Targeted Disruption of Guanosine Diphosphate–Dissociation Inhibitor for Rho-Related Proteins, GDID4: Normal Hematopoietic Differentiation but Subtle Defect in Superoxide Production by Macrophages Derived From In Vitro Embryonal Stem Cell Differentiation

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The Rho subfamily of small guanosine triphosphate (GTP)-binding proteins, through their role in cytoskeletal organization, is involved in diverse cellular functions, including cell motility and morphologic changes during differentiation. Rac also has a special role in the production of superoxide, a key component in phagocytic antimicrobial function. Guanosine diphosphate (GDP)-dissociation inhibitors (GDIs) belong to one of three classes of proteins that regulate the critical cycling of GTP-binding proteins between the inactive and active states. Two homologous GDIs for the Rho subfamily have been identified. GDID4 is preferentially expressed in hematopoietic cells, while RhoGDI is ubiquitously expressed. Whether different physiologic functions are served by the two GDIs is unknown. We have derived embryonal stem (ES) cells with targeted disruption of both alleles of the GDID4 gene and examined hematopoiesis and phagocytic functions of macrophages derived from in vitro ES-cell differentiation. GDID4 ES cells develop like wild-type cells into colonies that contain heterogeneous populations of progenitor cells and differentiated erythromyeloid cells. GDID4+ cells express no GDID4 protein, but have normal levels of RhoGDI. GDID4+–macrophages phagocytose yeasts and antibody-opsonized erythrocytes as effectively as wild-type macrophages. However, a slight but consistent reduction in their capacity to generate superoxide was observed, which suggests new insight into the cellular role of GDID4. The minimal phenotypic effect of a loss of function of GDID4 also indicates a significant redundancy of function between GDID4 and RhoGDI. Their functional repertoire may be better revealed by a disruption of both genes. The use of hematopoietic cells derived in vitro from genotypically altered ES cells avoids the difficulties inherent in generating knockout animals and is a useful complementary approach for evaluating the gene function.

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tion of superoxide\textsuperscript{10,11,18} in activated neutrophils and macrophages. Phagocytic respiratory burst oxidase plays a central role in the inflammatory response, as reflected by severely disabling diseases in instances of null mutations in components of the oxidase machinery.\textsuperscript{19} An absolute requirement for Rac as a component of NADPH oxidase was shown in a cell-free system.\textsuperscript{20,21} Furthermore, antisense oligonucleotide against rac was shown to be capable of causing a decrease of oxidase activity in neutrophils\textsuperscript{22} and, in a cell-free system, RhoGDI appears to inhibit NADPH oxidase activities.\textsuperscript{23} Extensive investigations of this pathway have demonstrated the requirement for translocation of various protein components from the cytosol to be assembled at the membrane before superoxide generation can occur.\textsuperscript{18,19,24,25} It was also shown that biologically active lipids can regulate the release of Rac from cytosolic complexes with RhoGDI in vitro.\textsuperscript{26} The postulate is that, upon cellular activation, Rac is released from RhoGDI to be translocated to the membrane to form an active NADPH complex.\textsuperscript{27,28} Whether GDID4 modulates oxidase activity in the same manner is not known.

To complicate the issue, RhoGDI has also been shown to be an inhibitor of the intrinsic and guanosine adenosine triphosphate (GAP)-stimulated GTP hydrolysis function of CDC42,\textsuperscript{29} Rac,\textsuperscript{30,31} and Rho,\textsuperscript{31} and thereby to possess the ability to maintain these proteins in the GTP-bound active form. This activity is apparently opposite to the end result of its GDI activity. Thus, RhoGDI appears to be a molecule with ambidextrous capacity to modulate cellular levels of active GTP-bound Rho proteins. Whether GDID4 can act also as a GAP inhibitor is not known.

An unanswered question is whether RhoGDI and GDID4 govern different physiologic functions. Although both functions as GDIs for the same spectrum of substrates, GDID4 has been shown to bind to the rho subfamily of proteins with a significantly lower affinity compared with RhoGDI. Thus, under in vitro conditions, to achieve the same level of GDI activity, the concentration of GDID4 required is 20 to 30 times higher than RhoGDI.\textsuperscript{13} In addition, the difference between the two GDIs in their binding affinity to Rho-related proteins may be determined by only a few residues in the carboxyl terminus of the proteins.\textsuperscript{22} For example, a replacement of the domain around residue 169 to 178 of GDID4 with the homologous region from RhoGDI can change its GDI activity to resemble that of RhoGDI, and vice versa. These data suggest that GDID4 and RhoGDI may perform subtly different cellular functions, but with some functions that overlap.

As a way to resolve this question and to define better the functional importance of the GDIs, we took the genetic approach of disrupting the GDID4 gene in murine embryonal stem (ES) cells using homologous recombination techniques.\textsuperscript{33,34} The effect on hematopoiesis and function of selected hematopoietic cells was then examined, using in vitro culture systems that have been shown to promote hematopoietic differentiation from ES cells.\textsuperscript{35,36} Surprisingly, null mutation of GDID4 does not affect hematopoietic differentiation. Furthermore, in using in vitro--derived macrophages to examine two key phagocytic functions in which Rho proteins are implicated, we found that a loss of function of GDID4 does not significantly affect phagocytosis, but appears to decrease the rate of production of superoxide. This report also demonstrates that the methodology can provide significant information about gene function in the absence of or before the derivation of mutant animals.

**MATERIALS AND METHODS**

**Cloning and mapping of the GDID4 gene.** Three genomic phage clones were isolated from a mouse strain 129 library in XFixII (Stratagene, La Jolla, CA) by screening with the murine GDID4 cDNA. A restriction map was derived with selected fragments cloned into pUC18 for restriction mapping and DNA sequencing.

**Construction of targeting vector.** A SalI/SmaI genomic piece (SalI site from vector) was subcloned into Bluescript KSII\textsuperscript{[3]} plasmid (Stratagene). The phosphoglycerate kinase (PGK)-promoter neomycin-resistance (PGKneo) cassette\textsuperscript{27} was directionally cloned into the EcoRI/KpnI site (Fig 1A and B) to disrupt the first two exons, creating the partial SalI/SmaI vector. Next, a XhoI/EcoRI fragment was subcloned into pBluescript KSII plasmid. The genomic Accl and EcoRI sites were deleted by restriction enzyme digestion, blunted, and the truncated vector religated. A XhoI/NorI (NorI site from the cloning vector) piece was then cloned into the XhoI site of the SalI/SmaI vector to give rise to the targeting vector pNeo-6D4 (Fig 1B). A StuI/EcoRI fragment outside of the targeting vector was selected as the diagnostic probe, p (Fig 1C). Wild-type DNA digested with EcoRI and hybridized with the probe should reveal a 10.0-kb EcoRI fragment (Fig 1C and E). A homologous recombination with the targeting vector was expected to yield an 8.6-kb fragment (Fig 1D). The vector was linearized by NorI before electroporation into ES cells. An alternative targeting vector, pPuro-6D4, was made the same way using a PGK-puramycin cassette (kindly provided by Dr Fred Alt, Harvard Medical School) instead of PGKneo.

**Transfection and screening of ES cells.** CCE ES cells were maintained on primary embryonic fibroblasts as described.\textsuperscript{35} For targeting with pNeo-6D4, cells were electroporated under conditions as described\textsuperscript{33,34} and selected in G418 (350 \mu g/mL). For targeting with the pPuro-6D4 vector, cells were selected in puramycin (5 \mu g/mL). Genomic DNAs were digested with EcoRI, separated on Southern blots, and probed with the 0.5-kb StuI/EcoRI diagnostic probe p.

**In vitro culture for ES-cell differentiation.** ES cells maintained on primary embryo fibroblasts were passaged on gelatin-coated plates in the presence of 1,000 U/mL leukemia inhibitory factor (LIF) (Genetics Institute, Cambridge, MA) 1 day before each differentiation experiment. In vitro culture of ES cells was performed as previously described\textsuperscript{33,34} in the presence of 0.9% methyl cellulose, interleukin-3 (IL-3; 100 U/mL), erythropoietin (2 U/mL), ker ligand (50 ng/mL), 1% albumin, and 20% fetal calf serum (FCS) with ES cells at a concentration of 2,000 to 3,000 cells/mL. The mixture was cultured in bacterial plates and incubated at 37°C in a humidified chamber with 5% CO\textsubscript{2}.

**Western analysis of ES colonies.** ES-cell--derived colonies were pooled and harvested at different days after culture. Cells were rinsed free of methyl cellulose before disruption in lysing buffer to generate Western blots as described.\textsuperscript{33} The filters were analyzed sequentially with antibodies against GDID4 and RhoGDI. A polyclonal rabbit antiserum specific for GDID4 had been previously generated using GDID4\textsuperscript{33,35} synthetic polypeptides (unpublished data, January 1995). RhoGDI-specific antibody was obtained from Santa Cruz Biotech, Santa Cruz, CA. The enhanced chemiluminescent (ECL) method (Amersham, Arlington Heights, IL) was used to localize antibodies.

**Phagocytosis assay.** The assay for phagocytosis of yeasts was
Saccharomyces cerevisiae (Fleischmann, San Francisco, CA) were suspended in phosphate-buffered saline (PBS), autoclaved, and washed three times in PBS. Just before use, yeasts were triturated to disrupt clumps and diluted in Hanks’ balanced salt solution (PBS), autoclaved, and washed three times in PBS. Just before use, the cells were resuspended in ice-cold PBS that contained 1% FCS. Adherent cells were washed three times with PBS just before addition of yeasts.

Yeasts in HHB were added at a 1,000:1 yeast to cell ratio, and the cells were incubated for 1 hour at 37°C in 5% CO₂ humidified incubator. After incubation, cells were gently washed twice with PBS to remove unbound yeasts and permeabilized and stained as described. The coverslips were mounted and examined by light microscopy. All cells with at least one intracellular particle were counted. At least 100 cells were counted in duplicate for each condition by a blinded, experienced observer.

**Quantification of oxidase activity.** ES colonies were pooled on day 15 to 18 and rinsed free of methyl cellulose with ice-cold PBS. The cells were resuspended in ice-cold PBS that contained 1% albumin until needed. The activation of an oxidative burst was measured by means of FcOxyburst (Molecular Probes Inc, Eugene, OR), a reduced fluorescein-labeled high-valency immune complex (DCFH-2’7’-dichlorodihydrofluorescein-labeled BSAnanti-bovine serum immunoglobulin G [IgG]). The complex is slightly fluorescent in the reduced state, which permits evaluation of binding to the cells. Upon activation of the oxidative burst, the fluorescein is oxidized and its fluorescence increases in proportion to the concentration of peroxide produced. Measurements were made on a Becton Dickinson 440 fluorescence-activated cell sorter (FACS 440; Becton Dickinson, San Jose, CA) equipped with an argon laser for excitation at 488 nm. Emission was evaluated with a narrow band pass filter. DCFH-IC was injected at a concentration of 180 µg/2 × 10⁶ cells/mL in Krebs-Ringer phosphate buffer (pH 7.4) at 4°C for 2 minutes in order to measure binding alone without accompanying phagocytosis and oxidative burst initiation. The cells were then warmed rapidly to 37°C and stirred. Because the number of available cells was low, fluorescence measurements were taken at timed intervals, ie, not continuously, for approximately 6,000 cells. Data taken 10 minutes after warming are shown here.

**Derivation of mast cells from ES cells and mast-cell degranulation assay.** In vitro ES colonies were refed at day 11 by gently overlaying culture plates with 1 mL of fresh methyl-cellulose growth medium. On day 18, colonies were pooled and single-cell suspension of colonies cultured in growth condition similar to primary culture except without IL-1. By day 8 to 10, many secondary mast-cell colonies were obtained. Colonies were pooled, washed free of methyl...
cellulose, and disaggregated by vigorous pipetting to obtain single-cell suspensions. The cells were then cultured in liquid suspension in T25 flasks in the presence of IL-3 (10 ng/mL) and kit ligand (50 ng/mL). Mast cells were expanded, and after long-term culture lasting more than 2 months became established lines. The degranulation assay was performed as described.22-24 Mast cells were washed and resuspended in Tyrodes buffer and incubated with a saturating concentration of ascertes from the IgE anti-trinitrophenyl (TNP) hybridoma (IgKL2, ATCC TIB142; Rockville, MD) for 60 minutes. Cells were then washed and resuspended at a concentration of 1 x 10⁶ cells/mL and plated into 96-well V-bottom plates. Kit ligand (10 ng/mL) was then added where indicated and the plates incubated for 10 minutes at 37°C. TNP-BSA, prepared as described, was added to a final concentration of 10 ng/mL and the cells incubated at 37°C for an additional 10 minutes. The release of the enzyme β-hexosaminidase was measured by standard methods29 as a measure of degranulation. Controls were run in each experiment to determine the unstimulated (no kit ligand or TNP-BSA added), as well as the 100% release values (cells lysed by addition of 1% Triton-X 100).

RESULTS

Disruption of GDID4 gene. ES clones that survived G418 selection after electroporation with the pNeo-ΔD4 targeting vector were found to contain hemizygous deletion of a single allele at a mean frequency of one in 10 clones. Figure 1E shows examples of hemizygous clones (+/-) with the expected mutant 8.6-kb band in addition to the 10-kb fragment from the wild-type allele in Southern analysis of EcoR1-digested DNAs probed with the diagnostic probe. Several clones were selected for in vitro differentiation, all of which behaved similarly to wild-type cells in colony frequency and hematopoietic differentiation. Our attempt to derive targeted disruption of both alleles by selecting hemizygous clones under increasing dose of G418 as described30 was not successful. Therefore, we resorted to using a second targeting vector to achieve disruption of the second allele in a hemizygous clone. A GDID4+/− clone, A20, was selected for reelectroporation with the pPuro-ΔD4 vector. More than 350 puromycin resistant clones were analyzed and a clone, B2, was identified that contained only a single mutant 8.6-kb band (clone marked −/− in Fig 1E), indicating that both alleles had been disrupted.

In vitro hematopoietic differentiation of ES cells. GDID4+/− ES cells maintained on stromal cells or on culture dishes supplemented with LIF showed no difference from wild-type or the parental A20 hemizygous clone in adherence, spreading on culture dishes, morphology, or proliferation rate. When cultured in methyl cellulose, GDID4+/− ES cells developed typical embryoid bodies so that by day 10 to 12, greater than 80% of the colonies manifested hematopoiesis with a mixture of erythroid and myeloid cells as in wild-type colonies (Fig 2A). The colony-forming efficiency of GDID4+/− ES cells was approximately 150 colonies per 2,000 cells, similar to that observed for wild-type ES cells (150 to 180 colonies/2,000 cells). Histochemoanalysis of day 12 GDID4+/− colonies revealed the same heterogenous composition of erythroid and myeloid cells at different stages of maturation as in wild-type colonies (Fig 2B). The frequency of erythroid and myeloid cells, as assessed by Wright’s stain, also appeared similar to wild-type colonies. In particular, the dominating mature myeloid cells were erythroid cells with macrophages ranging from 10% to 15% and less than 0.5% of neutrophils. Immunostaining with antibody against the macrophage antigen Mac-1 (murine homolog of CD11b) confirmed the frequency of macrophages. These observations are consistent with our previously published results.39 Colonies were pooled and harvested at different times in culture, and total RNA extracted to generate Northern blots. These were probed for expression of embryonic globin βh1, adult β-globin, and CD11b as markers of erythroid and myeloid terminal differentiation. The temporal profile of expression of these genes in GDID4−/− colonies was similar to that seen in wild-type colonies (data not shown) as previously demonstrated.39 Furthermore, replating of primary colonies generated secondary erythroid and myeloid hematopoietic colonies as described for wild-type ES cells34 (data not shown), including the derivation of mast cells as described later. Therefore, there appeared to be no apparent abnormality in the development of hematopoietic precursors from GDID4−/− ES cells and in their capacity to undergo maturation into terminally differentiated erythroid and myeloid cells.

Western analysis of GDID4 protein in GDID4−/− hematopoietic cells. To confirm the disruption of the GDID4 gene, we examined ES cells and ES-derived hematopoietic cells for expression of GDID4 protein using a GDID4-specific polyclonal antiserum that does not cross-react with RhoGDI protein. The expression of GDID4 and RhoGDI in GDID4−/− cells is similar to wild-type cells. Figure 3 illustrates a comparison between (+/−) and (−/−) cells. Undifferentiated GDID4−/− ES cells, express a low but detectable level of GDID4 (Fig 3A). Upon differentiation, the level of GDID4 increases noticeably by day 4, which corresponds to the development and increasing percentage of hematopoietic cells within the colonies. Figure 3C shows that the homozygous disruption of the GDID4 gene completely abrogated production of the protein in ES cells and in phenotypically normal hematopoietic cells derived from GDID4−/− ES cells throughout in vitro culture. In contrast to GDID4, the RhoGDI protein is detected at higher levels in undifferentiated GDID4−/− ES cells, with little or no change in the level throughout in vitro culture, which reflects the ubiquitous expression of the protein in tissues (Fig 3B). The level of RhoGDI protein in GDID4−/− ES cells was similar to wild-type or GDID4+/− cells and, likewise, the RhoGDI protein level remained unchanged throughout ES-cell differentiation into hematopoietic cells (Fig 3D). These results also indicate that the loss of GDID4 did not induce an upregulation of RhoGDI protein.

Assay of phagocytosis. Phagocytosis was evaluated by counting the number of yeast particles in cells with any internalized yeasts, ie, phagocytes. Extracellular yeasts that stain dark purple are easily distinguished from phagocytosed yeasts, which stain light bluish-pink.39 An average number of internalized particles per phagocyte was calculated and used to compare internalization in the mutant and wild-type ES-derived macrophages. Figure 2C, a photomicrograph of a representative field, shows that GDID4−/− cells preserve
the capacity for vigorous phagocytic activity. Several separate experiments were performed to generate and quantify phagocytic activity from in vitro-differentiated ES cells. The average number of yeast particles per positive cell was slightly higher in the GDID4−/− cells than in the wild-type cells (Fig 4). The distribution of particles per phagocytic cell would be expected to conform to a chi-square distribution. Biostatistical analysis (David Zurakowski, Children’s Hospital) concluded that the difference between the chi-square and normal distributions in this data was small enough to allow Student’s t-test to be used to assess the potential significance of differences in the mean particles per phagocytic cell between GDID4+/+ and GDID4−/− populations. The differences observed were not significant using the t test (P > .05). We therefore concluded that the development and phagocytic activity of macrophages are not adversely affected by a loss of function of GDID4.

**NADPH oxidase activity.** Direct in situ assays of the colonies with the NBT test showed that GDID4−/− cells retain the ability to generate superoxide (Fig 2D). A blue
TARGETED DISRUPTION OF GDID4

Fig 3. Western blot analysis of GDID4 and RhoGDI in ES-derived colonies on different days (6, 8, and 10) after initiation of culture. (A) GDID4+/− cell lysates probed with anti-GDID4 antiseraum. Note the low level of GDID4 in undifferentiated ES cells (day 0) and the subsequent increase in GDID4 protein level with increasing percentage of hematopoietic cells in the ES colonies. The same amount of protein (4 μg) was loaded per lane. Controls are murine erythroleukemia cells (MEL) and cervical cancer cells (HELA). (B) Same filter as (A) probed with anti-RhoGDI antibody. (C) Colonies from GDID4+/− ES cells probed with anti-GDID4 antibody. No protein was detected at any stage of differentiation. (D) Same filter as (C) probed with anti-RhoGDI. Note the same level of protein as seen in hemizygous cells.

precipitate, which indicates oxidation of the NBT, was visible in cells within some colonies by 30 minutes after addition of the NBT substrate. In a typical day 12 culture dish, the maximum number of colonies (>80%) that turned blue was attained within 4 to 5 hours. To better quantitate the oxidase activity among wild-type versus mutant cells, ES-derived phagocytes were evaluated flow cytometrically with an oxidizable phagocyte agonist, FcOxyburst. As described in the Methods, this agonist binds to the cells’ Fc receptors, even at 4°C, but does not become oxidized; at 37°C, phagocytosis occurs and the oxidative burst is activated, which leads to a large increase in fluorescence as the reduced fluorescein label on Oxyburst becomes oxidized.29,30

It is clear that this activity was more extensive in GDID4+/+ than GDID4−/− cells, although in both lines only a fraction of the total number of cells responded. The peaks are not sufficiently distinct to permit ‘‘gating’’ and evaluation of the number of cells responding, but a difference between the mean channel fluorescences of the labeled (cold) and activated (10 minutes, 37°C) cells can be calculated. Figure 5 shows the composite data for five comparable experiments; while the absolute magnitude of the respiratory burst varied from experiment to experiment, in every case the wild-type activity exceeded that in the (−/−) cells. Since the frequency of macrophages in GDID4−/+ colonies was similar to that in wild-type colonies, the difference in oxidase activity observed was due to a reduction in cellular activity and not to a difference in the frequency of macrophages.

GD1 expression and IgE-induced degranulation in ES-derived mast cells. Recent experimental evidence has indicated that the Rho and Rac GTP-binding proteins may be involved in the degranulation of mast cells.45,46 The expression of GDIs in mast cells has not been previously evaluated. Mast cells were derived from secondary plating of GDID4+/− and GDID4−/− primary ES embryoid colonies in the presence of IL-3 and kit ligand. These mast cells were morphologically similar to bone marrow– or peritoneal-derived mast cells (by granular staining with toludene blue) and remained factor-dependent even after more than 2 months in culture. Western analysis of wild-type, heterozygous, and GDID4−/−

Fig 4. Phagocytosis of unopsonized yeasts by ES-derived macrophages. Mean yeast particles per phagocytic cell; (□) wild-type cells; (□) GDID4+/−; (□) GDID4−/−. Data are the means of duplicate counts, except as indicated (*), where single counts are shown.
cells showed that mast cells do not express GDID4, whereas RhoGDI is abundantly detected (Fig 6). To use the availability of such a homogenous population of ES-derived hematopoietic lineage in functional assays to further support the validity of functional observations about the other ES-derived lineages described earlier, we evaluated the capacity of ES-derived mast cells to degranulate. Hemizygous GDID4+/− mast cells showed a rapid release of granular contents as measured by β-hexosaminidase activity (Fig 7). Mast cells saturated with antigen-specific IgE responded to antigenic challenge, maximally releasing contents within 5 minutes. Compared with total contents (measured by lysing control cells), these mast cells are able to release greater than 60% of contents via IgE receptor activation (Fig 7). The release is slightly but significantly higher when primed with kit ligand before activation. When cells were stimulated with the calcium ionophore A23187, a lower but substantial release of contents was also observed. Similarly, a high level of degranulation triggered with IgE and antigen was observed when GDID4−/− mast cells were challenged. Furthermore, an augmentation of the release as a result of priming by kit ligand was observed and response to A23187 was also intact. Thus, mast cells derived from ES cells degranulate and respond to IgE-receptor–mediated stimulation in a manner similar to that described for animal-derived mast cells.45

**DISCUSSION**

While RhoGDI and GD4 both function in vitro as GDIs for rho, rac, and CDC42, it is not known whether distinct cellular functions are regulated by the two proteins or whether they perform unique plus overlapping functions. Screening of cDNA libraries with GDID4 as bait for putative interacting proteins in the double hybrid yeast system47 has
thus far identified Rho A and Rho C, Rac1, Rac2, and CDC42 as GDID4-binding proteins with no novel GTPases identified (CN Adra and B Lim, unpublished data, June 1995). The in vivo importance of the two GDIs and why two similar GDIs are required remains unclear. A recent report that the IL-1 \( \beta \)-convertase enzyme (ICE) cleaves GDID4 but not RhoGDI indicates that the two GDIs may be processed and regulated differently. Furthermore, data concerning the role of GDI in NADPH oxidase activity have been obtained with RhoGDI only. Our objective was to see whether a phenotypic effect might emerge from a loss of function of the GDID4 gene, thus revealing a distinct function for GDID4.

GDID4-/- ES cells were indistinguishable from normal ES cells morphologically and in their growth rate. Various parameters to evaluate hematopoiesis, including colony-forming efficiency, presence of secondary progenitors, and expression of hematopoiesis-related genes, also show no defect in double mutant ES cells. Since in vitro hematopoietic differentiation of ES cells has been shown to resemble closely in vivo ontogeny of the hematopoietic system, it is likely that animals with loss of function of GDID4, if not lethal during embryogenesis, will also manifest normal development of erythroid and myeloid lineages in the adult animal. Since our in vitro method does not allow evaluation of lymphoid cells, we cannot make any conclusions with regard to lymphopoiesis.

Because of the role of Rho-related proteins in cytoskeletal reorganization, we sought for evidence of abnormalities in this function in several ways. Embryoid colonies after ES-cell differentiation, when dispersed into single cells and plated on culture dishes, give rise to an adherent layer of heterogenous cells that include macrophages and other unidentified epithelioid and fibroblastoid cells. GDID4-/--derived cells spread and adhere on culture dishes as well as wild-type cells. We attempted to use the adherent cells to examine stress fibers formation and membrane-ruffling in response to stimuli (such as platelet-derived growth factor [PDGF] and serum) after starvation, as described for 3T3 fibroblasts. Although we were able to detect the presence of stress fibers in both wild-type and GDID4-/- cells (data not shown), we found that the cell layers we derived cannot be used to yield any meaningful comparative results.

We then exploited the fact that a substantial percentage of the cells within differentiated ES colonies consists of macrophages to examine two phagocytic functions in which the role of Rac and Rho proteins have been implicated, namely, phagocytosis and the generation of superoxide. Colonies pooled at day 12 to 14 of in vitro culture typically contain 10% to 15% macrophages. Thus, by pooling the colonies and allowing the cells to adhere overnight on poly-L-lysine-coated dishes, sufficient numbers of macrophages are available to quantify and compare phagocytic activity between macrophages derived from different ES cells. Similarly, suspensions of cells from pooled colonies contain a sufficient number of macrophages that can be labeled, sorted, and displayed as a distribution of fluorescence activity. Respiratory burst activity by macrophages from normal and mutant ES cells may thus be compared.

Macrophages generated from ES cells and cultured as described displayed avid phagocytosis compared with peritoneal macrophages elicited from animals. GDID4-/- macrophages function as well as wild-type macrophages when tested for their ability to ingest unopsonized yeasts (Fig 2C and Fig 4) and antibody-coated erythrocytes (data not shown). In all experiments, GDID4-/- macrophages manifested a higher phagocytic index than wild-type cells; however, this effect was not statistically significant overall. Therefore, a null mutation of GDID4 does not appear to adversely affect some of the cytoskeletal reorganization functions attributed to the Rho-related proteins.

Rac has been shown to be an essential component of a protein complex required for the activation of NADPH oxidase. This function appears to be independent of the cytoskeleton. A markedly enhanced superoxide formation by neutrophils was observed in null-mutant cells purified from bcr-/- animals. This effect is consistent with the fact that bcr functions as a GAP for Rac to limit Rac activity by catalyzing the hydrolysis of GTP-bound Rac to the inactive GDP-bound form. Therefore, removal of bcr would be akin to the release of a brake for oxidase activity. It has also been shown, in a cell-free system, that RhoGDI inhibits oxidase activity. This would be consistent with the role of RhoGDI acting as a GDI to suppress the exchange of GDP for GTP, inhibiting the level of active Rac proteins, or to sequester Rac, making it unavailable for active complexing with other oxidase components. A loss of RhoGDI activity might therefore be expected to cause an increase in respiratory burst activity similar to a loss of bcr. However, it is possible that within the cellular context, GDID4, although homologous to RhoGDI, may perform a function of different importance.

Direct in situ NBT tests with colonies demonstrated that the production of superoxide is intact in GDID4-/- macrophages. Since it was difficult to obtain pure cell populations from these colonies, we used the single-cell measurement capabilities of flow cytometry to evaluate the accumulated activity of cells in GDID4-/- versus GDID4-/- colonies, which were capable of mounting an oxidative burst response to a cross-linking phagocytic and degranulatory stimulus acting via the Fc receptor. As the flow cytometer measures only cell-associated fluorescence, the formation of phagocytic vacuoles and consequent trapping of oxidative entities leads to a less delayed and much greater fluorescence than would be measured with a cytoplasmic probe. This permits us to observe differences in a rate of oxidative product release, within the first few minutes of stimulation, rather than rely on a static extent of oxidation measurement after a long period of time. Although the fraction of cells capable of this response was small and similar in both groups of cells, the rate of oxidation was clearly higher in the wild-type ES-derived macrophages than in GDID4-/- cells in every experiment. Since the measurement of oxidase activity in this method is dependent on phagocytosis, and since the phagocytic activity of GDID4-/- cells was, if anything, more avid than that of wild-type cells, the magnitude of superoxide production in (+/+) cells would be underestimated compared to GDID4-/- cells. Therefore, our observation that
oxidase activity in GDID4−/− cells is lower than (+/+) cells becomes more significant. Five separate experiments were performed to evaluate oxidase activity. Within the limits of the experimental technique, the differences were subtle but repeatable. Experiments with large numbers of pure macrophages and neutrophils derived from null-mutant animals should provide further clarification of the result. It should also be interesting to see if the subtle defects we detected translate into any significant abnormalities of immune and inflammatory response in whole animals. If confirmed, the result has several implications. It suggests that GDID4 may play an accessory role in facilitating the generation of active oxidase complex rather than inhibiting the process. Since an active GTP-bound form of rac is required for oxidase activity, it would also suggest that the primary role of GDID4 may not be to inhibit the level of GTP-bound rac. One possible way to explain our result is that GDID4 is required for facilitating rac transport to membrane sites of oxidase activity, while RhoGDI may be the important protein for regulating the level of active GTP-bound rac. Another possible explanation is that GDID4, like RhoGDI, may also have the capacity to inhibit the intrinsic and GAP-stimulated GTPase activity of rho-related proteins.28,29 A loss of function of GDID4 may therefore reduce the cellular mechanism to sustain levels of GTP-bound active Rac, resulting in a reduction in the rate or overall reduction of superoxide production.

Recently, experimental evidence has indicated that Rac and Rho may also be involved in the exocytosis of mast cells.56,67 Constitutively active mutant proteins of Rho and Rac enhanced secretion from permeabilized mast cells, while inhibition of endogenous Rac and Rho reduced the secretory response of mast cells to stimuli. The mechanism of granular secretion in mast cells may be analogous to the role of RabGDIs and rab proteins in the secretion of neuronal vesicles.68 However, the expression of GDIs in mast cells has not been previously addressed. We took advantage of the fact that large numbers of pure mast cells may be generated from in vitro–differentiated ES colonies.35 We found RhoGDI protein to be present in mast cells as evaluated by our methods, may be attributed to a considerable overlap and redundancy of function between RhoGDI and GDID4. A deletion of both genes may be necessary to better reveal their functional scope. The significance of having two quite similar GDIs remains elusive and indicates the fundamental importance of these regulatory proteins. Finally, our experiments demonstrate that the in vitro culture system of ES cells can provide a rapid means of deriving significant information about hematopoietic cell gene function before the more expensive and elaborate in vivo experiments, including, as others have shown, yielding useful insight concerning the cellular function of genes whose disruption may be lethal to the embryo or fetus.

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