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A Role for a RhoA/ROCK/LIM-Kinase Pathway in the Regulation of Cytotoxic Lymphocytes

Zhenkun Lou,²* Daniel D. Billadeau,²† Doris N. Savoy, † Renee A. Schoon, † and Paul J. Leibson³†

Polarization of lipid rafts and granules to the site of target contact is required for the development of cell-mediated killing by cytotoxic lymphocytes. We have previously shown that these events require the activation of proximal protein tyrosine kinases. However, the downstream intracellular signaling molecules involved in the development of cell-mediated cytotoxicity remain poorly defined. We report here that a RhoA/ROCK/LIM-kinase axis couples the receptor-initiated protein tyrosine kinase activation to the reorganization of the actin cytoskeleton required for the polarization of lipid rafts and the subsequent generation of cell-mediated cytotoxicity. Pharmacologic and genetic interruption of any element of this RhoA/ROCK/LIM-kinase pathway inhibits both the accumulation of F-actin and lipid raft polarization to the site of target contact and the subsequent delivery of the lethal hit. These data define a specialized role for a RhoA→ROCK→LIM-kinase pathway in cytotoxic lymphocyte activation. The Journal of Immunology, 2001, 167: 5749–5757.

Interestingly, the redistribution of lipid rafts is a signal-driven process dependent upon the activation of proximal Src- and Syk-family PTKs (17). However, the intracellular signaling molecules involved in the regulation of lipid raft polarization downstream of these PTKs are unknown.

Studies over the past decade have implicated the Rac/Rho family of GTPases in the control of several cellular processes, including the reorganization of the actin cytoskeleton, transcription factor regulation, and cellular transformation (18, 19). We have previously shown that the guanine nucleotide exchange factors for the Rac/Rho family of GTPases, Vav-1 and Vav-2, are involved in the regulation of cell-mediated killing by cytotoxic lymphocytes (20, 21). In addition, it has been shown that inactivation of the Rac/Rho family of GTPases by pharmacological or genetic approaches significantly impairs the development of cell-mediated killing by cytotoxic lymphocytes (20, 22). However, the identification of specific Rac/Rho family effector molecules or the mechanism by which these GTPases regulate cellular cytotoxicity is unknown.

Among the characterized Rho-effector molecules are the serine/threonine kinases p160ROCK and its homolog ROCK II (23–26). They have been shown to interact specifically with GTP-bound RhoA and to influence the formation of focal adhesions and stress fibers in nonhematopoietic cells (27). ROCK proteins elicit their activity downstream of Rho by phosphorylation of numerous downstream substrates (28–31). Among these substrates, p160ROCK has been shown to directly phosphorylate and activate LIM-kinase 1 (LIMK1) (29, 30). LIMK1 is a serine/threonine kinase that phosphorylates and inactivates the actin-depolymerization factor cofolin, thereby regulating actin cytoskeletal reorganization (32, 33). The involvement of p160ROCK in the regulation of the actin cytoskeleton downstream of RhoA and the known role for RhoA in regulating the development of cellular cytotoxicity led us to investigate the role of the p160ROCK/LIMK1 pathway in the regulation of cell-mediated killing by cytotoxic lymphocytes. To this end, we have found that LIMK1 is activated in a p160ROCK-dependent manner after anti-FcR cross-linking of NK clones. In addition, using pharmacological inhibitors and dominant-negative versions of RhoA, p160ROCK and LIMK1, we found that a functional RhoA/ROCK/LIM-kinase pathway is required for actin polymerization and lipid raft polarization at
the effector/target interface and the subsequent development of cell-mediated killing. These results provide a novel mechanism for the RhoA→p160ROCK→LIMK1 pathway in the activation of cytotoxic lymphocytes.

Materials and Methods

Reagents, cells, and Abs

Unless otherwise stated, all chemicals were from Sigma (St. Louis, MO). The K562 erythroid leukemia cell line and murine mastocytoma cell line P815 were obtained from American Type Culture Collection (Manassas, VA). Human NK cells and CD8⁺ T cells were cloned and passaged as previously described (20). Yoshitomi Pharmaceutical Industries (Osaka, Japan) generously provided the Y-27632 p160ROCK inhibitor. NK clones or peripheral blood leukocytes were infected with the indicated amount of Y-27632 for 1 h at 37°C unless otherwise indicated in the figure legend. C3 exoenzyme was obtained from Calbiochem (La Jolla, CA). Anti-FcγRIIIA mAb, 3G8, and anti-CD3 mAb, OKT3, were purified from ascites by affinity chromatography over protein A-agarose. Rabbit polyclonal anti-serum to p160ROCK and LIMK1 were obtained from Cocalico Biologi cal, Inc. (Reamstown, PA) after immunization of rabbits with keyhole limpet hemocyanin-conjugated LIMK1 peptide 279–304 (TSPGAEAGSSQPKVLRSIDRSPG).

DNA constructs and recombinant vaccinia generation

The recombinant FLAG-tagged RhoA.WT (F.RhoA.WT) and dominant-negative RhoA (F.N19RhoA) vaccinia have been previously described (20). To obtain a recombinant vaccinia virus expressing a dominant-negative version of p160ROCK (ROCK, ROCK1), amino acids 1081–1354 were amplified by PCR from a p160ROCK cDNA generously provided by S. Narumiya (Kyoto University, Kyoto, Japan) (24) using the forward (5′-AATAGCTTCAAGTCGACCTGGGACCGCC-3′) and reverse (5′-GGCCGTCCTAATGAGTTTTTGGTCCAGTGTTGC-3′) oligonucleotides. The LIMK1 cDNA was amplified by PCR from p160ROCK cDNA using the forward (5′-GATCAAGCTTATGGAGGTTCAGGCTTCTTG-3′) and reverse (5′-GCGGCCGCCTAGTCAGGTGGGATGATCACAAACCGAAGCCACCGACATGCCGGGCTCAGGGGT-3′) oligonucleotides. The PCR-amplified products were sequenced and subcloned into the pSH11.FLAG vector, and recombinant vaccinia viruses were produced as described previously (20). The catalytically inactive version of LIMK1 (LIMK1-KD, D460A) was generated using the Clontech (Palo Alto, CA) site-directed mutagenesis kit and the oligonucleotide (ATGACATCTACCCAGGCGCTAACATCTCAACTCCA CAACTGCG) as previously described (20). The GST-collin fusion protein was generated by PCR amplification using the collagen-specific forward (5′-CCCCAGGTCTTGGCCTTCGGAAGCTCT-3′) and reverse (5′-CCAGGCTTCAACGAGGGTCCAGTGTTGC-3′) oligonucleotides. The amplified product was subcloned into the pIEX-2G cloning vector to produce the GST-collin fusion protein. Nucleotides in bold represent restriction sites, or point mutations that were engineered into the oligonucleotide primer.

Cytotoxicity assays

In some cases, NK cells or PBLs were treated with the indicated concentration of C3 exoenzyme (overnight at 37°C) and the ROCK inhibitor (1 h at 37°C), or they were infected with the indicated recombinant vaccinia virus (6 h at 37°C). The cells were then assayed for their cytolytic activity toward K562 or anti-FcγR-coated P815 cells using the 10°C-release assay as previously described (20). In all cases, spontaneous release did not exceed 10% of maximum release. In redirected cytotoxicity assays, NK clones were only able to kill the P815 target cell in the presence of anti-FcR mAb. Lytic units were calculated based on 20% cytotoxicity (34).

In vivo labeling and in vitro kinase assay

NK clones were placed in phosphate-free media overnight in the absence of serum. They were subsequently labeled with [35S]orthophosphate (200 μCi/ml) for 2 h at 37°C. One hour before the end of the incubation, the cells were split in half and one half was treated with Y=27632 (50 μM). After the incubation, the cells were stimulated by cross-linking the FcR for the indicated time and lysed as previously described (20). LIMK1 was immunoprecipitated from the cell lysate, separated by SDS-PAGE, transferred to a nylon membrane, and analyzed using a STORM imaging system (Molecular Probes, Eugene, OR), using one of the indicated antibodies against LIMK1 during natural cytotoxicity. NK clones (1×10⁶/sample) were serum-starved overnight and then incubated at 37°C with 1% paraformaldehyde-fixed K562 cells (5×10⁶/sample). After the cell stimulation, LIMK1 was immunoprecipitated and assayed for kinase activity using 5 μg of GST-collin as previously described (30). Analysis of LIMK1 activity after FcR cross-linking was performed essentially as described above, except that one-half of the cells were treated for 1 h with Y=27632 (50 μM) or C3 exoenzyme (50 μg/ml) and then stimulated through the FcR as indicated. Where indicated, whole cell lysates were prepared from 1×10⁶ NK clones and analyzed for extracellular signal-related kinase (ERK) activation using an anti-phosphoERK-specific Ab.

Conjugate analysis

Quantification of effector-target conjugates was performed as previously described (20). Briefly, NK cells were labeled intrinsically for 1 h at 37°C with 100 μM sulfofluoroescein (Molecular Probes, Eugene, OR), and the K562 target cells were labeled intracellularly for 1 h at 37°C with 40 μg/ml hydroethidium (Polysciences Inc., Warrington, PA). In addition, the NK cells either were left untreated or were treated for 1 h at 37°C with 50 μM Y=27632 as indicated in the figure legends. The cells were then washed and resuspended at a concentration of 5×10⁶ cells/ml. The effectors and targets (25 μl of each) were mixed together, pelleted, and allowed to incubate at 37°C for 10 min. The pellet was gently resuspended and transferred to 1 ml of ice-cold RPMI 1640 medium. Conjugate formation was assessed using a FACSscan (BD Biosciences, San Jose, CA) and is revealed by the simultaneous emission of green and red fluorescence. Results are expressed as the percentage of total NK cells that formed conjugates.

Lipid raft polarization assay

In some instances NK cells were assayed for lipid raft polarization or F-actin content after infection for 5–6 h with the indicated recombinant vaccinia virus, a 1 h incubation with Y=27632 (50 μM), or an overnight treatment with C3 exoenzyme (50 μg/ml). The assessment of lipid raft polarization during the development of cell-mediated killing was done as previously described (17). In brief, NK cells were stained for 45 min on ice with FITC-cholera toxin B subunit (CTxB) (8 μg/ml). The labeled cells were washed twice in PBS containing 0.5% BSA and resuspended at a final concentration of 10⁶ cells/ml for NK cells and 5×10⁶ cells/ml for the target cells. Equal volume of 50 μl of NK cells and target cells were mixed, briefly pelleted, and then incubated for 5 min at 37°C. The cells were then fixed and transferred to glass slides by cytoospin. For analysis of F-actin content, the slides were stained with a 0.02 μM concentration of rhodamine-phalloidin (Molecular Probes). NK cells that had formed conjugates (25 μl of each) were fixed together, pelleted, and air-dried. F-actin content was revealed using the immunofluorescence microscope (Carl Zeiss, Jena, Germany). A total of 100 conjugates were evaluated per slide, and the individual performing the assay was blinded to the sample identities.

Immunofluorescence

NK cells (5×10⁶) were labeled with FITC-CTxB and incubated with the K562 target cell as described above. The cells were then fixed and transfixed with glass slides by cytoospin. The cells were permeabilized in 0.2% Triton X-100 in PBS for 2 min, rinsed three times in PBS, and placed in blocking buffer (PBS containing 5% glycerol, 5% goat serum, and 0.04% sodium azide) for 1 h. The cells were stained using a 1/250 dilution of anti-p160ROCK, anti-LIMK1 polyclonal rabbit antisera, or 0.02 μM rhodamine-phalloidin in blocking buffer for 2 h. After this primary incubation, the cells were washed three times in PBS and then incubated with a 1/250 dilution of anti-rabbit IgG goat anti-rabbit Texas Red–conjugated Ab (Molecular Probes) in blocking buffer. The cells were washed three times in PBS and mounted in Fluoresoal (Molecular Probes, Eugene, OR), and coverslips were applied. Individual NK cells, or those that had formed conjugates, were assessed for raft redistribution and p160ROCK, LIMK1, and F-actin using a fluorescence microscope (Carl Zeiss). Images were analyzed using the KS 400 image analysis software (Carl Zeiss).

Results

A RhoA/ROCK/LIM-kinase pathway is involved in the regulation of cellular cytotoxicity

Inactivation of the RhoA GTPase signaling pathway in NK cells by either preincubation of the NK cells with C3-exoenzyme or overexpression of a dominant-negative form of the GTPase (N19RhoA) inhibits the development of natural cytotoxicity toward the NK-sensitive target cell line K562 (Fig. 1A). The critical role for RhoA in the regulation of cellular cytotoxicity led us to investigate downstream effectors of RhoA that might be involved in regulating cell-mediated killing. One such candidate effector...
molecule of RhoA is p160ROCK. p160ROCK is a multidomain protein containing a serine/threonine kinase domain, a Rho-binding domain, a pleckstrin homology domain, cysteine-rich regions, and an amphipathic α-helical region. It has been shown that the kinase activity of p160ROCK is required for its ability to regulate events downstream of RhoA (35), and most recently it has been

FIGURE 1. Activation of LIMK1 during NK cell activation. A, Cloned NK cells (2 × 10⁶) were left untreated or incubated with 50 μg/ml C3-exoenzyme (left panel), or infected with the indicated recombinant vaccinia virus (right panel). The NK cells were then assayed for natural cytotoxicity by incubating them with ³ⁱCr-labeled K562 target cells (NK + K562). Data are expressed in lytic units. This is a representative example of three separate experiments. B, Cloned NK cells (1 × 10⁷) were left untreated or were treated for 1 h with 50 μM Y-27632 and were labeled internally with [³²P]orthophosphate and treated as described in Materials and Methods. LIMK1 was immunoprecipitated at the indicated times after anti-FcR cross-linking, separated by SDS-PAGE, and transferred to a nylon membrane, and incorporated [³²P]phosphate was measured using a STORM imaging system. The levels of LIMK1 protein were assessed by Western blotting followed by densitometry and were used to calculate the fold increase in phosphorylation. C, NK cells (1 × 10⁶) were left untreated or were treated for 1 h with 50 μM Y-27632. At the indicated times after anti-FcR cross-linking, whole cell lysates were prepared and analyzed for ERK activation using a phospho-specific anti-ERK antisera. The levels of ERK2 protein were assessed by reprobing the membrane with anti-ERK 2 antisera, and the protein levels were used to calculate the fold increase in FcR-initiated phosphorylation. The basal level of phosphorylation was set as one. This is a representative example of three separate experiments. D, Cloned NK cells (1 × 10⁷/sample) were serum-starved overnight, and then one-half of the sample was treated with Y-27632 (50 μM). After the indicated times of anti-FcR cross-linking, LIMK1 was immunoprecipitated from cell lysates and subjected to an in vitro kinase assay using GST-cofilin as the substrate. The fold increase in GST phosphorylation is indicated below the upper autoradiogram. The levels of LIMK1 protein were assessed by Western blotting the same membrane. Comparable levels of GST-cofilin were assessed by staining the membrane with amido black. This is a representative example of four separate experiments. E, Cloned NK cells (1 × 10⁷/sample) were serum-starved overnight and then one-half of the sample was treated with Y-27632 (50 μM). After the indicated times of anti-FcR cross-linking, LIMK1 was immunoprecipitated from cell lysates and subjected to an in vitro kinase assay using GST-cofilin as the substrate. The fold increase in GST phosphorylation is indicated below the upper autoradiogram. The levels of LIMK1 protein were assessed by Western blotting the same membrane. Comparable levels of GST-cofilin were assessed by staining the membrane with amido black. This is a representative example of four separate experiments. F, Cloned NK cells (1 × 10⁷/sample) were serum-starved overnight and incubated at 37°C with paraformaldehyde-fixed K562 cells (5 × 10⁶/sample). After the indicated times of incubation, the cells were lysed and LIMK1 was specifically immunoprecipitated using anti-LIMK1 polyclonal rabbit antisera or normal rabbit serum as a control. LIMK1 activity was analyzed in an in vitro kinase assay as described above. The levels of LIMK1 protein and GST-cofilin were assessed by Western blotting the same membrane with anti-LIMK1 polyclonal rabbit antisera and amido black staining, respectively.
shown to directly phosphorylate and activate the serine/threonine kinase LIMK1 (29, 30). Using rabbit polyclonal anti-p160ROCK and anti-LIMK1 antisera, we have found that both proteins are expressed in a variety of hematopoietic cell lines including cytotoxic lymphocytes (data not shown). Recently, a compound was identified, Y-27632, that inhibits p160ROCK kinase activity (29, 36). The specificity of Y-27632 has been shown and the IC50 is hundredsfold lower for p160ROCK than for other serine/threonine kinases, including protein kinase C, protein kinase A, and myosin L chain kinase (36). To determine whether LIMK1 undergoes FcR-initiated phosphorylation by p160ROCK, we immunoprecipitated LIMK1 from [32P]orthophosphate-labeled NK clones that had been left untreated or were pretreated with Y-27632. We observed a greater than 2.5-fold increase in the level of LIMK1 phosphorylation by 0.5 min of FcR cross-linking (Fig. 1B). FcR-initiated LIMK1 phosphorylation was absent in the NK clones that had been treated with Y-27632. Other FcR-initiated phosphorylation events, such as the formation of phospho-ERK (Fig. 1C), remained intact after treatment with Y-27632. Importantly, the increase in in vivo LIMK1 phosphorylation after FcR cross-linking correlated with a 2-fold increase in its kinase activity as measured by an in vitro kinase assay using GST-cofilin as a substrate (Fig. 1D). The activity of LIMK1 peaked around 10 min after FcR cross-linking and declined to basal levels between 15 and 30 min (Fig. 1D and data not shown). LIMK1 from Y-27632-treated NK clones contained little in vitro kinase activity (Fig. 1D). In addition, NK clones infected with the dominant-negative RhoA GTPase demonstrated little increase in LIMK1 activity after FcR cross-linking compared with the control virus (Fig. 1E). FcR-mediated LIMK1 activity was also diminished in NK clones that had been pretreated with C3-exoenzyme (data not shown). We also observed an increase in the activation of LIMK1 after the incubation of NK clones with the NK-sensitive K562 target cell line (Fig. 1F). In fact, there was more than a 2-fold increase in LIMK1 activity after 1 min of incubation with the K562 target cell line, with LIMK1 activity decreasing to basal levels by 5 min (Fig. 1F). Taken together, these data suggest that a RhoA/p160ROCK/LIMK1 pathway is activated in NK clones after triggering through the FcR and killer cell-activating receptors that regulate natural cytotoxicity toward the K562 target cell line.

To determine whether p160ROCK was required for the development of cell-mediated killing by cloned human NK cells, NK clones were incubated with increasing concentrations of Y-27632 for 1 h at 37°C. Preincubation of NK clones with Y-27632 resulted in a dose-dependent inhibition of the development of natural cytotoxicity toward the NK-sensitive K562 target cell line, with an IC50 between 5 and 10 μM (Fig. 2A). Similarly, the development of Ab-dependent cellular cytotoxicity, as measured in a reverse-Ab-dependent cellular cytotoxicity using the FcR-bearing P815 cell and anti-FcR mAb, was also inhibited (Fig. 2A). Natural cytotoxicity of Y-27632-treated NK clones toward other targets such as the B lymphoblastoid cell lines 721 and C1R was similarly inhibited (data not shown). Moreover, Y-27632 treatment of a human CD8+ cytotoxic T cell line showed a similar dose-dependent inhibition of cell-mediated killing of anti-CD3-coated P815 (data not shown). Importantly, high concentrations of Y-27632 did not affect cell viability as assessed by trypan blue exclusion (data not shown), ERK phosphorylation (Fig. 1C), other cellular events such as the formation of phospho-ERK (Fig. 1C), remained intact after treatment with Y-27632.

**FIGURE 2.** p160ROCK and LIMK1 regulate NK cell-mediated killing. A. Cloned NK cells (2 × 10⁶) were left untreated or were incubated with the indicated concentration of the p160ROCK inhibitor Y-27632 for 1 h at 37°C. The NK cells were then assayed for both natural cytoxicity as described above and FcR-mediated killing by incubating them with 51Cr-labeled P815 cells coated with 0.15 μg/ml anti-FcR mAb 3G8. Data are expressed in lytic units. This is a representative example of four separate experiments. B. Total mononuclear PBLs were left untreated or were treated with the indicated concentration of Y-27632 for 1 h at 37°C. The cytolytic function of the PBLs was assayed for both natural cytotoxicity and FcR-mediated killing as described above. This is a representative example of two separate experiments. C. Preincubation of NK clones with Y-27632 resulted in a dose-dependent inhibition of cell-mediated killing by cloned human NK cells, NK clones were incubated with increasing concentrations of Y-27632 for 1 h at 37°C. Preincubation of NK clones with Y-27632 resulted in a dose-dependent inhibition of the development of natural cytotoxicity toward the NK-sensitive K562 target cell line, with an IC50 between 5 and 10 μM (Fig. 2A). Similarly, the development of Ab-dependent cellular cytotoxicity, as measured in a reverse-Ab-dependent cellular cytotoxicity using the FcR-bearing P815 cell and anti-FcR mAb, was also inhibited (Fig. 2A). Natural cytotoxicity of Y-27632-treated NK clones toward other targets such as the B lymphoblastoid cell lines 721 and C1R was similarly inhibited (data not shown). Moreover, Y-27632 treatment of a human CD8+ cytotoxic T cell line showed a similar dose-dependent inhibition of cell-mediated killing of anti-CD3-coated P815 (data not shown). Importantly, high concentrations of Y-27632 did not affect cell viability as assessed by trypan blue exclusion (data not shown), ERK phosphorylation (Fig. 1C), other cellular events such as the formation of phospho-ERK (Fig. 1C), remained intact after treatment with Y-27632.
as conjugate formation (Fig. 3), or IFN-γ secretion (data not shown). Taken together, these data suggest a role for p160ROCK in the specific regulation of cell-mediated killing by cytotoxic lymphocytes.

To determine whether the effect of Y-27632 on the inhibition of killing by cloned NK cells was specific to activated cytotoxic lymphocytes, we incubated freshly isolated PBLs with increasing doses of Y-27632 and assayed their cytolytic potential. Compared with untreated PBLs, those treated with increasing doses of Y-27632 show a dramatic inhibition of natural cytotoxicity toward the K562 target cell line and killing initiated by the NK FcR (Fig. 2B). Interestingly, cell-mediated killing by PBLs was significantly more sensitive to Y-27632 than the cloned NK cells, demonstrating an IC₅₀ between 0.1 and 0.5 μM (Fig. 2B). This suggests that resting NK cells are more sensitive to inhibition of p160ROCK activity than are the activated clones (compare Fig. 2, A and B).

Although the above data using the Y-27632 inhibitor suggests that p160ROCK plays a fundamental role in the development of cellular cytotoxicity, we wanted to rule out any nonspecific inhibition of cytotoxicity by the drug. To do this, we generated a recombinant vaccinia virus expressing a truncated version of p160ROCK (ROCK.1081, amino acids 1081–1354) that has been previously shown to function as a dominant-negative mutant (35). We infected NK clones with either ROCK.1081-expressing vaccinia or the parental virus WR as a control. Significantly, overexpression of ROCK.1081 inhibits the development of both forms of cellular cytotoxicity compared with the WR control virus (Fig. 2C). The ability of ROCK.1081 to inhibit natural cytotoxicity toward K562 was not specific to this cell target because NK clones infected with ROCK.1081 were also inhibited in the killing of the NK-sensitive B-lymphoblastoid cell line 721 (data not shown). Moreover, overexpression of ROCK.1081 in CD8⁺ T cell lines inhibited killing initiated through the TCR (D. D. Billadeau and P. J. Leibson, unpublished observation). Taken together, the data based on pharmacological and genetic inhibition of p160ROCK identify p160ROCK as an effector molecule downstream of RhoA that is involved in the regulation of cell-mediated killing by cytotoxic lymphocytes.

To determine whether LIMK1 is involved in the regulation of cellular cytotoxicity, we generated recombinant vaccinia virus expressing FLAG epitope-tagged wild-type LIMK1 or a kinase-inactive version (LIMK1-KD). Significantly, when compared with control virus (WR) or overexpression of wild-type LIMK1, overexpression of LIMK1-KD inhibited the development of both natural cytotoxicity and killing initiated through the FcR (Fig. 2D). The expression of both LIMK1 and LIMK-KD were similar as determined by Western blotting. In addition, LIMK-KD was also found to inhibit TCR-mediated cellular cytotoxicity by cytotoxic T lymphocytes (D. D. Billadeau and P. J. Leibson, unpublished observation). Taken together, these data suggest that the RhoA-regulated p160ROCK/LIMK1 pathway is involved in the regulation of cell-mediated killing by cytotoxic lymphocytes.

**Inhibition of p160ROCK does not affect conjugate formation**

We have previously shown that one mechanism by which RhoA regulates the development of cell-mediated killing is partly through its influence on the formation of stable conjugates between the NK cell and the target cell (20). In an attempt to determine the mechanism by which p160ROCK influences the development of cellular cytotoxicity, we performed conjugate analyses using untreated NK clones or NK clones treated with 50 μM Y-27632. Treatment of NK clones with the p160ROCK inhibitor did not affect their ability to form conjugates (Fig. 3, left panel). However, the same Y-27632-treated NK clones were significantly inhibited in their ability to mediate natural cytotoxicity of the K562 target cell line (Fig. 3, right panel). These data suggest that the role of p160ROCK downstream of RhoA is at a site distal to the formation of stable effector-target conjugates.

**p160ROCK localizes to the effector/target interface during the generation of cell-mediated killing**

Initial experiments with cytochalasin D, a potent inhibitor of actin polymerization, indicated that rearrangement of the actin cytoskeleton was required for the polarization of lipid rafts to the site of target contact during the generation of cellular cytotoxicity (Z. Lou and P. J. Leibson, unpublished observation). To determine the intracellular distribution of p160ROCK and LIMK1 within isolated and target-engaged NK cells, we stained NK cells that had been incubated with the K562 target cell for p160ROCK or LIMK1 protein using specific polyclonal rabbit antisera. In addition, NK cells were incubated with CTxB-FITC to stain lipid rafts at the cell surface. Analysis of individual NK clones revealed a mostly cytoplasmic staining of p160ROCK, with some accumulation in the plasma membrane (Fig. 4A). In stark contrast, the majority of LIMK1 is associated with the NK plasma membrane, with very little protein staining within the cytoplasm (Fig. 4E). As has been previously shown (17), lipid rafts stained with FITC-CTxB polarize to the site of target contact (Fig. 4, C and G). Although the initial reaction with CTxB-FITC is at the cell surface, it appears that some components of the polarized lipid rafts become cytoplasmic, which would be consistent with ongoing endocytosis at the site of target contact. Interestingly, upon engagement of a susceptible target, there is a redistribution of p160ROCK from the cytoplasm to the area of contact between the NK clone and the K562 target cell that correlates with the redistribution of lipid rafts (Fig. 4, B–D). The increase in p160ROCK localization to the effector/target interface was not observed in control cells stained with normal rabbit serum (data not shown). In contrast to the redistribution of p160ROCK, there appears to be very little change in the amount of LIMK1 associated at the site of target contact (Fig. 4F). Taken together, these data suggest that p160ROCK is redistributed to the effector/target interface during the generation of cellular cytotoxicity, where it can interact with and activate the primarily plasma membrane-localized LIMK1.
The RhoA-p160ROCK-LIMK1 pathway regulates lipid-raft polarization during the development of cell-mediated killing

Because p160ROCK and lipid rafts localize to the interface between the NK and target cell during the generation of cell-mediated killing, we next sought to determine whether an activated RhoA/p160ROCK/LIMK1 pathway is required for the polarization of NK cell lipid rafts to the site of target recognition. To do this, we used a previously described technique for the visualization and quantitation of polarized lipid rafts (17). When visualized by fluorescence microscopy, the NK cell lipid rafts (FITC-CTxB-labeled, green fluorescence) can be visualized as well as the intracellularly labeled K562 target cell (hydroethidine, red fluorescence). NK cells that are not interacting with a target cell show a diffuse staining of lipid rafts throughout the plasma membrane (Fig. 5A). In contrast, when an NK cell forms a conjugate with a K562 target cell, there is a reorganization of the lipid rafts into “macroraf ts” at the interface between the NK cell and the K562 target cell (Fig. 5B). Again, there appear to be some lipid raft components endocytosed at the site of target contact.

To determine whether RhoA is involved in the regulation of lipid raft polarization during the development of cell-mediated killing, we incubated NK clones with C3-exoenzyme. Incubation of three separate NK clones with C3-exoenzyme dramatically decreased the number of conjugates containing polarized lipid rafts (Figs. 5C and 6A, left panel). To demonstrate the involvement of RhoA directly in this process, we infected NK clones with recombinant vaccinia virus expressing dominant-negative RhoA (F.N19RhoA), wild-type RhoA, or the control parental virus and assayed lipid raft polarization. NK clones overexpressing F.N19RhoA demonstrated a significant decrease in the percentage of conjugates with polarized lipid rafts compared with those over-expressing wild-type RhoA or the control-infected population (Figs. 5D and 6A, right panel). Importantly, when compared with uninfected cells, control-infected NK clones (WR) and NK clones infected with wild-type RhoA (F.RhoA.WT) displayed comparable levels of conjugates with polarized lipid rafts (Fig. 6A, right panel, and data not shown).

Because p160ROCK was also a key regulator of NK cellular cytotoxicity, we wondered whether the control of lipid raft polarization by RhoA was mediated by p160ROCK. To test this, we initially incubated NK clones with 50 μM Y-27632 and assayed lipid raft polarization (Fig. 5E). Treatment of NK clones with this concentration of Y-27632 severely diminishes the number of conjugates containing polarized lipid rafts (Fig. 6B, left panel). Importantly, the inhibition of lipid raft polarization observed using the pharmacological inhibitor of p160ROCK was also seen when NK clones infected with ROCK.1081 were assayed for lipid raft polarization (Figs. 5F and 6B, right panel). Lastly, we examined the role of LIMK1 in the regulation of lipid raft polarization during the generation of cell-mediated killing. Clearly, overexpression of LIMK1-KD led to a decrease in the number of conjugates with polarized lipid rafts, compared with the control-infected cells (Figs. 5G and 6C). Taken together, these data suggest a link between activation of the RhoA/p160ROCK/LIMK1 pathway and the regulation of lipid raft polarization during the development of cell-mediated killing.

Inhibition of the p160ROCK/LIMK1 pathway results in decreased actin polymerization at the effector/target interface

The observation that actin polymerization is required for lipid raft movement and the known role for the p160ROCK/LIMK1 pathway in the reorganization of the actin cytoskeleton led us to investigate the effect that inactivation of the p160ROCK/LIMK1 pathway would have on actin polymerization at the effector/target interface. First, we analyzed NK clones for F-actin content at the...
FIGURE 6. Inhibition of the RhoA/p160ROCK/LIMK1 signaling pathway abrogates lipid raft polarization to the effector-target interface. Cloned NK cells were left untreated, were treated overnight with 50 μg/ml C3-exoenzyme (A, left panel) or for 1 h at 37°C with 50 μM Y-27632 (B, left panel), or were infected with the indicated recombinant vaccinia virus (A, right panel; B, right panel; and C). In each experiment, lipid raft polarization was analyzed using K562 as the target. Lipid raft polarization was measured by fluorescence microscopy as described in Materials and Methods. The assay was performed with the individual scoring the polarized lipid rafts blinded to the identities of the samples, and only NK cell-target cell conjugates were scored for lipid raft polarization. One hundred conjugates were scored per slide, and the data are presented as the percentage of conjugates with polarized lipid rafts. The data presented are representative examples of at least three experiments performed using three separate NK clones.

FIGURE 7. Dominant-negative p160ROCK and LIMK1 affect F-actin polymerization at the effector/target interface. Cloned NK cells were analyzed for polymerized-actin content and lipid rafts as described in Materials and Methods. Images were recorded during confocal microscopy as follows: A, F-actin; B, lipid rafts; and C, merged images. D, NK clones were infected with the indicated recombinant vaccinia virus for 5 h at 37°C. In each experiment, F-actin polymerization was analyzed using K562 as the target and was measured as described in Fig. 5. One hundred conjugates were scored per slide, and the data are presented as the percentage of conjugates with polarized F-actin. The data presented are representative examples of three experiments performed using three separate NK clones.

dominate at the zone of interaction between the NK cell and the target cell.

Discussion

There is increasing evidence that lipid rafts are involved in the initiation and organization of signaling cascades downstream of activating receptors on lymphocytes and are primary sites of tyrosine phosphorylation (1, 2). Consistent with this, disruption of lipid rafts by sequestration or removal of cholesterol severely compromises initial activation events such as tyrosine phosphorylation and calcium mobilization downstream of activating receptors on lymphocytes (6, 11, 37, 38). We have recently shown that inactivation of Src- or Syk-family PTKs by pharmacological inhibitors or inhibition of PTK-activated signaling cascades by activation of the killer Ig-related receptor-associated SH2-domain containing phosphatase-1 blocks lipid raft polarization to the site of target contact (17). Taken together, these data suggest that there is a positive feedback loop between raft aggregation and signal propagation, with the initial triggering of lymphocytes through their activating receptors inducing a small-scale raft aggregation and initiation of signal transduction pathways. This initial small-scale activation could then lead to the movement of more rafts to the site of target contact, resulting in the formation of “macrorafths” and full-scale activation. This hypothesis suggests that movement of lipid rafts to the area of contact between the lymphocyte and target cell is an active signal-driven process requiring the initial activation of lipid rafts in the vicinity that would then begin an amplification process leading to the subsequent recruitment of other lipid rafts and their associated molecules. Our data highlight a role for the RhoA→ROCK→LIMK1 pathway in the regulation of lipid raft movement.
Recent data have implicated Vav-1, an upstream activator of Rac/Rho-family GTPases, as a critical regulator of actin-induced TCR capping (39). In addition, using T cells from mice carrying homozygous or heterozygous deletions of Vav-1, it was found that the concentration of class II MHC/TCR clusters at the T cell/APC interface was significantly diminished after stimulation with either superantigen or specific peptide/MHC II complexes (40). Moreover, pretreatment of T cells with C3-exoenzyme blocks both LFA-1 activation and inhibits sustained levels of intracellular calcium and the production of IL-2 in response to TCR cross-linking (41–43). Taken together, these data suggest that repositioning of cell surface molecules at the T cell/APC interface and the polarization of lipid rafts to the site of contact between the NK and target cell involve the regulation of the actin cytoskeleton.

Our data suggest that the regulation of the actin cytoskeleton by RhoA/p160ROCK/LIMK1 in cytotoxic lymphocytes is an important event in the development of cell-mediated killing and the polarization of lipid rafts to the effector/target interface. We have shown that LIMK1 is activated during natural cytotoxicity and upon FcR engagement and that this activation can be blocked by pretreatment of the NK clone with the p160ROCK inhibitor Y-27632 (Fig. 1, B and D–F). In addition, we have shown in NK clones that, in contrast to the p160ROCK protein, which appears to primarily cytosolic, LIMK1 is localized to the plasma membrane (Fig. 4E). This is interesting because previous analysis of LIMK1 localization in neurons and epithelial cells has shown a primarily cytoplasmic distribution with some nuclear staining (44). Localization of LIMK1 to the plasma membrane was also observed in the myeloid cell line K562 (Fig. 4F), but whether or not this is a common feature of cells of the hematopoietic lineage remains to be determined. The localization of LIMK1 to the plasma membrane in lymphocytes could allow a local activation of LIMK1 after the recruitment of p160ROCK to the effector/target interface that would serve to phosphorylate and activate LIMK1 locally to provide a polarized polymerization of F-actin at the cell-cell contact site. Significantly, we have observed an increase in F-actin content at the cell-cell contact area that can be decreased by inhibition of the p160ROCK or LIMK1 pathways.

Although there are no data implicating myosin activity in the control of cellular cytotoxicity, it is clear that the transport of receptors to the T cell immunological synapse requires myosin activity (45). Interestingly, ROCK has recently been found to both directly and indirectly influence myosin activity through the phosphorylation of an inhibitory site on the myosin-binding subunit of myosin phosphatase and through the phosphorylation of the regulatory L chain of myosin (28, 46). Therefore, it will be of interest to determine whether another mechanism by which ROCK controls lipid raft polarization and the development of cell-mediated killing is through its regulation of myosin.

The data presented in this paper, as well as our previous data, identify the RhoA signaling pathway as a critical regulator of cell-mediated killing by cytotoxic lymphocytes (20, 22). However, it is clear that other low-m.w. GTPases, such as Rac1 and CDC42, also control the generation of cellular cytotoxicity (D. D. Billadeau and P. J. Leibson, unpublished observation) (20, 47). However, neither the mechanism nor the effector molecules downstream of these GTPases involved in the regulation of these specific lymphocyte processes have been identified. Intriguingly, p21-activated kinase, an effector molecule of both Rac1 and CDC42, has recently been shown to directly phosphorylate and activate LIMK1 (48). However, whether or not Rac1 and CDC42 control the development of cell-mediated killing by activating a p21-activated kinase-LIMK1 pathway remains to be tested. Furthermore, the partial inhibition of F-actin polymerization at the site of target engagement that we observe by overexpression of dominant-negative p160ROCK and LIMK1 might be explained by the activation of other RhoA, Rac, or CDC42 effector molecules involved in the reorganization of the actin cytoskeletonal network. The identification of other Rho/Rac effector molecules in the regulation of cell-mediated killing remains to be determined.

We identify a previously undefined role for the RhoA–p160ROCK–LIMK1 pathway in the development of cell-mediated killing. Further characterization of other molecules downstream of RhoA and p160ROCK that are involved in the regulation of lipid raft polarization during the development of cellular cytotoxicity will be a key step in understanding how this pathway regulates such a critical lymphocyte function. Lastly, understanding how this pathway regulates not only the development of cellular cytotoxicity, but also the regulation of cytoskeleton-dependent activation of other cells within the immune system, may lead to the development of small molecule inhibitors that could be used for immunomodulation.

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References


