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A Pak1-PP2A-ERM signaling axis mediates F-actin rearrangement and degranulation in mast cells

Karl Staser^{1,2}, Matthew A Shew¹, Elizabeth G Michels¹, Muithi M Mwanthi^{1,3}, Feng-Chun Yang^{1,4}, D Wade Clapp^{1,2,3,#}, and Su-Jung Park^{1,2}

¹Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University School of Medicine

²Department of Biochemistry, Indiana University School of Medicine

³Department of Microbiology and Immunology, Indiana University School of Medicine

⁴Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana

Abstract

Mast cells coordinate allergy and allergic asthma and are crucial cellular targets in therapeutic approaches to inflammatory disease. Allergens cross-link IgE bound at high-affinity receptors on the mast cell's surface, causing release of pre-formed cytoplasmic granules containing inflammatory molecules, including histamine, a principal effector of fatal septic shock. Both p21 activated kinase 1 (Pak1) and protein phosphatase 2A (PP2A) modulate mast cell degranulation, but the molecular mechanisms underpinning these observations and their potential interactions in common or disparate pathways are unknown. Here, we use genetic and other approaches to show that Pak1's kinase-dependent interaction with PP2A potentiates PP2A's subunit assembly and activation. PP2A then dephosphorylates threonine 567 of Ezrin/Radixin/Moesin (ERM), molecules that have been shown to couple F-actin to the plasma membrane in other cell systems. In our study, the activity of this Pak1-PP2A-ERM axis correlates with impaired systemic histamine release in *Pak1*^{-/-} mice and defective F-actin rearrangement and impaired degranulation in *Ezrin* disrupted (*Mx1Cre⁺Ezrin^{flox/flox}*) primary mast cells. This heretofore unknown mechanism of mast cell degranulation provides novel therapeutic targets in allergy and asthma and may inform studies of kinase regulation of cytoskeletal dynamics in other cell lineages.

Introduction

Mast cells closely interact with cells in dermal, gastrointestinal, and other tissues, functioning as key sensors of multiple foreign substances including allergens, bacteria, parasites, toxins, and venoms [1]. Mast cells coordinate allergic diseases and asthma via activation of high-affinity IgE receptors (Fcε RI), resulting in immediate release of preformed cytoplasmic granules [2]. Granule trafficking and plasma membrane fusion require the coordinated reorganization of the filamentous-(F)-actin comprising the cytoskeleton. We have recently observed that the p21 activated kinase 1 (Pak1) modulates both allergen- and stem cell factor-induced F-actin rearrangement and subsequent mast cell degranulation [3, 4]. However, the molecular underpinnings of Pak1's control of F-actin dynamics are not known.

#Corresponding author: Dr. Wade Clapp, MD, Cancer Research Institute, 1044 West Walnut Street, Building R-4, Room 414, Indianapolis, Indiana 46202, dclapp@iupui.edu, Phone (317)-278-9290, Fax (317)-274-8679.

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Pak1, a member of the Group A Pak protein family, is a serine/threonine kinase activated downstream of lipid and Rho-family GTPase signaling [5]. In multiple cell systems, the Rho GTPase Rac1/2 and Cdc42 bind to and modulate the activation of Pak1 through an N-terminal p21 GTPase-binding-domain (PBD) [6]. GTPase binding at the PBD interferes with Pak1 dimerization, induces a conformational change, and destabilizes the inhibitory switch domain, exposing the kinase domain for catalytic activity. Auto-phosphorylation at threonine 423 further activates Pak1's kinase domain and maintains its catalytically active open conformation [7, 8]. Activated Pak1 can phosphorylate numerous targets involved in gene transcription and the cell cycle, including phosphorylation of Raf1 at serine 338 and Mek1 at Serine 298, a signal which potentiates mitogen-activated protein kinase (MAPK) cascades [9, 10]. In addition, through a series of incompletely understood interactions, Pak1 serves to critically regulate cytoskeletal rearrangement and cell motility through numerous potential effector molecules [5].

Previous studies found that Pak1 and protein phosphatase 2A (PP2A), a phosphatase regulating the dephosphorylation of numerous phosphoprotein substrates, can physically interact in certain cell types [11-15]. Moreover, pharmacologic studies using okadaic acid (OA), an inhibitor that influences multiple targets including PP2A, have suggested that membrane-localized PP2A modulates degranulation in cultured mast cells [16, 17]. Mechanistic details have remained elusive, though, in no small part due to PP2A's promiscuous biochemical roles in diverse cell types. Indeed, PP2A dephosphorylates hundreds of targets with variable specificity, principally contingent upon binding partners, subunit assembly, and subcellular localization [15, 18-20].

Here, we reveal a novel Pak1-PP2A-dependent mechanism of F-actin rearrangement and degranulation. Specifically, we show that in allergen-induced primary mast cells, Pak1 interacts with protein phosphatase 2A at its catalytic subunit C (PP2A_C), promoting assembly with PP2A subunit A (PP2A_A). Active PP2A then dephosphorylates threonine 567 on Ezrin/Radixin/Moesin (ERM) proteins, which appear to uncouple the plasma membrane from the actin cytoskeleton prior to F-actin rearrangement and degranulation. We further show that conditional deletion of *Ezrin* impairs F-actin dynamics and mast cell degranulation, implicating a functional role for at least one of the ERM proteins. While these insights further our understanding of mast cell-mediated allergy and anaphylaxis, they additionally reveal a novel mechanism of cytoskeletal control potentially conserved across diverse cell types.

Materials and methods

Mice

Targeting constructs and PCR protocols for the *Pak1*^{-/-} mice and *Ezrin*^{flox/flox} mice are previously described [4, 21]. *Ezrin*^{flox/flox} mice were crossed with *Mx1-Cre*⁺ transgenic mice [22]. To induce recombination in *Mx1-Cre*⁺*Ezrin*^{flox/flox} mice, *Mx1-Cre*⁺ and *Mx1-Cre*⁻ littermates were injected in the peritoneum five times, every other day, with 15-20 µg/g body weight of polyIC (Sigma) dissolved at 2 mg/mL in PBS. Bone marrow for mast cell culture was harvested approximately two weeks after the last dose of polyIC. Animal use was monitored by the Indiana University Laboratory Animal Resource Center.

Mast cell generation and culture

Bone marrow was isolated from the femurs, tibias, and iliac bones by flushing each bone three times with 2% fetal bovine serum (FBS)/IMDM using a 23-gauge needle. Low density mononuclear cells were isolated from this bone marrow by density gradient (Histopaque, Sigma). Mast cells were then generated by sub-culturing non-adherent low density

mononuclear cells for 4 to 8 weeks in 10% FBS/IMDM supplemented with 7.5 ng/mL IL-3 (Peprotech), as previously described [3]. Purity of mast cells was assessed by Giemsa histology and flow cytometry using anti-CD117-FITC and anti-FcεRI-PE antibodies (BD Biosciences). RBL-2H3 cells were obtained from the American Type Culture Collection and maintained in 10% FBS/DMEM, according to the supplier's protocol.

RNA interference

PP2A_c knockdown RBL-2H3 cells were generated by incubation with siRNA construct SASI-Rn01-00089612 (Sigma) or with scrambled SiRNA in siPORT NeoFx reagent (Ambion). Three siRNA constructs were tested, and one was chosen for further experimentation. Experiments were performed approximately 72 hours following transfection.

Plasmid construction, virus generation, and cell transformation

The human Pak1 and K299RPak1 (a gift from Jonathan Chernoff [23]) constructs were cloned in fusion to the enhanced green fluorescent protein construct (from pEGFP-C1, Clontech) and subcloned into either the lentiviral (LV) transfer plasmid PCL1 or PCL11. The PCL1 and PCL11 vectors as well as the packaging plasmid pCD/NL-BH, providing the Gag, Pol, Tat, and Rev constructs, and the envelope-coding plasmid pcoPE01, providing the vesicular stomatitis virus glycoprotein gene (VSV-G), were all kind gifts from Dr. Helmut Hannenberg. Pak1-EGFP and K299RPak1-EGFP LV particles were produced by polyethylenimine transfection into HEK293T cells, as previously described [24]. Supernatants containing particles were collected, filtered by PES 0.22 μm membrane (Millipore), and concentrated by ultracentrifugation (120 minutes, 30,000 × g). The infectivity of the concentrated viral vector stock was determined on HT1080 cells and scored by flow cytometry analysis of EGFP expression. Titers ranged from 10⁷ to 10¹⁰ infectious particles per mL. RBL-2H3 cells were plated in 10 cm plates 24 hours before infection. Growth media was replated with 3.5 mL of virus at approximately five infectious particles per one RBL-2H3 cell (5 MOI). Cells were then incubated for 4 hours at 37°C. Viral media was aspirated then replaced with fresh growth media. 48h after infection, cells were collected and the immunoprecipitation performed as described. CD63-EGFP virus generation and progenitor transduction were performed similar to above and as previously described [4].

Reagents and degranulation

IPA-3 was a gift from Jonathan Chernoff. Okadaic Acid (OA). Anti-DNP IgE and DNP were from Sigma. Calyculin A (CA) was from Cell Signaling. Primary bone marrow cultured mast cells were primed for four hours with 1.5 μg/mL IgE and stimulated with 30 ng/mL DNP. RBL-2H3 cells were primed with 50 ng/mL IgE and stimulated with 50 ng/mL DNP. As indicated, cells were pretreated with 30 μM IPA-3, 100 nM CA, or 1 μM OA for 15 minutes, 20 minutes, or 1 hour, respectively. β-hexosaminidase release was measured by colorimetric techniques, as described previously [4]. Briefly, pellets from IgE/DNP-treated cells were solubilized, the supernatants and pellets incubated with 4-nitrophenyl *N*-acetyl-beta-D-glucosaminide (Sigma), stopped with sodium bicarbonate solution, and color change read by spectrophotometer at 405 nm. Systemic histamine release was induced by tail vein injection of 3 μg IgE in 100 μL PBS and, 24 hours later, with 300 μg of DNP in 200 μL PBS. After 90 seconds, blood was harvested, and serum was extracted and analyzed for histamine release by ELISA (Genway), according to the manufacturer's protocol.

Antibodies, immunoblotting, and immunoprecipitation

Antibodies were from Cell Signaling, diluted 1:1000, with these exceptions: anti-pT423-Paks (1:200; Santa Cruz), anti-PP2A_C (1:1000, Millipore), and anti-pY307 PP2A_C (1:200; Santa Cruz). SDS sample buffer was added to equivalent cell numbers or, for RBL-2H3 cells, protein lysis buffer (Proteojet, Fermentas) supplemented with phosphatase/protease inhibitors. When using protein lysis buffer, protein supernatants were quantified and standardized by BCA, as described [3, 4]. For Pak1 immunoprecipitation, brain was homogenized, sonicated, BCA quantified, and equivalent quantities incubated with anti-Pak1 antibody (Santa Cruz) in protein-A/G-agarose. Samples were electrophoresed and probed with anti-PP2A_C antibody. Input was approximately 50% of total sample. PP2A phosphatase assay was performed with a PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate/Millipore), according to the manufacturer's protocol. Mast cells were lysed, sonicated, clarified, BCA quantified, and equivalent quantities incubated with anti-PP2A_C antibody and protein-A/G-agarose. After washing, samples were incubated with threonine phosphopeptide in ser/thr assay buffer then with detection solution and color change read at 650 nm.

Pak1 and PP2A_C direct interaction assay

Recombinant Pak1 and PP2A_C proteins were purchased from Genway Biotech and Cayman Chemicals, respectively. 2 µg Pak1 and 2 µg PP2A_C were incubated together in binding buffer (20 mM Tris-Cl pH 7.5, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 0.05% NP-40, 0.5 mM DTT, 1× protease inhibitor cocktail (Roche)) with species appropriate 4 µg IgG and either 4 µg anti-Pak1 or 4 µg anti-PP2A_C antibody for 2 hours at 4°C. Subsequent detection steps were performed as described above.

Microscopy

Cells were fixed in paraformaldehyde, spun to slides (or, for RBL-2H3 cells, grown in chamber slides), permeabilized in 0.3% Triton X-100/PBS, blocked with 5% FBS/PBS, and incubated with primary antibody (1:200) then with anti-rabbit or mouse Alexa Fluor 568 or 488 (Molecular Probes), as described [3, 4]. Alexa Fluor 488- or rhodamine-phalloidin was used to detect F-actin. Samples were mounted in Vectashield with DAPI (Vector Labs). Acquisition was performed on a Zeiss LSM 510 confocal laser scanning system or on an Applied Precision DeltaVision (deconvolution) system. Fluorescence quantification was performed using NIH free software (ImageJ).

Results

In prior work we made the observation that genetic disruption of Pak1 (*Pak1*^{-/-}) leads to a 2-3 fold reduction in IgE/DNP mediated degranulation [4]. To begin mechanistically characterizing these observations, we first examined the consequence of *Pak1* deletion on the cytoskeleton and, subsequently, on in vivo systemic histamine release. We induced degranulation by sensitizing primary cultured mast cells with recombinant anti-DNP IgE followed by DNP stimulation. DNP-induced *Pak1*^{-/-} cells demonstrated an abnormal persistence of cortical F-actin structure, as shown by increased phalloidin-FITC signal at high-magnification on deconvolution microscopy (Figure 1A and 1B). To directly visualize degranulation, we generated mast cells carrying a *GFP-CD63* transgene, finding that DNP-induced *Pak1*^{-/-} mast cells abnormally retained CD63⁺ granules (Supplement Fig 1). To ascertain the importance of these findings in vivo, we performed histamine release assays on *Pak1*^{-/-} mice. Mice were sensitized with anti-DNP IgE and subsequently injected intravenously with DNP. In these assays, *Pak1*^{-/-} mice demonstrated impaired serum histamine release, as measured by ELISA (Figure 1C). Thus, Pak1 modulates allergen-

induced mast cell F-actin rearrangement, degranulation, and systemic histamine release in vivo.

To test our hypothesis that Pak1 signals through PP2A, we examined phosphatase-inhibited primary mast cells, finding that okadaic acid impairs F-actin rearrangement temporally and spatially reminiscent of the defect observed in *Pak1*^{-/-} mast cells (Figure 2A). Next, to test if Pak1 and PP2A_C directly interact, we incubated recombinant Pak1 and PP2A_C, immunoprecipitated each protein, then probed with either anti-Pak1 or anti-PP2A_C antibodies. In these experiments, we found strong evidence of a direct interaction in vitro (Figure 2B). Although we were unable to detect a Pak1-PP2A interaction in primary mast cells – possibly due to low Pak1 protein expression or due to the short duration of the interaction – we genetically validated a Pak1-PP2A_C interaction in live cells by two methods (Figure 2C). First, we immunoprecipitated Pak1 from WT and *Pak1*^{-/-} brain tissue followed by detection with anti-PP2A_C antibody, finding high-intensity signal in only WT samples. Next, we expressed EGFP-Pak1 in RBL-2H3 cells, a rat mast cell line expressing FcεRI and c-kit [25], finding that PP2A_C could be detected by western blot on immunoprecipitated EGFP. We then assessed whether Pak1 directly influences PP2A_C activity by immunoprecipitating PP2A_C from stimulated primary mast cells and performing phosphatase activity assays. As shown in Figure 2D, allergen-induction increased phosphatase activity in WT but not *Pak1*^{-/-} mast cells, suggesting that Pak1 enhances PP2A_C activity during mast cell degranulation.

We initially hypothesized that *Pak1*^{-/-} mast cells would demonstrate higher phosphorylation levels of PP2A_C's tyrosine 307, a highly-conserved motif transiently inhibiting phosphatase activity and thus enhancing activity within multiple signaling networks [26-28]. IgE-priming increased pY307-PP2A_C to a similarly mild degree in WT and *Pak1*^{-/-} mast cells (Figure 2E), implying that temporal PP2A inhibition readies downstream degranulatory mechanisms. Allergen-induction reduced pY307-PP2A_C, suggesting immediate disinhibition during degranulation. However, we did not detect increased pY307-PP2A_C in *Pak1*^{-/-} mast cells through multiple replicates and conditions, as previously hypothesized. As shown at the one minute DNP-induction time point in Figure 2E, we were more likely to observe decreased pY307-PP2A_C in stimulated *Pak1*^{-/-} cells. These data suggest that the phosphorylation state of pY307-PP2A_C does not account for Pak1's modulation of PP2A activity. Rather, this phosphorylation state may depend on Src family kinases via canonical FcεRI pathways [29].

Hence, we sought an alternate and preponderant mechanism for Pak1's modulation of PP2A activity. In doing so, we found that activated Pak1 promotes PP2A_C's interaction with PP2A_A, a subunit assembly which is required for phosphatase activation [15]. PP2A_C immunoprecipitated from WT mast cell protein extracts and probed with anti-PP2A_A antibody demonstrated increased signal within one minute of DNP-induction. This signal dissipated within five minutes (Figure 2F), temporally corresponding to microscopic observations of F-actin rearrangement and functional assessments of degranulation. In addition to the data shown in Figures 2D and 2F, we assessed other immediate and prolonged time points following DNP-induction in *Pak1*^{-/-} mast cells, but we were unable to find substantially enhanced PP2A_C-PP2A_A interactions or phosphatase activity as compared to WT samples. Intriguingly, OA-inhibition of IgE/DNP-treated cells prevented dephosphorylation of pT423-Paks (Supplement Figure 2A), and increased the quantity of PP2A_A bound to PP2A_C (Supplement Figure 2B). These findings suggest that activated PP2A directly dephosphorylates pT423-Paks and facilitates PP2A subunit disassembly in a self-catalyzed negative feedback loop.

We also tested whether Pak1's promotion of PP2A subunit assembly depended on Pak1's kinase activity, as compared to its structural presence alone. To accomplish this, we transduced a well-characterized dominant negative kinase-dead Pak1 mutant (K299R)[30, 31] into RBL-2H3 cells. Cells transduced with WT Pak1 demonstrated a DNP-induced association of PP2A_C with PP2A_A similar to the interaction observed in primary mast cells (Figure 2G). By contrast, the RBL-2H3 cells transduced with the kinase-dead Pak1 construct did not demonstrate DNP-induced association of PP2A_C with PP2A_A, resembling the findings from *Pak1*^{-/-} primary mast cells shown in Figure 2F. Together, these data argue that mast cells require Pak1's kinase activity to promote allergen-induced PP2A subunit assembly, which precipitates F-actin rearrangement.

Based on our observation that Pak1 regulates F-actin rearrangement, promotes PP2A subunit assembly, and enhances PP2A_C activity, we next assessed potential downstream targets of this Pak1-PP2A interaction. Cofilin is an actin regulatory protein with a known PP2A dephosphorylation motif and suspected roles in mast cell cytoskeletal organization [32, 33], and Pak1-mediated phosphorylation of LIMK regulates cofilin-dependent lamella/lamellipodium formation in epithelial cells [34]. In our experiments, we found rapid dephosphorylation of pS3-Cofilin during DNP-induction. However, in multiple experiments under various stimulation conditions, we were unable to consistently detect any substantial differences in pS3-Cofilin levels between WT and *Pak1*^{-/-} mast cells (Figure 3A). Although our data suggest that mast cell degranulation involves pS3-Cofilin signaling, its immediate dephosphorylation does not appear to depend upon Pak1-PP2A interactions.

We next investigated the possibility that Pak1-PP2A's modulation of F-actin rearrangement proceeds through ERM (Ezrin/Radixin/Moesin) family proteins. We specifically considered Ezrin, which, upon dephosphorylation at threonine 567, uncouples from its position as an actin-to-membrane linker element [35], an event which some studies have shown to be PP2A- and phospholipase-dependent [36, 37]. Indeed, we found that allergen-induction substantially reduced pT567-ERM in WT but not *Pak1*^{-/-} mast cells (Figure 3B). To further evaluate these findings, we examined F-actin and pERM levels and localization using confocal microscopy. Allergen-induction diminished F-actin and pT567-ERM signal in WT cells to a greater degree than *Pak1*^{-/-} cells (Figure 3C). pERM and F-actin signal co-localized at the cortical membrane, concurring with these proteins' putative membrane coupling function. Moreover, OA treatment prevented both F-actin depolymerization and pT567-ERM dephosphorylation (Figure 3C, bottom row). Immunoblots of non-stimulated and OA-treated pERM levels can be found in Supplement Figure 3A.

To complement our kinase-dead Pak1 study of PP2A subunit assembly, we assessed ERM phosphorylation in primary mast cells after the application of IPA-3, a chemical inhibitor of Pak kinase activity [38]. IPA-3 prevented pT567-ERM dephosphorylation, inhibited phosphorylation of pS298-Mek1/2 (a Paks-specific phosphorylation site), and reduced Erk1/2 phosphorylation concomitant with the inhibition of Pak-potentiated Raf-Mek phosphorylation (Figure 3D), as observed in SCF-mediated pathways [3]. Although potential non-selective effects of this chemical inhibitor cannot be ruled out, these data reinforce our finding that Pak1's kinase activity promotes PP2A subunit assembly and extend the implication that this activity leads to the dephosphorylation of ERM proteins in the allergen-induced mast cell.

To further assess the relationship between Pak1, PP2A, and pERM, we further examined IgE/DNP signaling events in RBL-2H3 cells. As seen in primary mast cells, phosphatase inhibition with either OA or calyculin A (CA) prevented PP2A_C-PP2A_A disassembly (Figure 4A). By contrast, we detected little PP2A_A bound to PP2A_C in IPA-3 treated RBL-2H3 cells. Moreover, all three inhibitors prevented DNP-induced pERM

dephosphorylation (Figure 4B). In all, these data buttress our previous genetic findings suggesting that, in the DNP-induced mast cell, Pak1 kinase activity promotes PP2A_C-PP2A_A interactions, that Pak1-PP2A interactions lead to pERM dephosphorylation, and that PP2A phosphatase activity catalyzes its own subunit disassembly.

We then used RNA interference to knockdown PP2A_C in RBL-2H3 cells. The ~45% reduction in PP2A_C diminished DNP-induced pT567-ERM dephosphorylation (Figure 4C), and deconvolution microscopy revealed persistent cortical patterns of pT567-ERM and disrupted F-actin rearrangement in DNP-induced, PP2A_C siRNA-treated cells (Figure 4D, fourth column).

Finally, we tested whether Ezrin, a specific ERM family member, directly regulates F-actin rearrangement and degranulation in primary mast cells. Primary mast cells were cultured from bone marrow harvested from *Mx1-Cre⁺Ezrin^{fllox/fllox}* and *Ezrin^{fllox/fllox}* mice approximately two weeks after the last dose of polyIC (Cre inducer). As assessed by immunoblot and flow cytometry of maturation markers, *Ezrin* disruption was stable and apparently not compensated by increased expression of moesin (Figure 5A). Importantly, *Ezrin*-disrupted bone marrow progenitor cells could give rise to phenotypically normal mast cells in IL-3 culture, as shown by flow cytometry of mast cell surface protein expression (Fig 5B) and assessed by Giemsa cytology (not shown).

However, *Ezrin* disrupted cells displayed abnormal cortical F-actin structure in resting and IgE-primed states, as well as persistent cortical F-actin after allergen-induction (Figure 5C). These data recapitulate aspects of F-actin dysregulation observed in the various experiments of Pak1 and PP2A inhibition, as described above. Moreover, β -hexosaminidase degranulation assays revealed compromised degranulation in *Ezrin* disrupted mast cells (Figure 5D). Ezrin, then, appears to regulate F-actin rearrangement and degranulation, implicating functional importance for this ERM family member protein in mast cell physiology.

Discussion

Our data delineate a Pak1-PP2A-ERM signaling axis with a clear role in the Fc ϵ RI-mediated pathways that coordinate F-actin rearrangement and mast cell degranulation. In our proposed model, allergen cross-linking of IgE bound to Fc ϵ RI induces Pak1 binding to, assembly, and activation of PP2A subunits, which, in turn, dephosphorylate ERM proteins at threonine 567. Dephosphorylated ERMs may then decouple the cortical membrane from F-actin, facilitating cytoskeletal rearrangement and granule extrusion, as suggested by our study of *Ezrin* deficient mast cells (Fig 6).

Our initial interest in Pak1 stems both from previous observations of Pak1's importance in SCF and IgE-mediated F-actin dynamics in the mast cell as well as Pak1's importance in regulating cytoskeletal dynamics of diverse cell types [23, 39-41]. Although our previous study had shown a critical role for Pak1 in IgE-mediated mast cell degranulation [4], the downstream effector molecules had been heretofore unknown. According to established findings, we had initially hypothesized that Pak1 modulates mast cell cytoskeletal dynamics through cofilin/LIMK signaling. We did not find evidence to substantiate this hypothesis and, instead, turned to PP2A, a ubiquitous phosphatase shown to interact with Pak1 with important functional implications in brain tissue and cardiomyocytes [11, 13, 42, 43]. Accordingly, we have presented evidence that Pak1 and PP2A can interact in a Pak1 kinase-dependent manner and that this interaction influences mast cell degranulation.

These findings necessitate further inquiry, including more detailed investigation into the temporal effects of *Pak1* disruption on allergen-induced PP2A activity and mast cell degranulation. While *Pak1* disruption impaired in vivo histamine release at the tested time point, it is plausible that total degranulatory activity over time remains unaffected, possibly due to redundant or complementary mechanisms. Moreover, we are interested in determining the kinase(s) responsible for PP2A_C's phosphorylation state at tyrosine 307, as we had initially hypothesized that this event could be mediated through the activity of a kinase dependent on Pak1's activity. Our data did not support this hypothesis. We are also interested in the upstream activators of Pak1, which, as suggested by other cell-signaling systems, likely include Rac1 and Rac2 [44-46]. Our preliminary studies in *Rac*-deficient mast cells have revealed similar findings to those observed in *Pak1*-deficient mast cells, reinforcing the reasonable supposition that Rac directly serves as the upstream effector of Pak1 in this proposed signaling pathway. If established, these observations would provide a direct link between PI-3K signaling with our proposed Pak1-PP2A-ERM axis. Of note, recent studies have shown critical roles for Fyn/Lyn/Syk-activated PI-3K and phosphatidylinositol triphosphate in mast cell degranulation [47, 48].

We are also interested in further study of Ezrin as well as of the remaining ERM family members, Moesin and Radixin. While we observed severe cytoskeletal defects and a moderate diminution in the degranulatory capacity of *Ezrin*-deficient mast cells, we suspect that additional functional consequences would be found in targeted deletions of *Radixin* and/or *Moesin* in primary mast cells. To date, little is known about the specific roles of these proteins in mast cell cytoskeletal control.

Regardless of the individual contribution of each ERM protein, we surmise that the Pak1-PP2A-ERM signaling axis comprises an important portion of the tightly-regulated mast cell degranulation machinery. These insights may facilitate the development of new therapies for allergy, asthma, and anaphylaxis. Moreover, data revealed in this primary cell model could have implications in other cell systems and diseases. Recent studies have examined Pak1-mediated cytoskeletal events in multiple cell types, including platelets [49], endothelial cells [14], epithelial cells [34], and metastatic cancer cells (reviewed in [50]). Using primary cultured mast cells, which depend on complex cytoskeletal machinery to coordinate motility and degranulation, we reveal mechanistic consequences of Pak1-PP2A interactions, and we report that Pak1's well-described regulation of LIMK and Cofilin may not apply to all cell types. Thus, our finding of a Pak1-PP2A-ERM signaling axis opens new avenues in broader studies of cytoskeletal control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Pak1 regulates mast cell cytoskeletal rearrangement, degranulation, and systemic histamine release

High resolution deconvolution microscopy reveals that *Pak1* disrupted cells retain an abnormal cortical F-actin ring at five minutes following allergen-induction (a, quantified in b)(n=4 *Pak1*^{+/+} and n=3 *Pak1*^{-/-}, ***p<0.001, 2-way ANOVA with Bonferroni correction, ns indicates p>0.05 by student's unpaired two-tailed t-test). Systemic histamine release depends on Pak1, as measured by serum ELISA after intravenous administration of anti-DNP IgE followed by DNP (c) (for both genotypes, n=3 in IgE/PBS and n=7 in IgE/DNP; **p<0.01, 2-way ANOVA with Bonferroni correction).

Figure 2. Pak1 promotes PP2A subunit assembly and activation

Okadaic acid (OA), an inhibitor of phosphatase activity, prevents F-actin depolymerization at five minutes following allergen-induction in mast cells (a) ($n=4$ for IgE/DNP condition and $n=3$ for OA condition, $**p<0.01$, $***p<0.001$, one-way ANOVA with Bonferroni correction, ns indicates $p>0.05$ by student's unpaired two-tailed t-test). Pak1 and PP2A_C interact directly in vitro, as shown by immunoprecipitation and western blot experiments using recombinant Pak1 and PP2A_C proteins (b). Pak1 and PP2A_C interact in murine tissue, as shown by immunoprecipitation of Pak1 and probing with anti-PP2A_C antibody in *Pak1*^{+/+} and *Pak1*^{-/-} samples, and EGFP-Pak1 expression in RBL-2H3 cells allows for the immunoblotting of PP2A_C from EGFP immunoprecipitates (c). Loss of *Pak1* diminishes allergen-induced PP2A_C activity, as shown by a phosphatase activity assay of PP2A_C immunoprecipitated from *Pak1*^{+/+} and *Pak1*^{-/-} mast cells (d) ($n=3$, $***p<0.001$, 2-way ANOVA with Bonferroni correction). *Pak1* disruption does not increase phosphorylation of PP2A_C at tyrosine 307, an inhibitory site (e), but it diminishes allergen-induced PP2A subunit assembly, as demonstrated by PP2A_C immunoprecipitation followed by anti-PP2A_A antibody probe (f). Transduction of the dominant negative Pak1K299R construct into RBL-2H3 cells prevents the ability to immunoblot PP2A_A from PP2A_C immunoprecipitate after allergen induction (g). Western blot experiments in c, e, and f were performed a minimum of three times, with results representative of typical findings. The experiment in g was performed twice on a stably transduced cell line, with representative results shown.

Figure 3. *Pak1* disruption inhibits pT567-ERM dephosphorylation but not cofilin dephosphorylation

Changes in the level of pS3-Cofilin was similar between *Pak1*^{+/+} and *Pak1*^{-/-} cells across multiple conditions, including those shown here (a). By contrast, *Pak1* disruption diminishes allergen-induced pT567-ERM dephosphorylation (b) (n=3, ***p<0.001 at DNP 1-minute, 2-way ANOVA with Bonferroni correction, ns indicates p>0.05 by student's unpaired two-tailed t-test). Phospho-T567-ERM and F-actin localize at the mast cell cortical membrane, as shown by confocal microscopy (c, first row). F-actin and pT567-ERM signals persist five minutes following allergen-induction in *Pak1*^{-/-} cells (c, second row) (n=3, p=0.010 by student's unpaired two-tailed t-test), and OA prevents F-actin depolymerization and pERM dephosphorylation (c, third row). IPA-3, a Pak kinase inhibitor, prevents DNP-induced pT567-ERM dephosphorylation, pS298-Mek1/2 phosphorylation (a Pak-dependent site), and mildly depresses pErk1/2 (d).

Figure 4. Pak1 and PP2A mediate pT567-ERM dephosphorylation and F-actin rearrangement in RBL-2H3 cells

IPA-3, OA, and CA treatment of RBL-2H3 cells recapitulated findings from primary mast cells, demonstrating that activated Paks promote PP2A subunit assembly, that phosphatase activity catalyzes subunit disassembly (a), and that activated Pak1 and PP2A modulate pT567-ERM dephosphorylation (b). siRNA knockdown of PP2A_C impairs allergen-induced pT567-ERM dephosphorylation (c). As shown by deconvolution microscopy, PP2A_C knockdown results in persistent pT567 and abnormal F-actin signals after DNP-induction, recapitulating findings from primary mast cells (d). Three distinct siRNA constructs were tested for PP2A_C knockdown efficiency. In the selected construct, similar results were obtained in three RBL-2H3 cultures, assayed 72 hours after transfection.

Figure 5. Ezrin modulates mast cell cytoskeleton and degranulation

Mice bearing *Ezrin* conditional knockout alleles (*Ezrin*^{flox/flox}) were intercrossed with *Mx1-Cre*⁺ mice, generating *Mx1Cre*⁺*Ezrin*^{flox/flox} mice. Two weeks after Cre induction *in vivo*, mast cells were cultured from bone marrow cells in IL-3 containing media. Immunoblot demonstrates that *Mx1-Cre*⁺ primary mast cells contain no detectable Ezrin protein, with no apparent compensation in total Moesin protein (a) (two samples shown each genotype). Flow cytometry of c-kit and FcεRI expression shows similar mast cell populations generated from WT and *Ezrin*^{-/-} bone marrow cells (b). Flow cytometry is representative of five independent cultures for each genotype. *Ezrin*-KO mast cells (cultured from *Mx1Cre*⁺*Ezrin*^{flox/flox} bone marrow) have aberrant F-actin organization and persistent F-actin polymerization after allergen-induction (c). *Ezrin* disruption impairs degranulation, as measured by a β-hexosaminidase release assay (d) (n=8, *p<0.01, 2-way ANOVA with Bonferroni correction; derived from four biologically-independent samples at two culture ages).

Figure 6. Proposed model for a Pak1-PP2A-ERM axis regulating allergen-induced mast cell degranulation

In our model, allergen-induction promotes a Pak1-PP2A interaction, which leads to PP2A subunit assembly, activation, and dephosphorylation of pT567-ERM, facilitating F-actin rearrangement preceding mast cell degranulation. Dashed lines indicate other potential/unknown events (c).