Differential regulation of mast cell degranulation versus cytokine secretion by the actin regulatory proteins Coronin1a and Coronin1b

Niko Föger,1 André Jenckel,1 Zane Orinska,1 Kyeong-Hee Lee,1 Andrew C. Chan,3 and Silvia Bulfone-Paus1,2

1Department of Immunology and Cell Biology, Research Center Borstel, 23845 Borstel, Germany
2School of Translational Medicine, The University of Manchester, M13 9PT Manchester, England, UK
3Department of Immunology, Genentech, South San Francisco, CA 94080

Mast cell (MC) activation via aggregation of the high affinity IgE receptor (FcεRI) causes degranulation and release of proinflammatory mediators in a process that involves the reorganization of the actin cytoskeleton. However, the regulatory pathways and the molecular links between cytoskeletal changes and MC function are incompletely understood. In this study, we provide genetic evidence for a critical role of the actin-regulatory proteins Coronin1a (Coro1a) and Coro1b on exocytic pathways in MCs: Coro1a/−/− bone marrow–derived MCs exhibit increased FcεRI–mediated degranulation of secretory lysosomes but significantly reduced secretion of cytokines. Hyperdegranulation of Coro1a/−/− MCs is further augmented by the additional loss of Coro1b. In vivo, Coro1a/−/− Coro1b/−/− mice displayed enhanced passive cutaneous anaphylaxis. Functional reconstitution assays revealed that the inhibitory effect of Coro1a on MC degranulation strictly correlates with cortical localization of Coro1a, requires its filamentous actin–binding activity, and is regulated by phosphorylation of Ser2 of Coro1a. Thus, coronin proteins, and in turn the actin cytoskeleton, exhibit a functional dichotomy as differential regulators of degranulation versus cytokine secretion in MC biology.

Abbreviations used: BMMC, BM-derived MC; DNP-HSA, DNP human serum albumin; F-actin, filamentous actin; MC, mast cell; mRNA, messenger RNA; PCA, passive cutaneous anaphylaxis; PFA, paraformaldehyde.

© 2011 Föger et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

A. Jenckel and Z. Orinska contributed equally to this paper.
A.C. Chan and S. Bulfone-Paus contributed equally to this paper.

Supplemental Material can be found at:
/content/suppl/2011/06/11/jem.20101757.DC1.html
RESULTS AND DISCUSSION

To investigate the impact of coronins on MC function, we first determined the expression pattern of coronins. Real-time PCR analysis revealed expression of Coro1a, Coro1b, Coro1c, Coro2a, and Coro7 messenger RNA (mRNA) in MCs, whereas the other coronin family members, Coro2b and Coro6, could not be detected (Fig. S1 a). Within the classical actin regulatory type I coronins, expression was highest for Coro1a and Coro1b, and our further experiments focused on these two coronin proteins. Expression of Coro1a and Coro1b was confirmed on the protein level by Western blotting (Fig. 1 a). Confocal microscopy revealed that Coro1a is primarily localized at the filamentous actin (F-actin)—rich cell cortex in MCs but also exhibits some punctuate cytoplasmic staining, which only minimally colocalized with CD107a (Lamp1)+ secretory lysosomes (Fig. 1 b, 1–9; Fig. S1 f; and Table S2). Cortical localization was instead less pronounced for Coro1b (Fig. 1 b, 10–18).

Importantly, MC stimulation via antigen-specific cross-linking of FcεRI induced the transient phosphorylation of Coro1a and Coro1b on Ser residues, including the regulatory Ser at position 2 (Ser2) of Coro1b (Fig. 1, c and d; and Fig. S1 g), suggesting a regulatory role of Coro1a and Coro1b on Ser residues, including the regulatory

MC development is not impaired in the absence of Coro1a and Coro1b.

To evaluate whether Coro1a and Coro1b regulate MC function in vivo, we examined passive cutaneous anaphylaxis (PCA), an MC-dependent, IgE–mediated experimental model which reflects in situ MC mediator release (Inagaki et al., 1986; Wershil et al., 1987). PCA was induced by antigen (DNP human serum albumin [DNP-HSA]) challenge of mice that had been sensitized by intradermal ear injections with anti-DNP IgE. The subsequent assessment of IgE-dependent ear swelling revealed that Coro1a−/−Coro1b−/− mice developed a significantly enhanced PCA reaction compared with WT mice (Fig. 2 a), thus indicating a physiological function of coronin proteins in MC biology and allergic responses.

To provide a cellular basis for the increased cutaneous anaphylaxis in Coro1a−/−Coro1b−/− mice, we examined FcεRI-mediated MC degranulation in coronin-deficient BMMCs by measuring the release of β-hexosaminidase from prestored MC granules. Interestingly, antigen-mediated cross-linking of FcεRI resulted in significantly enhanced release of β-hexosaminidase activity from Coro1a−/−BMMCs as compared with WT cells (Fig. 2 b). Although Coro1b−/−BMMCs showed normal degranulation, the additional loss of Coro1b further increased hyperdegranulation in Coro1a−/−BMMCs. For maximal activation, cells were also stimulated with PMA/ ionomycin, which overrides the observed hyperdegranulation phenotype of Coro1a−/−Coro1b−/− BMMCs.

Increased FcεRI-mediated degranulation of Coro1a−/− and Coro1a−/−Coro1b−/− BMMCs was further confirmed by assessing the up-regulation of CD107a cell surface expression, which is a marker for granule exocytosis (Fig. 2 c). Again, hyperdegranulation was more pronounced in Coro1a−/−Coro1b−/− BMMCs compared with Coro1a−/− BMMCs, whereas Coro1b−/− cells showed no significant difference from WT cells. Together, these data identify Coro1a as a negative regulator of FcεRI-mediated MC degranulation. Furthermore, because the double deficiency of Coro1a and Coro1b exacerbates the Coro1a−/− hyperdegranulation phenotype, Coro1b also contributes an overlapping role in regulating MC activities.

To further determine a role of coronins in FcεRI-mediated cytokine secretion, supernatants of BMMCs sensitized with anti-DNP IgE and stimulated with DNP-HSA were collected and tested for cytokine production. IL-6 and TNF secretion was not significantly affected by the single loss of Coro1b (Fig. 2 d). However, in striking contrast to the observed hyperdegranulation of Coro1a−/− BMMCs, the release of both IL-6 and TNF was substantially reduced in Coro1a−/−BMMCs compared with WT cells (Fig. 2 d), indicating a critical role for Coro1a in FcεRI-mediated cytokine secretion. The additional loss of Coro1b in Coro1a−/−Coro1b−/− BMMCs had no effect on cytokine production. Notably, the reduced detection of IL-6 and TNF in cell supernatants of Coro1a−/− and Coro1a−/−Coro1b−/− BMMCs was not secondary to reduced synthesis of these cytokines, as intracellular flow cytometric staining revealed normal to slightly enhanced conservation features and functions. However, individual family members may have developed additional selective and specific functions. Based on their phylogenetic relationship, mammalian coronins have been divided into three different types: type I (Coro1a, Coro1b, Coro1c, and Coro6), type II (Coro2a and Coro2b), and type III (Coro7). In humans and mice, mutation or deletion of Coro1a, which is preferentially expressed in hematopoietic cells, results in a severe combined immunodeficiency that has mainly been attributed to defective actin regulation in T lymphocytes (Föger et al., 2006; Mugnier et al., 2008; Shiow et al., 2008). Coro1a is also required for the survival of mycobacteria in phagosomes of infected macrophages (Jayachandran et al., 2007). The role of coronins in the regulation of MC activities is largely unknown.
expression of IL-6 and TNF in Coro1a<sup>−/−</sup>, Coro1b<sup>−/−</sup>, and Coro1a<sup>−/−</sup>Coro1b<sup>−/−</sup> BMMCs (Fig. 2 e and Fig. S3, e and f). Consistent with these findings, FceRI-mediated induction of IL-6, IL-13, and TNF mRNA was similar between WT and coronin-deficient BMMCs (Fig. 2 f).

To examine whether coronins are required for IgE-dependent signaling events, FceRI-induced phosphorylation of the mitogen-activated protein kinases Erk1/Erk2 (Thr202/Tyr204), p38 (Thr180/Tyr182), and JNK1/JNK2 (Thr183/Tyr185) in coronin-deficient and WT BMMCs was investigated. As shown in Fig. 2 g, similar phosphorylation levels and comparable kinetics were observed among BMMCs of all four genotypes. Similarly, normal phosphorylation of the protein kinases Akt (Ser473) and p90Rsk (Ser380) was observed. Because Coro1a has been implicated in the regulation of Ca<sup>2+ </sup>signaling in T lymphocytes (Mueller et al., 2008), we examined the FcεRI-mediated Ca<sup>2+ </sup>influx in Coro1a<sup>−/−</sup>Coro1b<sup>−/−</sup> BMMCs and compared it with the one induced in WT BMMCs. However, FceRI-mediated Ca<sup>2+ </sup>influx was comparable between Coro1a<sup>−/−</sup>Coro1b<sup>−/−</sup> and WT BMMCs (Fig. S3 d).
Figure 2. Coro1a/Coro1b deficiency leads to enhanced IgE-dependent MC degranulation and impaired cytokine production. (a) Enhanced PCA reaction. Indicated mice received intradermal injections of anti-DNP IgE (or PBS as control) into the ears. After sensitization, mice were i.v. challenged with DNP-HSA as described in Materials and methods. PCA was quantified by monitoring ear thickness. Data are means ± SEM (WT, n = 5; DKO, n = 4; control treatment, 3 mice each). Results are representative for four independent PCA experiments. *, P < 0.001 compared with WT. (b) Increased FcεRI-induced β-hexosaminidase release. WT, Coro1a−/− (C1a.KO), Coro1b−/− (C1b.KO), and Coro1a−/− Coro1b−/− (DKO) BMMCs were sensitized with anti-DNP IgE and stimulated for 10 min with the indicated concentrations of DNP-HSA. Alternatively, cells were stimulated with PMA/ionomycin. Degranulation was assessed by measuring the release of β-hexosaminidase activity as described in Materials and methods. Data are means ± SD of duplicate cultures and representative of at least three independent experiments. *, P < 0.001 compared with WT. (c) Increased FcεRI-induced up-regulation of CD107a surface expression. IgE-sensitized BMMCs were stimulated for 10 min with the indicated concentrations of DNP-HSA. Degranulation was assessed by flow cytometric analysis of CD107a cell surface expression. Numbers shown are the percentage of CD107a-positive cells. Data are representative of at least three independent experiments. (d) Decreased FcεRI-mediated cytokine secretion. Sensitized BMMCs were stimulated for 4 h with the indicated concentrations of DNP-HSA. Release of IL-6 and TNF into the supernatant was measured by ELISA. Data are mean ± SEM from four cultures each. *, P < 0.05 compared with WT cells at the indicated antigen concentrations. Data are representative of five independent experiments. (e) Intracellular cytokine production. Sensitized BMMCs were stimulated for 4 h with the indicated concentrations of DNP-HSA in the presence of Brefeldin A. Cells were analyzed for intracellular IL-6 or TNF by flow cytometry, and graphs show the percentage of positive cells ± SEM from two independent experiments. (f) Cytokine gene transcription. Sensitized BMMCs were stimulated for 1 h with DNP-HSA. mRNA levels for IL-6, TNF, and IL-13 were measured by real-time quantitative PCR. The expression level of nonstimulated WT BMMCs was set as 1. Data are mean ± SEM from two independent experiments. (g) FcεRI-induced signaling. Sensitized BMMCs were stimulated with 10 ng/ml DNP-HSA for the indicated times. Whole cellular lysates were subjected to immunoblot analysis using the indicated antibodies. Data are representative of at least three independent experiments.
Together, our data suggest a differential role of coronins in FcεRI-mediated degranulation and cytokine secretion. Furthermore, Coro1b is able to substitute for some functions of Coro1a because hyperdegranulation is aggravated in Coro1a−/− Coro1b−/− BMMCs compared with Coro1a−/− cells. In addition, our findings indicate that Coro1a and Coro1b do not affect FcεRI-dependent signaling and cytokine expression in MCs.

To understand whether changes in the actin cytoskeleton are responsible for the differential effect on FcεRI-mediated degranulation and cytokine secretion, we tested the effect of the actin modulatory drug latrunculin B on FcεRI-mediated degranulation. Consistent with previous
of mRNA encoding for IL-6, IL-13, and TNF was similar to slightly enhanced upon treatment of BMMCs with latrunculin B (Fig. 3 f). Collectively, these results suggest that the actin cytoskeleton regulates diverse aspects of MC function. Notably, treatment of BMMCs with latrunculin B mimicked the opposing effects of coronin deficiency on MC degranulation and cytokine secretion. Coro1a has been linked to the control of the cellular steady-state F-actin content in T cells (Föger et al., 2006). However, in contrast to the massive accumulation of F-actin in Coro1a−/− T cells (Föger et al., 2006; Mugnier et al., 2008; Shiow et al., 2008), only minor differences in cellular F-actin levels were observed between WT, Coro1a−/−, Coro1b−/−, and Coro1a−/− Coro1b−/− BMMCs (Fig. S4, a and b), reinforcing the idea of cell type- and/or context-specific differences in the actin regulatory activities of coronin proteins (Gandhi et al., 2009). To gain further insights into the role of the actin cytoskeleton and coronin proteins for MC function, we next investigated the intracellular F-actin structure, as well as the subcellular localization of Coro1a and Coro1b upon MC activation by confocal microscopy. MC stimulation induced characteristic changes in cell morphology that are associated with granule release, including cell spreading and the formation of filopodia, membrane ridges, and craters (Fig. 4 a, bottom). Furthermore, contrary to its enhancing effect on MC degranulation, treatment of BMMCs with latrunculin B mimicked the opposing effects of coronin deficiency on MC degranulation and cytokine secretion. Coro1a has been linked to the control of the cellular steady-state F-actin content in T cells (Föger et al., 2006). However, in contrast to the massive accumulation of F-actin in Coro1a−/− T cells (Föger et al., 2006; Mugnier et al., 2008; Shiow et al., 2008), only minor differences in cellular F-actin levels were observed between WT, Coro1a−/−, Coro1b−/−, and Coro1a−/− Coro1b−/− BMMCs (Fig. S4, a and b), reinforcing the idea of cell type- and/or context-specific differences in the actin regulatory activities of coronin proteins (Gandhi et al., 2009). To gain further insights into the role of the actin cytoskeleton and coronin proteins for MC function, we next investigated the intracellular F-actin structure, as well as the subcellular localization of Coro1a and Coro1b upon MC activation by confocal microscopy. MC stimulation induced characteristic changes in cell morphology that are associated with granule release, including cell spreading and the formation of filopodia, membrane ridges, and craters (Fig. 4 a, bottom). Furthermore, MC activation resulted in the disassembly of the cortical F-actin ring (Fig. 4 a and b). As seen before in unstimulated BMMCs (Fig. 1 b), Coro1a predominantly exhibited a characteristic ring-like staining pattern at the cell periphery, where it colocalized with cortical F-actin (Fig. 4 a). However, after activation of BMMCs, by either stimulation with PMA/ionomycin or antigen-specific studies (Nishida et al., 2005; Sasaki et al., 2005), BMMCs pre-treated with latrunculin B exhibited an enhanced degranulation in response to FcεRI cross-linkage, as assessed by measuring β-hexosaminidase release (Fig. 3 a) and by determining the increase in CD107α cell surface staining (Fig. 3 b). Furthermore, contrary to its enhancing effect on MC degranulation, treatment of BMMCs with latrunculin B resulted in decreased secretion of IL-6 and TNF in response to FcεRI stimulation (Fig. 3, c and d). However, intracellular production of these cytokines was not affected by the treatment with latrunculin B, as intracellular FACS staining revealed comparable percentages of IL-6− and TNF−producing cells in latrunculin B− and control-treated BMMCs (Fig. 3 e). Also, the induction of mRNA encoding for IL-6, IL-13, and TNF was similar to slightly enhanced upon treatment of BMMCs with latrunculin B (Fig. 3 f). Collectively, these results suggest that the actin cytoskeleton regulates diverse aspects of MC function. Notably, treatment of BMMCs with latrunculin B mimicked the opposing effects of coronin deficiency on MC degranulation and cytokine secretion.
cross-linkage of FcεRI, Coro1a relocalized from the cell cortex into the cytoplasm (Fig. 4 a, 6; and Fig. S4 d). Similarly, cortical Coro1b staining also got lost upon MC activation (Fig. 4 b, 6).

An association of Coro1a and Coro1b with the actin cytoskeleton in unstimulated MCs and subcellular relocation of these coronin proteins in response to MC activation was further supported by biochemical fractionation experiments. In nonstimulated BMMCs, Coro1a and Coro1b were predominantly recovered in the detergent-insoluble F-actin–rich pellet (cytoskeletal fraction; Fig. 4 c). Activation of BMMCs by cross-linkage of FcεRI, Coro1a relocalized from the cell cortex into the cytoplasm (Fig. 4 a, 6; and Fig. S4 d). Similarly, cortical Coro1b staining also got lost upon MC activation (Fig. 4 b, 6).

An association of Coro1a and Coro1b with the actin cytoskeleton in unstimulated MCs and subcellular relocation of these coronin proteins in response to MC activation was further supported by biochemical fractionation experiments. In nonstimulated BMMCs, Coro1a and Coro1b were predominantly recovered in the detergent-insoluble F-actin–rich pellet (cytoskeletal fraction; Fig. 4 c). Activation of BMMCs by
stimulation with PMA/ionomycin or antigen-specific triggering of FcεRI induced the transient relocation of a substantial amount of Coro1a and Coro1b from the particulate fraction into the cytosolic fraction (Fig. 4 c). The kinetics of the subcellular redistribution of Coro1a and Coro1b upon MC activation resembled the phosphorylation kinetics of Coro1a and Coro1b. Moreover, the subcellular relocation of Coro1b correlated with the detection of Coro1b phosphorylation on Ser2 in the cytosolic fraction (Fig. 4 c). Together, these data suggest that the intracellular distribution of Coro1a and Coro1b in MCs might be determined by their phosphorylation status.

To test this hypothesis, we reconstituted *Coro1a***−/−**/BMMCs with Flag-tagged Coro1a mutants that affect Coro1a Ser2 phosphorylation and/or its ability to bind F-actin and examined their subcellular localization. As expected, WT Coro1a mainly localized to the F-actin–rich cell cortex (Fig. 5 a, 13) and was predominantly detected in the detergent-insoluble cytoskeletal fraction (Fig. 5 b, lane 2). Similarly, the Coro1a-S2A mutant, which cannot be phosphorylated at Ser2, also exhibited strong colocalization with the actin cytoskeleton (Fig. 5 a, 14). In striking contrast, the phosphomimetic Coro1a-S2D mutant, which lacks Arp2/3-binding activity (Föger et al., 2006), was mainly found in the detergent-soluble cytosolic fraction and showed severely compromised cortical staining (Fig. 5 a, 15). The F-actin–binding mutant Coro1a-R29E (Tsujita et al., 2010) and the Coro1a-S2D-R29E double mutant were almost exclusively detected in the cytoplasmic fraction and exhibited mainly cytoplasmic staining (Fig. 5 a, 16 and 17). Although poorly expressed, Coro1a-ΔCCI, which lacks the C-terminal coiled-coil domain, was also primarily found in the cytosolic fraction. Collectively, our data suggest that the cortical localization of Coro1a in BMMCs is largely determined by Ser2 phosphorylation and the F-actin–binding activity of Coro1a.

Next, we investigated the ability of Coro1a mutants to negatively regulate FcεRI-induced degranulation in BMMCs. Reconstitution of *Coro1a***−/−**/*Coro1b***−/−**/BMMCs with WT Coro1a or Coro1a-S2A resulted in a profound reduction in degranulation, as assessed by CD107a surface staining on transfected (GFP+) cells (Fig. 5 c). In marked contrast, hyperdegranulation of *Coro1a***−/−**/*Coro1b***−/−**/BMMCs was minimally restored by expression of mutant Coro1a-S2D and, even less so, by Coro1a-R29E and Coro1a-S2D-R29E (Fig. 5 c). Together, these results demonstrate that Coro1a negatively regulates MC degranulation via an actin-dependent mechanism.

Our study reveals a dual function of coronin proteins and the actin cytoskeleton on secretory processes in MCs: regulation of MC degranulation and facilitation of cytokine release. Hyperdegranulation of *Coro1a***−/−**/*Coro1b***−/−**/deficient BMMCs correlates with enhanced cutaneous anaphylaxis in vivo, suggesting a role of coronin proteins in the pathophysiology of allergic disorders. The negative regulatory function of Coro1a on MC degranulation strictly correlates with cortical localization of Coro1a and requires the F-actin–binding site of Coro1a. Interestingly, no alterations in MC function were detected in MCs expressing a truncated mutant of Coro1a (Q262X Coro1a; Arandjelovic et al., 2010), possibly because of some residual regulatory activity of the mutant Coro1a protein. We propose that Coro1a exhibits no major effects on steady-state actin polymerization in unstimulated MCs but rather stabilizes the cortical network of actin filaments via its actin-binding and/or -bundling activity, thereby entrapping secretory granules and preventing unwanted granule release. This idea is consistent with the recently described F-actin decoration and stabilization function of Coro1A (Galkin et al., 2008). Actin-dependent processes have further been implicated in the regulation of membrane trafficking from the ER to the Golgi and the plasma membrane (Müsch et al., 1997), a cellular transport route which is also used for release of newly synthesized cytokines. Normal synthesis but reduced secretion of cytokines in *Coro1a***−/−**/MCs thus suggests that owing to its actin–regulatory function, Coro1a is involved in the control of vesicular trafficking in MCs. Together, our data provide genetic evidence to support the longstanding hypothesis that the cortical actin network acts as a physical barrier to prevent docking and fusion of secretory granules to the plasma membrane (Aunis and Bader, 1988).

Exocytosis is accompanied by a regulated reorganization of cortical actin filaments, which is then thought to allow for recruitment of granules to exocytic sites (Eitzen, 2003). Our data suggest that Coro1a and Coro1b are functionally involved in the stimulus-dependent control of the stability and the dynamics of the cortical actin network. FcεRI triggering induces the subcellular relocation of Coro1a and Coro1b from the actin–rich cell cortex to the cytoplasm, thereby likely contributing to reduced cortical stability and better access of secretory granules to the plasma membrane upon cellular activation. This intracellular redistribution of Coro1a and Coro1b in MCs is regulated by FcεRI-mediated phosphorylation of Coro1a and Coro1b on the critical Ser2. This phosphorylation event has previously been demonstrated to reduce the interaction with the Arp2/3 complex (Cai et al., 2005; Föger et al., 2006), which is a central component of the actin cytoskeleton. Thus, Ser2-phosphorylated coronin exhibits reduced binding to the cortical actin network, which likely annihilates the actin–dependent negative regulatory function of coronin on MC degranulation. Despite the high degree of sequence conservation between coronin family members, Coro1b only partially compensates for Coro1a deficiency in MC degranulation and, in contrast to Coro1a, has no discernable effect on cytokine release. Thus, our findings indicate that coronin family members have developed both common and specialized functions.

In conclusion, distinct secretory pathways have fundamentally different requirements on coronin proteins and the actin cytoskeleton in MCs. These findings likely represent common regulatory themes that also apply to other secretory cells. Additional mechanistic studies will further reveal the specific modalities of actin regulation for exocytic processes under normal and pathophysiological conditions.
MATERIALS AND METHODS

Mice. Coro1a-deficient mice were reported previously (Föger et al., 2006). Coro1b-deficient mice were generated using homologous recombination in embryonic stem cells as described in Fig. S2. Heterozygous mice were backcrossed for >10 generations onto C57BL/6N mice. Coro1a/Coro1b double-deficient mice were obtained by interbreeding Coro1a- and Coro1b-deficient mice. Mice were housed under specific pathogen–free conditions at the Animal Care Facility of the Research Center Borstel. Animal experiments were performed in accordance with institutional guidelines and were approved by the local authorities (Ministry of Agriculture, the Environment, and Rural Areas, Schleswig-Holstein).

Antibodies and reagents. Fluorescent labeled antibodies specific for c-Kit, FcγR1α, TNF-α, CD11b, CD11c, CD107a, and B220 were all obtained from eBioscience, and anti-T1/ST2 was obtained from R&D Systems. Standards were purchased from R&D Systems. For the detection of intracellular cytokines, cells were stimulated for 4 h in the presence of 3 µg/ml anti-DNP IgE (SPE-7) as a stimulus for the detection of intracellular cytokines and cytokines in cell supernatants were analyzed by ELISA. Coating and detection antibodies for TNF and IL-6 and protein standards were purchased from R&D Systems. For the detection of intracellular cytokines, cells were stimulated for 4 h in the presence of Brefeldin A (eBioscience) for blockade of Golgi function. Cells were fixed with 4% PFA, permeabilized, and subjected to intracellular FACS staining using antibodies against IL-6 and TNF.

IMMUNOFLUORESCENCE MICROSCOPY. Cells were fixed with 4% PFA and permeabilized with 1% Triton X-100 in Tyrode’s buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, and 0.1% [vol/vol] BSA). Supernatant or cell pellets solubilized with 0.1% Triton X-100 in Tyrode’s buffer were incubated with p-nitrophenyl-N-acetyl-D-glucosamidase (Sigma-Aldrich; 1.3 mg/ml in 0.1 M citrate, pH 4.5), and color was developed for 30 min at 37°C. The enzyme reaction was stopped by the addition of 0.2 M Gly–NaOH, pH 10.7, and the absorbance at 405 nm was measured. The percentage of β-hexosaminidase released was calculated by dividing the absorbance in the supernatant by the sum of the absorbance in the supernatant and detergent-solubilized cell pellet. Statistical analysis was performed by two-tailed Student’s t test. Degranulation was also assessed by flow cytometric measurement of the surface expression of CD107a (Lamp1).

Measurement of cytokines. Sensitized BMMCs were stimulated with DNP-HSA for 4 h at 37°C, and cytokines in cell supernatants were analyzed by ELISA. Coating and detection antibodies for TNF and IL-6 and protein standards were purchased from R&D Systems. For the detection of intracellular cytokines, cells were stimulated for 4 h in the presence of Brefeldin A (eBioscience) for blockade of Golgi function. Cells were fixed with 4% PFA in PBS, permeabilized, and subjected to intracellular FACS staining using antibodies against IL-6 and TNF.

RT-PCR and real-time quantitative RT-PCR analysis. Total RNA was purified from BMMCs using the RNeasy system (QIAGEN) according to the manufacturer’s instructions and reverse transcribed using the SuperScript II first-strand synthesis system (Invitrogen). For some experiments, the FastLan e cDNA kit (QIAGEN) was used. Primers and conditions for standard PCR to detect MC proteases were described previously (Orinska et al., 2010). Mouse MCP-1 was detected with sense (5’-AACGCTTCTGACATTTCTAC-3’) and antisense (5’-AGCCTGCTGGTATAGGTC-3’) primers. PCR products were separated on an agarose gel, stained with ethidium bromide, and photographed. For real-time quantitative RT-PCR, IL-6, TNF and IL-13 gene expression was measured relative to HPRT using the Universal ProbeLibrary (Roche) TaqMan-based system. Amplification was performed in a fluorescence temperature cycler (Light Cycler 2.0; Roche). Primers and the Universal ProbeLibrary ID numbers are listed in Table S1.

Western blotting and immunoprecipitation. After stimulation of sensitized BMMCs with DNP-HSA or PMA/ ionomycin, cells were lysed in cell extraction buffer: 1% NP-40, 50 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 10 mM Na fluoride, 1 mM Na orthovanadate, and protease inhibitors (complete protease inhibitor cocktail; Roche). For immunoprecipitation experiments, cell lysates were preclarified with protein A beads and subsequently incubated for 1–2 h with protein A beads covalently coupled with anti-Coro1a mAb. Immune complexes were washed four times with cell extraction buffer. Eluted samples or whole cellular lysates were resolved by SDS–PAGE, and proteins were detected by Western blotting using the...
isolated antibodies. Denstometric analysis was performed using ImageJ software (National Institutes of Health; Tables S3–S9).

Isolation of the cytoskeleton-rich fraction. Subcellular fractionation and isolation of the cytoskeleton-containing detergent-insoluble fraction was performed according to Gatfield et al. (2005). In brief, cell pellets (10^6 cells) were suspended in 0.3 ml of ice-cold cytoskeletal isolation buffer (1% Triton X-100 in 80 mM Pipes, pH 6.8, 5 mM EGTA, and 1 mM MgCl2) and immediately centrifuged at 3,000 g for 3 min to obtain the detergent-insoluble cytoskeleton-containing fraction and the detergent-soluble cytosolic fraction. Equal cell equivalents were subjected to SDS-PAGE and Western blotting.

Expression constructs and transient transfection of BMMCs. Coro-
nin expression constructs were cloned into the bicistronic expression vector pIRES2-EGFP (Takara Bio Inc.). Full-length mouse Coro1a (aa 1–461) carrying a C-terminal Flag tag, as well as Coro1a-S2A and Coro1a-S2D mutants previously (Föger et al., 2006). The actin-binding mutant Coro1a-R29E (Tsujita et al., 2010) and the Coro1a-S2D-R29E double mutant were generated using the QuickChange site-directed mutagenesis kit (Agilent Technologies). Constructs were transfected into BMMCs using the Nucleofector system (solution T and program T-16) according to the method described by Jeffrey et al. (2006). After transfection, cells were allowed to recover for 24 h in MC medium containing 1 ng/ml IL-3. Cells were then sensitized with anti-DNP-HSA and stimulated with DNP-HSA. MC degranulation was as-
sessed using the QuikChange site-directed mutagenesis kit (Agilent Technologies). Constructs were transfected into BMMCs using the Nucleofector system (solution T and program T-16) according to the method described by Jeffrey et al. (2006). After transfection, cells were allowed to recover for 24 h in MC medium containing 1 ng/ml IL-3. Cells were then sensitized with anti-DNP-HSA and stimulated with DNP-HSA. MC degranulation was as-

Online supplemental material. Fig. S1 shows mRNA expression levels of coronin family members in different tissues and cell types, demonstrates the specificity of the Coro1a and Coro1b staining in confocal microscopy ex-

REFERENCES


Brown, J.M., T.M. Wilson, and D.D. Metcalfe. 2008. The mast cell and aller-


Clemen, C.S., V. Rybakin, and L. Eichinger. 2008. The coronin family of pro-


Inagaki, N., S. Goto, M. Yamasaki, H. Nogai, and A. Koda. 1986. Studies on vascular permeability increasing factors involved in 48-hour homolo-
gous PCA in the mouse ear. J. Immunol. 139:253–266. doi:10.4049/jimmunol.139.1.253


acquisition of the cytoskeleton-rich fraction. Subcellular fractionation and isolation of the cytoskeleton-containing detergent-insoluble fraction was performed according to Gatfield et al. (2005). In brief, cell pellets (10^6 cells) were suspended in 0.3 ml of ice-cold cytoskeletal isolation buffer (1% Triton X-100 in 80 mM Pipes, pH 6.8, 5 mM EGTA, and 1 mM MgCl2) and immedi-

2010. Published August 15, 2011

Submitted: 23 August 2010
Accepted: 7 July 2011
Brief Definitive Report


