated with or without nocodazole (1 μ g/ml) for 1.5 h. **(a)** The adherent cells were further fixed and subjected to immunofluorescence staining as described above. Representative confocal images of the immunostaining (Bar, 4 μ m) and phase-contrast photo of the treated cells were shown as indicated. **(b)** PMA-induced attached (PMA-att) and nocodazole-treated adherent (PMA-att + Noc.) D2 cells as described above were collected for subcellular fractionation and RhoA activity assay. By densitometric scanning, the intensity ratio of RhoA in GTP-bound form to total RhoA in lysate of PMA-induced attached cells (PMA-att) was set to 1, and relative ratio was shown below from two individual experiments. **(c)** LacZ^{shRNA} and GEF-H1^{shRNA} transduced D2 cells plated on regular dish were treated with PMA for 2 h, after which the adherent cells were treated with or without nocodazole for 1.5 h and were lysed for RhoA activity assay

shown that whether phorbol ester treatment relays a signal to induce apoptosis is dependent on the RhoA/ROCK signaling pathway, which cooperates with PMA-induced PKC activation to cause contraction and apoptosis. In the current study, we determined the cellular context contributing to RhoA activation in this cell type. We showed that GEF-H1, a specific RhoA activator, is highly expressed in D2 cells. By fractionation and immunofluorescent staining, we found that in D2 cells GEF-H1 is mainly distributed in the cytoplasm. By lentiviral-mediated expression of shRNAi of GEF-H1 to deplete GEF-H1 expression in D2 cells, we demonstrated that cytosolic GEF-H1 is indeed responsible for the sustained activation of RhoA in D2 cells under serum-free incubation condition. Depletion of GEF-H1 expression, therefore, prevented D2 cells from PMA-induced apoptosis under this condition. Since GEF-H1 activity is inhibited by its association with microtubules,²² we concluded that the elevated level of GEF-H1 in cytoplasm, free from inhibition by microtubule association, is the major cellular factor contributory to

elevation of RhoA activity, thus conferring ROCK-mediated contractility in PMA-induced apoptosis.

GEFs of the Dbf family are multifunctional molecules, which play a crucial role in the transduction of signals leading to the activation of Rho. Among them, GEF-H1/Lfc is well known in its association with cytoskeletal proteins, microtubules in HeLa, Cos-7, NIH-3T3 and lung endothelial cells, actin filaments in human MRC-5 fibroblasts, and F-actin in tight junctions (TJs) of MDCK and human intestinal Caco-2 cells,^{22–24,27} suggesting its cell type-dependent localization in regulation of RhoA signaling. A recent study has shown that its association with microtubules promotes mitotic spindle formation, implicating its functional role in mitosis.²⁹ The association of GEF-H1 with F-actin in TJs changes TJ function by upregulating RhoA activation, which in turn confers paracellular permeability of MDCK cell monolayers. On the other hand, a recent report has shown that in confluent culture of MDCK cells, the GEF activity of GEF-H1 associated with F-actin in TJ can be inhibited by its interaction with the

Figure 6 Effect of taxol treatment on subcellular distribution of GEF-H1 and RhoA activity. Suspended D2 cells plated on hydrogel-coated dishes were treated with or without taxol (1 μ M) for 1.5 h, after which cells were collected for subcellular fractionation and RhoA activity assay

F-actin binding junction adaptor, cingulin, thereby resulting in inhibition of G1/S transition of these confluent monolayer cells.³⁰ In neurons, Lfc, the murine homologue of GEF-H1, was found to release from microtubules and translocate to F-actin upon depolarization stimulation by activation of NMDA receptor and L-type Ca^{+2} channel.³¹ During stimulation, the microtubule-released Lfc specifically interacts with neuron-specific F-actin binding proteins, neurabin/spinophilin, by which RhoA is activated, leading to F-actin stabilization with cytoskeletal alterations in spine morphology. In addition to its interaction with F-actin binding protein in different cell types, phosphorylation status of GEF-H1 has been shown to affect its association with microtubules. It has been shown that PAK1 can phosphorylate GEF-H1 at Ser-885 site.³² Co-expression of dominant active form of PAK1 (T423E) with GEF-H1 promotes complex formation with 14-3-3 proteins and enhances microtubule localization of this complex in HeLa cells. Furthermore, a recent report showed the interaction between PAK4 and GEF-H1 *in vitro* and their colocalization *in vivo*.³³ Phosphorylation sites of GEF-H1 by PAK4 were found to be at both Ser-143 and Ser-885, and coexpression of activated PAK4 with GEF-H1 in NIH3T3 fibroblast resulted in the remarkable redistribution of GEF-H1 into the cytoplasm, while the structure of microtubules remained intact. Altogether, these results suggest that not only the interacting proteins of GEF-H1 but also its phosphorylation status is involved in its functional role in regulating RhoA activity, and the mechanism determining its subcellular localization seems to be complicated and diverse in various cell types and cell cycle stages.

Figure 7 Expression of GEF-H1(1-572) prevented PMA-induced adhesion through activating RhoA. (a) D2 cells were transfected with 4 μ g of pEGFP, pGFP-RhoAV14, pEGFP – GEF-H1(1-572) or pEGFP – GEF-H1(Y393A) expression plasmid. After transfection for 48 h, cells were washed with PBS and incubated in serum-free medium in the presence of PMA (32 nM) for 2 h. Number of GFP-positive attached cell was determined by fluorescent microscope observation and expressed as relative to the cells transfected with the control pEGFP vector. The transfection efficiency was 5–10% in all experiments and was counted by plating cells on fibronectin-coated dish. All results are means $\pm$ S.D. from three independent experiments. (b) Cells transfected with pEGFP or pEGFP – GEF-H1(1-572) for 2 days were pretreated with or without Tat-C3 proteins (20 μ g/ml) for 30 min in serum-free medium prior to PMA treatment for 2 h. The relative attached cell number was calculated as described above from three independent experiments

In our case of myeloid D2 cells, GEF-H1 is stimulated to translocate onto microtubules by PMA treatment of adherent cells. Treating these adherent cells with microtubule depolymerization reagent to release GEF-H1 not only increased RhoA activity but also reinstated PMA-induced contraction. These results clearly demonstrated that subcellular localization of GEF-H1 plays a major role in regulating RhoA activity in PMA-induced differentiation and apoptosis of D2 cells. However, it should be mentioned that adhesion is insufficient for GEF-H1 association with microtubules, since the majority of GEF-H1 still stayed in the cytoplasm of D2 cells when adhered to fibronectin-coated dishes (data not shown). Given that GEF-H1 did not translocate to microtubules in PMA-induced proapoptotic cells that remained in suspension, certain PMA-induced molecular change(s) together with the adhesion signal are required for translocation of GEF-H1 onto microtubules in D2 cells. Although Pak1 was preferentially activated in PMA-induced differentiating cells, we found no difference in the extent of phosphorylation at Ser-885 of GEF-H1 in PMA-induced proapoptotic and differentiating cells (data not shown). Taken together, it seems that neither phosphorylation on Ser-885 nor microtubule stabilization by itself is sufficient to localize GEF-H1 on microtubules. Although our

previous study has observed no significant differences in activation of various PKC isoforms, α , β , γ , δ , ϵ and θ in both PMA-induced differentiating and proapoptotic fractions of D2 cells,² it is still possible that the cytoskeletal reorganization during adhesion may make the difference in downstream events of PKC activation, thereby affecting the localization of GEF-H1. At present, the mechanism underlying the differential subcellular localization of GEF-H1 in PMA-induced proapoptotic and differentiating cells remains to be investigated.

Considering that RhoA activity can also be regulated by RhoGAPs, which inactivate RhoA by promoting its GTP hydrolysis activity, there is a possibility that RhoGAP could also play a role in differential regulation of RhoA activity in PMA-induced apoptotic and differentiating cells. For example, it has been shown that protein level of p190RhoGAP is downregulated in late mitotic phase of breast cancer cells, where RhoA activation is increased for contracting cleavage furrow during division.³⁴ In mitotic HeLa cells, increase of RhoA activity is associated with inactivation of p190RhoGAP, which has decreased phosphotyrosine and an altered electrophoretic mobility due to increased phosphoserine or phosphothreonine.³⁵ However, in PMA-induced adherent and apoptotic cells we did not detect tyrosine phosphorylation of p190RhoGAP. Nor did we find any difference in total amount or electrophoretic mobility shift of p190RhoGAP in these two populations of cells (data not shown). Therefore, it is unlikely that there is a correlation between differential regulation of RhoA and molecular alteration of p190RhoGAP during PMA-induced adhesion and apoptosis.

In summary, the results of this study revealed that elevated expression level of cytosolic GEF-H1 without binding to microtubules in suspension D2 cells constitutes a cellular context for sustained activation of RhoA, which is required for ROCK-dependent contraction in response to PMA stimulation. In PMA-induced differentiating cells, GEF-H1 is sequestered by the well-organized microtubules and is unable to activate RhoA/ROCK signaling, enabling cells to survive with adhesion and spreading.

Materials and Methods

Cell culture

D2, K562 and U937 cells were maintained in RPMI 1640 (Gibco-BRL; Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Hyclone, Logan, Utah), 2 mM L-glutamine, 100 U of penicillin G per ml and 100 U of streptomycin per ml. TF-1 cells were maintained in the same medium with 1 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml. HeLa, 293T, IMR90 and human 293FT cells were maintained in DMEM (Gibco-BRL; Gaithersburg, MD) containing 10% FBS, 2 mM L-glutamine, 100 U of penicillin G per ml and 100 U of streptomycin per ml.

Plasmids

Complementary DNA encoding wild-type RhoA sequence was amplified by PCR from the cDNA of HeLa cells and subcloned into the pGEX-3X vector. EGFP – GEF-H1(1-572) and EGFP – GEF-H1(Y393A) constructs in the pCMV5-EGFP expression vectors have been described

previously.²² The pGFP-RhoAV14 and pGST-RBD plasmids were kindly provided by Dr GG Gundersen (Columbia University, New York, NY, USA) and Dr MA Schwartz (University of Virginia, Charlottesville, VA, USA), respectively.

SDS/PAGE and immunoblotting

Protein samples were separated by 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore). Rabbit anti-GEF-H1 was kindly provided by Dr K Matter²⁴ (Institute of Ophthalmology, University College London, London, UK). The antibodies used and their dilutions were as follows: rabbit polyclonal antibodies against GEF-H1 (1:1000); mouse monoclonal antibodies against β -tubulin (Sigma) (1:3000); mouse monoclonal antibodies against vimentin (Santa Cruz) (1:3000); mouse monoclonal antibodies against RhoA (Santa Cruz) (1:1000). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or anti-mouse IgG antibody (Santa Cruz) was used for the detection of the primary antibodies. The ECL detection for HRP reaction was performed according to the vendor's instructions.

Endogenous RhoA activity assay

Endogenous RhoA activity was assayed as described by Ren *et al.*³⁶ In brief, cells were washed and lysed in a buffer containing 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 10 mM MgCl₂, 1 mM PMSF and protease inhibitor cocktail (Sigma). After centrifugation at 13 000 $\times$ g for 10 min at 4°C the supernatants of the lysates were incubated at 4°C for 1.5 h with GST-RBD-coupled glutathione-Sepharose beads. The beads were washed four times with buffer containing 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 0.1 mM PMSF and protease inhibitor cocktail. The bound RhoA protein was detected by Western blotting with a monoclonal antibody against RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Expression of GST-RhoA proteins

Glutathione S-transferase (GST)-fused RhoA proteins were expressed in the BL21(DE3)pLysS strain of *Escherichia coli*. After induction with 0.2 mM IPTG at 28°C for 3 h, cells were pelleted and resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 0.5% NP40, 1 mM DTT and 1 mM PMSF) for sonication and centrifugation, the cleared supernatant was mixed with glutathione-Sepharose beads (Amersham Biosciences) at 4°C for 2 h. After centrifugation, the beads were then washed five times with the washing buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂ and 0.5% NP40) to remove unbound proteins. Finally, the beads were resuspended gently in the wash buffer containing 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail and 10% glycerol and were stored at 4°C.

GST-RhoA affinity capture

The beads containing GST (48 μ g) or GST-RhoA^{wt} (60 μ g) were each preloaded with nucleotide depletion buffer (0.5 mg/ml. BSA, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) for 10 min at room temperature.³⁷ D2 cells were pelleted at 4°C and washed with ice-cold PBS, after which cells were lysed on ice with lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 100 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and protease inhibitor cocktail). The cell lysates (10 mg of protein) were precleared by incubation with GST immobilized beads for 20 min at 4°C, and was then mixed with GST-RhoA

or control GST beads in the buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 0.1% Triton X-100 and protease inhibitor cocktail. After 2 h of additional rotation at 4°C, beads were washed five times in wash buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂ and 1 mM DTT). The retained proteins associated with beads were boiled and separated by SDS-PAGE (8% gel).

Protein identification

Preparative SDS-PAGE gels were silver stained using the fixation protocol without glutaraldehyde, and the protein bands were excised from gels. The in-gel digestions were performed according to Shevchenko *et al.*³⁸ The proteins were digested with modified porcine trypsin (Promega), and the peptides were eluted with 5% TFA/50% acetonitrile. The in-gel digests were then separated and analyzed by the Nanoflow capillary liquid chromatography-tandem mass spectrometric (LC/MS/MS) system using a capillary LC system (Ultimate, LC Packings, Amsterdam, Netherlands) coupled to a Q-TOF (quadrupole time-of-flight) mass spectrometer (QSTAR Pulsar i, Applied Biosystem/MDS Sciex, Foster City, CA). Peptide product ion spectra generated by LC/MS/MS were searched against the SwissProt protein database using the Mascot search program sequence database searching engine.³⁹

Knockdown of GEF-H1 by RNAi

To generate stable cells for GEF-H1 RNAi analysis, the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA) was used to stably express shRNA in D2 cells. Nucleotides (nt) 217–237 of the GEF-H1 open reading frame (ORF) were chosen as the target sequence. In order to form the shRNA, one strands of oligonucleotide containing the target sequence followed by a 7 nt short loop and another 21-mer of nucleotide sequence complementary to the target sequence was designed. The forward and reverse strands of oligonucleotides were then synthesized and annealed, followed by insertion into pENTR™/U6 vector containing a U6 RNAi cassette to generate an entry construct expressing shRNA targeting the GEF-H1 gene. The LacZ double-stranded control oligomer supplied in the kit was also cloned as a nonsilencing control siRNA construct. The lentiviral construct was then cloned by the recombination of the U6 RNAi cassette from the pENTR™/U6 entry construct into the pLenti6/BLOCK-iT™-DEST vector. The 293FT producer cells (6×10^6 cells) were then co-transfected with 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti6/BLOCK-iT™-DEST expression construct containing the U6 RNAi cassette to produce a replication-incompetent lentivirus, which can then be transduced into D2 cells. The virus infected cells were further selected using 2 µg/ml nucleoside antibiotic Blasticidin to produce stable cells expressing shRNAs.

Protein extraction and subcellular fraction

Cells were washed twice with PBS at 37°C and then extracted by microtubule-stabilizing buffer (100 mM PIPES, pH 6.9, 5 mM MgCl₂, 2 mM EGTA, 2 M glycerol, 0.1% NP40, 10 mM β-glycerophosphate, 50 mM NaF, 0.3 µM Okadaic acid (Roche, IN), 1 mM PMSF and protease inhibitor cocktail) for 15 min at RT.⁴⁰ This extract was separated at RT by 5 min spins at 1000 × g, which is sufficient to pellet both the unextractable and the microtubule-enriched cytoskeleton components. Soluble cytosolic supernatant contained lipids, small molecules, and major parts of the total cell protein including unassembled tubulin.

Immunofluorescence staining

Cells on coverslip were fixed with 4% paraformaldehyde (Merck) in PBS at 37°C for 30 min, and then permeabilized with 0.5% Triton X-100 in PBS at RT for 5 min. Cells were washed with PBS twice and blocked by incubation with 3% bovine serum albumin (BSA)/PBS for 1 h. Cells were then incubated with rabbit anti-GEF-H1 and mouse anti-β-tubulin (Sigma) antibodies in PBS containing 1% BSA for 4 h at RT. After PBS wash, cells were incubated with Rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG antibodies (Sigma) in 3% BSA/PBS for 1 h at RT. Cells were then washed with PBS four times and mounted for analysis with a Leica TCS SP2 confocal spectral microscope.

Transient transfection and GFP expression observation

D2 cells were transiently transfected with GFP expression vector by the DEAE-dextran method as described previously.² After transfection for 48 h, cells were washed with 8 ml of PBS three times, resuspended in medium for different treatment and were examined by phase-contrast microscopy and Olympus AX-70 fluorescence microscopy to detect green-fluorescent protein (GFP) expression.

Expression and purification of the Tat-C3 protein

Recombinant Tat-C3 protein was produced in *E. coli* and purified as previously described.⁴¹ In brief, the induced bacteria expressing Tat-C3 fusion protein were harvested and sonicated in a binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl, 6 M Urea, 1 mM PMSF and protease inhibitor cocktail). After removing cell debris by centrifugation, cell extracts were loaded with Ni-NTA agarose (Qiagen) for 1 h at RT, and the beads were washed with the binding buffer and the washing buffer (20 mM Tris-HCl, pH 7.9, 20 mM imidazole and 0.5 M NaCl). Proteins were then eluted with elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M imidazole and 0.5 M NaCl), followed by rapid dialysis against PBS buffer. After dialysis, the supernatant was supplemented with 10% glycerol and stored at –80°C.

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