The Vav–Rac1 Pathway in Cytotoxic Lymphocytes Regulates the Generation of Cell-mediated Killing

By Daniel D. Billadeau,* Kathryn M. Brumbaugh,* Christopher J. Dick,* Renee A. Schoon,* Xose R. Bustelo,‡ and Paul J. Leibson*

*From the *Department of Immunology, Mayo Clinic and Foundation, Rochester, Minnesota 55905; and the ‡Department of Pathology, State University of New York at Stony Brook, University Hospital, Stony Brook, New York 11794-7025*

Summary

The Rac1 guanine nucleotide exchange factor, Vav, is activated in hematopoietic cells in response to a large variety of stimuli. The downstream signaling events derived from Vav have been primarily characterized as leading to transcription or transformation. However, we report here that Vav and Rac1 in natural killer (NK) cells regulate the development of cell-mediated killing. There is a rapid increase in Vav tyrosine phosphorylation during the development of antibody-dependent cellular cytotoxicity and natural killing. In addition, overexpression of Vav, but not of a mutant lacking exchange factor activity, enhances both forms of killing by NK cells. Furthermore, dominant-negative Rac1 inhibits the development of NK cell–mediated cytotoxicity by two mechanisms: (*a*) conjugate formation between NK cells and target cells is decreased; and (*b*) those NK cells that do form conjugates have decreased ability to polarize their granules toward the target cell. Therefore, our results suggest that in addition to participating in the regulation of transcription, Vav and Rac1 are pivotal regulators of adhesion, granule exocytosis, and cellular cytotoxicity.

Key words: natural killer cell • granule exocytosis • Vav • Rac1 • signal transduction

The Vav protooncogene, a multidomain protein expressed primarily in hematopoietic cells, is tyrosine phosphorylated after the cross-linking of a number of cell surface receptors. Although it was initially identified due to a mutation which enables it to transform fibroblasts (1, 2), it has become increasingly clear that Vav is an important second messenger used by many receptors on hematopoietic cells (3, 4). For example, Vav undergoes tyrosine phosphorylation after the cross-linking of many multisubunit immune recognition receptors, including the B cell antigen receptor, T cell antigen receptor, and Fc receptors (5–10). Both Src family and Syk family protein tyrosine kinases (PTKs)[1] are involved in Vav phosphorylation (11–13). Additional modular domains in Vav include two src homology (SH)3 domains, an SH2 domain, a pleckstrin homology domain, and a *dbl* homology domain, all of which potentially facilitate its interaction with other second messenger pathways (3, 4, 14). Recent analyses have focused on the ability of Vav, through its *dbl* homology domain, to act as a guanine nucleotide exchange factor (GEF) for the Rho family low molecular weight GTPase, Rac1 (12, 13, 15, 16).

The Rho family of GTP-binding (G) proteins Rho, Rac, and CDC42 has been implicated in regulating a number of cellular processes, including cytoskeletal alterations (17), transcription factor regulation (18–21), cell-cycle progression (22, 23), and cellular transformation (24–27). Using genetic and biochemical approaches, RhoA and CDC42 have been associated with a number of receptor-initiated events in hematopoietic cells, including FcεRI-mediated membrane ruffling in a basophilic leukemia cell line (28), FcR-mediated phagocytosis by macrophages (29, 30), IL-8 receptor–induced integrin adhesion in lymphocytes (31), and growth factor–dependent actin organization and cell adhesion in macrophages (32). Furthermore, inactivation of CDC42 in a $CD4^+$ T cell hybridoma inhibits the polarization of the microtubule organizing center (MTOC) toward the APC, and botulinum toxin–mediated inactivation of

[1]*Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; G protein, GTP-binding protein; GEF, guanine nucleotide exchange factor; KAR, killer cell–activating receptor; MTOC, microtubule organizing center; OV, recombinant vaccinia virus encoding oncogenic-Vav; PV, recombinant vaccinia virus encoding proto-Vav; PIP_2, phosphatidylinositol 4,5 bisphosphate; PTK, protein tyrosine kinase; SH, src homology; WR, wild-type vaccinia virus.

RhoA in cytotoxic T cells and NK cells inhibits natural cytotoxicity toward sensitive target cells (33, 34). These observations indicate that certain Rho family G proteins are involved in various specific leukocyte-mediated activation events. However, it is unclear how these G proteins are activated during receptor signaling, what GEFs are involved, and whether the specific Rho family member Rac1 plays any role in the generation of cell-mediated killing.

In this study, we have analyzed the role of Vav and its downstream target, Rac1, in the regulation of NK cell–mediated killing. NK cells represent a subpopulation of lymphocytes that mediate lysis of virus-infected and tumor cells through either natural cytotoxicity or FcγRIIIA-mediated antibody-dependent cellular cytotoxicity (ADCC; reference 35). Proximal signaling events initiated during ADCC and natural cytotoxicity include both Src and Syk family PTK activation (36–39). Vav is one of the proteins that becomes rapidly tyrosine phosphorylated after FcR ligation, but its regulatory role in the generation of ADCC or natural cytotoxicity remains unknown. We find that Vav is tyrosine phosphorylated after the incubation of NK cells with NK-sensitive target cells or with FcR-specific agonists. In addition, overexpression of Vav results in enhanced killing, whereas a mutation in Vav which has been shown to block its ability to mediate GTP for GDP exchange on Rac1 abrogates this enhancement. Also, overexpression of dominant-negative Rac1 inhibits NK cell–mediated cytotoxicity. NK cells expressing dominant-negative Rac1 have a decreased ability to form conjugates with targets, and those that do form conjugates have decreased ability to polarize their cytolytic granules toward the target cell. Together, our data highlight a regulatory role for Vav and Rac1 in the generation of cell-mediated killing.

Materials and Methods

Reagents, Antibodies, and Cells. Unless otherwise noted, reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies used in these studies included anti-Vav polyclonal rabbit antisera (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-FLAG murine mAb FLAG-M2 (Eastman Kodak Co., New Haven, CT), antiphosphotyrosine mAb 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), and goat anti–mouse IgG $F(ab')_2$ (ICN Biomedicals, Inc., Aurora, OH). Purified anti-CD94 mAb was isolated from the hybridoma HP-3B1 (provided by Miguel Lopez-Botet, La Princesa Hospital, Autonomous University of Madrid, Madrid, Spain) as described previously (40). Anti-FcγRIII mAb 3G8 (anti-CD16; reference 41) was purified by affinity chromatography over protein A–agarose. Cloned human NK cells were isolated from the defibrinated peripheral blood of healthy donors as described previously (42). The K562 human erythroid leukemia cell line and the murine mastocytoma cell line P815 were obtained from the American Type Culture Collection (Rockville, MD).

Recombinant Vaccinia Virus Generation and Infection. Proto-Vav (PV) and oncogenic-Vav (OV) cDNAs were excised from pCDNA3 using HindIII and NotI (12), and then directionally subcloned into the HindIII/NotI cloning site of pSHN11. pSHN11 is a derivative of the original vaccinia virus expression vector pSC11 (43). To tag the NH_2-terminal end of Vav with the FLAG amino acid sequence, a silent mutation was engineered into the internal NcoI site of proto-Vav using the mutagenic oligonucleotide 5′-CCCTGTGGTCGGCATGGGCAAGATTTCGC-3′, the pSHN11 selection oligonucleotide 5′-CGACGGGATCCCACGTGGAATTC-3′, and the transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA). Next, the 5′ NcoI site encompassing the Vav start site was used to add the FLAG amino acid sequence after digestion with NcoI and ligation of the phosphorylated and annealed NcoI FLAG-adaptor oligonucleotides 5′-CATGGACTACAAGGACGACGATGACAAGGC-3′ (+) and 5′-CATGGCCTTGTCATCGTCGTCCTTGTAGTC-3′ (−). The FLAG-tagged proto-Vav construct containing the C464S mutation (FLAG.PV.C529S) was generated by subcloning a Pst1/Not1 fragment from the OV.C464S construct into a Pst1/NotI-digested FLAG.proto-Vav vaccinia vector. cDNAs encoding wild-type rac1, N17-rac1, wild-type rhoA, and N19-rhoA were provided by J. Silvio Gutkind (National Institute of Dental Research, National Institutes of Health, Bethesda, MD; reference 18). The coding sequences were isolated from pCDNA3 using BamHI and NotI, blunted using Klenow fragment, and subcloned into the Sma1 cloning site of pSC11. The cDNAs within the recombinant pSHN11 and pSC11 vectors were then introduced into the WR strain of vaccinia via homologous recombination (44). Semipurified recombinant vaccinia virus was used to infect cloned human NK cells (2×10^6 cells/ml) for 1 h in serum-free medium at a multiplicity of infection of 20:1. The remainder of the infection (3–5 h) was carried out at 10^6 cells/ml in RPMI 1640 containing 10% bovine calf serum.

Cytotoxicity Assays. The ^{51}Cr-release assays were performed as described previously (42). In all cases, spontaneous release did not exceed 10% of maximum release. In redirected cytotoxicity assays, NK clones were only able to lyse P815 target cells when antibodies to specific NK cell triggering receptors were added. Lytic units were calculated based on 20% cytotoxicity (45).

Immunoprecipitations and Immunoblot Analysis. For experiments in which NK cells were activated by target cells, 5×10^6 cloned NK cells were briefly pelleted with 2.5×10^6 target cells and then incubated at 37°C for the indicated period of time. In experiments involving specific cell surface receptor cross-linking, 5×10^6 NK clones were incubated for 3 min on ice with either anti-FcR mAb (3G8, 10 μg/ml) or anti-CD94 mAb (HP-3B1, 30 μg/ml). Washed cells were then incubated with goat anti–mouse IgG $F(ab')_2$ at 37°C for the indicated period of time. After stimulation, the cells were lysed on ice for 10 min in 1 ml of buffer containing 20 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 0.1% BSA, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. Cellular debris was removed by centrifugation at 14,000 rpm for 5 min at 4°C. FLAG-Vav or endogenous Vav were immunoprecipitated from the lysate for 1–2 h at 4°C using 5 μg of anti–FLAG-M2 mAb bound to goat anti–mouse IgG–agarose beads, or 5 μg of anti-Vav rabbit antisera bound to protein A–Sepharose beads, respectively. Protein complexes were washed four times in wash buffer (lysis buffer lacking BSA). Bound proteins were then eluted in 40 μl of SDS-sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Tyrosine-phosphorylated proteins were detected using the 4G10 mAb, followed by sheep anti–mouse IgG coupled to horseradish peroxidase (Amersham International, Little Chalfont, Buckinghamshire, UK) and the ECL detection system (Amersham International). Vav was detected using specific rabbit antisera to Vav, followed by protein A–horseradish peroxidase (Amersham International) and the ECL detection system.

Conjugate Analysis. Quantification of effector–target conjugates was performed as described previously (46). In brief, NK cells were labeled intracellularly for 1 h at 37°C with 100 μM sulfofluorescein (Molecular Probes, Inc., Eugene, OR), and the K562 target cells were labeled intracellularly for 1 h at 37°C with 40 μg/ml of hydroethidine (Polysciences Inc., Warrington, PA). The washed cells were then resuspended at a concentration of 5×10^6 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 (simultaneous green and red fluorescence) was quantitated using a FACScan® (Becton Dickinson, San Jose, CA). Results are expressed as the percentage of total NK cells that formed conjugates.

Granule Polarization Assay. The cytoplasmic granules of infected NK cells were labeled with acridine orange (5 μl, 1 mg/ml; Polysciences Inc.) for 30 min at 37°C in the dark. The cells were washed four times in PBS containing 1% BSA and resuspended in the same buffer at a concentration of 2×10^7 cells/ml. K562 target cells were washed in PBS containing 1% BSA and then resuspended at a final concentration of 10^7 cells/ml. Equal volumes (50 μl) of effector and target cells were briefly pelleted and then incubated at 37°C in the dark for 10 min. The pellets were gently resuspended, and 30 μl was placed on each slide. NK cells that had formed conjugates were assessed for granule polarization using a fluoromicroscope (Carl Zeiss, Jena, Germany). A total of 100 conjugates was evaluated per slide, and the evaluation was performed by an individual blinded to the various treatment groups.

Results

Vav Is Tyrosine Phosphorylated during the Generation of Cellular Cytotoxicity. Src family and Syk family tyrosine kinases provide early and requisite signals during the generation of NK cell–mediated killing. However, it has been difficult to determine the precise identities of tyrosine-phosphorylated substrates that regulate natural killing because a single prototypic triggering molecule on the NK cell has not been identified. Consistent with a previous report (10), Vav becomes tyrosine phosphorylated after FcR cross-linking on NK cells (Fig. 1 *A*). However, since it has been previously observed that different intracellular signals can be required for the generation of ADCC and natural killing (47), we wanted to determine if Vav is activated during natural killing. To do this, we generated a recombinant vaccinia virus encoding a FLAG-tagged proto-Vav (FLAG-Vav), which we used to selectively infect the NK effector cells. This allows us to distinguish Vav protein derived from NK cells from that of NK-sensitive target cells. Human NK cells were infected with the recombinant FLAG-Vav virus and then incubated with the prototypic NK cell target, K562. The cells were lysed, and recombinant FLAG-Vav protein was specifically immunoprecipitated using the anti-FLAG mAb and analyzed for tyrosine phosphorylation. As shown in Fig. 1 *B*, Vav undergoes a time-dependent increase in tyrosine phosphorylation during the incubation with target cells, peaking at 2 min and decreasing to background levels by 10 min. This increase in Vav tyrosine phosphorylation is also seen when endogenous Vav is immunoprecipitated from [^{32}P]orthophosphate-labeled NK cells after target cell incubation, as determined by autoradiography and phosphoamino acid analysis (Billadeau, D.D., C.J. Dick, and P.J. Leibson, unpublished observation). Furthermore, Vav becomes tyrosine phosphorylated after the incubation with other NK-sensitive targets such as the class I–deficient B lymphoblastoid cell lines 721 and CIR (data not shown). To determine if Vav activation is linked to specific killer cell–activating receptors (KARs), we analyzed a human NK clone that undergoes activation and killing of target cells upon cross-linking of the CD94–NKG2 complex on the cell surface (48). Indeed, cross-linking of the CD94–NKG2-activating complex on these NK clones results in a time-dependent increase in Vav tyrosine phosphorylation, peaking between 1 and 2 min and decreasing to background levels by 5 min (Fig. 1 *C*). Together, these data suggest that the activation of Vav, as measured by tyrosine phosphorylation, appears to be an integral part of signaling pathways activated by FcR cross-linking, CD94–NKG2-activating receptor cross-linking, and natural cytotoxicity.

Vav Overexpression Enhances Cell-mediated Cytotoxicity. The above data identify Vav as a signaling molecule that is biochemically altered during cell-mediated killing, but its role in regulating this process is unclear. It is conceivable that the only role for Vav is the regulation of transcription factors required for gene expression after NK cell activation. However, it is also possible that it plays a more immediate role in the generation of the cytotoxic response itself. To test this possibility, we evaluated cytotoxicity using NK

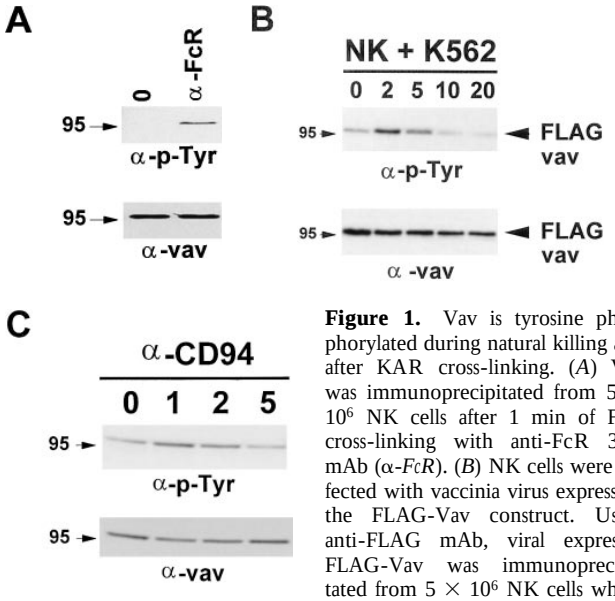

Figure 1. Vav is tyrosine phosphorylated during natural killing and after KAR cross-linking. (*A*) Vav was immunoprecipitated from 5×10^6 NK cells after 1 min of FcR cross-linking with anti-FcR 3G8 mAb (α-*FcR*). (*B*) NK cells were infected with vaccinia virus expressing the FLAG-Vav construct. Using anti-FLAG mAb, viral expressed FLAG-Vav was immunoprecipitated from 5×10^6 NK cells which had been incubated at 37°C for the indicated times (in minutes) with 2.5×10^6 K562 cells. (*C*) Vav was immunoprecipitated from 5×10^6 NK cells after cross-linking of the activating CD94–NKG2 complex for the indicated times (in minutes) at 37°C. In all cases, the immunoprecipitates were resolved by SDS-PAGE, transferred to a nylon membrane, and probed with either antiphosphotyrosine mAb (*upper panels*, α-*p-Tyr*) or anti-Vav polyclonal rabbit sera (*lower panels*, α-*vav*). This is a representative example from three separate experiments.

clones which had been infected with vaccinia virus expressing proto-Vav (PV), oncogenic-Vav (OV), or a mutant of proto-Vav (PV.C529S), which should putatively inhibit GEF activity (12). To determine if Vav is involved in regulating natural killing, we infected human NK clones with the different Vav-encoding recombinant vaccinia. Infection of NK clones with proto-Vav results in a significant increase in killing of the K562 target cells compared with cells infected with the wild-type virus alone (WR; Fig. 2 *A*, $P < 0.005$), whereas infection with oncogenic-Vav or the inactive proto-Vav mutant does not enhance natural killing compared with WR-infected clones (Fig. 2 *A*). In addition, using reverse ADCC and the Fc-bearing P815 target cell line, we found that Vav is involved in the regulation of cytotoxicity initiated through the FcR, and through the activating CD94–NKG2 complex on NK clones (Fig. 2, *B* and *C*). As observed with natural cytotoxicity, proto-Vav significantly enhanced killing initiated by either of these receptor complexes ($P < 0.005$ for each receptor), whereas expression of the inactive proto-Vav or oncogenic-Vav proteins had no effect (Fig. 2, *C* and *D*). The ability of proto-Vav to enhance cell-mediated cytotoxicity through natural killing or reverse ADCC was not unique to NK cells, as similar results were found after overexpression of the various Vav constructs in cloned human $CD8^+$ cytotoxic T cells using anti-CD3 stimulation in a reverse ADCC assay (Billadeau, D.D., and P.J. Leibson, unpublished observations). The ability of proto-Vav, but not the inactive proto-Vav, or oncogenic-Vav to enhance NK clone killing was not due to differences in protein expression levels, since all recombinant proteins were equivalently expressed as determined by immunoblotting (data not shown). Taken together, these data suggest that Vav is involved in regulating the NK cell cytolytic machinery during the generation of natural killing, FcR-mediated killing, and killing initiated by KAR cross-linking. Furthermore, since the oncogenic-Vav C464S (identical to C529S in proto-Vav) mutation has been previously shown to inhibit GTP→GDP exchange on Rac1, it is likely that Vav regulates, at least in part, cell-mediated cytotoxicity by enhancing Rac1 activation.

Rac1 Regulates NK Cell-mediated Cytotoxicity. Based on the observations that Vav is involved in regulating NK cell killing of target cells and that it is a known GEF for Rac1, we next investigated if Rac1 is involved in the generation of cell-mediated cellular cytotoxicity in NK cells. We assessed NK cell–mediated killing in NK clones infected with recombinant vaccinia virus expressing wild-type Rac1, RhoA, or dominant-negative versions of Rac1 (N17rac1) and RhoA (N19rhoA). It has previously been observed that inactivation of RhoA protein by ADP-ribosylation using C3 exoenzyme inhibits NK and CTL killing of target cells (33). Therefore, we used N19rhoA as a control for inhibition in our ^{51}Cr-release assays. Indeed, expression of N19rhoA in NK clones significantly inhibited killing of the K562 target cells during natural killing compared with WR ($P < 0.005$), whereas wild-type rhoA had no effect (Fig. 3 *A*). Expression of N17rac1 in NK clones also significantly inhibited killing of K562 (Fig. 3 *A*, $P < 0.005$) or killing initiated either through the FcR or the activating CD94–NKG2 complex (Fig. 3, *B* and *C*, $P < 0.005$ for both receptors). The inhibition of killing observed with dominant-negative Rac1 and RhoA was also observed in cloned human cytotoxic T cells after CD3 cross-linking in a reverse ADCC assay (Billadeau, D.D., and P.J. Leibson, unpublished observations). These data suggest that Rac1, one of the targets of Vav, regulates cell-mediated cytotoxicity initiated by a variety of "triggering" receptors.

Conjugate Formation between NK Clones and Target Cells Is Inhibited by Overexpression of Dominant-negative Rac1. The formation of a stable conjugate between an NK cell and its potential target cell is required for granule polarization and the generation of cell-mediated cytotoxicity. It is possible that dominant-negative Rac1 is affecting the ability of the NK cell to kill the target by interfering with formation of stable conjugates or by inhibiting granule polarization. To determine if NK clones expressing dominant-negative Rac1 were affected in their ability to form conjugates, we labeled uninfected or infected NK cells intracellularly with sulfofluorescein, and labeled K562 target cells intracellularly with hydroethidine. Using two-colored flow cytometry, we analyzed the NK cells (green fluorescence) for their ability to form conjugates with K562 (red fluorescence). Conjugates were scored based on simultaneous emission of both green and red fluorescence, and the results are ex-

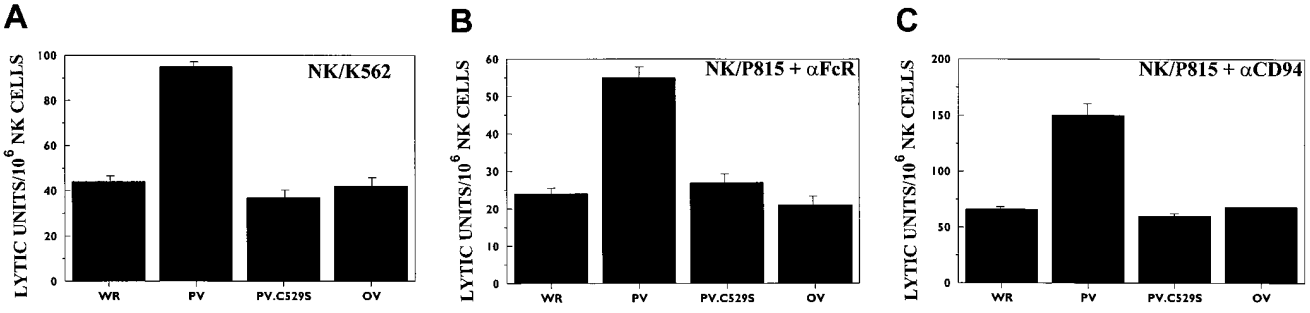

Figure 2. Vav is involved in cell-mediated cytotoxicity. NK clones were infected with the indicated recombinant vaccinia virus. Infected NK clones were incubated with ^{51}Cr-labeled K562 (*A*), P815 cells coated with 0.15 μg/ml of the anti-FcR mAb 3G8 (*B*), or P815 cells coated with 0.15 μg/ml of the anti-CD94 mAb HP-3B1 (*C*). This is a representative example of eight separate experiments. Data are expressed as lytic units/10^6 cells ± 1 SD.

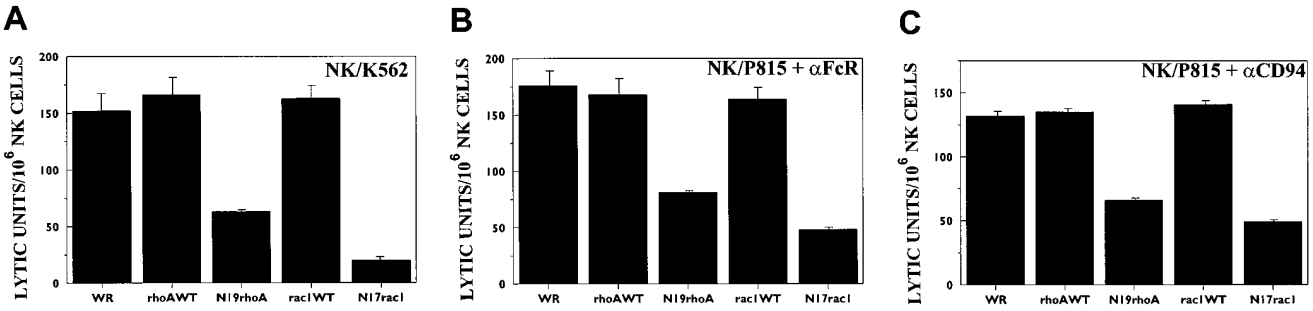

Figure 3. Rac1 regulates cell-mediated cytotoxicity. NK clones were infected with the indicated recombinant vaccinia virus. Infected NK clones were incubated with ^{51}Cr-labeled K562 (*A*), P815 cells coated with 0.15 μg/ml of the anti-FcR mAb 3G8 (*B*), or P815 cells coated with 0.15 μg/ml of the anti-CD94 mAb HP-3B1 (*C*). This is a representative example of eight separate experiments. Data are expressed as lytic units/10^6 cells ± 1 SD.

pressed as the percentage of total NK cells that formed conjugates. In all experiments, ^{51}Cr-release assays were performed as controls to measure inhibition and enhancement of killing by the infected NK clones (data not shown). Infection of NK clones with wild-type vaccinia virus (WR) did not significantly affect their ability to form conjugates compared with uninfected cells (Fig. 4, and data not shown). Although we consistently observed an enhanced killing of target cells by NK clones overexpressing proto-Vav (see Fig. 2, *A–C*), we never observed a significant increase in the percentage of NK clones forming conjugates compared with cells infected with WR (see Fig. 4). However, we did consistently observe a significant decrease in the number of conjugates formed by NK cells overexpressing the dominant-negative N17rac1. RhoA has previously been implicated in influencing LFA-1–dependent adhesion (31). Therefore, as a positive control, we also tested the influence of dominant-negative N19rhoA. Similar to N17rac1, N19rhoA significantly inhibited conjugate formation (Fig. 4). These data suggest that at least for N17rac1 and N19rhoA, one mechanism by which they inhibit NK clone killing of a target cell during natural killing is by decreasing the formation of a stable effector to target cell interaction.

NK Cells Expressing Dominant-negative Rac1 Have a Decreased Ability to Polarize Granules after Conjugate Formation. Directed delivery of granule components to the target cell is controlled by actin polymerization and formation of an MTOC. Since Rac1 and RhoA have been implicated as major regulators of the actin cytoskeleton in other cell types, it is possible that N17rac1 and N19rhoA may also be working at the level of granule polarization to inhibit killing. In addition, proto-Vav, which we have consistently found to significantly enhance killing, does not appear to enhance conjugate formation but may be involved in enhancing granule polarization. Therefore, we analyzed the effects of these proteins on granule polarization, as this is a requisite step in cell-mediated cytotoxicity. Since neither N17rac1 nor N19rhoA completely blocks conjugate formation, we directly analyzed NK cells and target cells that had formed conjugates to determine if they had polarized their granules toward the target cell. Acridine orange is a weak base that can cross plasma membranes and is taken up rapidly into acidic granules. Once inside NK cells, this fluorochrome becomes protonated and trapped within the cytolytic granules, where it fluoresces red (49). The fluorescent granules can be visualized within the cells using fluorescence microscopy, and the position of the granules within the cell in relation to the target cell can be observed. In nonconjugated NK cells, the granules are located diffusely throughout the cytoplasm. However, once a stable conjugate is formed and the cytolytic machinery is activated, the MTOC forms, and the granules become positioned adjacent to the target cell. The upper panel of Fig. 5 shows a confocal microscopic image of an acridine orange–labeled NK clone conjugated to the K562 target cell in which the granules of the NK cell are not polarized toward the target, but remain diffusely located throughout the cytoplasm. The lower panel of Fig. 5 shows a conjugate where the granules of the NK cell have polarized toward the target cell. To determine if the dominant-negative Rac1 and RhoA proteins are affecting granule polarization, we infected NK clones with the recombinant viruses indicated in Table 1, labeled their cytolytic granules with acridine orange, incubated them with the K562 target cell, and then using fluorescence microscopy scored the number of

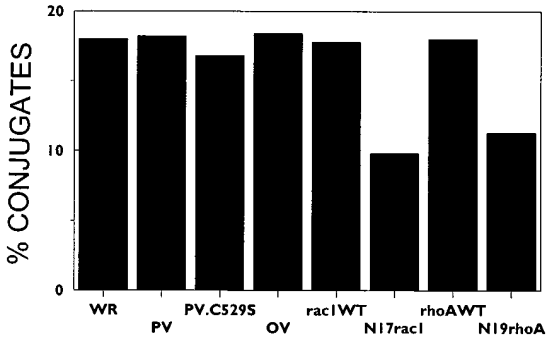

Figure 4. Dominant-negative Rac1 inhibits conjugate formation. NK clones infected with the indicated recombinant vaccinia virus were stained intracellularly with sulfofluorescein (green fluorescence), and then incubated for 10 min at 37°C with K562 target cells which had been stained intracellularly with hydroethidine (red fluorescence). Using flow cytometry, the percentage of NK cells forming conjugates were scored based on the simultaneous emission of both green and red fluorescence. 10^4 events were analyzed per sample. The data presented are a representative example of five different experiments.

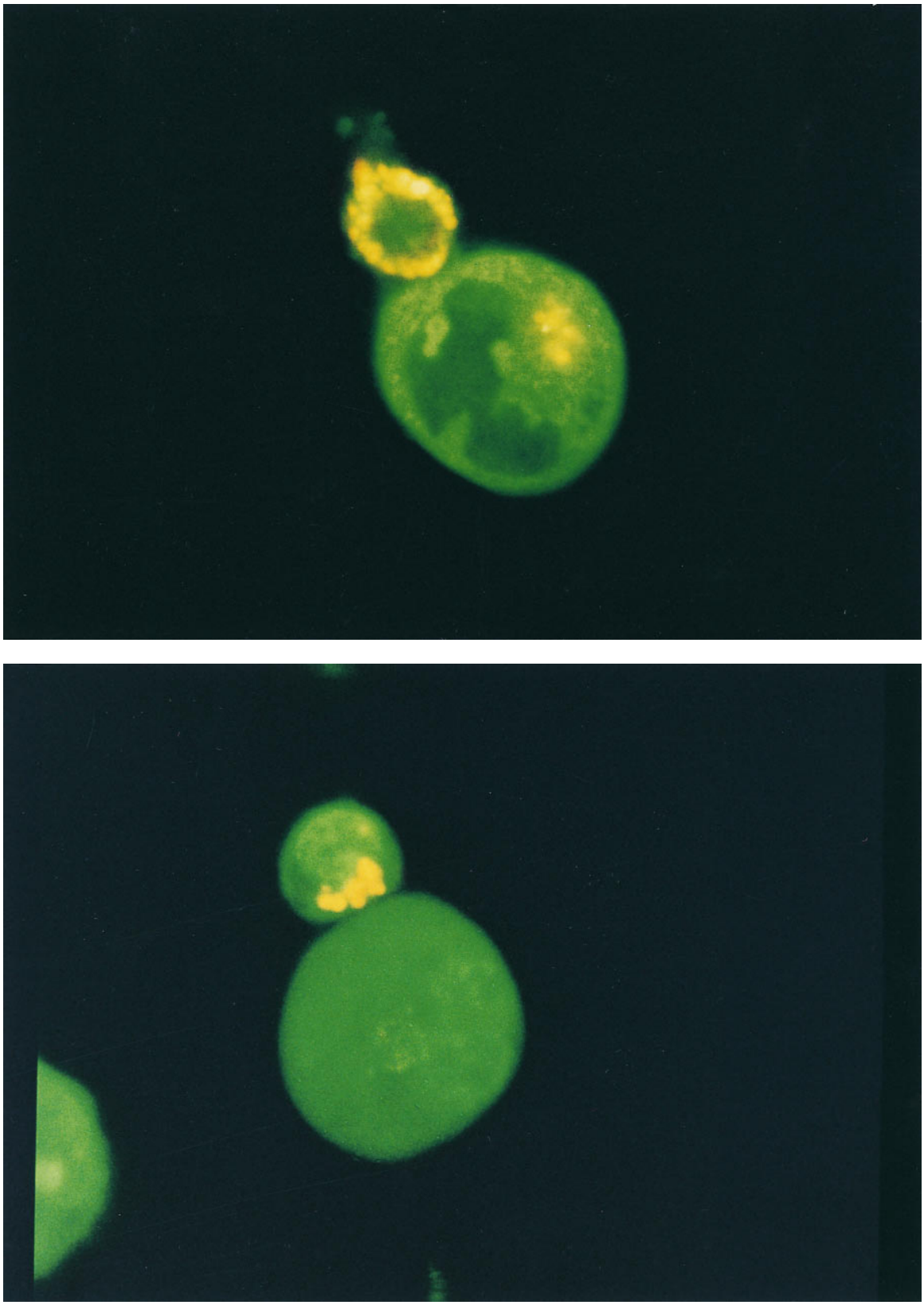

Figure 5. Visualization of acridine orange–stained granules in NK cells by fluorescence microscopy. Acridine orange–labeled NK clones were incubated with K562 target cells as described in Materials and Methods. After this incubation, the cells were placed on a slide, and the fluorescent granules were observed using fluorescence microscopy. (*Top*) Representative example of a conjugate in which the cytolytic granules of the NK cell have not polarized with respect to the K562 target cell. (*Bottom*) Conjugate in which the granules in the NK cell have been polarized toward the K562 target cell. Scale: 0.1 μM/1 pixel; picture width = 512 pixels).

Table 1. *NK Cell Granule Polarization Is Regulated by Vav and Rho Family G Proteins*

NK cell infection with recombinant vaccinia virus	Exp. 1	Exp. 2	Exp. 3
		*polarized/total**	
WR	58/100	52/100	51/100
PV	82/100	77/100	76/100
PV.C529S	53/100	54/100	48/100
N17rac1	27/100	28/100	22/100
N19rhoA	31/100	35/100	29/100

Exp., Experiment.
*Polarized to total number of conjugates counted.

conjugates containing polarized granules. Simultaneously, ^{51}Cr-release assays were performed as a measure of enhancement and inhibition of killing by the infected NK clones (data not shown). In each experiment (Table 1), we observed an increase in granule polarization of NK clones overexpressing proto-Vav. This parallels our finding that proto-Vav overexpression enhances cell-mediated cytotoxicity (see Fig. 2, *A–C*). Similar to our findings in ^{51}Cr-release assays, expression of PV.C529S abrogates granule polarization compared with proto-Vav, suggesting that it requires its GEF activity in order to enhance killing. Interestingly, overexpression of N17rac1 and N19rho results in a decrease in granule polarization of NK cells that have formed conjugates compared with WR, which is consistent with their ability to inhibit killing (see Fig. 3 *A*). These data suggest that Vav and Rac1 can potently regulate cell-mediated killing by influencing granule polarization in NK cells which have bound to susceptible targets.

Discussion

The killing of virus-infected or tumor target cells by CTLs is a tightly regulated process involving receptor–ligand interactions between the CTL and the target cell, and the subsequent activation of multiple biochemical signaling pathways. In this study, we have provided both biochemical and genetic evidence that the Vav–Rac1 pathway regulates cell-mediated killing by CTLs. We found that the GEF Vav is tyrosine phosphorylated during ADCC and natural killing, and that overexpression of proto-Vav enhances cell-mediated killing, whereas expression of inactive proto-Vav or oncogenic-Vav has no effect on the killing of target cells. Vav has been previously shown to facilitate Rac1 activation (12). This suggests that Rac1 is a potential downstream target involved in the regulation of cell-mediated killing. Indeed, overexpression of dominant-negative Rac1 inhibits killing of target cells during ADCC and natural cytotoxicity. Furthermore, we show that overexpression of dominant-negative Rac1 abrogates conjugate formation, and that those NK cells that do form conjugates have a decreased ability to polarize their cytolytic granules toward the target cell. Taken together, our data highlight a novel role for the Vav–Rac1 pathway that is distinct from its known role as a regulator of transcriptional activation.

It is clear that the proximal activation of PTKs is a critical and requisite step in the development of cell-mediated cytotoxicity (37–39), and that Vav is phosphorylated by both Src and Syk family PTKs after the cross-linking of many multisubunit immune receptors (11–13, 50, 51). In NK cells, we have found that Vav is a target of activated PTKs during natural killing as well as cross-linking of the activating CD94–NKG2 complex and FcγRIIIA (see Fig. 1, *A–C*), but the precise identity of the PTK(s) involved in its phosphorylation remains unknown. NK cells express many Src family PTKs, including Lck, Fyn, and Lyn, as well as both Syk family PTKs, ZAP-70 and Syk. Both Src and Syk family kinases are activated after cross-linking of FcγRIIIA (52–57) and CD94–NKG2 (40, 48), and we have recently shown that Syk is activated during natural killing (36). Among the predicted Vav SH2 domain binding sites (58), there are YESP motifs in Syk at position Y348 and in ZAP-70 at position 315. These tyrosines are required for the physical interaction of the Vav SH2 domain with the Syk family PTKs and also for the activation of Vav (50, 59–61). Since Syk is activated during natural killing and ADCC, it might be predicted that mutation of Y348F in Syk would impact Vav activation. Furthermore, mutation at the critical amino acid R696 in the Vav SH2 domain, which has been shown to inhibit Vav tyrosine phosphorylation (62) and subsequent Vav activity (59, 60), should negatively impact cell-mediated cytotoxicity when overexpressed in NK cells. Additional studies will be needed to test these predictions.

Our observation that overexpression of Vav results in enhanced cell-mediated cytotoxicity parallels results obtained in T cells where overexpression of Vav results in a synergistic increase in nuclear factor of activated T cells (NFAT)-dependent and NFIL-2–dependent transcription after TCR cross-linking (63). However, although expression of oncogenic-Vav, which is missing the first 65 amino acids at the NH_2 terminus of the protein, is oncogenic in NIH 3T3 fibroblasts (1), its expression does not result in a synergistic increase in transcriptional activation (63). Similarly, we found that expression of oncogenic-Vav in NK clones does not enhance NK cell killing (Fig. 2, *A–C*), suggesting that the NH_2 terminus of Vav is somehow required to get full Vav activity in normal hematopoietic cells. It has been reported that the NH_2-terminal region of Vav contains a potential helix-loop-helix domain followed by a leucine zipper (1, 2). However, recent protein alignment studies have suggested that this domain is more similar to a domain found in the F-actin binding protein, calponin (64). This is intriguing, as Vav has been found to associate with other proteins involved in cytoskeletal organization, such as tubulin (65) and zyxin (66). We have observed that both proto-Vav and oncogenic-Vav become tyrosine phosphorylated in a similar time-dependent fashion after FcR cross-linking (Fig. 1 *A*,

and data not shown). Therefore, it is possible that the interaction of Vav with cytoskeletal constituents after its activation might serve to localize Vav in a particular compartment where it can then activate its downstream effectors such as Rac1. Furthermore, since Vav has been found to associate with a number of signaling molecules through its SH2, SH3, and proline-rich regions (for a review, see reference 3), it is possible that incorrect localization of these proteins when bound to oncogenic-Vav results in nonfunctional signaling complexes. Interestingly, as shown in Fig. 2, *A–C*, overexpression of PV.C529S, which putatively lacks GEF activity (12), does not lead to enhanced target cell killing. Taken together, these results suggest that in order for Vav to enhance killing, it must have GEF activity, and it must retain structural determinants in its NH_2 terminus to be correctly localized during its activation.

The importance of Vav in T and B cell antigen receptor–mediated signal transduction was previously defined using the recombination activating gene (RAG)-$1^{-/-}$ blastocyst complementation technique and Vav-deficient embryonic stem cells (67–69). Those authors observed that in the absence of Vav, there was an overall decrease in the number of peripheral T cells and subpopulations of B cells, and both mature populations of cells had severe defects associated with antigen receptor signaling. The role of Vav in other cells of the immune system was not evaluated due to the observed embryonic lethality of these Vav-deficient mice (67–69). However, recent experiments have demonstrated that Vav-deficient mice are viable, and that Vav is involved in both the positive and negative selection of thymocytes (70). Data on NK cell function in these Vav-deficient mice have not been reported. Interestingly, in response to TCR stimulation, Vav-deficient thymocytes have a decreased ability to mobilize intracellular Ca^{2+} (70), a critical second messenger required for NK cell–mediated killing by ADCC and natural killing (42, 71). Furthermore, it has been demonstrated that the targets of Vav activation, the Rho family of GTPases, activate phosphatidylinositol 4-phosphate 5-kinase, leading to an increase in phosphatidylinositol 4,5 bisphosphate (PIP_2; references 72–74). PIP_2 is a known substrate for phospholipase C, which is activated during FcR cross-linking on NK cells (56, 75), and results in the subsequent release of intracellular Ca^{2+} after the generation of inositol 3,4,5 trisphosphate. Additionally, PIP_2, which has been shown to interact and modulate the function of a number of cytoskeleton-associated proteins (76–78), may directly influence cytoskeletal reorganization during the generation of cell-mediated killing. Data on phosphoinositide metabolism and calcium signaling in NK cells with impaired Rho family G protein function have not been reported.

The Rho family of G proteins was initially identified as regulating the cytoskeleton, including the formation of stress fibers, membrane ruffles, filopodia, and lamellipodia (for a review, see reference 17). Our data clearly indicate that in CTLs, Rac1 is involved in the regulation of granule exocytosis initiated by cross-linking of activating receptors (see Fig. 3, *A–C*). It was previously observed using pharmacologic inhibition of RhoA with C3 exoenzyme that RhoA is involved in the generation of cell-mediated killing (33). Using a genetic approach, we found that expression of a dominant-negative form of RhoA (N19rhoA) also inhibits killing elicited through activating receptors. In addition, although we have not tested whether CDC42 is involved in NK cell–mediated killing, it has been shown in a T cell line that expression of dominant-negative CDC42 inhibits MTOC formation after binding to APCs (34). These data suggest that in lymphocytes, cytoskeletal rearrangements and granule exocytosis are controlled in part through the activation of multiple Rho family members (i.e., Rac1, CDC42, and RhoA). However, it remains unclear whether these proteins serve overlapping functions during granule exocytosis, or if they regulate separate portions of the involved signaling pathways. Indeed, different Rho family members have been observed to control various aspects of the actin cytoskeleton and also to influence activation of the other family members (79, 80).

The Rho family of G proteins has been shown to regulate events in leukocytes that require rearrangement of the cytoskeletal network, including FcR-induced phagocytosis by macrophages (29, 30), degranulation in mast cells (28), and monocyte spreading (81, 82). Interestingly, inhibition of RhoA function in macrophages with C3 exoenzyme inhibits Ca^{2+} signaling through the FcR, and the clustering of receptors in response to opsonin, both of which are critical steps in FcR-induced signaling and phagocytosis (30). Moreover, cadherin is a Ca^{2+}-dependent adhesion molecule involved in cell–cell interactions, and its ability to interact with the actin cytoskeleton is inhibited by expression of dominant-negative Rac1 and RhoA (83). Cell–cell contacts and the aggregation of activated receptors are required for the development of cell-mediated cytotoxicity. Indeed, humans and mice lacking the β2-containing integrin LFA-1 (CD11a/CD18) have deficient NK cell function (84, 85). Although we observed a significant inhibition of conjugate formation in NK cells expressing N17rac1 and N19rhoA, we did not observe a significant increase in conjugate formation in cells overexpressing proto-Vav (see Fig. 4). It is possible that although expression of the inactive proteins does not completely interfere with conjugate formation, it compromises the quality of the cell–cell contact and thereby influences the development of cell-mediated killing. The granule polarization studies demonstrate that conjugates are formed with cells expressing the dominant-negative proteins, but the majority of these cells fail to polarize their granule toward target cells (see Table 1). In addition, whereas overexpression of proto-Vav leads to an increase in cell-mediated killing, it has no impact on conjugate formation. However, the data from Table 1 suggest that overexpression of proto-Vav results in an increase in the number of conjugates in which the NK cells have polarized their granule toward the target cell. Therefore, it is possible that proto-Vav does not increase the overall adhesion between the two cells, but influences more directly the machinery involved in granule polarization.

Our data suggest that the GEF Vav, a target of PTKs after activation through a variety of cell-surface receptors on

NK cells, and its target, Rac1, are involved in the regulation of granule exocytosis in CTLs. These results suggest a novel role for Vav distinct from its ability to act as a regulator of transcription factors. In addition, our results provide insight into how the engagement of ligands on the cell surface of CTLs can control the development of cell-mediated killing through the activation of Rho family GTPases. This experimental system can now be used to evaluate proximal interactions influencing Vav–Rac1 activation and the downstream effectors critical for the generation of effective cell-mediated cytotoxicity.

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Address correspondence to Paul J. Leibson, Department of Immunology, Mayo Clinic, 200 First St., Rochester MN 55905. Phone: 507-284-4563; Fax: 507-284-1637; E-mail: leibson.paul@mayo.edu

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