Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase–independent manner

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Caspase activation in target cells is a major function of granzyme B (grB) during cytotoxic lymphocyte granule–induced apoptosis. grB-mediated cell death can occur in the absence of active caspases, and the molecular targets responsible for this additional pathway remain poorly defined. Apoptotic plasma membrane blebbing is caspase independent during granule exocytosis–mediated cell death, whereas in other instances, this event is a consequence of the cleavage by caspases of the Rho effector, Rho–associated coiled coil–containing protein kinase (ROCK) I. We show here that grB directly cleaves ROCK II, a ROCK family member encoded by a separate gene and closely related to ROCK I, and this causes constitutive kinase activity and bleb formation. For the first time, two proteins of the same family are found to be specifically cleaved by either a caspase or grB, thus defining two independent pathways with similar phenotypic consequences in the cells. During granule–induced cell death, ROCK II cleavage by grB would overcome, for this apoptotic feature, the consequences of deficient caspase activation that may occur in virus-infected or malignant target cells.

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Abbreviations used: grB, granzyme B; GST, glutathione S-transferase; MLC, myosin regulatory light chain; PS, phosphatidlyserine; ROCK, Rho-associated coiled coil–containing protein kinase; z-VAD-fmk, z-Val-Ala-Asp(Ome)-fluoromethylketone.

NK cells and cytotoxic T lymphocytes activate the latent death machinery in their target cells by two basic mechanisms (1, 2). One is triggered by cross-linking of cell-surface death receptors of the TNF receptor superfamily expressed on the target and consists of a classical initiator-to-effector caspase cascade, starting with procaspase-8 autoprocessing. Then, caspase-8 activates effector caspases, such as caspase-3, either directly or via an amplification loop involving the release of mitochondrial proapoptotic factors into the cytosol and subsequent activation of caspase-9.

The second pathway consists of intracellular delivery of exogenous proteins into the target by exocytosis of preformed cytotoxic granules. The major death effectors contained in the granules, a family of serine proteases called granzymes, are internalized in the target and released from the endosome through the action of another granule protein, perforin. Granzyme B (grB), the most potent proapoptotic protease contained in the granules, displays an aspartase specificity, which is a property reminiscent of that of caspases. Once in the cytosol, grB pro-

cesses procaspase-3 between the large and small subunits at a site also used by caspase-8 (3). In most cases, grB-mediated mitochondrial alteration is required to fully activate caspase-3 (4), and the triggering of this mitochondrial loop involves the cleavage of Bid, a proapoptotic, BH3-only Bcl-2 family member (5). In this context, granzyme B function is comparable with that of the apical caspase-8.

More than 200 intracellular substrates of effector caspases, such as caspase-3, have been identified, and the cleavage of some of these substrates is responsible for specific cellular alterations that characterize the death phenotype (6). We and others have shown that plasma membrane blebbing, a ubiquitous morphological change observed at the onset of apoptosis, results from caspase-3–induced cleavage and activation of the serine/threonine kinase Rho-associated coiled coil–containing protein kinase (ROCK) I, an effector of the GTPase Rho (7, 8). Deregulated ROCK I increases the phosphorylation of myosin regulatory light chain (MLC) and stimulates the actomyosin contraction that is required for bleb protrusion (9).

Even though caspase activation is a major pathway of the apoptosis induced by cytotoxic cells, granule exocytosis can induce cell death in the absence of active caspases (10, 11). In addition to cleaving Bid-like caspase-8, grB has been shown to directly cleave some caspase-3 substrates (12, 13), and these proteolytic events contribute to the caspase-independent component of lymphocyte granule-induced cell death. Accordingly, Henkart and colleagues have reported the caspase independence of some cytoplasmic apoptotic features induced in target cells by cytotoxic lymphocytes, including bleb formation (11). In the present study, we provide a molecular explanation to this latter observation by showing that grB directly cleaves and activates ROCK II, a

ROCK family member encoded by a separate gene and closely related to ROCK I (65% overall homology and 92% in the kinase domain). This cleavage is shown to be responsible for cytotoxic cell–induced, caspase-independent plasma membrane blebbing in target cells.

RESULTS
ROCK II is cleaved during apoptosis induced by the cytotoxic granule pathway in a caspase–independent manner
The stimulus for this investigation was the observation that plasma membrane blebbing was abrogated by the broad caspase inhibitor z–Val–Ala–Asp(Ome)–fluoromethylketone (z–VAD–fmk) in various cell types exposed to a series of apop-
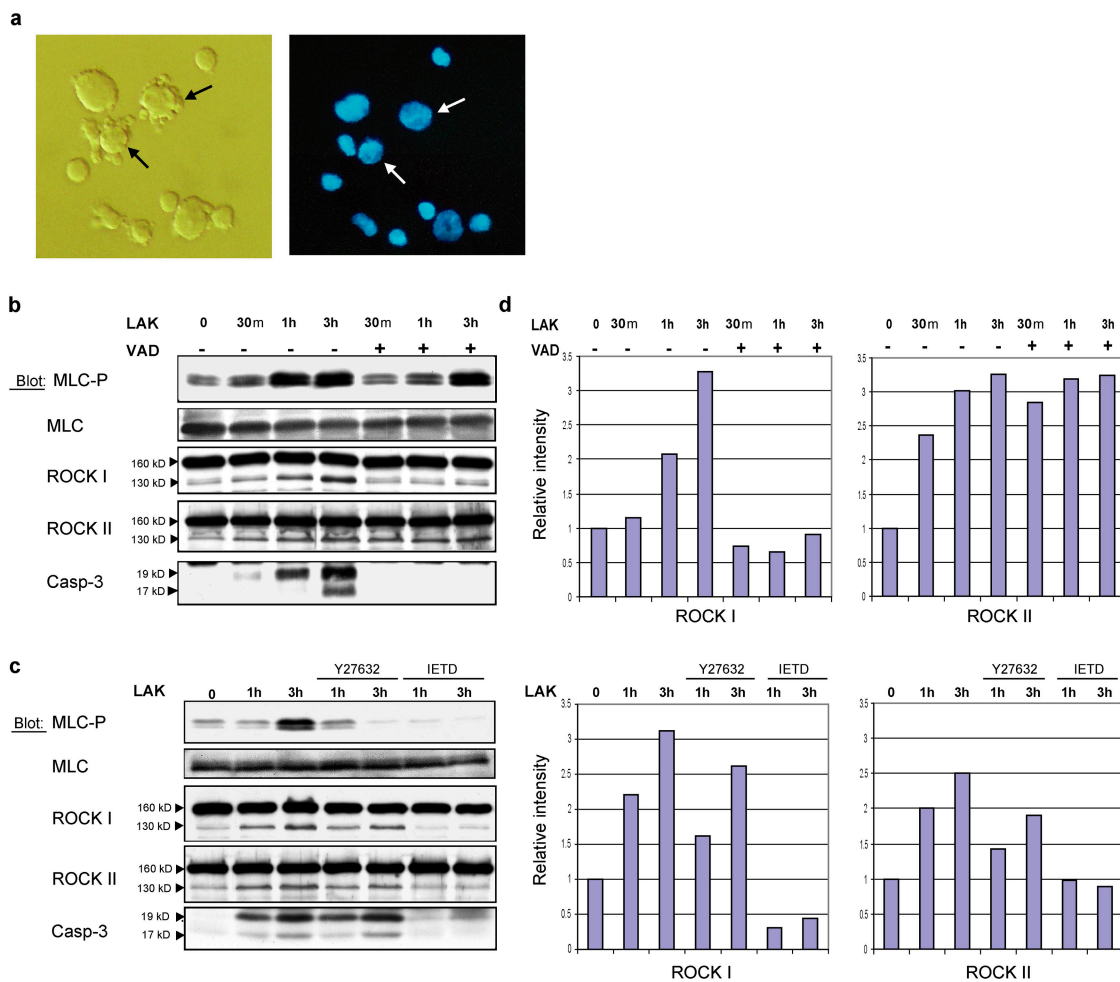

Figure 1.   ROCK II cleavage during cytotoxic granule–mediated apoptosis. (a) K562 cells incubated with LAK cells for 5 h in the presence of 20 μM z-VAD-fmk, and then stained with 5 μg ml⁻¹ Hoechst 33342. Photomicrographs of the same field were obtained by phase-contrast microscopy (left) and by microscopy with ultraviolet irradiation (right). Target cells are much larger than LAK cells. (arrows) Blebbing target cells; no chromatin condensation is detected as caspases are inhibited. (b) Western blot analysis of the indicated proteins (MLC, myosin regulatory light chain; MLC-P, phosphorylated MLC; Casp-3, caspase-3 active fragments) in lysates of K562 cells exposed for the indicated times to LAK cells in the presence or absence of 20μM z-VAD-fmk. (c) Western blot analysis of the indicated proteins in lysates of K562 cells exposed to LAK cells in the presence or absence of 10 μM Y-27632 and in the presence or absence of 20 μM z-IETD-fmk. (b and c) Molecular mass is indicated in kilodaltons. The anti-MLC antibody was used also as the loading control. Antibodies used include H-85 anti–ROCK I and clone 21 anti–ROCK II. Results are representative of three independent experiments. (d) Quantification of the cleavage fragments. Images of the cleavage bands of ROCK I and ROCK II were acquired by densitometry scanning (Gel Doc; Bio-Rad Laboratories) and analyzed with Quantity One software. Band intensity relative to the baseline signal (set as 1) is shown in arbitrary units.

tosis inducers (7), with the notable exception of apoptosis induced by cytotoxic granule exocytosis. This is illustrated in Fig. 1 a where the K562 cell line, which is specifically sensitive to this pathway (14), was used as a target for LAK cells in the presence of z–VAD-fmk.

Because we had demonstrated previously that caspase-mediated cleavage of the serine/threonine kinase ROCK I was responsible for apoptosis-associated membrane blebbing through increased MLC phosphorylation (7), we examined the phosphorylation status of MLC in K562 cells exposed to LAK effector cells. As shown in Fig. 1 b, MLC phosphorylation increased over the 3-h observation period. This increase was only moderately affected by the presence of z–VAD-fmk. In this setting, z–VAD-fmk efficiently prevented appearance of the ROCK I 130-kD cleavage fragment as well as that of the p19 and p17 forms of active caspase-3. These observations indicated that MLC phosphorylation in K562 cells exposed to LAK effector cells was, at least in part, a caspase-independent event. Interestingly, Western blot analysis of ROCK II detected a peptide whose size ($\sim$130 kD) was similar to the ROCK I cleavage fragment in addition to the full–length 160-kD protein. This peptide accumulated over time and this accumulation was not abrogated by z–VAD-fmk (Fig. 1 b). Quantification of ROCK cleavage fragments confirmed that z–VAD-fmk reduced ROCK I cleavage to baseline, but completely spared that of ROCK II (Fig. 1 d, top). These observations suggested that granule-mediated apoptosis had the unique property of inducing the cleavage of both ROCK proteins and that only ROCK I cleavage was sensitive to caspase inhibitors.

To further document the nature of the caspase-independent blebbing of target cells exposed to LAK cells, cytotoxicity assays were performed in the presence of either a ROCK inhibitor, Y-27632, or a permeant grB inhibitor, z-IETD-fmk (15). These two inhibitors prevented both MLC phosphorylation (Fig. 1 c) and apoptotic blebbing (not depicted). Neither the previously described caspase-mediated cleavage of ROCK I nor the newly identified cleavage of ROCK II was significantly affected by the presence of Y-27632. In contrast, inhibition of grB prevented the appearance of their cleaved form (Fig. 1 c and Fig 1 d, bottom), indicating that grB activity was required for both events. As expected, grB inhibition also prevented caspase-3 activation (Fig. 1 c).

Together, these observations indicated that death triggered by the cytotoxic granule pathway led to cleavage of both ROCK proteins under the dependence of grB. However, only ROCK I cleavage appeared to depend on z–VAD-fmk–sensitive proteases. Moreover, MLC phosphorylation was not abrogated by caspase inhibitors but was suppressed by either ROCK or grB inhibitors.

ROCK II is a granzyme B substrate whereas ROCK I is not

The ability of grB to directly cleave ROCK proteins was tested by using an in vitro assay. To avoid any cleavage by residual caspase-3 activity, ROCK I and ROCK II were im-
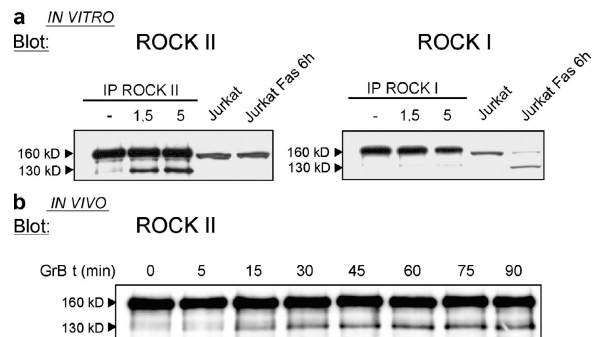

Figure 2. ROCK II in vitro and in vivo cleavage by purified grB.
(a) ROCK II or ROCK I was immunoprecipitated from MCF-7 cells, and then incubated with the indicated concentrations of grB for 60 min at 37°C before performing Western blot analysis of ROCK II (left) and ROCK I (right). Western blot analysis of Jurkat cells, either left untreated (Jurkat) or treated with an agonistic anti-Fas antibody for 6 h (Jurkat Fas 6h) was used for controls. (b) Purified grB was introduced into MCF-7 cells treated with Chariot reagent. At the indicated times (minutes) after grB addition, cells were lysed and ROCK II was analyzed by Western blotting using clone 21 antibody.

munoprecipitated from caspase-3–deficient MCF-7 cells. Fig. 2 a shows that ROCK II is efficiently cleaved by grB at a concentration as low as 1.5 nM, generating a cleavage fragment of similar size to that induced by LAK cells (Fig. 2 a). In contrast, grB did not cleave ROCK I (Fig. 2 a), even at the highest tested concentration (e.g., 90 nM; not depicted). Consistent with our previously published data (7), ROCK I but not ROCK II was cleaved in lysates of anti-Fas–treated Jurkat cells used as controls.

To further document these in vitro results, purified grB was introduced into MCF-7 cells with the Chariot reagent (16). In these conditions, a ROCK II fragment of a molecular mass comparable with that observed in vitro was detected, starting 15 min after grB addition (Fig. 2 b). After 30–45 min, the ROCK II fragment did not accumulate anymore, suggesting that the protein had been cleaved in all permeabilized cells (Fig. 2 b). In the same type of experiments, ROCK I remained in its native form (not depicted). Together, these results demonstrate that ROCK II is a direct substrate for granzyme B in vitro and in vivo, whereas ROCK I is not.

Granzyme B cleaves ROCK II at D[1131]

We previously identified the caspase-3 cleavage site in ROCK I as DETD[1113] (7). A consensus cleavage sequence for grB, which has been established as I/V/L-G/E-X-D (15), was identified in ROCK II COOH terminus (IGLD[1131] sequence) missing in ROCK I (Fig. 3 a). We used site-directed mutagenesis to substitute the aspartic acid 1131 of ROCK II by an alanine. WT and mutated D1131A ROCK II tagged with the AU1 peptide in NH$_2$ terminus were transiently transfected in MCF-7 cells. Exogenous ROCK II, immunoprecipitated with an anti-AU1 antibody, was exposed to purified grB in an in vitro cleavage assay. Mutated D1131A ROCK II was not cleaved by grB (Fig. 3 b), indicating that ROCK II cleavage occurred at aspartic acid 1131.
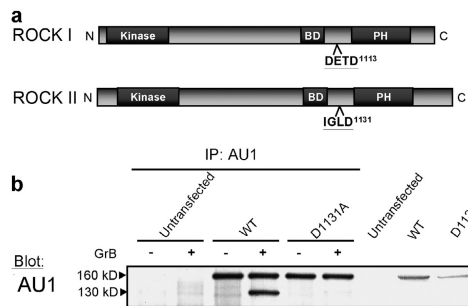
Figure 3.   Identification of ROCK II cleavage site. (a) Schematic representation of the structure of ROCK I and ROCK II. BD, Rho-binding domain; PH, pleckstrin homology domain. DETD[1113] has been demonstrated to be a caspase-3–mediated cleavage site in ROCK I (reference 7). (b) MCF-7 cells were transiently transfected with either a wild-type (WT) or a mutated (D1131A) ROCK II (the two constructs were tagged in the NH_2 terminus with the AU1 peptide). 20 h after transfection, AU-1–tagged proteins were immunoprecipitated, exposed to 5 nM grB for 60 min, and analyzed by Western blotting using an anti-AU1 antibody. Western blot analysis of transiently transfected cell lysates was used as the control.

Consequences of ROCK II cleavage by granzyme B

We have shown previously that ROCK I NH_2-terminal fragment (130 kD) produced by caspase-3 cleavage retained its ability to bind the GTP active form of Rho (7). We performed an in vitro pull-down assay using glutathione *S*-transferase (GST)–Rho loaded with either GDP or GTP by using extracts of MCF-7 transiently transfected with either WT, mutated D1131A ROCK II, or truncated ROCK II (ΔROCK II, truncated at aspartic acid 1131) constructs tagged with the AU1 peptide. Retained proteins were separated by SDS-PAGE, and exogenous ROCK II was revealed with the anti–AU1 antibody. The three proteins bound preferentially to Rho-GTP, although a weaker signal was obtained with ΔROCK II (Fig. 4 a, top). Equal expression for the three ROCK II forms was assessed by Western blot analysis. Reprobing the blot with the anti–ROCK II antibody directed against the COOH terminus sequence showed that the level of precipitated endogenous ROCK II was similar in untransfected and ΔROCK II–transfected cells (Fig. 4 a, bottom), indicating homogenous pull-down efficiency. We conclude that the 130-kD, NH_2-terminal fragment of ROCK II generated by grB cleavage still interacts with active Rho, albeit with an apparent lower affinity than WT and D1131A ROCK II proteins.

Caspase-3–mediated removal of the ROCK I COOH terminus inhibitory domain was shown to generate a constitutively active kinase (7, 8). WT and ΔROCK II were transiently expressed in 293T cells, purified by immunoprecipitation, and tested in an in vitro kinase assay. ΔROCK II exhibited a higher kinase activity than WT ROCK II (Fig. 4 b). The extent of activation of ΔROCK II compared with WT ROCK II was 2.4-fold, and thus comparable with ROCK I activation by caspase cleavage (2.1-fold; not depicted).

To determine whether ΔROCK II was able to induce plasma membrane blebbing, 293T cells were transiently cotransfected with either WT or ΔROCK II together with a
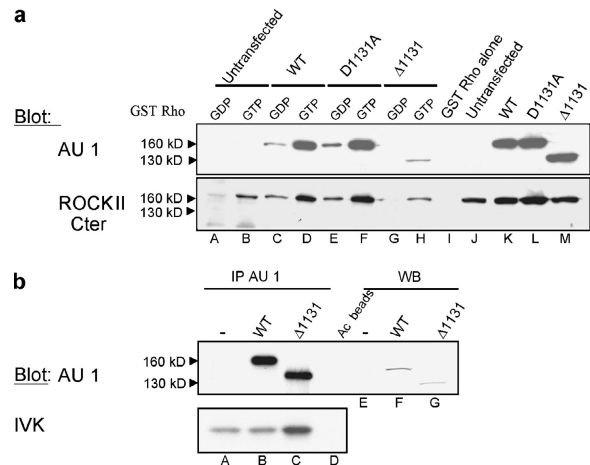

Figure 4.   Properties of cleaved ROCK II. (a) MCF-7 cells were either left untransfected (lanes A, B, and J) or transiently transfected with either wild-type (WT; lanes C, D, and K) or mutated D1131A (lanes E, F, and L) or truncated Δ1131 (lanes G, H, and M) ROCK II constructs tagged in NH_2 terminus with the AU1 peptide. 22 h after transfection, in vitro pull-down assay was performed: lysates were incubated with GDP-bound (A, C, E, and G) or GTP-bound (B, D, F, and H) GST-RhoA, and precipitated proteins were analyzed by Western blotting with anti-AU1 (top) or anti–ROCK II COOH terminus (bottom) antibody. Controls are Western blot analysis of GST Rho alone (I) and lysates of cells left untransfected (J) or transfected with either WT (K) or D1131A (L) or Δ1131 (M) AU-1–tagged constructs, indicating equal expression of the three transfected constructs in the tested cells. The blot was reprobed with the anti–ROCK II COOH terminus antibody to verify homogenous pull-down efficiency. (b) In vitro kinase assay. 293T cells were either left untransfected (lanes A and E) or transiently transfected with either WT (lanes B and F) or truncated Δ1131 (lanes C and G) AU1-tagged ROCK II constructs. 24 h later, cells were lysed, and exogenous ROCK II proteins were immunoprecipitated using the anti-AU1 antibody (IP AU1) and assayed for kinase activity in vitro with myelin basic protein as a substrate (bottom, IVK, in vitro kinase assay). Beads alone were used as a control (lane D, Ac beads). The efficiency of immunoprecipitation was determined by Western blotting using an anti-AU1 antibody (top, lanes A–D) with simultaneous analysis of constructs expression with an aliquot of cell lysates as controls (lanes E–G).

plasmid encoding the EGFP protein, in the presence of z-VAD-fmk. As illustrated in Fig. 5, ΔROCK II, but not WT ROCK II, triggered the formation of numerous membrane blebs in transfected cells. Indeed, virtually all the cells transfected with ΔROCK II and EGFP that were EGFP positive underwent blebbing. Hoechst labeling indicated that the nuclei of blebbing cells had a normal appearance; e.g., with no apoptotic chromatin condensation (not depicted). Likewise, blebs were not labeled by annexin V, indicating that active ROCK II does not induce phosphatidylserine externalization (not depicted). Membrane blebbing was insensitive to C3 exoenzyme, a specific inhibitor of Rho, but was totally abrogated by Y-27632, a specific inhibitor of ROCK (unpublished data). Finally, expression of ΔROCK II, but not of WT ROCK II, led to a major increase in MLC phosphorylation, which was reduced to baseline by Y-27632 but not by exoenzyme C3 (Fig. 5 b). Efficient ADP ribosylation and, thus, inhibition of Rho was verified by a mobility shift
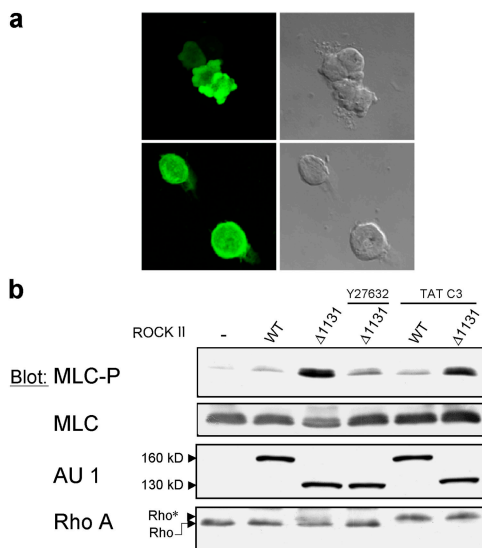
Figure 5. **Functional activity of cleaved ROCK II.** (a) 293T cells were transiently cotransfected with ROCK II and EGFP plasmids in the presence of 40μM z-VAD-fmk for 20 h. Images were obtained by phase-contrast (right) and fluorescence (left) confocal microscopy of identical fields for each population. (top) ΔROCK II; (bottom) WT ROCK II. (b) 293T cells were either left untransfected or transiently transfected with the indicated ROCK II constructs in the absence or presence of 10 μM Y-27632 or 50 μg/ml TAT-C3 for 20 h. Then, lysates were probed with antibodies against phosphorylated MLC (MLC-P), MLC (charge control), or AU1 (transfection efficiency control). (bottom) A gel shift assay performed with an anti-RhoA antibody that identifies non–ADP-ribosylated (Rho) and ADP-ribosylated (Rho*) Rho.

assay (Fig. 5 b). Together, these observations indicated that ΔROCK II exhibits a constitutive kinase activity that is sufficient to trigger membrane blebbing, outside of an apoptotic context, in a Rho- and caspase-independent manner.

ROCK II is not cleaved in target cells exposed to granzyme B-deficient cytotoxic cells

A grB cleavage site similar to that detected in human ROCK II (IGMD[1131] instead of IGLD[1131]) was identified in rat and mouse ROCK II. By using in vitro assays, we observed that grB could cleave mouse ROCK II with a pattern and an efficacy similar to that observed when studying the human protein (unpublished data). Thus, GrB-deficient mice were used to validate our observations in the murine model. The NK target cells YAC-1 were exposed to IL-2–activated splenocytes from C57Bl/6J (WT) or B6.129S2-*Gzmb^{tm1Ley}*/J (*gzmb*−/−) mice for various times before cell lysis and analysis by Western blotting. As shown in Fig. 6, both ROCK I and ROCK II were gradually cleaved in YAC-1 cells cultured with cytotoxic cells derived from WT mice. GrB-deficient splenocytes were still able to induce cleavage of ROCK I, albeit with a reduced efficiency that is most likely related to caspase-3 activation through the death receptor pathway. In contrast, grB-deficient cytotoxic cells were unable to induce ROCK II cleavage in YAC-1 targets, thus showing that ROCK II is a direct grB substrate.
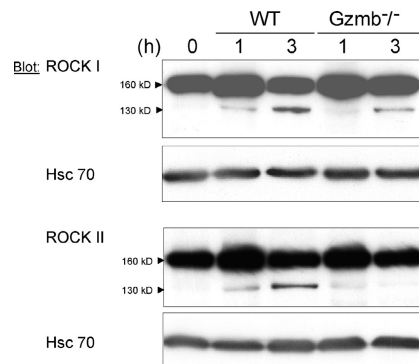

Figure 6. **Granzyme B–deficient murine cytotoxic cells fail to induce ROCK II cleavage in their target.** IL-2 activated splenocytes from WT or grB-deficient (*gzmb*−/−) mice were incubated with YAC-1 targets for the indicated times at a 2:1 ratio before cells lysis and analysis of ROCK I and ROCK II cleavage by Western blotting. Hsc70 was used as a loading control.

DISCUSSION

We have demonstrated that ROCK II is cleaved by grB during granule-mediated killing of target cells. ROCK II cleavage by grB removes an inhibitory domain similar to that deleted in ROCK I by caspase-3 cleavage (7, 8). Consequently, the NH_2-terminal fragment of ROCK II contains the entire kinase domain, is constitutively active, and is sufficient to trigger MLC phosphorylation and plasma membrane blebbing in a Rho-independent manner. Therefore, the consequences of ROCK II cleavage by grB are similar to that of ROCK I cleavage by caspases. This is the first time that two proteins of a same family, with no functional differences, are found to be specifically cleaved by either a caspase or grB, thus defining two specific pathways with similar phenotypic consequences in the cells.

Several substrates common to caspases and grB have been previously identified. Most often, grB and caspase-3 process these common substrates at different sites, usually within an ~15–amino acid span, thus generating distinct cleavage fragments. With few exceptions such as topoisomerase-I or DNA-PK, inhibition of caspases is necessary to detect grB-mediated cleavage of the common targets (17). Granule-mediated apoptosis is associated with grB-mediated cleavage of both ROCK proteins, and we show here that whereas ROCK I cleavage is indirect and requires the activation of caspases, grB cleaves ROCK II directly. This grB-mediated cleavage of ROCK II is very efficient in that it is readily detectable after 30 min of K562 cells being exposed to killer cells, in the absence or presence of caspase inhibitors. This observation is in accordance with our in vitro data showing that a minimal amount of grB is sufficient to process ROCK II.

Preventing caspase activation during granule-mediated apoptosis largely inhibits nuclear changes such as chromatin condensation or DNA oligonucleosomal fragmentation. In contrast, phosphatidylserine (PS) externalization, mitochondrial potential decrease and plasma membrane blebbing in target cells are not inhibited, suggesting the involvement of cytoplasmic caspase-independent events (11, 14). PS expo-

sure could signal the phagocytosis of target cells despite incomplete nuclear apoptotic changes. Mitochondrial depolarization is likely caused by grB either as a consequence of Bid cleavage or via an alternative, Bid-independent pathway (18). We show here that plasma membrane blebbing is another phenotypic feature of target cell apoptosis that can be directly mediated by grB through direct cleavage of ROCK II, thus ensuring the occurrence of this apoptotic change in cells with either defective or inhibited caspase activation.

Apoptotic bleb formation may have important functional consequences. Impaired clearance of apoptotic cells by macrophages is known to contribute to the development of autoimmune and inflammatory diseases (19–22). Apoptotic membrane blebs express externalized PS and are a preferred site for complement C1q and C-reactive protein binding, events which are implicated in the noninflammatory clearance of dying cells (21, 23–25). Interestingly, most of the substrates cleaved by both caspase-3 and by grB are nuclear autoantigens that redistribute and cluster in blebs and/or apoptotic bodies (17, 26–28). Bleb recognition and engulfment by phagocytes permits the efficient removal of packaged caspase and/or grB substrates, and may participate in the maintenance of tolerance to self proteins.

The autoantigenic status of grB substrates, as opposed to the vast majority of caspase substrates, might be due to the exposure of cryptic and potentially more immunogenic epitopes that require swift uptake to avoid triggering of an autoimmune response (27). During granule-mediated cell death, ROCK II cleavage by grB would maintain plasma membrane bleb formation and autoantigen clustering, even in the face of incomplete apoptotic signaling caused by deficient caspase activation in virus-infected or malignant target cells.

MATERIALS AND METHODS

Cells and reagents. LAK cells were prepared by overnight culture of human peripheral blood lymphocytes in the presence of 100 U/ml rIL-2 (Cetus). z-VAD-fmk was purchased from Bachem and Y-27632 was provided by A. Yoshimura (Yoshitomi Pharmaceuticals, Iruma, Japan). Plasmid construction and purification of TAT-C3 protein were performed as described previously (7). The following ROCK II antibodies were used: anti-ROKα (clone 21; BD Biosciences) and anti–ROCK II COOH terminus (C-20; Santa Cruz Biotechnology, Inc.). The following ROCK II antibodies were used: anti–ROCK II (H-85; Santa Cruz Biotechnology, Inc.) and anti–Rock II COOH terminus (C-19; Santa Cruz Biotechnology, Inc.); anti-cleaved (Asp175) caspase-3 (Cell Signaling); anti-AU1 (Babco); anti-MLC (MY21; Sigma-Aldrich); and anti–MLC-PP, a gift from J.M. Straddon (University College, London, UK). Purified grB was provided by C. Froelish (Northwestern University, Evanston, IL), and KIAA0619 was provided by T. Nagase (Kazusa DNA Research Institute, Chiba, Japan).

Mice. C57BL/6J mice were obtained from the Jackson Laboratory, and B6.129S2-$Gzmb^{tm1Ley}$/J ($grB-/-$) mice (29) were provided by A. Senik (INSERM U542, Villejuif, France). Mice were bred and maintained in specific, pathogen-free, accredited facilities at the Centre National de la Recherche Scientifique Unit 44. Male mice were used at 6 wk of age.

Western blots. Cells were lysed in Triton X-100 buffer for ROCK detection and in Laemmli buffer for caspases and MLC detection before electrophoresis was performed on SDS-PAGE. Mobility shift of ADP-ribosylated Rho was analyzed as previously described (7).

In vitro grB cleavage assay. Subconfluent MCF-7 cells were lysed in Triton X-100 buffer. ROCK immunoprecipitation was performed with 300 μg of protein per 4 μg anti–ROCK I (sc-H-85) or anti–ROCK II (sc-C20) at 4°C for 6 h followed by protein G–Sepharose at 4°C for 1 h. In vitro grB cleavage assay was performed as described previously (13).

In vivo granzyme B cleavage assay. Subconfluent MCF-7 cultured in 6-well microplates were washed twice in serum-free medium, treated with Chariot reagent (Active Motif; reference 16), and mixed with 2 μg of purified grB. Cells were lysed with Triton X-100 buffer before Western blot analysis was performed.

ROCK II plasmid constructions. cDNA coding for human ROCK II (KIAA0619) was used as a template for PCR amplification with the following primers: forward, 5′-ATGAGCCGGCCCCCGCCGACG-3′; and reverse, 5′-TTAGCTAGGTTTGTTTGGGGCAAGCTG-3′. Human ROCK II cDNA was cloned in mammalian expression vector pcDNA$_3$-AU1 at BamHI and EcoRV sites. Uncleavable ROCK II was obtained by mutation of asp$_{1131}$ to alanine using the primer 5′-CCTTGCATATTGGTCTGGC-CAGTTCCAGTATAGGCAG-3′, and ROCK II Δ1131 was obtained by asp$_{1131}$ mutation in amber codon using the primer 5′-GCCTTGCATA-TTGGTCTGGGCTAGCTCCAGTATAGGCAGTGGACCAGGG-3′, with their respective complementary oligonucleotides by overlap extension, using the QuikChange site-directed mutagenesis kit (Stratagene). Both mutations were verified by sequencing.

GST-RhoA pull–down assay. *Escherichia coli* expression of RhoA was achieved by the use of pGEX-2T (Amersham Biosciences) encoding GST fusion proteins. pGEX-transformed *E. coli* were grown to the mid-exponential phase, induced for 5 h with 1 mM IPTG, and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 150 mM NaCl, 10 mM MgCl$_2$, 170 μg ml^{-1} PMSF, and 1 μg ml^{-1} of protease inhibitors [aprotinin, leupeptin, and pepstatin]). The GST-RhoA was purified from the bacterial lysate by incubating with glutathione-coupled Sepharose beads (Amersham Biosciences), elution in a buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl$_2$, 100 mM CaCl$_2$, 10 mM DTT, and 10 mM reduced glutathione and dialysis against PBS/2 mM DTT.

50 μg GST-RhoA bound to glutathione-coupled Sepharose beads were incubated with 0.1 mM GDP or 0.1 mM GTPγ in PBS buffer supplemented with 1 mM EDTA and 0.5 μM MgCl$_2$ for 1 h at 4°C, and the bound nucleotide was stabilized with 10 mM MgCl$_2$. MCF-7 cells, transiently transfected with AU-1 tagged ROCK II constructs with Lipofectamine 2000 (Invitrogen), were lysed in Triton X-100 buffer and 2 mg of cell lysate was added to the GST-RhoA-GDP or -GTP for 1 h at 4°C. Beads were washed in lysis buffer, and the proteins associated with the GST-RhoA bound to the beads were boiled and separated on SDS-PAGE. Immunoblotting was performed with the anti-AU1 or anti–ROCK II (C20) antibodies.

ROCK II in vitro kinase assay. 293T cells, transfected with ROCK II AU1-tagged constructs with Lipofectamine 2000, were used for in vitro kinase assay as described previously (30). In brief, 24 h after transfection, cells were lysed with Triton X-100 buffer and AU1-tagged ROCK II proteins were immunoprecipitated with the anti-AU1 antibody. A kinase reaction was performed with the immunoprecipitated proteins in the presence of γ-[^{32}P]ATP and myelin basic protein for 30 min. After boiling, the preparation was run on SDS-PAGE and transferred on polyvinylidene difluoride membrane. For autoradiography, signals from ^{32}P-labeled proteins were acquired quantitatively with a phosphorimager (Molecular Dynamics), and for Western blotting analysis of immunoprecipitated proteins, the membranes were probed with the anti-AU1 antibody.

Detection of ΔROCK II–induced membrane blebbing. 293T cells seeded on glass coverslips were cotransfected with ROCK II and pcDNA3-EGFP plasmids, cultured for 20 h in the presence of z-VAD-fmk and fixed in paraformaldehyde (4%) before performing analysis on a laser scanning confocal microscope imaging system (Carl Zeiss MicroImaging, Inc.).

ROCK cleavage in YAC-1 cells exposed to activated murine NK cells. Red cell-depleted splenocytes of C57BL/6J (WT) or B6.129S2-Gzmb[tm1Ley]/J (*gzmb*−/−) mice were cultured for 8 d in the presence of 500 U/ml rIL-2 (Cetus). Activated splenocytes were incubated with YAC-1 target cells at a ratio of 2:1 for the times indicated in the figures. Cell mixtures were lysed in Triton X-100 buffer before performing electrophoresis on SDS-PAGE and blotting with anti-ROCK antibodies. Hsc-70 was used as a loading control.

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