Rictor Negatively Regulates High-Affinity Receptors for IgE-Induced Mast Cell Degranulation

Daniel Smrz, Glenn Cruse, Michael A. Beaven, Arnold Kirshenbaum, Dean D. Metcalfe and Alasdair M. Gilfillan

J Immunol published online 5 November 2014
http://www.jimmunol.org/content/early/2014/11/05/jimmunol.1303495

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/11/05/jimmunol.130349
5.DCSupplemental

Why The JI? Submit online.
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Rictor Negatively Regulates High-Affinity Receptors for IgE-Induced Mast Cell Degranulation

Daniel Smrz,* Glenn Cruse,* Michael A. Beaven,† Arnold Kirshenbaum,* Dean D. Metcalfe,* and Alasdair M. Gilfillan*

Rictor is a regulatory component of the mammalian target of rapamycin (mTOR) complex 2 (mTORC2). We have previously demonstrated that rictor expression is substantially downregulated in terminally differentiated mast cells as compared with their immature or transformed counterparts. However, it is not known whether rictor and mTORC2 regulate mast cell activation. In this article, we show that mast cell degranulation induced by aggregation of high-affinity receptors for IgE (FcεRI) is negatively regulated by rictor independently of mTOR. We found that inhibition of mTORC2 by the dual mTORC1/mTORC2 inhibitor Torin1 or by downregulation of mTOR by short hairpin RNA had no impact on FcεRI-induced degranulation, whereas downregulation of rictor itself resulted in an increased sensitivity (∼50-fold) of cells to FcεRI aggregation with enhancement of degranulation. This was linked to a similar enhancement in calcium mobilization and cytoskeletal rearrangement attributable to increased phosphorylation of LAT and PLCγ1. In contrast, degranulation and calcium responses elicited by the G protein-coupled receptor ligand, C3a, or by thapsigargin, which induces a receptor-independent calcium signal, was unaffected by rictor knockdown. Overexpression of rictor, in contrast with knockdown, suppressed FcεRI-mediated degranulation. Taken together, these data provide evidence that rictor is a multifunctional signaling regulator that can regulate FcεRI-mediated degranulation independently of mTORC2. The Journal of Immunology, 2014, 193: 000–000.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that has diverse regulatory functions in multiple cell types (1). Two mTOR signaling complexes, mTOR complex 1 (mTORC1) and mTORC2, exist, each containing unique and shared partners (2). These two complexes are characterized by the association of mTOR with either raptor (mTORC1) or rictor (mTORC2) as their unique partner and by their differences in specificity toward target substrates (3, 4). Activation of mTORC1, for example, leads to the phosphorylation of 4E-BP1 and p70 S6K to regulate mRNA translation (5–8), whereas activation of mTORC2 leads to the phosphorylation of Akt(Ser473) (9–12) and PKCα (3, 13).

The activities of the mTOR complexes are regulated at multiple levels primarily through stimulatory or inhibitory phosphorylations of mTOR and its binding partners within the mTOR complex (2). The catalytic activity of mTOR is dependent on its autophosphorylation of Ser2481 (14). In addition, mTOR is phosphorylated on Ser2448 in insulin-stimulated cells (15) and was presumed to regulate mTOR activity (16). Later studies demonstrated that both phosphorylations occur only when mTOR is part of the intact mTOR complex, with mTOR being phosphorylated predominantly on Ser2448 in TORC1 and on Ser2481 in TORC2 (17). It is suggested that both phosphorylations could serve as markers to distinguish between mTORC1 and mTORC2. The regulation of mTORC2 via rictor is also mediated through several phosphorylation sites on rictor (18), one of which, Thr1135, is targeted by mTORC1-p70 S6K (18–20). There are reports, however, that some of the mTORC components may function independently of mTOR. This is particularly true for the mTORC2 component rictor, which was shown to associate with other complexes and function independently of mTORC2 (21–26).

Our studies have demonstrated that stimulation of mast cells with Kit ligand, stem cell factor (SCF), or through Ag-mediated aggregation of IgE-occupied high-affinity receptors for IgE (FcεRI) activates mTOR catalytic activity (27, 28). Mast cells are immune cells of hematopoietic origin that contribute to host defense mechanisms through receptor-mediated release of inflammatory mediators (29), as well as the inflammatory reactions associated with allergic disorders such as asthma and anaphylaxis (30, 31). The expansion of CD34+/CD117 (KIT)+ bone marrow mast cell progenitors, their subsequent maturation in tissues, and the homing of mast cells to sites of inflammation is largely regulated by SCF (32–34), whereas the activation of mast cells and ensuing degranulation and mediator release is largely a consequence of Ag/IgE-induced aggregation of FcεRI (35). Our studies revealed that mTORC1 promotes SCF-mediated mast cell migration, cytokine production, and survival (27, 28), whereas mTORC2 primarily regulates mast cell expansion (28), and G protein-coupled receptor (GPCR)–mediated mast cell migration and chemokine production (36). Whether mTORCs regulate FcεRI-mediated responses, however, is unknown. mTOR does not appear necessary for Ag-induced degranulation, despite its activation, as neither the mTORC1 inhibitor, rapamycin (27), nor the dual mTORC1/mTORC2 inhibitor, Torin1 (37), blocked degranulation in

*Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication January 2, 2014. Accepted for publication October 13, 2014.

This work was supported by Division of Intramural Research programs within the National Institute of Allergy and Infectious Diseases (to D.S., G.C., A.K., D.D.M., and A.M.G.) and the National Heart, Lung, and Blood Institute (to M.A.B.).

Address correspondence and reprint requests to Dr. Daniel Smrz at the current address: Institute of Immunology, 2nd Medical School and Motol University Hospital, Charles University, V Úvalu 84, 150 06 Prague 5, Czech Republic. E-mail address: daniel.smrz@fimotol.cuni.cz

The online version of this article contains supplemental material.

Abbreviations used in this article: FcεRI, high-affinity receptors for IgE; GPCR, G protein-coupled receptor; huIgE, human myeloma IgE; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; SA, streptavidin; SCF, stem cell factor; shRNA, short hairpin RNA; S6RP, S6 ribosomal protein.
mature human mast cells (28). Expression of mTORC components and their activities is nevertheless substantially diminished in mature nonproliferating mast cells as compared with their expanding immature or transformed counterparts (28) in which the more highly expressed mTORCs might otherwise regulate degranulation.

In this study, we examined the role of mTORC2 components on FceRI-induced degranulation in LAD2 human mast cells in which expression and activity of mTORC2 components are elevated compared with nonproliferating human mast cells. We found that suppression of mTORC2 activity and short hairpin RNA (shRNA)-induced knockdown of mTOR in these cells had no significant effect on FceRI-mediated degranulation. Surprisingly, knockdown of rictor markedly enhanced the sensitivity of cells to FceRI aggregation with respect to degranulation without significantly affecting mTORC2 catalytic activity as monitored by the phosphorylation of the mTORC2 downstream substrates, Akt(Ser473) and 4E-BP1(Thr37/46). Rictor knockdown appeared instead to increase FceRI-mediated signaling including LAT and PLCγ1 phosphorylation, resulting in enhanced calcium signaling and F-actin rearrangement. These data and the effects of rictor overexpression revealed a novel mode of action of rictor within mast cells in that it may regulate specific cellular functions independently of its regulation of mTOR activity.

Materials and Methods

Cell culture

The LAD2 human mast cell line (38) was maintained in StemPro-34 SMF with supplement (In Vitrogen, Carlsbad, CA) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), and recombinant human SCF (100 ng/ml; PeproTech, Rocky Hill, NJ).

Abs and reagents

Human myeloma IgE (huIgE; Calbiochem, La Jolla, CA) was biotinylated in the National Institute of Allergy and Infectious Diseases Core Facility. The protein-specific Abs used in confocal microscopy were mTOR (10343; IBL, Minneapolis, MN) and rictor (NB100-612; Novus Biologicals, Littleton, CO). The β-actin–specific Ab for immunoblotting was from Cell Signaling Technology (Beverley, MA) and Lyn-specific Ab for immunoblotting from Santa Cruz Biotechnology, West Grove, PA). HRP-conjugated rabbit IgG (Amersham Biosciences, Piscataway, NJ), and mouse IgG Fc-specific Ab (Sigma-Aldrich). The phosphoprotein-specific Abs for immunoblotting were p-mTOR(Ser2448), p-mTOR(Ser2481), p-akt(Thr308), p-akt(Ser473), p-LAT(Tyr171), and p-β-actin–specific Ab (Sigma-Aldrich). The phophoprotein-specific Abs for immunoblotting were p-mTOR(Ser2448), p-mTOR(Ser2481), p-akt(Thr308), p-akt(Ser473), p-LAT(Tyr171), and p-β-actin–specific Ab (Sigma-Aldrich).

Calcium measurement, immunoblotting, and F-actin analysis by flow cytometry

To monitor the calcium response in activated cells, we loaded sensitized cells with Fura-2 AM to monitor changes in cytosolic Ca2+ concentrations as described previously (46). Immunoblotting was performed on lysates of activated cells according to previous protocols (41). For densitometric analysis, the chemiluminescence from immunoblots was acquired on Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA). Images acquired within a linear range of saturation were then analyzed using Quantity One software (Bio-Rad). For studies of rictor overexpression specifically, the Li-Cor Odyssey CLX system was used for immunoblot analysis. Phospho-proteins (rabbit Abs) were measured using goat anti-rabbit 800CW secondary Ab (Li-Cor), in the green channel, and β-actin loading control (mouse Ab) was measured using goat anti-mouse 680RD secondary Ab (Li-Cor) in the red channel. A correction factor for loading was applied to increase FcεRI-mediated signaling including LAT and PLCγ1 phosphorylation, resulting in enhanced calcium signaling and F-actin rearrangement. These data and the effects of rictor overexpression revealed a novel mode of action of rictor within mast cells in that it may regulate specific cellular functions independently of its regulation of mTOR activity.

Calcium measurement, immunoblotting, and F-actin analysis by flow cytometry

To monitor the calcium response in activated cells, we loaded sensitized cells with Fura-2 AM to monitor changes in cytosolic Ca2+ concentrations as described previously (46). Immunoblotting was performed on lysates of activated cells according to previous protocols (41). For densitometric analysis, the chemiluminescence from immunoblots was acquired on Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA). Images acquired within a linear range of saturation were then analyzed using Quantity One software (Bio-Rad). For studies of rictor overexpression specifically, the Li-Cor Odyssey CLX system was used for immunoblot analysis. Phospho-proteins (rabbit Abs) were measured using goat anti-rabbit 800CW secondary Ab (Li-Cor), in the green channel, and β-actin loading control (mouse Ab) was measured using goat anti-mouse 680RD secondary Ab (Li-Cor) in the red channel. A correction factor for loading was applied to increase FcεRI-mediated signaling including LAT and PLCγ1 phosphorylation, resulting in enhanced calcium signaling and F-actin rearrangement. These data and the effects of rictor overexpression revealed a novel mode of action of rictor within mast cells in that it may regulate specific cellular functions independently of its regulation of mTOR activity.

shRNA knockdown

The MISSION shRNA system (Sigma-Aldrich) was used to knock down the specific proteins in the studied cells. The following TRC1-pLKO-puro vectors were used: two targeting sequences for mTOR, designated as mT1 (5’-CCGCCGCTAGTCTGGGATTTTCGAGAAATAAACCTCCCTACTAGGGGTTTGTG3’; TRCN0000038674) and mT2 (5’-CCGGGCGAGTAAATGTACCTACTGAGCAAGAATGAGTCTGTCCGGATGATGCCTTGTG3’; TRCN0000038675); and two targeting sequences for rictor, designated as Ric1 (5’-CTCTGCGATGTTTCACTGGAAGTTCGAGGAAGAAGATTTCGAGGATGCTGGTCTG3’; TRCN0000074289) and Ric2 (5’-CCGGCCGAGGTTCTACAAGAAATTACTCCGATTCTGTCAGAAAATATTCTG3’; TRCN0000074291). The TRC1-pLKO-puro vectors with nontarget sequence, (5’-CCGGCAACAAGATGAGGAGCAGCACTGCTGAGAAGATACGATGTTTCTTTGTTT-3’; SHC002), and with luciferase targeting sequence (5’-CCCGCCTGAGTACTTGGAAATGTCGAGGATCGAAGATCTACGGCTGGT-3’; SHC007) were used as negative controls. The virus for cell transduction was prepared using MISSION Lentiviral Packaging Mix (SHC0001; Sigma-Aldrich) according to the manufacturer’s instructions. The first harvest of the virus was filtered (0.45-μm pore size), then frozen and stored at −80°C. Before transduction, the virus was thawed and diluted (1:20-50) with the cell suspension of the targeted cells and cells cultured for 48 h. Studies were conducted on the cells after a subsequent 48- to 72-h positive selection (puromycin: 1 μg/ml).

Overexpression of rictor

LAD2 cells were transfected with human myc-tagged rictor in a pRK5 plasmid kindly supplied by Dr. D.D. Sarbassov (Department of Molecular and Cellular Oncology, University of Texas, MD Anderson Cancer Center, Houston, TX) (39) as described previously (40). In brief, 2 × 106 LAD2 cells were transfected using the Amaxa Nucleofector II system and then plated out in StemPro-34 medium supplemented with SCF and 100 ng/ml biotinylated human IgE for 6 h. This time point was chosen to achieve near-maximal expression of rictor without discernible effects on cell morphology or viability. The extent of overexpression was 25%, and typical results are described and shown in the Supplemental Fig. 1. After transfection, degranulation and activation studies were performed as described below.

Cell activation and degranulation

LAD2 cells were sensitized with biotinylated huIgE (100 ng/ml) overnight. The cells were then harvested and processed as described previously (41) using streptavidin (SA) to activate the cells via aggregation of biotinylated anti-IgE, bound-FcεRI, C3a to activate the cells via the C3R GPCR (42), or thapsigargin to activate the cells in a receptor-independent, calcium-dependent manner (43, 44). After stimulation of the cells for 30 min, degranulation was determined as a percentage of total (cellular and released) β-hexosaminidase that was released into the medium (45).

Confocal microscopy

After addition of stimulants as indicated, cell activation was stopped and the cells were further treated as described previously (47) using mTOR- and rictor-specific Abs of, respectively, mouse and rabbit origin in conjunction with donkey Cy3-labeled mouse and AlexaFluor488-labeled rabbit IgG-specific Abs. Initial investigation revealed that knockdown of rictor by 40–50% resulted in a 25% decrease in cellular fluorescence. The mTOR Ab (from IBL) used in this study was the only one tested that gave a sufficient fluorescent signal for confocal microscopy of LAD2 cells. The confocal images were acquired on a Leica SP5 confocal microscope (Leica Microsystems, Exton, PA) with 63× oil immersion objective and numerical aperture 1.4. The Huygens Essential software (Scientific Volume Imaging BV, Hilversum, the Netherlands)–deconvoluted images were processed using Imaris software (Bitplane AG, Zurich, Switzerland).

Statistics

The statistical significance was computed from the number (n) of values indicated. The statistical significance between the control and the test sets
of values was determined using unpaired two-tailed Student t test. The p values <0.05 were considered significant.

Results

Localization of mTOR and rictor in LAD2 human mast cells

The presence of rictor in the mTORC2 complex is critical for the ability of mTOR to phosphorylate its substrates. We therefore examined the relative cellular distribution of rictor and mTOR in LAD2 human mast cells before and after FceRI aggregation induced by cross-linking of receptor-bound biotinylated huIgE with SA. Under resting conditions, both mTOR and rictor were localized in punctate-like regions largely within the cytosol; neither appeared to be associated with the plasma membrane (Fig. 1A). However, simultaneous Ab staining of both molecules revealed that these molecules are only partially colocalized in resting cells (Fig. 1B, 1C). After FceRI aggregation for 2 min under optimal conditions for degranulation (48), there was only a small redistribution and increase in the association of rictor with mTOR (Fig. 1B, 1C). These data thus indicate that, as reported in a limited number of other cell types (49), rictor exists in two pools in LAD2 cells: one associated with mTOR presumably as part of the mTORC2 complex and the other localized in cellular compartments independently of mTOR. This suggested, therefore, that rictor would have the potential to regulate cellular process, not only in the context of mTORC2 activation, but also independently of mTOR activity. Two approaches were adopted to explore these possibilities: inhibition of mTOR activity by the dual mTORC1/mTORC2 inhibitor Torin1 (28, 37) and shRNA-induced knockdown of mTOR and rictor.

FIGURE 1. Localization of mTOR and rictor in resting and FceRI-activated LAD2 cells. (A) Sensitized (biotin-huIgE) cells were stained with mouse-origin mTOR- or rabbit-origin rictor-specific Abs. The bound Abs were visualized with a mix of donkey-origin Cy3-labeled mouse and Alexa Fluor 488–labeled rabbit IgG-specific Abs and the nuclei stained with DAPI. (B) Sensitized cells were activated with SA (100 ng/ml) for 2 min. The cells were then simultaneously stained with mouse origin mTOR- and rabbit origin rictor-specific Abs. The bound Abs and nuclei were visualized as in (A). (C) The correlation of mTOR and rictor in deconvolved images of the resting (nonactivated) and activated cells in (B) was calculated. (A and B) Typical deconvolved images from four independent experiments are shown. Scale bar, 5 μm. (C) Data show mean, SEM, and statistical significance (n = 4; *p < 0.05, Student t test) from four independent experiments in each of which four to six cells were analyzed.

FIGURE 2. mTORC stimulation in FceRI-activated LAD2 cells. (A) Sensitized cells were activated with SA (100 ng/ml) for time periods indicated. Cells were lysed and the indicated phosphoproteins analyzed by immunoblotting. Lyn content was used as a loading control. (B) Densitometric data from (A) were normalized. (A) Typical immunoblots from three independent experiments are shown. (B) Data represent mean and SEM, and statistical significance between activated and nonactivated samples for three independent experiments is indicated (n = 3; *p < 0.05, Student t test).
FcεRI-mediated degranulation is independent of mTOR activity

To explore the role of rictor in the context of regulation of mTORC2 function in LAD2 cells, we initially examined whether mTOR and rictor are activated by measuring the extent of phosphorylation of mTOR (Ser2481 and Ser2448) and rictor (Thr1135) after FcεRI aggregation. As shown in Fig. 2, FcεRI aggregation induced by cross-linking of receptor-bound biotinylated huIgE with SA increased phosphorylation of mTOR and, to a greater extent, rictor. These phosphorylations reached a maximum at 10 min and then declined. We next determined whether mTORC2 activation regulated degranulation by examining the outcome of its inhibition with Torin1. As shown in Fig. 3A, Torin1 had little, if any, effect on degranulation in SA-stimulated LAD2 cells at a concentration (200 nM) that maximally inhibits activating phosphorylations of mTOR and downstream substrates in CD34+-derived human mast cells (see Supplemental Material) (28). This result was also in accord with our previous studies (28) where mTORC2 activity was increased after FcεRI aggregation but was not required for degranulation as the latter was not inhibited by Torin1. As further verification, we knocked down expression of mTOR in LAD2 cells. For these studies, we used two control shRNA vectors and four shRNA constructs: two that targeted mTOR and another two rictor. As shown in Fig. 3B and 3C, the control vectors minimally impaired mTOR expression, although in all these knockdown experiments, one of the control vectors (non-target2 in Fig. 3C) elevated the expression of rictor. Nevertheless, one of the mTOR-targeted shRNAs (i.e., mT2 in Fig. 3C) reduced mTOR expression by ~50% in these cells without significantly affecting rictor or FcεRI expression as indicated by IgE bound to the cell surface (Fig. 3D). Despite the partial knockdown of mTOR with mT2 shRNA, degranulation was not significantly different from that induced in the control vector (non-target1 and 2) or mT1-treated cells (Fig. 3E). However, consistent with experiments described later in this article, the non-target2 transduced cells with the highest rictor levels (as shown in Fig. 3C) exhibited significantly reduced degranulation compared with non-target1 transfected cells and control non-transfected cells with lower rictor levels. This pattern was consistent in all individual experiments. Overall, these data further support the conclusion that mTOR activity, and presumably rictor in the context of mTORC2, had little or minimal role in the regulation of FcεRI-mediated mast cell degranulation.

Rictor knockdown increases sensitivity to FcεRI aggregation with respect to degranulation

We next used the shRNA constructs to knock down rictor specifically to see whether it regulated degranulation independently of mTORC2 in LAD2 cells. As shown in Fig. 4A and 4B, this strategy significantly downregulated expression of rictor with minimal change in the expression of mTOR, FcεRI (as assessed

**FIGURE 3.** Role of mTOR in degranulation of FcεRI-activated LAD2 cells. (A) Sensitized cells were pretreated with Torin1 (200 nM) or vehicle alone (Ctrl) for 30 min. The cells were then activated with SA (0–1000 ng/ml) for 30 min and degranulation determined. (B) LAD2 cells were, or not (Ctrl), transduced with two nontargeting (Non-target1 and Non-target2) and two mTOR-targeting (mT1 and mT2) shRNA plasmids, and expression of mTOR and rictor was determined by immunoblotting. β-Actin content was used as a loading control. (C) Normalized densitometric data from all immunoblots are shown. (D and E) The transduced cells in (B) and (C) were sensitized overnight with biotin-huIgE and evaluated for biotin-huIgE binding by flow cytometry (D) or activated with SA (0–1000 ng/ml) for 30 min to measure degranulation (E). (B) Typical immunoblots from three independent transductions are shown. (A and C) Data show mean and SEM from three independent transductions, and statistical significance between nontransduced and transduced cells is indicated (n = 3; *p < 0.05, Student t test). (E) Bars for SEM are shown except where they fall within the data points. Significant reductions were observed for non-target2 compared with non-target1 and controls (p < 0.01), and for mT1 and mT2 transduced cells compared with non-target1 and controls (p < 0.05) at 100 and 1000 ng/ml SA (data not shown).
by IgE binding in Fig. 4C), and other proteins (see later). However, rictor knockdown resulted in a marked (∼50-fold) increase in sensitivity of the LAD2 cells to SA with respect to degranulation as indicated by the leftward shift in the concentration–response curve (Fig. 4D). This shift was in contrast with the relatively small differences noted for degranulation with knockdown of mTOR in Fig. 3E.

To examine whether the enhanced sensitivity was specific for FcεRI-mediated signaling, degranulation was examined after challenge with C3a, which activates mast cells via the GPCR, C3R, and thapsigargin, which induces receptor-independent calcium-dependent degranulation. As shown in Fig. 4E, there were no differences in the responses between control shRNA- and rictor-specific shRNA-treated cells to either stimulant, thus revealing that the regulation of degranulation by rictor was restricted to FcεRI-mediated stimulation.

**Increased sensitivity to FcεRI aggregation is associated with enhanced calcium signaling**

To identify signaling events that are responsible for the increased sensitivity to FcεRI-mediated stimulation, we next examined the increase in cytosolic calcium, a critical signaling event for mast cell degranulation (50, 51). As shown in Fig. 5A, when mast cells were stimulated with a concentration of SA that induced maximal degranulation, there was a small, although statistically significant, increase in the calcium response in rictor knockdown cells as compared with control cells. However, with lower concentrations of SA, the calcium response in control cells was diminished accordingly, whereas the response in the rictor knockdown cells still remained high with an even greater difference from that of control cells. This difference was most apparent with concentrations of SA (1 ng/ml) that induced minimal calcium response in the control cells (Fig. 5A, right panel). In contrast, when the cells were stimulated with C3a, the calcium response in rictor knockdown and control cells were comparable (Fig. 5B). These data thus show that the calcium response in SA-activated cells is negatively regulated by rictor. However, as with degranulation, rictor does not influence the calcium response elicited by the GPCR agonist, C3a.

**Rictor negatively regulates F-actin reorganization after FcεRI aggregation**

The calcium signal in activated mast cells initiates necessary events for degranulation (50, 51). Among these, the reorganization of F-actin plays a key role in the process of granule extrusion and passage through the juxta-membrane F-actin barrier (47, 52–55). We therefore selected one control and one rictor-targeting vector to determine whether rictor knockdown affected F-actin-mediated stimulation.

**FIGURE 4.** Role of rictor in degranulation of FcεRI-activated LAD2 cells. (A and B) LAD2 cells were or were not (Ctrl) transduced with the nontargeting or rictor-targeting shRNA plasmids as described in Fig. 3. Expression of mTOR and rictor was determined by immunoblotting. β-Actin content was used as a loading control. Typical immunoblots are shown in (A) and normalized densitometric data for all immunoblots in (B). The transduced cells were sensitized for measurement of biotin-huIgE binding (C) and degranulation after activation with SA (0–1000 ng/ml) as described for Fig. 3 (D). (E) The transduced sensitized cells were also activated with C3a (0–2000 ng/ml) or thapsigargin (0–1 nM) for 30 min, and degranulation was determined. (B–E) Data show mean and SEM (except where bars fall within data points) from three independent transductions, and statistical significance between non-transduced and transduced cells is indicated (n = 3; *p < 0.05, Student t test). The asterisks have been stacked vertically where two data points overlap as in panels (D) and (E).
RAPIDLY RETURNED TO THOSE OBSERVED BEFORE ACTIVATION, WHEREAS THE RICTOR KNOCKDOWN CELLS MAINTAINED ELEVATED F-ACTIN LEVELS FOR UP TO 30 MIN. WHEN CELLS WERE STIMULATED WITH A CONCENTRATION OF SA THAT INDUCED NO DEGRANULATION IN CONTROL CELLS BUT STILL SUBSTANTIAL DEGRANULATION IN RICTOR KNOCKDOWN CELLS (FIG. 4D), ONLY THE RICTOR KNOCKDOWN CELLS DISPLAYED ENHANCED F-ACTIN LEVELS (FIG. 6A, RIGHT PANEL). IN CONTRAST, STIMULATION VIA GPCR USING C3A REVEALED NO DIFFERENCE IN THE ELEVATION OF F-ACTIN BETWEEN THE RICTOR KNOCKDOWN AND CONTROL CELLS (FIG. 6B). THESE DATA ARE CONSISTENT WITH THE CONCLUSION THAT RICTOR ALSO FUNCTIONS AS A NEGATIVE REGULATOR OF F-ACTIN REORGANIZATION, AS IS THE CASE FOR THE CALCIUM RESPONSE.

RICTOR NEGATIVELY IMPACTS EARLY FcεRI-INDUCED SIGNALING EVENTS

WE NEXT FOUCUSED ON THE MECHANISMS THROUGH WHICH RICTOR MAY ACT ON UPSTREAM SIGNALING EVENTS REGULATING THE CALCIUM RESPONSE AND DOWNSTREAM F-ACTIN REMODELING, AS WELL AS DEGRANULATION. THE FcεRI-MEDIATED CALCIUM SIGNAL IS PRODUCED VIA PHOSPHORYLATION OF LAT, WHICH ENABLES RECRUITMENT, PHOSPHORYLATION, AND ACTIVATION OF PLCγ1. THE EUISING RELEASE OF INTRACELLULAR CALCIUM FROM THE ENDOPLASMATIC RETICULUM AFTER PRODUCTION OF INOSITOL 1,4,5-TRISPHOSPHATE BY PLCγ1 IS COUPLED TO INFUX OF INTRACELLULAR CALCIUM AND ACTIVATION OF PHOSPHOINOSITIDE 3-KINASE 3-KINASE (56). ACCORDINGLY, WE ANALYZED THESE BIOCHEMICAL EVENTS IN THE CONTROL AND RICTOR KNOCKDOWN CELLS STIMULATED BY SA AT A LOW CONCENTRATION (1 ng/ml) THAT PRODUCED NO OR MINIMAL CALCIUM SIGNAL, DEGRANULATION, AND ALTERATION OF F-ACTIN LEVELS IN CONTROL CELLS, BUT SUBSTANTIAL RESPONSES IN RICTOR KNOCKDOWN CELLS (FIG. 4D, 5A, 6A). AS SHOWN IN Fig. 7A AND 7B, THE EXTENT OF PHOSPHORYLATION OF BOTH LAT AND PLCγ1 WAS CONSIDERABLY GREATER IN THE RICTOR KNOCKDOWN CELLS THAN CONTROL CELLS UNDER THESE CONDITIONS. A SIMILAR AND SIGNIFICANT ENHANCEMENT WAS ALSO NOTED IN THE PHOSPHORYLATION OF AKT(THR308), A SUBSTRATE OF mTORC2 (9), 4E-BP1(THR37/46), A SUBSTRATE OF mTORC1 (28, 37), AND S6RP(SER240/244) (58), A SUBSTRATE FOR mTORC1-REGULATED p70 S6 KINASE (59) (Fig. 7B). HOWEVER, PHOSPHORYLATION OF S6RP(SER240/244) REMAINED SUBSTANTIALLY HIGHER IN RICTOR KNOCKDOWN CELLS THAN IN CONTROL TRANSFECTED CELLS AT ALL TIME POINTS, WHICH MIGHT REFLECT, IN PART, THE RECIPROCAL ACTIVITIES OF THE mTORCs IN REGULATING CELL ACTIVITIES. NEVERTHELESS, THESE RESULTS OVERALL IMPLY THAT AT LOW LEVELS OF FcεRI-MEDIATED ACTIVATION, EVENTS MAY BE LINKED TO TEMPORARY INHIBITION OF mTORC-DEPENDENT PHOSPHORYLATION OF AKT(THR308) INDEPENDENTLY OF ITS LINK TO mTORC2-DEPENDENT PHOSPHORYLATION OF AKT(SER473).

FIGURE 5. Role of rictor in the calcium response in FcεRI-activated LAD2 cells. LAD2 cells were transduced, or not (Ctrl), with nontargeting or rictor-targeting shRNA plasmids and sensitized overnight with biotin-huIgE as in previous figures. The sensitized cells were loaded with Fura-2 AM, stimulated with SA (100, 10, and 1 ng/ml) (A) or with C3a (2000, 500, 100 ng/ml) (B), and the calcium responses determined. Data show mean and SEM from three independent transductions. The differences between rictor- and vector-transduced cells were statistically significant at almost all time points shown in (A) (p < 0.05 to <0.001, Student t test).

FIGURE 6. Role of rictor in F-actin depolymerization/repolymerization in FcεRI-activated LAD2 cells. (A) LAD2 cells were transduced with nontargeting (Non-target1; Non-target) or rictor-targeting (Rict1; Rictor KD) shRNA plasmids and sensitized. After activation with SA (10 and 1 ng/ml) for time periods indicated, cells were fixed, permeabilized, and F-actin content was analyzed after staining with FITC-phalloidin by flow cytometry. (B) Changes in F-actin were similarly determined in C3a-stimulated (500 ng/ml) cells. Values for staining intensity were normalized and show mean and SEM from three independent transductions, and statistical significance between nontransduced and transduced cells at each time point is indicated (n = 3; *p < 0.05, Student t test).

FIGURE 7. Role of rictor in F-actin depolymerization/repolymerization in FcεRI-activated LAD2 cells. (A) LAD2 cells were transduced with nontargeting (Non-target1; Non-target) or rictor-targeting (Rict1; Rictor KD) shRNA plasmids and sensitized. After activation with SA (10 and 1 ng/ml) for time periods indicated, cells were fixed, permeabilized, and F-actin content was analyzed after staining with FITC-phalloidin by flow cytometry. (B) Changes in F-actin were similarly determined in C3a-stimulated (500 ng/ml) cells. Values for staining intensity were normalized and show mean and SEM from three independent transductions, and statistical significance between nontransduced and transduced cells at each time point is indicated (n = 3; *p < 0.05, Student t test).
To verify that rictor negatively regulated mast cell activation, the effects of overexpression of rictor were examined in LAD2 cells. We were able to achieve overexpression of rictor (~25%), and although modest, this increase in rictor levels resulted in significantly reduced degranulation at 100 ng/ml SA. This trend was apparent, but not significant, at lower concentrations of SA (Supplemental Fig. 1A). As for rictor knockdown, degranulation in response to thapsigargin was unaffected (Supplemental Fig. 1B), to suggest again that the inhibitory effect of rictor was related to FcεRI-mediated signaling rather than the mechanics of exocytosis. The increase in rictor levels was also associated with diminished SA-induced phosphorylation of Akt(Thr308), whereas phosphorylation of Akt(Ser473) was unchanged (Supplemental Fig. 1C, 1D), as was true for rictor knockdown. Therefore, the data further support the conclusions that rictor inhibits FcεRI-mediated degranulation and that the inhibitory action of rictor on PI3K/PDK1-dependent phosphorylation of Akt(Thr308) occurs independently of TORC2-dependent phosphorylation of Akt(Ser473).

Discussion
In this study, we have demonstrated that rictor can function as a negative regulator of signaling events responsible for FcεRI-mediated mast cell degranulation independently of mTOR and by implication mTORC2. Although our studies reveal a complex and unclear interrelationship between mTORC1 and mTORC2 that warrants further examination, the focus of this study is the role of rictor in FcεRI-mediated activation of mast cells. Rictor was originally described as an integral component of mTORC2, a complex that comprises mTOR and several other binding partners (61). The signaling function of this complex is directly linked to the kinase activity of mTOR as demonstrated by recently developed highly specific inhibitors of mTOR (62). We have previously shown that inhibition of mTORC2 catalytic activity by a dual mTORC1/mTORC2 kinase inhibitor Torin1 (37) inhibits proliferation of developing and neoplastic mast cells (28). However, because this inhibitor failed to block FcεRI-induced mast cell degranulation, we concluded that mTORC2 catalytic activity was not required for this response (28). This conclusion is further supported by our current observation that, in addition to Torin1, shRNA-mediated downregulation of mTOR had no effect on degranulation in FcεRI-activated LAD2 human mast cells.

Previously, we had noted that, at various stages of development of mast cells from CD34+ progenitors, the ratio between mTOR and rictor varied (28). This variable stoichiometry suggested that these mTORC components may function, not only in the context of mTORC2, but also independently of each other. Our confocal examination of mast cells demonstrates that rictor is localized in at least two discernible pools regardless of the activation status of the cells: one colocalized with mTOR and the other not. Using rictor-specific shRNA, we further demonstrated that downregulation (~70%) of rictor in LAD2 mast cells had no effect on mTORC2 catalytic activity in FcεRI-stimulated cells because there was no impairment of the phosphorylation of mTORC2 substrate Akt(Ser473) (9) and the mTORC1/mTORC2 downstream signaling target 4E-BP1(Thr37/46) (7, 28, 37). These data might be indicative of insufficient knockdown of rictor to affect mTORC2 activity but are in contrast with findings in other systems where rictor downregulation was usually associated with loss of mTORC2 activity (9, 63). Nevertheless, our data are consistent with reports...
of rictor forming complexes with other proteins or functioning independently of mTORC2 (21–25).

The marked increase in sensitivity of LAD2 human mast cells after rictor knockdown to SA, which leads to enhanced degranulation without alteration in mTORC2 signaling, also supports our conclusion that rictor can function independently of mTOR complexes. This increase in sensitivity was associated with corresponding changes in critical FcεRI-mediated signaling events such as the generation of the calcium signal and resulting F-actin rearrangement. In fact, these signals and degranulation were apparent in the rictor knockdown cells at concentrations of SA that failed to stimulate degranulation and were minimally effective in eliciting a calcium signal or actin remodeling in control cells. Consistent with the earlier results, overexpression of rictor diminished degranulation and PI3K-dependent phosphorylation of Akt(Thr308) without affecting mTORC2 signaling via phosphorylation of Akt(Ser473).

The resistance of C3a and thapsigargin stimulation to rictor knockdown or overexpression indicated that the negative effects of rictor were restricted to FcεRI-specific signaling events. These include the phosphorylation of LAT and PLCγ, that precede calcium mobilization and the activation of PI3K activity, as indicated by increased phosphorylation of Akt(Thr308), which helps maintain early FcεRI-mediated signaling. LAT is a plasma membrane adaptor protein that, by virtue of phosphorylation of specific tyrosine residues and subsequent recruitment and assembly of key signaling molecules including PLCγ (64), is the nexus for signal propagation for FcεRI (65) and other receptors including TCR (66) and pre-BCR (67). Whether rictor negatively regulates LAT signal propagation from these other receptors or is restricted to FcεRI-mediated activation specifically in mast cell function is unknown and needs further investigation.

In contrast with the comparable enhancements in FcεRI-mediated phosphorylation of Akt(Thr308), LAT, and PLCγ after rictor knockdown, the increased constitutive phosphorylation of S6RP(Ser240/244) in resting rictor knockdown or overexpression indicated that the negative effects of rictor were restricted to FcεRI-specific signaling events. These include the phosphorylation of LAT and PLCγ, that precede calcium mobilization and the activation of PI3K activity, as indicated by increased phosphorylation of Akt(Thr308), which helps maintain early FcεRI-mediated signaling. LAT is a plasma membrane adaptor protein that, by virtue of phosphorylation of specific tyrosine residues and subsequent recruitment and assembly of key signaling molecules including PLCγ (64), is the nexus for signal propagation for FcεRI (65) and other receptors including TCR (66) and pre-BCR (67). Although the precise reason for the upregulation of phosphorylation of S6RP in the rictor knockdown LAD2 cells remains unclear, it may reflect competition between rictor and raptor for a finite pool of mTOR for the formation of mTOR complexes into functional units. Regardless, our data show that, with a specific type of activation such as FcεRI stimulation in LAD2 mast cells, rictor can act independently of mTORC2 and contribute to the regulation of the activation pathway that is controlled by PI3K/mTORC1 signaling (56).

As noted earlier, rictor levels are significantly increased at the early stages of mast cell development and that, upon maturation, these levels gradually decline to a minimal level in terminally differentiated nondividing mast cells (28). Furthermore, the proliferation capacity of developing and neoplastic mast cells correlates with and is controlled by rictor expression (28). In this article, we report that, in addition to cell proliferation, rictor may also temper the sensitivity of mast cells to FcεRI stimulation, especially at low levels of stimulation, to prevent inappropriate activation of the developing mast cells during the maturation phase. Such control of the sensitivity of mast cells to FcεRI-mediated signals is particularly important upon migration of mast cells to tissues where terminal mast cell differentiation takes place. The diminution in expression of rictor in mature cells may then allow a more responsive phenotype to Ag stimulation. Collectively, these findings indicate that rictor expression may be an important player in the regulation of mast cell numbers, their distribution, and their activation phenotype in vivo, and that abnormal rictor expression or function could result in dysfunctional mast cell proliferation and activation in disorders of mast cell proliferation, anaphylaxis, and atopy.

In summary, our data demonstrate for the first time, to our knowledge, that rictor participates in the regulation of the FcεRI-mediated cell activation independently of mTORC2. This regulation is executed at the early stages of FcεRI signaling to negatively regulate critical signaling events such as calcium mobilization and cytoskeletal rearrangement. Studies with C3a and thapsigargin suggest that the actions of rictor might be specific for FcεRI-mediated activation in mast cells. This specificity points to rictor as being a novel therapeutic target to control the responsiveness of the mast cells in allergic disease.

Acknowledgments
We thank Dr. Nathanael S. Gray and Dana-Farber Cancer Institute for providing Torin1. We thank the clinical research staff for assistance and the Biological Imaging Facility (Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health) for technical assistance in confocal image production, including Juraj Kabat for assistance in image postprocessing and analysis.

Disclosures
The authors have no financial conflicts of interest.

References

The Journal of Immunology