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J Immunol 2007; 179:8322-8331; doi: 10.4049/jimmunol.179.12.8322
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Rap1a Null Mice Have Altered Myeloid Cell Functions Suggesting Distinct Roles for the Closely Related Rap1a and 1b Proteins

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The Ras-related GTPases Rap1a and 1b have been implicated in multiple biological events including cell adhesion, free radical production, and cancer. To gain a better understanding of Rap1 function in mammalian physiology, we deleted the Rap1a gene. Although loss of Rap1a expression did not initially affect mouse size or viability, upon backcross to C57BL/6J mice some Rap1a<sup>−/−</sup> embryos died in utero. T cell, B cell, or myeloid cell development was not disrupted in Rap1a<sup>−/−</sup> mice. However, macrophages from Rap1a null mice exhibited increased haptotaxis on fibronectin and vitronectin matrices that correlated with decreased adhesion. Chemotaxis of lymphoid and myeloid cells in response to CXCL12 or CCL21 was significantly reduced. In contrast, an increase in FcR-mediated phagocytosis was observed. Because Rap1a was previously copurified with the human neutrophil NADPH oxidase, we addressed whether GTPase loss affected superoxide production. Neutrophils from Rap1a<sup>−/−</sup> mice had reduced FMLP-stimulated superoxide production as well as a weaker initial response to phorbol ester. These results suggest that, despite 95% amino acid sequence identity, similar intracellular distribution, and broad tissue distribution, Rap1a and 1b are not functionally redundant but rather differentially regulate certain cellular events. The Journal of Immunology, 2007, 179: 8322–8331.
whether this lethality was solely due to the loss of Rap1 activity (21–24).

To better understand the role of Rap1 in mammalian cell biology and to address possible redundancy between the 1a and 1b proteins, we generated a mouse lacking the rap1a gene. rap1A−/− mice initially exhibited no overt phenotype. However, upon backcrossing mice into the C57BL/6J strain, some embryonic lethality was observed. Because of the high abundance of Rap1a in human platelets (25) and Rap1a in human neutrophils (26) where it associates with the phagocyte NADPH oxidase (5, 27), we examined several properties of Rap1a-null white blood cells. Although there were no obvious defects in blood cell development, changes in macrophage adhesion, haptotaxis, and phagocytosis were observed and neutrophils produced less superoxide in response to formyl peptide and phorbol ester challenge despite the presence of Rap1b in these cells. These data support the role of Rap1 in cell adhesion and migration, provide a unique model for studying Rap1 function, and suggest that the Rap1a and 1b proteins have both redundant and unique biological functions.

Materials and Methods

Generation of Rap1A targeting vector

A 19 kb partial Rap1a genomic clone isolated from a 129Sv/J mouse genomic library (28) was sequenced to generate a restriction map and identify exons. The clone was found to encode exons 3 through 6 of rap1a that encode residues 43–184. To generate the targeting vector, a 0.95 kb PvuII-NdeI fragment, located upstream of exon 4, was blunted and inserted into the SacI site of the pBluescript II/NeoTK vector (Fig. 1A). This vector contains the phosphoglycerokinase promoter driving the neo gene and the herpes simplex virus thymidine kinase gene both being expressed in the opposite direction to the Rap1a sequence. An 8.6 kb NdeI-StuI fragment downstream of exon 4 was inserted at the XhoI site of the vector. By this strategy, exon 4 (codons 62–108) and surrounding sequence was replaced by the neomycin resistance gene. The targeting vector was linearized using NotI and electroporated into embryonic stem (ES) cells.

Generation of Rap1A knockout (KO) mice

Culture of ES cells and isolation of homologous recombinants were performed according to standard protocols (30). Screening of 261 G418/gancyclovir double resistant ES clones for homologous recombinants was performed by PCR methods as described (30) using 5′-CCCTAAATGCTTCTGCTTAAAGCT-3′ and 3′ end primers are shown as well restriction enzymes used in gene manipulation or analysis. BglII, NdeI, PstI, PvuII, StuI, SphI, and NeoI were used as markers. Genomic DNA from ES cells was prepared with the Puregene DNA isolation kit (Genta). Genomic DNA was isolated from mouse tails by the proteinase K digestion method. Mouse tail pieces were clipped and digested in a solution containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1% SDS, 200 μg of proteinase K per ml at 57°C overnight. After two chloroform-isooamy alcohol (24/1) extractions, the DNA was precipitated with ethanol. Genomic DNA (10–20 μg) digested with SpeI or PstI (–10 U/μg) was resolved by electrophoresis in 0.5% agarose gels and transferred to nitrocellulose membranes (BA-85; Schleicher and Schuell). DNA fragment probes were radiolabeled with 32P using the Megaprime DNA labeling system (GE Biosciences).

Flow cytometry

All Abs were purchased from BD Biosciences. Surface expression on the indicated cell populations was analyzed by flow cytometry (FACSscan or FACSComp; Becton Dickinson). The percent positive population was evaluated by the CellQuest program (BD Biosciences).

Blood cell proliferation assays

Cells were isolated from spleens of the indicated genotypes of mice. Cells were then plated in triplicate at 5 × 104 cells/well in a round-bottom 96-well plate in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone) and stimulated with 2.5 μg/ml platebound anti-CD3 (for T cells) or 2.5 μg/ml anti-IgM ± 5 ng/ml IL-4 (for B cells) for 72 h. Cells were pulsed for the last 16–18 h with 1 μCi per well of [3H]thymidine. The incorporation of [3H]thymidine was measured with a liquid scintillation counter.

FIGURE 1. Rap1a deletion strategy and detection. A, KO strategy showing replacement of exon 4 with neo resistance gene. Positions of genotyping primers are shown as well restriction enzymes used in gene manipulation or analysis. BglII; N, NdeI; Pst, PstI; PvuII, PvuII; S, SphI; Stu, StuI. Numbered regions indicate location of exons, solid black bars show short and long arms used in homologous recombination. B, PCR analysis of Rap1a null mice. Using a common primer 5′ of the targeted sequence (in exon 3) and 3′ primers complementary to either exon 4 or the neo gene, PCR detected Rap1a +/+ (upper band only) −/− (lower band only) or +/- (both bands) mice. Con, control plasmid template; F1, chimeric founder mouse. C, Western blot of Rap expression in mouse neutrophils. Cells were blotted for Rap1a, total Rap1, or Rap2 expression using specific Abs. GAPDH was used as an internal loading control. Data are shown for three separate Rap1a+/+ and −/− mice.
**Th cell differentiation assay**

CD4+ cells were purified from spleen and lymph node cells by MiniMacs positive selection and plated in RPMI 1640 plus 10% FBS. Cells were plated at 1 × 10^6/ml stimulated with 4 μg/ml platebound anti-CD3, 0.5 μg/ml anti-CD28 in either Th1 (2 ng/ml IL-12 + 10 μg/ml anti-IL-4) or Th2 (10 ng/ml IL-4 + 10 μg/ml anti-IFN-γ) culture conditions. After 5 days in culture, cells were washed and restimulated with 2 μg/ml platebound anti-CD3 alone. Supernatants were collected after 24 h and tested for levels of IL-4, IL-5, IL-13, and IFN-γ.

**Macrophage isolation, cell migration, and phagocytosis adhesion assays**

Macrophages were prepared as previously described (31). Cell migration assays were performed on polycarbonate membranes using Transwell migration chambers (pore size 8 μm; Costar) as previously described (31). The underside of the membrane to which cells migrate was coated with 20 μg/ml of either vitronectin, or α4β1 integrin-binding fragment of fibronectin, H296, in PBS for 1 h at 37°C. Surfaces were subsequently blocked with heat-denatured BSA. Cell adhesion assays were performed using flat-bottom 96-well poly styrene plates coated with 20 μg/ml of either vitronectin, or the H296 fibronectin peptide. Adherent cells were fixed with 3.5% formaldehyde and stained with 0.1% crystal violet. The stain was eluted with 10% acetic acid, and absorbance was determined at 600 nm with a microplate reader (31). Phagocytosis of serum opsinized-zymosan-H296 was obtained from Collaborative Biomedical. Vitronectin was purified with 10% acetic acid, and absorbance was determined at 600 nm.

**Superoxide anion measurement**

Superoxide production in response to phorbol ester (200 ng/ml anti-CD3 for 48 h and proliferation was assessed by [3H]thymidine incorporation. As shown in Fig. 2C, both WT and Rap1a-deficient T cells proliferated to a similar extent in response to anti-CD3. To further test T cell function, CD4+ T cells were purified from WT and Rap1a-deficient mice and differentiated to Th1 and Th2 phenotypes using cytokine and Ab mixtures as described in Materials and Methods. The resulting populations were then washed, counted, and restimulated with anti-CD3 alone. WT and Rap1a-deficient Th1 cells secreted similar levels of IFN-γ and minimal levels of Th2 cytokines (Fig. 2D). Conversely, Th2 cells derived from both genotypes secreted similar levels of the

**Detection of Rap1 proteins in mouse BM neutrophils**

Neutrophils were suspended in PBS to no greater than 5 × 10^7/ml. A total of 2.7 mM dithioerythritol, a serine protease inhibitor, was added and incubated for 10 min on ice. After two washes in PBS, 5 × 10^7 cells were lysed in 1 ml IPF buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 ng/ml chymostatin, 2 mM PMSF, 10 mM leupeptin, 1 mM 4-c2-aminoethyl benzenesulfonyl fluoride) with 30 min incubation on ice. Cell debris were centrifuged down at 16,000 × g for 2 min at 4°C. Cleared lysates were mixed with 2× sample buffer and boiled for 5 min. Thirty micrograms of lysate protein was loaded onto SDS-PAGE gels. Rap1a protein was detected with mAb T22, a gift of Dr. Yoshimi Takai (Osaka University, Osaka, Japan) (26), and total Rap1a/b by anti-Rap1a (H-C. Chang and M. Kaplan, unpublished observations). To determine whether Rap1a is required for normal T cell development, we first analyzed thymocytes from WT and Rap1a−/− mice. Thymi from both genotypes had similar cellularity and flow cytometry revealed similar percentages of cells in the CD4 and CD8 double-negative, double-positive, and single-positive populations (Fig. 2A). Within the double negative population, there were similar percentages of cells expressing CD25 and CD44 (H-C. Chang and M. Kaplan, unpublished observations). These results suggest that Rap1a-deficiency does not adversely affect T cell development.

**Results**

**Generation of Rap1a−/− mice**

To explore the biological role of Rap1a, a targeting construct that replaces the fourth exon of the murine GTfase/with a neomycin resistance gene cassette was developed and used to disrupt the Rap1a gene in ES cells by homologous recombination. Proper integration of the cassette was established by Southern blot (Y. Li and L. Quilliam, unpublished observations) and PCR analysis (Fig. 1B). Embryonic stem cells were injected into mouse blastocysts. Chimeric mice were then bred to obtain germine transmission of the disrupted allele. Heterozygous mice were further bred to obtain Rap1a-deficient mice. Purified neutrophils were blotted for Rap1a expression using an isoform-specific mAb (26). Rap1a was not detected in these cells (Fig. 1C), or in various organs derived from −/− mice (Y. Li and L. Quilliam, unpublished observations). Furthermore, a ~16 kDa protein that could potentially have arisen from splicing of exons 3 and 5 was not detected. This was consistent with the lack of Rap1 detection from an engineered Rap1a cDNA, missing the equivalent 47 codons encoded by exon 4, upon transfection of 293T cells (residues 62–108; Y. Li and L. Quilliam, unpublished observations). Western blotting with a pan Rap1 Ab that detects Rap1a and 1b, but not Rap2 indicated a ~25% decrease in total Rap1 protein expressed in neutrophils following deletion of Rap1a. Only modest changes in neutrophil Rap2 were detected (Fig. 1C).

**Rap1a−/− mouse viability depended on background strain**

Rap1a−/− mice were viable, healthy, and fertile, suggesting that the Rap1a gene is not required for mouse development or breeding. Separation of the mutant Rap1a allele followed Mendelian inheritance (24%:51%:25% ratio of +/+:+/+:+:−/− from 446 mice born from heterozygote mating) with +/+ and −/− mice weight and the organ/body weight ratio of their brain, heart, liver, and lungs being indistinguishable between genotypes (Y. Li and L. Quilliam, unpublished observations). These mice were used for the initial characterization of Rap1a function. However after backcrossing for six generations into the C57BL/6J mouse strain, the number of Rap1a−/− pups generated from heterozygote crosses was significantly reduced (30.3:54.8:14.9% ratio of +/+:+/+:+:−/− from 241 mice) with no sex bias. Dissection of pregnant Rap1a−/− mice at E9.5–14.5 indicated that the lethality occurred much earlier than E9.5 due to no trace of embryos in the deciduas (H. Chen and W. Shou, unpublished observations).

**Normal T cell development and function in the absence of Rap1a**

Rap1a is highly expressed in various cells of hematopoietic lineage (13, 26, 36, 37). Therefore, we next examined whether the loss of Rap1a impacted T or B cell function. Rap1 is activated in T cells by TCR engagement variably resulting in decreased intracellular signals and enhanced integrin-mediated adhesion (13, 37–39). However, the role of Rap1 in T cell development had not been examined. To determine whether Rap1a is required for normal T cell development, we first analyzed thymocytes from WT and Rap1a−/− mice. Thymi from both genotypes had similar cellularity and flow cytometry revealed similar percentages of cells in the CD4 and CD8 double-negative, double-positive, and single-positive populations (Fig. 2A). Within the double negative population, there were similar percentages of cells expressing CD25 and CD44 (H-C. Chang and M. Kaplan, unpublished observations). These results suggest that Rap1a-deficiency does not adversely affect T cell development.
FIGURE 2. Analysis of T cell development and function. A, Flow cytometric analysis of CD4 and CD8 expression on thymocytes from WT and Rap1A-deficient mice. B, Flow cytometric analysis of CD4 and CD8 expression on WT and Rap1A-deficient spleen (top) and peripheral blood (bottom). C, Splenocytes from WT and Rap1A-deficient mice were stimulated with 2.5 μg/ml anti-CD3 in microwells. Cultures were pulsed with [3H]thymidine for the last 18 h of a 72 h incubation. D, CD4+ cells were purified from WT or Rap1A-deficient spleen and stimulated with 4 μg/ml platebound anti-CD3, 0.5 μg/ml anti-CD28, or 10 ng/ml IL-4 plus 10 μg/ml anti-IFN-γ, for Th2 cultures or 2 ng/ml IL-12 plus 10 μg/ml anti-IL-4 for Th1 cultures. After 5 days in culture, cells were restimulated with anti-CD3 alone and supernatants were recovered after 24 h for cytokine testing by ELISA. Data represent mean ± SEM from six +/+ and five −/− mice.

Th2 cytokines IL-4, IL-5, and IL-13 with little IFN-γ (Fig. 2D). Together, these results indicate that Rap1a is not required for T cell development, migration to peripheral lymphoid organs, proliferation, or Th cell differentiation.

Normal B cell development and function in Rap1a-deficient mice
Rap1 is also activated by BCR engagement (40). To determine whether Rap1a is required for B cell development, we first analyzed B cells in the bone marrow of WT and Rap1a-deficient mice. Percentages of B220+ and B220+CD43+ populations were indistinguishable between the two genotypes (Fig. 3A). Similarly, the percentages of B220+ cells in the spleen, lymph nodes, and peripheral blood of Rap1a-deficient mice did not indicate any defect in B cell development or migration into the periphery (Fig. 3B and unpublished observations).

To test the function of Rap1a-deficient B cells, splenocytes from WT and Rap1A−/− mice were stimulated in vitro with anti-IgM in the presence or absence of IL-4. Similar levels of proliferation were observed in WT and Rap1A-deficient cultures stimulated with anti-IgM or anti-IgM plus IL-4 (Fig. 3C). Serum levels of IgM, IgG1, and IgG2a were also indistinguishable between WT and Rap1A-deficient cells (Fig. 3D). Together, these data indicate that Rap1A is not required for B cell development or function.

Normal macrophage and granulocyte development in Rap1a-deficient mice
Monocytes and polymorphonuclear leukocytes are stimulated by several factors that activate Rap1 and, thus, Rap1a may be required for development or function (41). To examine the content of these cells in marrow and spleen, we analyzed the expression of CD11b and Gr-1 on cells in the bone marrow and spleen of WT and Rap1A-deficient mice. As shown in Fig. 4, the percentages of CD11b+ and CD11b+Gr-1+ cells are similar between these genotypes, suggesting that Rap1a is not required for normal production of these cells.

Macrophages from Rap1a−/− mice exhibited increased haptotaxis, migration, and phagocytosis
Rap1 has recently been implicated in inside-out signaling to regulate integrin-mediated adhesion (12, 14, 42–45). To address whether Rap1a was required for macrophage adhesion, isolated bone marrow-derived cells were incubated in fibronectin peptide H296-coated 96-well plates for 4 h and bound cells were measured. This is a measure of α5β1 integrin-dependent adhesion. As seen in Fig. 5A, Rap1a null cells bound to this fibronectin fragment less effectively. Similar results were found with adhesion to vitronectin, an αv-specific extracellular matrix protein (P. De and D. Durden, unpublished observations). The levels of Rap1a and total Rap1 were measured using appropriate Abs. Although Rap1a, as expected, was absent from the cells, total Rap1 levels were similar to that of WT mice (Y. Li and L. Quilliam, unpublished observations). These findings suggest Rap1a and 1b play differential roles in signaling pathways required for provisional integrin dependent migration.

To further address the role of Rap1a in integrin-mediated events, we generated mouse embryo fibroblasts (MEFs) from E13.5 embryos and immortalized them using SV40-T (46). A modest but statistically significant reduction in adhesion was noted (30% reduction in adhesion to fibronectin and collagen I, p < 0.001).

Because macrophages function by migrating to and phagocytopsing foreign material within the body, we examined whether the modest reduction in macrophage adhesion noted above affected their ability to function. Bone marrow-derived (BMD) and splenic
macrophages were isolated and random movement (haptotaxis) examined following plating on 8-μm porous filters. As shown in Fig. 5B, BMD macrophages from Rap1a−/− mice migrated considerably faster than those obtained from Rap1a+/- littermates. Similar results were obtained using either vitronectin or the H296 fibronectin-derived peptide (Fig. 5B) and were qualitatively similar in splenic cells (PD and DLD, not shown). This suggests that the reduced adhesion to matrix may have permitted cells to migrate more effectively. Using flow cytometric analysis, surface expression levels of αv (CD51), α5 (CD49e), Mac-1 (CD11b), β2 (CD18), and α4 (CD49d) integrins or the macrophage marker F4/80 were not significantly different between WT and Rap1a−/− cells (J. Yan, V. Munugulavadla, and R. Kaqur, unpublished observations) suggesting that altered integrin levels were not responsible for the reduced adhesion.

Rap1 has been implicated in phagocytosis in both Dictostelium and macrophages and localized to endocytic vesicles as well as the specific granules of the neutrophil (47–50). Therefore, we next compared the ability of macrophages from WT and Rap1a−/− mice to take up IgG-coated sheep RBCs. The phagocytic index (number of ingested particles/100 macrophages) was greatly increased in Rap1a null cells implicating Rap1a in receptor signaling and/or phagocytosis (Fig. 5C). There was no difference noted in the percentage of macrophages forming sheep RBC rosettes nor the number of sheep RBCs bound per spleen-derived or BMD macrophage between Rap1a−/− and WT mice (P. De and D. Durden, unpublished observations). The above macrophage phagocytic activity had been measured in response to FcR stimulation that is mediated by Rac- and Cdc42-induced engulfment of particles (51). To examine the response to integrin activation, which is alternatively mediated by ρ (51), we further examined the ability of macrophages to engulf C3bi-opsonized sheep RBCs. Surprisingly, there was no significant difference in the phagocytosis of C3bi-opsonized cells in macrophages derived from Rap1a−/- vs +/− mice, neither in terms of the percentage of cells taking up RBCs nor the number of RBC taken up by individual macrophages (Fig. 5D). Cell surface expression of FcγRII/III (CD16/32), as measured by fluorescence staining intensity, was not significantly different between Rap1a+/+ and −/− macrophages. Furthermore, all the above macrophage studies yielded comparable data using second or seventh generation backcross into the C57BL/6J mouse strain.

Loss of Rap1a resulted in decreased chemotaxis

Inside-out signaling involves the activation of integrin-extracellular matrix binding from within the cell. Several reports have implicated Rap1 in promoting increased affinity and/or avidity of integrins depending on the system studied (12). G-protein-coupled chemokine receptors promote integrin activation and Rap1 has

FIGURE 3. Analysis of B cell development and function. A, Flow cytometric analysis of B220 and CD43 expression in bone marrow from WT and Rap1a-deficient mice. B, Flow cytometric analysis of B220 and CD3 expression on spleen and peripheral blood cells from WT and Rap1a-deficient mice. C, Splenocytes from WT and Rap1a-deficient mice were stimulated with 2.5 μg/ml anti-IgM in the presence or absence of 5 ng/ml IL-4 in microwells. Cultures were pulsed with tritiated thymidine for the last 18 h of a 72 h incubation. D, Serum was isolated from WT and Rap1a-deficient mice. Concentrations of individual Ig isotypes were tested by ELISA.

FIGURE 4. Analysis of macrophage and granulocyte development. Flow cytometric analysis of CD11b and Gr-1 expression on bone marrow and spleen cells from WT and Rap1a-deficient mice.
been implicated as a downstream effector in several G protein-mediated events (44, 52, 53). We therefore examined the ability of T cells, B cells, and myeloid cells to migrate toward SDF1/CXCL12 and CCL21 chemokines. As seen in Fig. 6, a decrease in basal and chemokine-induced migration was observed upon loss of Rap1a. A similar decrease in chemotaxis was seen in Rap1a−/−BMD T and B cells as well as macrophages (K. Christopherson, J.}

**FIGURE 5.** Loss of Rap1a affects macrophage haptotaxis, adhesion and phagocytosis. A. Adhesion of WT and Rap1a−/− macrophages to the (αb, integrin binding) fibronectin H296 peptide or vitronectin-coated cell culture wells was determined 90 min post plating following fixing and staining of adherent cells. B. Haptotaxis of WT and Rap1a−/− macrophages through fibronectin H296 peptide-coated Transwell filters was measured at indicated time points. C. Phagocytosis of IgG-coated sheep RBC by BMD macrophages was measured at the indicated time points after exposure. D, Phagocytosis of C3bi-opsonized sheep RBCs by macrophages was measured 30 min after exposure. All data are mean ± SD for cells derived from three mice/genotype and are representative of at least four independent experiments (two experiments using a total of four mice were pooled for 5D).

**FIGURE 6.** Loss of Rap1a influences chemotaxis of leukocytes. The ability of B220+ splenic B cells (upper), CD4+ splenic T cells (middle), or Sca1+/lin- bone marrow hematopoietic stem cells (lower) from WT and Rap1a−/− mice to migrate toward CCL21 or CXCL12 chemokines was determined using a Transwell assay. Data represent mean ± SEM for cells derived from six mice (four for bone marrow). * p < 0.05 with a two-tailed Student’s t test.
was not statistically distinguishable between WT and KO mice had a slower, milder response. The onset of neutrophil superoxide production in response to PMA is typically slower but overall NADPH oxidase activity is much more pronounced and sustained. Although the sustained induction of superoxide production was not statistically distinguishable between WT and Rap1a−/− neutrophils, the initial rate of superoxide production was significantly reduced in Rap1a−/− mice (Fig. 7B). This was seen using isoluminol or cytochrome C assays to measure superoxide production. These data support previous findings for a role of Rap1 in NADPH oxidase regulation but indicate that, unlike Rac2 (56–58), Rap1a is not an essential component of the enzyme complex. Analysis of Rap1a and total Rap1 indicated that there was still a significant amount of Rap1b expressed in Rap1a−/− mouse neutrophils (Fig. 1C).

**Discussion**

Rap1 has been implicated in a number of biological events and is regulated by a broad spectrum of biological stimuli and pathways (10, 12–14). Furthermore, over-activation of Rap1 by KO of the Spa1 Rap GAP or a RasGRP2 translocation or expression of a mutant transgene, has been recently reported to promote several myeloproliferative disorders (16, 17, 59). Loss of the single Rap1 gene in Drosophila was lethal at the larval stage, suggesting its fundamental role in development (18). However, mammals have two copies of Rap1 (1a and 1b) and, although there is data indicating some differential expression, most notably high Rap1b expression in platelets (25), little was known about the specific functions of the two isoforms. Therefore, to better understand the biological functions of mammalian Rap1, we deleted the mouse Rap1a gene. The results of this study demonstrated that loss of Rap1a affected cell adhesion, migration, phagocytosis, and the oxidative burst of leukocytes. This occurred in cells that expressed significant levels of Rap1b protein, suggesting that the two Rap1 isoforms are not redundant. Additionally, defects in early embryonic development were observed that were strain dependent and exhibited only partial penetrance.

The Mendelian ratio of mice born from heterozygote crosses of mixed lineage (C57BL/6J:129Sv) animals was normal, consistent with the findings of a recent independent report of a Rap1a−/− mouse (60). However, our findings of embryonic lethality in
C57BL/6J back-crossed mice suggests that levels or timing of, for example, Rap1b expression may compensate for loss of Rap1a in the 129Sv but not the C57BL/6J strain. 129Sv-Rap1a−/− mice were not established from chimera founders. Loss of mice during early development is consistent with embryonic morphogenesis defects in Drosophila (18) and may be related to the role of Rap1 in cell-cell and/or cell-matrix adhesion (14, 15). Interestingly, the noncanonical Wnt pathway was recently demonstrated to regulate zebrafish and Xenopus gastrulation by activating casein kinase ε. This kinase then bound and phosphorylated the Rap GAP, Spak, resulting in Rap1-GTP accumulation (61). It is therefore possible that loss of Rap1a in mice resulted in insufficient Rap1 activity during gastrulation to mediate Wnt-regulated morphogenesis.

During the completion of this study, Chrzanowska-Wodnicka et al. (62) reported that Rap1b KO resulted in 85% embryonic and perinatal lethality. This difference from Rap1a null mice may, at least in part, be due to abnormal bleeding associated with reduced platelet function following Rap1b loss (62). Using the Rap1a−/− mice described in the current study, it was established that Rap1A-null platelets have normal aggregation responses (62), again supporting differential roles for the Rap1a and 1b isoforms in higher eukaryotes. Six of the nine residues that are divergent between Rap1a and 1b are located close to the C terminus. This is reminiscent of Rac 1 and 2 where 50% of divergent residues occur in the hypervariable domain adjacent to the prenylated CAAX cysteine. C-terminal tail swaps between Rac1 and 2 have demonstrated that these residues confer unique properties to these proteins (63, 64). Whether, like Rac1 vs Rac2, these differences between Rap1a and 1b are due to their divergent C termini remains to be established. Because the hypervariable region can influence the subcellular locale of Ras proteins (65–67), one might anticipate unique subcellular distribution. However, our findings expressing GFP-fusion proteins in Cos7 cells (Y. Li, J. Yan, and L. Quilliam, unpublished observations) and those of Pizon et al. (48) suggest that the two proteins have similar intracellular localization. This may however differ in specialized cells or situations. Most Rap1a or 1b specific Abs are of poor quality, detect only unprocessed protein, or are no longer available (68–70) making differential expression and localization of endogenous proteins difficult to monitor. We are currently attempting to generate isoform specific anti-sera to address such issues.

Analysis of blood cells revealed that Rap1a was not required for development, migration to peripheral organs, or many mature functions of T, B, or myeloid cells. This is consistent with a recent study by Duchniewicz et al. (60) who independently knocked out Rap1a. Duchniewicz et al. reported that primary hemopoietic cells isolated from the spleen or thymus had a diminished adhesive capacity on ICAM and fibronectin substrates. In addition, polarization of T cells from Rap1a−/− mice after CD3 stimulation was impaired. These cumulative data suggest that Rap1 isoforms are, at least in part, redundant. Total levels of Rap1 were only reduced by 25% in Rap1A−/− neutrophils, suggesting that Rap1b either is the major isoform or is up-regulated to compensate for the loss of Rap1A. In support of the former notion, PCR analysis of reverse transcribed neutrophil mRNA (Y. Li and L. Quilliam, unpublished observations) suggested that there was at least a two-fold excess of Rap1b over Rap1a message. Therefore it is all the more interesting that there were defects in the ability of Rap1a−/− cells to adhere and migrate. This finding further supports the notion that Rap1 isoforms serve distinct as well as common functions. Upon crossing, Rap1a−/− mice with the Rap1b−/− mice generated by Chrzanowska-Wodnicka et al. (62), Rap1a/1b double KO was more lethal than loss of either single isoform; no surviving null offspring were obtained (N. Paianavitana and L. Quilliam, unpublished observations). However, this finding can support either common or divergent function for Rap1 isoforms.

Because Rap1 has been demonstrated to activate various integrins, the reduction in macrophage binding to fibronectin and vitronectin was logical and consistent with reduced adhesion of MEFs from C3G KO mice (19, 20). Although C3G can also regulate Rap1b, Rap2, TC10, and R-Ras (21–24), our data suggest that the effect of GEF loss was at least in part due to decreased Rap1a activation. It is likely that the modest observed decrease in adhesion in the current study was responsible for the enhanced macrophage haptotaxis as was observed with C3G−/− fibroblasts (19, 20). This finding is supported by the decreased adhesion capacity of primary hemopoietic cells in this study and impaired polarization of T cells from Rap1a−/− mice reported by Duchniewicz et al. (60). Similar binding of cell surface integrins was observed on macrophages isolated from Rap1a+/+ and −/− mice in the current study, suggesting that altered adhesion was not because of changes in integrin composition.

In contrast to nondirectional haptotaxis, chemotaxis involves directed migration in response to a chemical gradient. Because Rap1 signaling has been found downstream of G protein coupled receptors (52, 53) and to couple CXCR4 to integrins (44), the reduced chemotactic response to CXCL12 or CCL21 was anticipated and was consistent with our findings. That the chemokines still elicited a significant response is presumably due to the continued presence of Rap1b. Alternatively, Rap2 has also been implicated in chemokine signaling (71). The decreased basal migration on plastic in Fig. 6 cannot be attributed to increased adhesion as Rap1a−/− macrophages bound slightly less than WT cells to tissue culture plastic (10% difference, p < 0.005) but still exhibited reduced basal and chemokine-induced migration (J. Yan, unpublished observation).

Because Rap1 controls integrin-mediated events through inside-out signaling (14) and specifically can control functional activation of the integrin-type CR3 receptor (αMβ2) (47), we anticipated that loss of Rap1a would impair phagocytosis of complement-coated particles. Because no effect of Rap1a deletion was observed, it is possible that Rap1a and 1b act redundantly in this process or that it is preferentially mediated by Rap1b. FcγR-mediated phagocytosis of Ig-coated particles is a Cdc42/Rac2-dependent process in macrophages (72). Because we have previously implicated Rap1 in Rac activation (73), it was again anticipated that loss of Rap1a might attenuate uptake of IgG-coated red cells. Interestingly, Rap1a-null macrophages exhibited enhanced FcγR-mediated phagocytosis. We and others have found that activation of Rap1 by its upstream GEFs is spatially regulated, resulting in selective activation of downstream targets at specific intracellular locales (74–78). It is tempting to speculate that in the absence of Rap1a, Rap1b can be more effectively activated in a locale that leads to phagocytosis. For example, activation of Rac by Vav2 at the cell periphery to promote cell spreading is dependent on Rap1-GTP (73). Drosophila macrophage function is dependent on the Rap GEF “Dizzy” (79). Because the localization of the mammalian homologue of Dizzy, PDZ-GEF1/RapGEF2, is regulated by its Rap1-GTP product (75), loss of Rap1a might lead to PDZ-GEF redistribution to more effectively activate Rap1b and enhance phagocytosis. The development of Rap1 isoform-specific Abs will help address this possibility. Because FcγR signaling in myeloid cells and α, integrin-mediated haptotaxis in macrophages requires Rac2 and the Syk tyrosine kinase, but not Rac1 (31), preferential interaction of Rap1a vs 1b with the Syk-Rac2 signaling axis in myeloid cells might occur. Future experiments will examine the impact of Rap1a loss on integrin-induced actin polymerization and macrophage polarization.

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Despite Rap1 identification within the phagocyte NADPH oxidase (5, 27), the GTP-dependent component of this enzyme complex was later determined to be Rac2 (56). In this study we show that, unlike Rac2, there is not an absolute requirement for Rap1a in order for neutrophils to respond to formylated peptide or phorbol ester. Because then the transient IMPL response and the early stages of the PMA response were attenuated by Rap1a loss, Rap1a may serve a function in enhancing complex assembly, such as NADPH recruitment (73) and/or Rac2 activation or specific granule trafficking. Although Rap1a is abundant in human neutrophils (26), there was considerable Rap1b detectable in the Rap1a−/− mouse phagocytes. Interestingly, despite a dependence on Rac2 for NADPH oxide regulation (58), mouse neutrophils express considerably more Rac1 vs Rac2 compared with humans (35). A similar scenario may exist with Rap1 isoforms in these species.

In summary, we have observed changes in blood cell adhesion, migration, and phagocytosis as well as embryogenesis in Rap1a−/− mice. Our findings are supportive of both novel and redundant functions for Rap1 isoforms. The use of these mice with novel small molecular weight GTP-binding protein with the same putative functions in embryonic stem cells: a gene-targeting tag-and-exchange strategy. This paper describes the results of our studies on Rap1a−/− mice. Our findings are supportive of both novel and redundant functions for Rap1 isoforms. The use of these mice should enable a better understanding of Rap1 function at the cellular and whole animal level. Additionally, because Rap1 activation has been implicated in the development of several leukemias (16, 17) and preventing solid tumor metastasis (80), it will be interesting to establish whether there is any Rap1 isoform specificity in the development of these diseases that are not discernable using dominant inhibitory mutants or Rap GAP over-expression.

Acknowledgments

We thank Dr. Yoshimi Takai, Osaka University, for generously providing the last 20 μl of Rap1a-specific Ab, Dr. Magda Chrzanska-Wodnicka, Blood Research Center, Milwaukee, for Rap1b−/− mice, and Bill Carter/ members of Indiana University transgenic core facility for generating Rap1a−/− mice.

Disclosures

The authors have no financial conflict of interest.

References


