Rab27a Is Required for Regulated Secretion in Cytotoxic T Lymphocytes✪

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Abstract. Rab27a activity is affected in several mouse models of human disease including Griscelli (*ashen* mice) and Hermansky-Pudlak (*gunmetal* mice) syndromes. A loss of function mutation occurs in the Rab27a gene in *ashen* (*ash*), whereas in *gunmetal* (*gm*) Rab27a dysfunction is secondary to a mutation in the α subunit of Rab geranylgeranyl transferase, an enzyme required for prenylation and activation of Rabs. We show here that Rab27a is normally expressed in cytotoxic T lymphocytes (CTLs), but absent in *ashen* homozygotes (*ash/ash*). Cytotoxicity and secretion assays show that *ash*/*ash* CTLs are unable to kill target cells or to secrete granzyme A and hexosaminidase. By immunofluorescence and electron microscopy, we show polarization but no membrane docking of *ash*/*ash* lytic granules at the immunological synapse. In *gunmetal* CTLs, we show underprenylation and redistribution of Rab27a to the cytosol, implying reduced activity. *Gunmetal* CTLs show a reduced ability to kill target cells but retain the ability to secrete hexosaminidase and granzyme A. However, only some of the granules polarize to the immunological synapse, and many remain dispersed around the periphery of the CTLs. These results demonstrate that Rab27a is required in a final secretory step and that other Rab proteins also affected in *gunmetal* are likely to be involved in polarization of the granules to the immunological synapse.

Key words: Rab27a • cytotoxic T lymphocyte • secretory lysosomes • immunological synapse • Arp2/3

Introduction

Cytotoxic T lymphocytes (CTLs)[1] destroy their target cells by regulated secretion from specialized secretory lysosomes known as lytic granules. Several other cell types possess secretory lysosomes, most of which are derived from the hemopoietic lineage. Several lines of evidence support the idea that the mechanisms regulating exocytosis of these lysosomal secretory compartments differ from those controlling conventional secretory granules (for review see Stinchcombe and Griffiths, 1999). The most compelling evidence comes from our previous studies on the immunodeficiency, Chediak-Higashi syndrome (CHS) in which we showed that the defect in CTL killing was due to a defect in lytic granule exocytosis (Baetz et al., 1995). The selective hemopoietic defects observed in CHS support the idea that these cells use a different mechanism of secretion than cells with conventional secretory granules (Griffiths, 1996). Curiously, CHS patients and the *beige* mouse model of the disease also show partial albinism, indicating that melanosome secretion is also impaired. Several other inherited syndromes exist which also demonstrate selective immunodeficiencies and pigmentary defects, including Hermansky-Pudlak (Boissy and Nordlund, 1997; Shotelersuk and Gahl, 1998) and Griscelli (Griscelli et al., 1978; Klein et al., 1994) syndromes.

The regulated secretion of the lytic granules from CTLs is triggered by T cell receptor (TCR) recognition of a target cell. Recognition results in kinesin-driven lytic granule movement along microtubules to the point of membrane contact and release of perforin and granzymes which trigger rapid destruction of the target (for review see Griffiths, 1995). The microtubule organizing center and the Golgi complex polarize toward this contact site and the cytosolic protein, talin, which can link membrane proteins to the actin cytoskeleton, accumulates at the synapse between the two cells (Kupfer et al., 1985, 1986). This "immunological synapse" has been extensively studied in $CD4^+$ T cells, conjugated to their antigen presenting cells. Proteins involved in this interaction segregate in a highly organized three-dimensional structure with TCRs clustered in the center surrounded by a ring of talin (Monks et al., 1998). The recruitment of Wiskott-Aldrich syndrome protein (WASP) and the Arp2/3 complex of proteins in re-

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[1]*Abbreviations used in this paper:* CHS, Chediak-Higashi syndrome; CTL, cytotoxic T lymphocyte; IMDM, Iscove's modified Dulbecco's medium; RGGT, Rab geranylgeranyl transferase; TCR, T cell receptor; WASP, Wiskott-Aldrich syndrome protein.

sponse to TCR activation are also thought to play a key role in the actin rearrangement seen at the synapse (Krause et al., 2000). It is not known whether a similar organization exists between CTLs and their targets and, if so, where the granules interact within the synapse.

Expression of the Ras-like GTPase Rab27a is particularly high in spleen, platelets, and melanocytes, consistent with a potential role in secretory lysosome secretion (Seabra et al., 1995; Chen et al., 1997). Rab27a has been found recently to be defective in patients with Griscelli syndrome and associated with impaired CTL-mediated killing (Menasche et al., 2000). Most if not all mutations reported to date in the *RAB27A* gene are predicted to result in absent expression of functional protein. In the corresponding mouse model, *ashen*, a splice site mutation is predicted to abolish or severely diminish Rab27a expression (Wilson et al., 2000). Rab27 function is also affected in *gunmetal*, a model for Hermansky-Pudlak syndrome. The mutation in *gunmetal* has been identified as a splice site mutation in the α subunit of Rab geranylgeranyl transferase (RGGT) (Detter et al., 2000). This mutation results in an 80% reduction in RGGT activity and a decrease in prenylation of a subset of Rab proteins in platelets, including Rab27a (Detter et al., 2000). Since prenylation is required for membrane attachment of Rab GTPases (Seabra, 1998), the reduction in prenylation of Rab27a will result in a reduction or loss of activity of Rab27a in these mice. Other Rab proteins are affected by this mutation (Swank et al., 1993; Detter et al., 2000), although they have yet to be identified.

Rab27a is therefore a strong candidate as a regulator of lysosome secretion. To address this possibility, we have examined CTL secretion and the polarization of lytic granules at the immunological synapse in these two natural mouse mutants in which Rab27a activity is affected. Our findings demonstrate an important role for Rab27a in the final stages of secretion and indicate that other Rab proteins may be involved in granule polarization.

Materials and Methods

Mice

Gunmetal mice (C57BL/6-*gm*/*gm*)were kindly supplied by Richard Swank (Roswell Park Cancer Institute, Buffalo, NY) (Detter et al., 2000). *Ashen* mice (C3H/HeSn-*ash/ash*) were purchased from The Jackson Laboratory. All were maintained and propagated under UK project license 70/5071 at the Central Biomedical Services of Imperial College, London.

Antibodies and Cells

The rabbit antiserum against cathepsin D was the generous gift of S. Kornfeld (Washington University, St. Louis, MO), the rat monoclonal recognizing granzyme A (7.1) was a gift from M. Simon (Max-Planck-Institute, Freiburg, Germany) (Fruth et al., 1987), and mouse monoclonal antibodies against talin were from Sigma-Aldrich. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories unless otherwise indicated. The rabbit antiserum against p34-Arc of the Arp2/3 complex was generated against a COOH-terminal peptide as described (Machesky et al., 1997) and affinity purified using the antigenic peptide cross-linked to Sepharose beads (Amersham Pharmacia Biotech).

Cell Culture

CTLs were derived by activation of 2.5×10^6 splenocytes from effector strains (C57Bl/6, *+/ash*, *ash/ash*, *+/gm*, and *gm/gm*) with 2.5×10^6 irradiated (3,000 rads) stimulator splenocytes from BALB/C mice in 10 ml Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS and 2-mercaptoethanol. After 5 d in culture at 37°C with 5.5% CO_2, CTLs were purified over Ficoll 1096 (Sigma-Aldrich), washed three times, and either assayed for lysis as described below or resuspended in culture medium supplemented with 100 U/ml interleukin 2. Cells were restimulated every 7–10 d for continued passage in culture for up to 7 wk. P815 mouse target cells were maintained in RPMI/10% FCS.

Immunoblotting

Frozen cell pellets were thawed, resuspended in lysis buffer (50 mM Hepes, pH 7.2, 10 mM NaCl, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml aprotinin and 5 μg/ml leupeptin) (Sigma-Aldrich), and mechanically disrupted by serial passages through a 21-gauge needle. The protein concentration of this homogenate was determined using Coomassie Plus Protein Assay Reagent (Pierce Chemical Co.). Equal amounts of total protein (13 μg) were subjected to SDS-PAGE on 12.5% acrylamide gels and transferred to PVDF membranes using a Hoeffer transfer apparatus (2 h at 500 mA). The membranes were allowed to dry and then blocked with 5% skimmed milk in PBS with 0.2% Tween 20 (PBS_T) for 1 h at room temperature. The membranes were then incubated with monoclonal anti–rat Rab27a antibody, 4B12 (Hume et al., 2001) or polyclonal anticalnexin (1:5,000; StressGen Biotechnologies) antibodies diluted in PBS. After washing, blots were incubated with either goat anti–rabbit or sheep anti–mouse peroxidase-labeled secondary antibodies (Dako and Amersham Pharmacia Biotech, respectively), diluted in blocking buffer, and developed using the Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) according to the manufacturer's directions.

In Vitro Prenylation Reactions and Immunoprecipitation after Prenylation

In vitro prenylation assays were performed essentially as described previously (Seabra et al., 1995; Detter et al., 2000). In brief, CTL lysates were prepared as for immunoblotting and spun at 100,000 *g* for 1 h. The supernatant (S100) was used for subsequent assays. Equal amounts (40 μg) of *gm/gm, +/gm*, or *+/+* (wild-type) cytosolic protein were added to a 25-μl reaction containing 50 mM sodium Hepes, pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 50 μM NP-40, 1 μM [^{3}H]GGPP (47,300 dpm/pmol), 0.5 μM recombinant Rab escort protein 1, and 0.5 μM recombinant RGGT. After incubation for 30 min at 37°C, samples were separated on a 17.5% polyacrylamide gel, transferred to a PVDF membrane, and exposed to a tritium-sensitive PhosphorImaging screen (Fuji) for ~2 wk. The radioactive pattern, representing Rabs to which [^{3}H]GGPP had been transferred in vitro, was visualized using a Cyclone PhosphorImaging system. The same membrane was then probed with anti-Rab27a 4B12 antibody as described above. For immunoprecipitation, *gm/gm* CTL cytosolic Rabs were prenylated in vitro as above in a 75-μl reaction and then incubated overnight at 4°C with protein A–sepharose beads bound to either polyclonal anti-Rab27 antibody (N688) (Hume et al., 2001) or preimmune serum as a negative control. Precipitated Rabs were eluted by boiling with SDS sample loading buffer, separated by SDS-PAGE on a 17.5% polyacrylamide gel, and visualized by autoradiography.

Cytotoxicity Assays

Cytotoxicity was assayed using a Cytotox 96 nonradioactive kit (Promega) following the instructions provided. In brief, Ficoll-purified T cells were plated in 96-well plates at the effector/target ratios shown using 10^4 P815 ($H2^d$) target cells per well in a final volume of 100 μl per well using RPMI lacking phenol red. Lactate dehydrogenase release was assayed after 4 h incubation at 37°C by removal of 50 μl supernatant from each well and incubation with substrate provided for 30 min and the absorbance read at 490 nm using the Thermomax plate reader (Molecular Devices). Percentage cytotoxicity = (experimental $effector_{spontaneous}$ − target spontaneous/$target_{maximum}$ − target spontaneous) × 100. All cytotoxicity assays were reproducible in at least three separate assays.

Secretion Assay

Round-bottomed 96-well plates (Falcon) were coated with the same batch of hamster anti–mouse CD3 antibody, 145.2C11 (+CD3), or left uncoated (−CD3) overnight at 4°C. Wells were then blocked with IMDM with 10% FCS for 1 h at room temperature. Cells were plated at 5×10^4/well in 50 μl serum-free IMDM. For measurement of total activity 20 μl of medium

was replaced with 20 μl 0.1% Triton X-100, and the final sample was resuspended completely before 20 μl was assayed for enzyme activity. After 1 h, plates were spun briefly at 1,000 rpm to ensure that all cells were pelleted and 20-μl aliquots were removed to test for hexosaminidase and granzyme A activity. Hexosaminidase activity was assayed by adding 20 μl test supernatant to 20 μl substrate buffer (34 mg *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma-Aldrich) in 20 ml 50 mM sodium citrate, pH 4.8, 0.2% Triton X-100). Samples were incubated at 37°C for 1 h before 200 μl stop buffer (33 mM glycine, 83 mM sodium carbonate, 67 mM sodium chloride, pH 10.7) was added. Absorbance at 415 nm was read. Granzyme A activity was assayed by incubation of 20-μl samples with 180 μl of substrate (PBS, 0.2 mM *N*-benzyloxy-carbonyl-L-lysine-thiobenzylester, 0.2 mM dithiobis-nitrobenzoic acid) for 30 min, and the absorbance was read at 415 nm. For both enzymes the percentage of total secretion was calculated as $100 \times (OD_{supernatant}/OD_{lysate})$.

Immunofluorescence and Electron Microscopy

CTLs taken at 5–8 d after restimulation were purified over Ficoll and either used directly or incubated overnight with 2 mg/ml HRP added to the growth medium. Cells were washed one to three times in RPMI (GIBCO BRL) lacking FCS (RPMI−), resuspended at ~5×10^6 cells/ml in RPMI−, and mixed 1:1 with either RPMI− alone or RPMI− containing 10^7 cells/ml P815 cells prewashed as above. Samples were left in suspension for 5 min then plated onto either uncoated glass multiwell slides (50 μl/well) or into 12-well tissue culture plates (Nunc) (1 ml/well) and incubated at 37°C for a further 30–55 min.

Cells on slides were fixed with either methanol precooled to 20°C or 2% paraformaldehyde (Electron Microscopy Sciences) in PBS and processed for immunofluorescence as described previously (Stinchcombe et al., 2000). Samples labeled with antibodies against Arp2/3 were fixed in 4% paraformaldehyde and then processed as above except that the washing and blocking steps between the Triton X-100 permeabilization and primary antibodies were excluded and all antibody incubations and washing stages were performed in PBS alone. Samples were examined using an MRC-1024 Bio-Rad Laboratories confocal microscope with the iris set at 1.4.

Cells in 12-well tissue culture plates were fixed by adding 1 ml of 2× fixative (3% glutaraldehyde [Agar Scientific Ltd.], 4% paraformaldehyde) directly to the medium. After 10–20 min, fixative was replaced with fresh 1× fixative (1.5% gluteraldehyde, 2% paraformaldehyde) for a further 10–20 min. Samples were then processed for HRP cytochemistry, postfixed with reduced osmium, and embedded in Epon as described previously (Stinchcombe et al., 2000).

Online Supplemental Material

Online supplemental materials can be found at http://www.jcb.org/cgi/content/full/152/4/825/DC1. Supplementary material is provided to show granule polarization of CTL from *+/gm* and *gm/gm* mice conjugated to P815 target cells in three dimensions. All images are stained with rabbit anti–cathepsin D (green) and antitalin (red) antibodies as described above and shown in Fig. 6, a and d. Optical sections (0.2 μm apart) were taken using the BioRad 1024 confocal microscope, and assembled using BioRad Lasersharp software.

Results

Rab27a Is Absent in Ashen and At Least Partially Inactivated in Gunmetal CTLs

To determine whether Rab27a is expressed in CTLs and whether there is a difference in Rab27a expression in CTLs derived from *ashen* and *gunmetal* mice, we used a monoclonal antibody specific for the Rab27a isoform (4B12) to probe CTL lysates (Fig. 1 A). The CTL lysates derived from *+/ash*, *+/gm*, and *gm/gm* mice show a band migrating just above 28 kD corresponding to endogenous Rab27a. However, *ash/ash* CTLs exhibit no detectable endogenous Rab27a. The same blot was probed with an antibody against calnexin (ER membrane–associated protein) to demonstrate that equivalent levels of protein had been loaded. These results demonstrate that Rab27a is normally expressed in

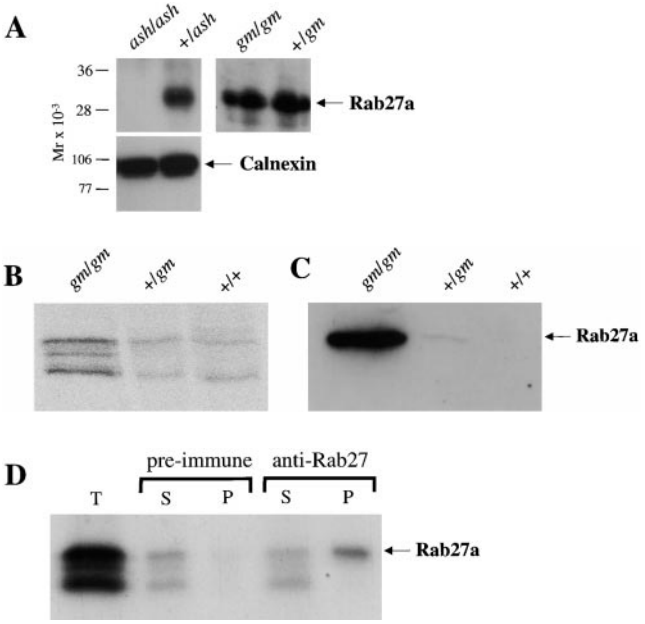

Figure 1. Rab27a is not expressed in *ashen* and at least partially inactivated in *gunmetal* CTLs. (A) Immunoblot of cell lysates from *ashen* and *gunmetal* CTLs using Rab27a-specific antibody (4B12). CTLs from *ash/ash*, *+/ash*, *gm/gm*, and *+/gm* were subjected to immunoblot analysis as described in Materials and Methods (top). A loading control using antibody anticalnexin (ER membrane–associated protein) shows that equal amounts of protein were loaded in each lane (bottom). (B) In vitro prenylation of *gm/gm*, *+/gm*, and *+/+* (wild-type) CTL cytosolic Rabs. Recombinant RGGT was used to transfer [^{3}H]GGPP to cytosolic Rabs as described in Materials and Methods. After the prenylation reaction, Rabs were separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by PhophorImager. (C) The same membrane was then immunoblotted using 4B12, a Rab27a-specific antibody. (D) Immunoprecipitation of Rab27 from the in vitro prenylation reaction. *gm/gm* CTL cytosolic Rabs were prenylated in vitro (total reaction, T) and precipitated with either preimmune serum (negative control) or an anti-Rab27 antibody (N688) bound to protein A–Sepharose beads. Proteins unbound (supernatant, S) and bound (pellet, P) to the beads were separated by SDS-PAGE and visualized by autoradiography.

CTLs, but that it is absent in *ashen* CTLs. The expression of Rab27b could not be detected in CTLs (data not shown).

As predicted, Rab27a is expressed at normal levels in *gunmetal* CTLs (Fig. 1 A). However, the deficiency of RGGT activity in these mice may result in underprenylation of Rab proteins in CTLs. To test this prediction, we employed an in vitro prenylation assay whereby recombinant RGGT will transfer [^{3}H]GGPP to any unprenylated Rab proteins present in cytosolic extracts. As shown in Fig. 1 B, *gm/gm* CTL cytosol shows an excess of Rabs available for prenylation compared with heterozygote *+ash/ash* and wild-type CTLs. An immunoblot performed on the same membrane using the Rab27a-specific antibody 4B12 (Fig. 1 C) reveals a band coinciding with the largest band of in vitro prenylated Rabs, suggesting that Rab27a is one of the few Rabs affected in *gm/gm* CTLs. No significant amount of Rab27a is detectable in either *+/gm* or *+/+* CTL cytosol, indicating that the majority of Rab27a is properly prenylated and membrane associated in these strains. To further

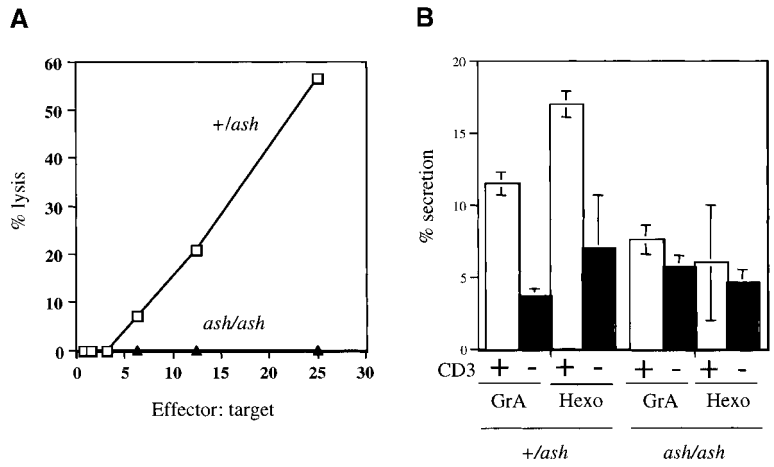

Figure 2. Ashen CTLs cannot lyse targets or secrete their granule contents. (A) Graph of percent lysis (y-axis) at various ratios of effector cells to a fixed number of target cells (x-axis) for heterozygous (*+/ash*, open squares) and homozygous (*ash/ash*, filled triangles) *ashen* mice. (B) Graph of percentage of total secretion from CTLs derived from *+/ash* and *ash/ash* mice in response to cross-linking with an antibody to the CD3 subunit of the TCR (white bars) or no stimulation (black bars). Secretion of both granzyme A (GrA) and hexosaminidase (Hexo) is shown.

demonstrate that one of the bands observed after in vitro prenylation of *gm/gm* CTLs is Rab27a, we subjected the in vitro prenylation reaction to immunoprecipitation with a specific anti-Rab27 antibody. Fig. 1 D shows that this antibody was able to precipitate a significant portion of the largest in vitro prenylated band. These data indicate that Rab27a is among several Rabs that appear underprenylated in *gunmetal* CTLs, thus remaining cytosolic and inactive.

CTL s from Ashen Mice Are Unable to Kill and Unable to Secrete Their Granule Contents

We next asked whether Rab27a is involved in CTL-mediated lysis by deriving CTLs from *ash/ash* and *+/ash* mice by stimulation with BALB/C spleens. This allowed us to assess the lytic ability of the T cells against the P815 target cell line, which shares the same class I major mistocompatibility complex as the BALB/C strain ($H2_d$). 5 d after activation, CTLs derived from *+/ash* killed P815 targets effectively. However, *ash/ash* CTLs were completely unable to kill targets (Fig. 2 A). These results were entirely reproducible in three separate experiments. The inability to kill arises from a lack of secretion of the lytic granules as shown by measuring the levels of the lysosomal hydrolase, hexosaminidase, and the CTL-protease, granzyme A, release in response to TCR cross-linking (Fig. 2 B). Using an antibody to the CD3 chain of the TCR immobilized on plates, we were able to demonstrate that granzyme A and hexosaminidase are secreted effectively in response to stimulation in heterozygote mice (*+/ash*). However, in the *ashen* homozygous mice (*ash/ash*) no secretion above background of unstimulated cells was detectable even though the total intracellular enzyme activity in heterozygote and homozygote CTLs was comparable (data not shown). This demonstrates that the lytic granules of *ash/ash* mice are not able to secrete their contents in response to TCR triggering.

CTLs from Gunmetal Mice Show Reduced Levels of Killing but Granule Secretion Is Not Dramatically Impaired

As shown in Fig. 1, *gunmetal* CTLs contain underprenylated Rab27a, among other Rabs. It was therefore possible that CTL-mediated killing might also be affected in *gunmetal* mice. Cytotoxicity was measured against P815 target cells and *gm/gm* CTLs possessed only weak killing activity compared with the parental strain, C57BL/6 (Fig. 3 A). These results suggest that defects in the RGGT perturb killing in *gunmetal* mice.

The ability of CTLs generated from C57BL/6, *+/gm*, and *gm/gm* to secrete their granule contents in response to TCR cross-linking was compared by examining the release of granzyme A and hexosaminidase (Fig. 3 B). The parental strain, C57BL/6, secrete both granzyme A and hexosaminidase in response to TCR cross-linking. *Gunmetal* heterozygous (*+/gm*) CTLs secrete at least as well as the parental strain (if not slightly better). Interestingly, the secretion from the homozygous mutants (*gm/gm*) is not significantly

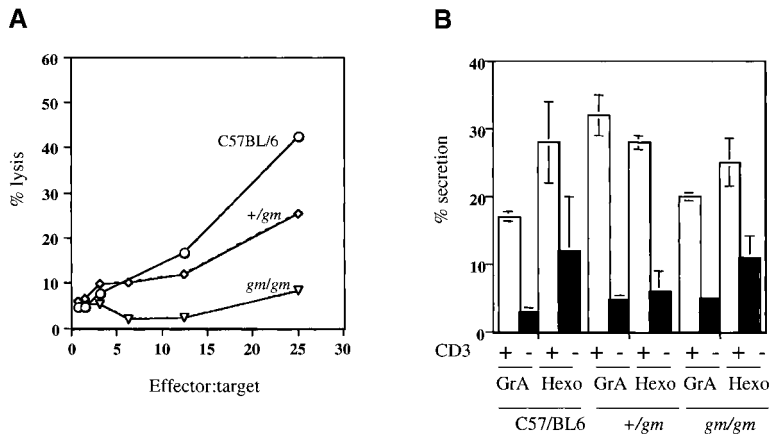

Figure 3. Gunmetal CTLs show reduced target cell lysis but normal secretion. (A) Graph of percent lysis (y-axis) at various ratios of effector cells to a fixed number of target cells (x-axis) for wild-type C57BL/6 (*+/+*, open circles), heterozygous (*+/gm*, open squares), and homozygous (*gm/gm*, open triangles) *gunmetal* mice. (B) Graph of percent of total secretion from CTLs derived from *+/+*, *+/gm*, and *gm/gm* mice in response to cross-linking with an antibody to the CD3 subunit of the TCR (white bars) or no stimulation (black bars). Secretion of both granzyme A (GrA) and hexosaminidase (Hexo) is shown.

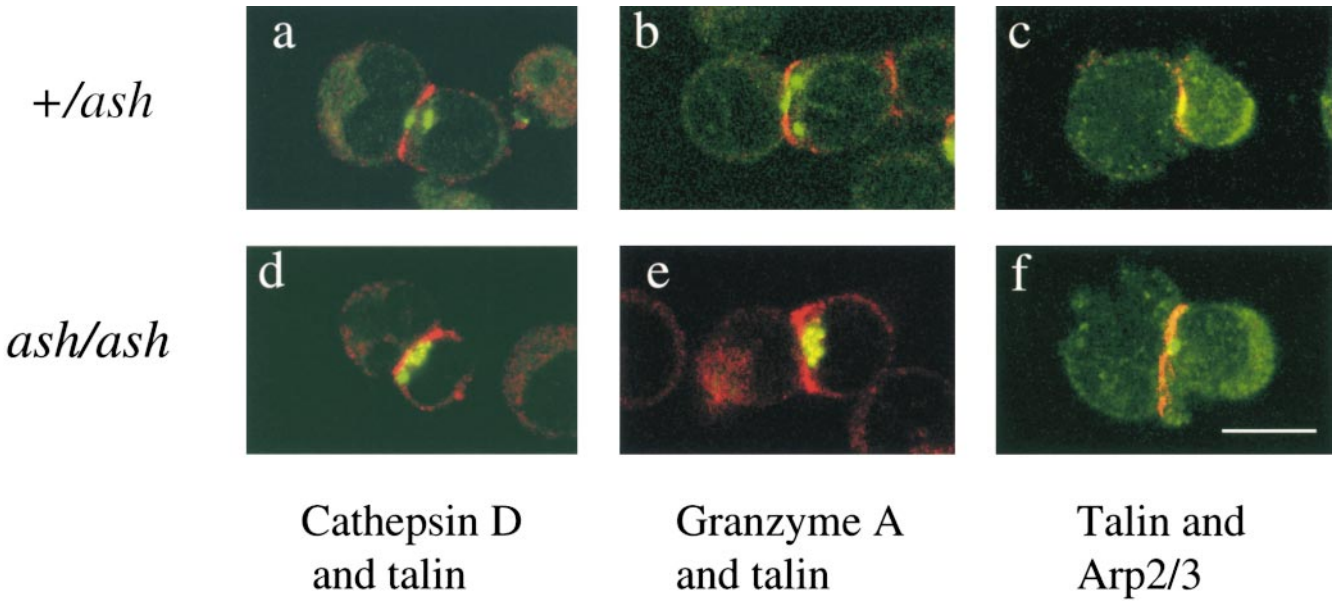

Figure 4. The lytic granules of *ashen* CTLs polarize at the immunological synapse. Allogeneic CTLs conjugated with P815 target cells, from *+/ash* (a–c) and *ash/ash* (d–f) stained with antibodies against cathepsin D (green, a and d), granzyme A (green, b and e), Arp2/3 (green, c and f), and talin (red). Bar, 10 μm.

impaired, with secreted levels of granyzme A and hexosaminidase comparable to those of wild-type C57BL/6 CTLs. The levels of hexosaminidase secretion for *+/gm* and *gm*/*gm* CTLs are comparable at 15, 30, and 60 min, being 0% for *gm*/*gm* and 3% for *+/gm* at 15 min and 10% for both at 30 min. This indicates that there are no significant differences in the rates of secretion. Since TCR cross-linking triggers not only degranulation but also new protein synthesis (Isaaz et al., 1995), small differences in secretion may not be observed. These results indicate that, although RGGT defect perturbs the lytic ability of *gunmetal* CTLs, regulated secretion from these cells is not significantly impaired.

The Lytic Granules from Ashen CTLs Polarize Correctly towards Target Cells

To determine at which step of secretion Rab27a functions, we examined CTL–target cell conjugates by immunofluorescence. We used antibodies against cathepsin D or granzyme A to detect the lytic granules and talin and the Arp2/3 complex to assess the formation of the immunological synapse.

In conjugates between *+/ash* and *ash/ash* CTLs and their targets (P815), we find that talin forms a ring at the site of target cell contact with a hole in the center. We find that Arp2/3 is focussed at the center of the talin ring and that in *+/ash*, the lytic granules are also focussed tightly within this ring (Fig. 4, a–c) very close to the contact site membrane. In CTLs derived from *ash/ash* mice, talin also forms a ring with Arp2/3 focussed in the center (Fig. 4 f). All of the lytic granules are found clustered towards the point of contact with the target, demonstrating that the lytic granules can move along microtubules to the point of membrane contact and polarization is not impaired (Fig. 4, d and e). Only two differences between *+/ash* and *ash/ash* conjugates are evident at this level of resolution. First, the granules always stain much more strongly at the synapse of *ash/ash*, consistent with the loss of content from *+/ash* but not *ash/ash* granules; second, that the *ash/ash* granules are not as tightly focussed within the central talin hole (for example, Fig. 4, d and e). These results show that granule polarization is normal in *ash/ash* mice lacking Rab27a and demonstrate that Rab27a is not required for microtubule-mediated movement to the point of contact. The observation that *ash/ash* granules are not tightly clustered in the central talin hole at the immunological synapse suggests that movement from the ends of the microtubules to the plasma membrane may be impaired in *ashen* mutants.

To address this possibility, we examined the localization of granules in *+/ash* and *ash/ash* CTLs conjugated to targets by EM. Fig. 5 shows the immunological synapse in *+/ash* (Fig. 5, a–c) and *ash/ash* (Fig. 5, d–f) conjugates. The Golgi complex is seen polarized very close to the site of membrane contact (Fig. 5, b, c, e, and f). Intriguingly, a central cleft is formed between two areas of tight membrane contact in both *ash/ash* and *+/ash* conjugates. Two important differences are observed between wild-type and mutant conjugates. First, in *+/ash* conjugates, granules are found docked at the plasma membrane (Fig. 5 a), whereas in *ash/ash* CTL conjugates, the granules do not dock at the membrane but rather line up behind the Golgi complex (Fig. 5 d). Second, the cleft at the contact site is filled with electron-dense and vesicular material in the *+/ash* conjugates consistent with the secretion of granule contents into this space. However, the cleft is devoid of content in the *ash/ash* conjugates (Fig. 5, e and f), indicating that the granule contents are not secreted. Curiously, endosomes from the target cell also appear to be recruited to the target side of the contact site in both strains (Fig. 5, a and d).

Granule Polarization Is Impaired in gm/gm CTLs

Our data shows that although killing by *gm*/*gm* CTLs is reduced, secretion is not (Fig. 3). We therefore examined gran-

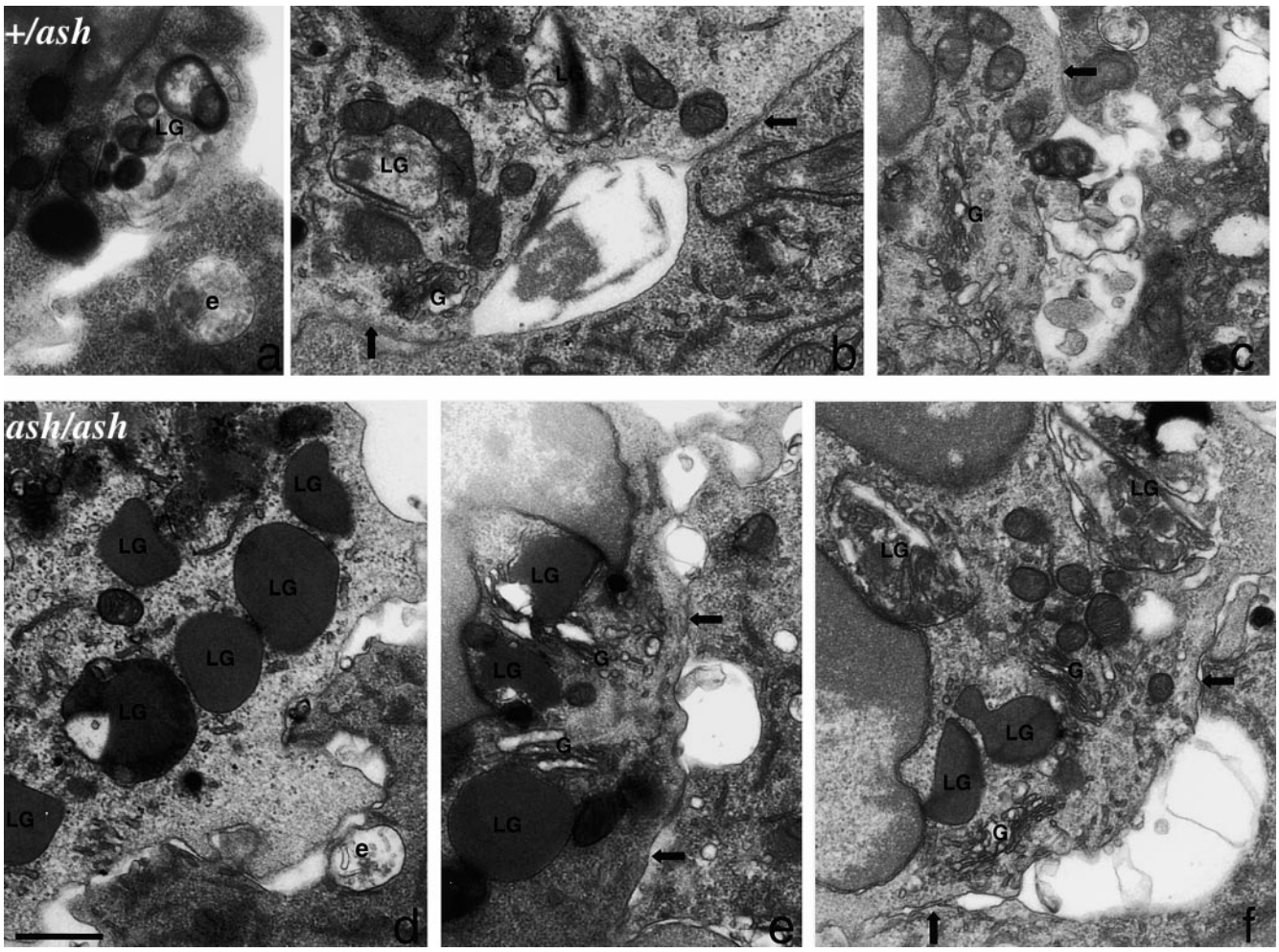

Figure 5. Lytic granules polarize with the Golgi complex but do not dock with the plasma membrane in *ash/ash* CTLs. Semithick (150–200 nm; a and e) and thin (50–60 nm; b–d and f) stained sections of *+/ash* (a–c) and *ash/ash* (d–e) CTLs (left) conjugated to P815 target cells (right). In both cells the Golgi complex (G) polarizes to the CTL plasma membrane at the immunological synapse. In both cell types the lytic granules (LG) have a heterologous appearance. In *+/ash* CTLs the lytic granules are either closely associated with the plasma membrane (e.g., a) or are absent (e.g., c) because the cells have already exocytosed, whereas in *ash/ash* CTLs (d–f) they accumulate behind the Golgi complex but fail to dock with the membrane. Note in b–c and e–f that in both cell types the CTLs and P815 cell plasma membranes are tightly aligned in some regions of the contact membrane (arrows) but that there is a gap between the two membranes directly opposite the polarized Golgi complex, probably corresponding to the talin hole. This gap contains crystalline and membranous material similar to that stored in lytic granules in *+/ash* cells but lacks it in *ash/ash* cells. Note in the sections shown in a and d in the target cells, endocytic structures (e) also appear to polarize to the contact sites. Bar, 500 nm.

ule polarization in CTL–target cell conjugates in order to determine whether synapse formation or polarization is impaired. In both *+/gm* and *gm/gm*, talin and Arp2/3 concentrate at the synapse between the cells, with talin forming an outer ring and Arp2/3 focussing in the center (Fig. 6, c and f). In *+/gm* CTL–target conjugates, the granules are tightly clustered at the center of the talin ring. However, in *gm*/*gm* conjugates the granules are incompletely polarized and many granules are left scattered around the perimeter of the cell (Fig. 6, d and e). This differs markedly from unpolarized cells in which the granules are scattered throughout the cell rather than just at the periphery. Only 12% of conjugates showed a tight polarization of the majority of the granules at the talin synapse in *gm*/*gm* compared with 86% in *+/gm* and >90% in *ash/ash* and *+/ash*. These results demonstrate that granule polarization is partially impaired in *gm*/*gm* CTLs. Since CTLs from *ash/ash* mice are able to polarize their granules completely, we conclude that Rab27a is not involved. As the defect in polarization observed here must be due to reduced prenylation, it may well be the result of the reduced activity of the other two Rab proteins which are underprenylated in the *gm*/*gm* CTLs (Fig. 1).

Discussion

This study describes several novel findings concerning the mechanisms that regulate the secretion of the lytic granules from CTLs. First, we demonstrate a critical role for Rab27a in the exocytosis of the lytic granules during killing. Second, we show that Rab27a is not involved in granule polarization to the immunological synapse but other Rab proteins are required for polarization. Third, we characterize the fusion of the lytic granules at the immunological synapse and demonstrate that the granules focus at the

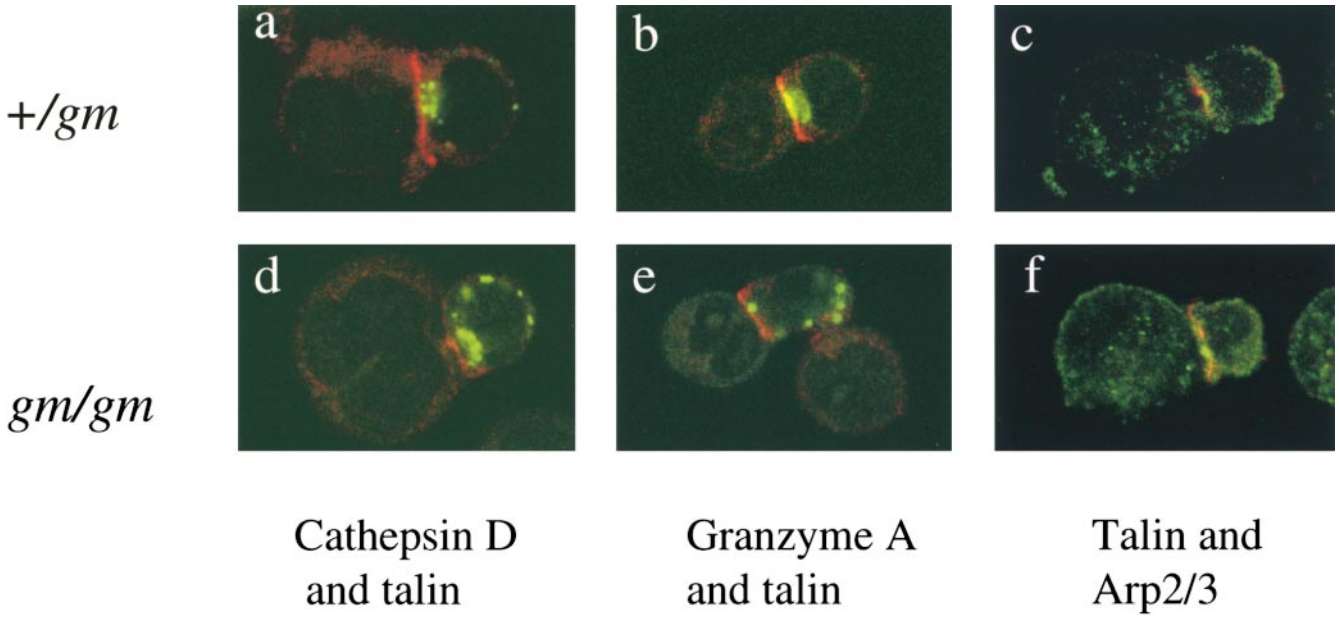

Figure 6. Lytic granule polarization is impaired in *gunmetal* mice. Allogeneic CTLs conjugated with P815 target cells from *+/gm* (a–c) and *gm/gm* (d–f) stained with antibodies against cathepsin D (green, a and d), granzyme A (green, b and e), Arp2/3 (green, c and f), and talin (red). Bar, 10 μm.

center of the talin ring where the actin polymerizing protein, Arp2/3, also polarizes.

The expression of Rab27a has been shown to be high in spleen, platelets, and melanocytes, suggesting a specialized role for this Rab GTPase. The recent report showing mutations in Rab27a in a subset of Griscelli syndrome patients in which CTL activity is impaired supports a role for Rab27a in CTL activity (Menasche et al., 2000). We have examined expression of Rab27a in CTLs from mutants where Rab27a is itself mutated (*ashen*) or in which the RGGT required to activate Rab27a for membrane binding is mutated (*gunmetal*). We show that Rab27a is expressed in heterozygous *ashen* CTLs but is completely absent in CTLs derived from *ashen* (Fig. 1). This is the first demonstration that the splice site mutation reported in *ashen* (Wilson et al., 2000) results in loss of expression of Rab27a, at least in CTLs, as expression studies were not reported previously. The loss of Rab27a in *ashen* CTLs results in complete inability to kill allogeneic targets matched by a lack of secretion in response to TCR cross-linking which mimics target cell recognition (Fig. 2).

We also show here that there is no major defect in polarization of lytic granules in the CTLs of *ashen* mice (Fig. 4). All of the lytic granules in *ashen* CTLs appear in the vicinity of the immunological synapse, although we were unable to find granules focussed in the center of the talin ring as observed in heterozygous *ashen* CTLs. This raises the intriguing possibility that the defect in secretion could arise from an inability to move into a docking position at the plasma membrane before secretion. Our EM studies of the conjugates support this idea. Fig. 5 shows that in *+/ash* conjugates the granules can be found polarized at the membrane, but in *ash/ash* mutants, the granules line up near the membrane but do not dock. This is reminiscent of the Rab3 mutants in *Caenorhabditis elegans* in which the synaptic vesicles are less tightly clustered around the presynaptic density (Nonet et al., 1997).

Recent reports suggested that Rab27a might be cooperating with myosinVa in the movement of vesicles, namely melanosomes in melanocytes. Mutations in either Rab27a or myosinVa genes give rise to similar phenotypes in mice (*ashen* and *dilute*) and in human Griscelli disease (Menasche et al., 2000; Wilson et al., 2000). Both proteins colocalize in melanosomes, coimmunoprecipitate, and cooperate to promote the peripheral distribution of melanosomes in melanocytes (Wu et al., 1997; Hume et al., 2001). At present, it is not clear whether myosinVa plays any role in CTL secretion. Both Griscelli patients (Menasche et al., 2000; Pastural et al., 2000) and *dilute* mice, with mutations in myosinVa (Haddad et al., 2001), show normal levels of CTL activity, suggesting that Rab27a can function in secretion in the absence of myosinVa. However, these observations need to be interpreted cautiously as some residual myosinVa activity may still be present in some cases or myosinVa activity may be redundant. In these situations, there could be enough activity to ensure proper movement of lytic granules to the plasma membrane. Our experiments do not address a role for myosinVa but future experiments should.

CTLs derived from *gunmetal* show reduced killing but curiously are not significantly defective in the ability to secrete their lytic granule contents (Fig. 3). Different possibilities could account for this. First, since several different Rab GTPases are affected by the loss of RGGT activity in *gunmetal* CTLs, it is possible that Rabs other than Rab27a are involved in correct sorting of lytic proteins to the granules. Alternatively, the C57BL/6 strain from which *gunmetal* are derived may simply be much better at responding to TCR cross-linking than the C3H strain from which *ashen* mice are derived. Our previous studies have shown that TCR cross-linking not only results in degranulation but also in rapid de novo synthesis of granule proteins, a large proportion of which are then secreted via the constitutive secretory pathway. This hypothesis is supported by the in-

creased maximal values of secretion observed in C57BL/6 (32%) (Fig. 3 b) compared with C3H/He (17%) mice (Fig. 2 b). It is possible that a slight decrease in regulated secretion might be masked. Three-dimensional reconstruction of conjugates shows tight polarization of granules at the synapse in *+/gm* CTLs (see http://www.jcb.org/cgi/content/full/152/4/825/DC1). In some *+/gm* CTL conjugates, the granules are absent, consistent with secretion from the synapse. However, in *gm*/*gm* CTLs two types of conjugates are seen: (a) those with some granules polarized and others dispersed around the periphery; and (b) those with no granules at the synapse but some left around the periphery. These observations suggest that the unpolarized granules in *gm*/*gm* CTL conjugates are unable to fuse with the plasma membrane and secrete their contents.

Immunofluorescence on CTL–target cell conjugates from *gunmetal* mice supports the idea that granules are secreted from *gm*/*gm* CTLs since a fraction of the granules are polarized towards the site of contact and some granules are seen within the talin ring. We show that although Rab27a prenylation is decreased in CTLs, the decreased killing could reflect the incomplete polarization of granules as well as the decrease in Rab27a activity. Since granules polarize completely in *ashen* mice, the polarization defects observed in *gunmetal* cannot be attributed to Rab27a and are likely to be the result of decreased prenylation of one or more other Rab GTPases. The identity of the Rab GTPase(s) involved in polarization is not known.

This study is the first to describe the fusion of the granules at the immunological synapse of CTLs. Kupfer's initial studies on T cell conjugates with target cells used CTLs and showed the concentration of talin, the microtubule organizing center, and Golgi complex at the contact site (Kupfer et al., 1985, 1986). Subsequent high resolution studies of this synapse have focussed on $CD4^+$ cells that do not contain lytic granules (Monks et al., 1998). Using antibody markers for lytic granule contents and confocal microscopy, we have been able to show the polarization of the granules to the central hole in the talin ring. We also provide the first demonstration that the Arp2/3 complex, an actin filament–initiating complex, localizes to the center of the talin ring close to the point where granules accumulate before secretion. Recent reports suggest that the Arp2/3 complex can induce the motility of endocytic vesicles in cells via the assembly of an actin comet tail which propels the vesicles through the cytoplasm (Rozelle et al., 2000; Taunton et al., 2000). This raises the interesting possibility that actin polymerization could be a driving force for propulsion of granules to the plasma membrane. The Arp2/3 complex is activated in cells by proteins of the WASP family (Machesky and Insall, 1999) and a similar arrangement of Arp2/3 complex associated with a ring of talin is observed in structures termed podosomes. These structures, largely of unknown function, are found in a variety of cells, including macrophages, dendritic cells, osteoclasts, and src-transformed cells. Podosomes also contain membrane invaginations and are rich in WASP (Linder et al., 1999) so there may be parallels between the immunological synapse and podosomes.

This study demonstrates the effects of the *ashen* and *gunmetal* mutations on the exocytosis of the secretory lysosomes (lytic granules) of CTLs. These are not the only cell types affected in these mice. Notably, melanosomes are affected since both mutants show hypopigmented coat colors. Also, platelet secretion has been shown to be impaired in *gunmetal* mice (for review see Swank et al., 1998), but few other cell types have been studied. Our studies raise the possibility that all cells with secretory lysosomes may be affected by these mutations and demonstrate that Rab27a is a key regulator of secretion in cells with secretory lysosomes.

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References

Baetz, K., S. Isaaz, and G.M. Griffiths. 1995. Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. *J. Immunol*. 154:6122–6131.

Boissy, R.E., and J.J. Nordlund. 1997. Molecular basis of congenital hypopigmentary disorders in humans: a review. *Pigment Cell Res*. 10:12–24.

Chen, D., J. Guo, T. Miki, M. Tachibana, and W.A. Gahl. 1997. Molecular cloning and characterization of rab27a and rab27b, novel human rab proteins shared by melanocytes and platelets. *Biochem. Mol. Med*. 60:27–37.

Detter, J.C., Q. Zhang, E.H. Mules, E.K. Novak, V.S. Mishra, W. Li, E.B. McMurtrie, V.T. Tchernev, M.R. Wallace, M.C. Seabra, R.T. Swank, and S.F. Kingsmore. 2000. Rab geranylgeranyl transferase alpha mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis. *Proc. Natl. Acad. Sci. USA*. 97:4144–4149.

Fruth, U., M. Prester, J.R. Golecki, H. Hengartner, H.G. Simon, M.D. Kramer, and M.M. Simon. 1987. The T cell-specific serine proteinase TSP-1 is associated with cytoplasmic granules of cytolytic T lymphocytes. *Eur. J. Immunol*. 17:613–621.

Griffiths, G.M. 1995. The cell biology of CTL killing. *Curr. Opin. Immunol*. 7:343–348.

Griffiths, G.M. 1996. Secretory lysosomes—a special mechanism of regulated secretion in haemopoietic cells. *Trends Cell Biol*. 6:329–332.

Griscelli, C., A. Durandy, D. Guy-Grand, F. Daguillard, C. Herzog, and M. Prunieras. 1978. A syndrome associating partial albinism and immunodeficiency. *Am. J. Med*. 65:691–702.

Haddad, E.K., X. Wu, J.A. Hammer III, P.A. Henkart. 2001. Defective granule exocytosis in Rab27a-deficient lymphocytes from *ashen* mice. *J. Cell Biol.* 152:835–841.

Hume, A.N., L.M. Collinson, A. Rapak, A.Q. Gomes, C.R. Hopkins, and M.C. Seabra. 2001. Rab27a regulates the peripheral distribution of melansomes in melanocytes. *J. Cell Biol.* 152:795–808.

Isaaz, S., K. Baetz, K. Olsen, E. Podack, and G.M. Griffiths. 1995. Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. *Eur. J. Immunol*. 25:1071–1079.

Klein, C., N. Philippe, F. Le Deist, S. Fraitag, C. Prost, A. Durandy, A. Fischer, and C. Griscelli. 1994. Partial albinism with immunodeficiency (Griscelli syndrome). *J. Pediatr*. 125:886–895.

Krause, M., A.S. Sechi, M. Konradt, D. Monner, F.B. Gertler, and J. Wehland. 2000. Fyn-binding protein (Fyb)/SLP-76–associated protein (SLAP), Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. *J. Cell Biol*. 149:181–194.

Kupfer, A., G. Dennert, and S.J. Singer. 1985. The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells. *J. Mol. Cell. Immunol.* 2:37–49.

Kupfer, A., S.J. Singer, and G. Dennert. 1986. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. *J. Exp. Med*. 163:489–498.

Linder, S., D. Nelson, M. Weiss, and M. Aepfelbacher. 1999. Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages. *Proc. Natl. Acad. Sci. USA*. 96:9648–9653.

Machesky, L.M., and R.H. Insall. 1999. Signaling to actin dynamics. *J. Cell Biol.* 146:267–272.

Machesky, L.M., E. Reeves, F. Wientjes, F.J. Mattheyse, A. Grogan, N.F. Totty, A.L. Burlingame, J.J. Hsuan, and A.W. Segal. 1997. Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins. *Biochem. J.* 328: 105–112.

Menasche, G., E. Pastural, J. Feldmann, S. Certain, F. Ersoy, S. Dupuis, N. Wulffraat, D. Bianchi, A. Fischer, F. Le Deist, and G. de Saint Basile. 2000. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat. Genet*. 25:173–176.

Monks, C.R., B.A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*. 395:82–86.

Nonet, M.L., J.E. Staunton, M.P. Kilgard, T. Fergestad, E. Hartwieg, H.R. Horvitz, E.M. Jorgensen, and B.J. Meyer. 1997. *Caenorhabditis elegans* rab-3 mutant synapses exhibit impaired function and are partially depleted of vesicles. *J. Neurosci*. 17:8061–8073.

Pastural, E., F. Ersoy, N. Yalman, N. Wulffraat, E. Grillo, F. Ozkinay, I. Tezcan, G. Gedikoglu, N. Philippe, A. Fischer, and G. de Saint Basile. 2000. Two genes are responsible for Griscelli syndrome at the same 15q21 locus. *Genomics*. 63:299–306.

Rozelle, A.L., L.M. Machesky, M. Yamamoto, M.H. Driessens, R.H. Insall, M.G. Roth, K. Luby-Phelps, G. Marriott, A. Hall, and H.L. Yin. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr. Biol*. 10:311–320.

Seabra, M.C. 1998. Membrane association and targeting of prenylated Ras-like GTPases. *Cell. Signal*. 10:167–172.

Seabra, M.C., Y.K. Ho, and J.S. Anant. 1995. Deficient geranylgeranylation of Ram/Rab27 in choroideremia. *J. Biol. Chem*. 270:24420–24427.

Shotelersuk, V., and W.A. Gahl. 1998. Hermansky-Pudlak syndrome: models for intracellular vesicle formation. *Mol. Genet. Metab*. 65:85–96.

Stinchcombe, J.C., and G.M. Griffiths. 1999. Regulated secretion from hemopoietic cells. *J. Cell Biol*. 147:1–6.

Stinchcombe, J.C., L.J. Page, and G.M. Griffiths. 2000. Cytotoxic T lymphocytes as a model system for studying secretory lysosome biogenesis. *Traffic*. 1:435–444.

Swank, R.T., S.Y. Jiang, M. Reddington, J. Conway, D. Stephenson, M.P. McGarry, and E.K. Novak. 1993. Inherited abnormalities in platelet organelles and platelet formation and associated altered expression of low molecular weight guanosine triphosphate-binding proteins in the mouse pigment mutant gunmetal. *Blood*. 81:2626–2635.

Swank, R.T., E.K. Novak, M.P. McGarry, M.E. Rusiniak, and L. Feng. 1998. Mouse models of Hermansky Pudlak syndrome: a review. *Pigment Cell Res*. 11:60–80.

Taunton, J., B.A. Rowning, M.L. Coughlin, M. Wu, R.T. Moon, T.J. Mitchison, and C.A. Larabell. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. *J. Cell Biol*. 148:519–530.

Wilson, S.M., R. Yip, D.A. Swing, T.N. O'Sullivan, Y. Zhang, E.K. Novak, R.T. Swank, L.B. Russell, N.G. Copeland, and N.A. Jenkins. 2000. A mutation in Rab27a causes the vesicle transport defects observed in *ashen* mice. *Proc. Natl. Acad. Sci. USA*. 97:7933–7938.

Wu, X., B. Bowers, Q. Wei, B. Kocher, and J.A. Hammer III. 1997. Myosin V associates with melanosomes in mouse melanocytes: evidence that myosin V is an organelle motor. *J. Cell Sci*. 110:847–859.