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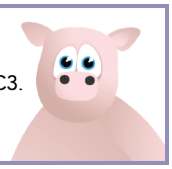

The Journal of Immunology

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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 PLCγ_1. 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(Ser^{473}) (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 Ser^{2481} (14). In addition, mTOR is phosphorylated on Ser^{2448} 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 Ser^{2448} in TORC1 and on Ser^{2481} 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, Thr^{1135}, 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

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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.

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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 FcεRI-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 FcεRI-mediated degranulation. Surprisingly, knockdown of rictor markedly enhanced the sensitivity of cells to FcεRI aggregation with respect to degranulation without significantly affecting mTORC2 catalytic activity as monitored by the phosphorylation of the mTORC2 downstream substrates, Akt(Ser^{473}) and 4E-BP1($Thr^{37/46}$). Rictor knockdown appeared instead to increase FcεRI-mediated signaling including LAT and PLCγ_1 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 (Invitrogen, 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 (Santa Cruz, CA). The conjugated protein-specific Abs were fluorophore-conjugated Abs (Jackson Immunoresearch Laboratories, 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(Ser^{2448}), p-mTOR(Ser^{2481}), p-rictor(Thr^{1135}), p-Akt(Thr^{308}), p-Akt(Ser^{473}), p-LAT(Tyr^{171}), p-S6 ribosomal protein(S6RP)($Ser^{240/244}$), p-4E-BP1($Thr^{37/46}$), and rictor (Cell Signaling Technology), and p-PLCγ_1(Tyr^{783}) (Invitrogen). The blocking donkey serum was from Santa Cruz Biotechnology, and Torin1 (37) was a gift of Dr. Nathanael S. Gray, Dana-Farber Cancer Institute, Harvard Medical School (Boston, MA). FITC-conjugated phalloidin and other chemicals and reagents were from Sigma-Aldrich.

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′-CCGGCCGCTAGTAGGGAGGTTTATTCTCGAGAATAAACCTCCC-TACTAGCGGTTTTTG-3′; TRCN0000038674) and mT2 (5′-CCGGGC-CAGAATCTATTCATTCTTTCTCGAGAAAGAATGAATAGATTCTGG-CTTTTTG-3′; TRCN0000038677); and two targeting sequences for rictor, designated as Rict1 (5′-CCGGCGTCGGAGTAACCAAAGATTACTCG-AGTAATCTTTGGTTACTCCGACGTTTTTG-3′; TRCN0000074291) and Rict2 (5′-CCGGCGGAGGTTCATACAAGAATTACTCGAGTAATTCTT-GTATGAACCTCCGTTTTTG-3′; TRCN0000074289). The TRC1-pLKO-puro vectors with nontarget sequence, (5′-CCGGCAACAAGATGAA-GAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′; SHC002), and with luciferase targeting sequence (5′-CCGGCGCTGAGTA-CTTCGAAATGTCCTCGAGGACATTTCGAAGTACTCAGCGTTTTT-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, 2×10^6 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 later.

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 biotin-huIgE–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).

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 Ca^{2+} 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 the fluorescence intensity of the phosphorylation bands to produce corrected phosphorylation intensity.

The activated cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with 1 μg/ml phalloidin-FITC as described previously, to determine changes in the content of F-actin (47). The stained cells were then analyzed by flow cytometry on FACSCalibur with CellQuest 3.3 software (BD Biosciences, San Jose, CA). The acquired data were analyzed using FlowJo 7.6 software (TreeStar, Ashland, OR).

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

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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 FcεRI 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 FcεRI 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.

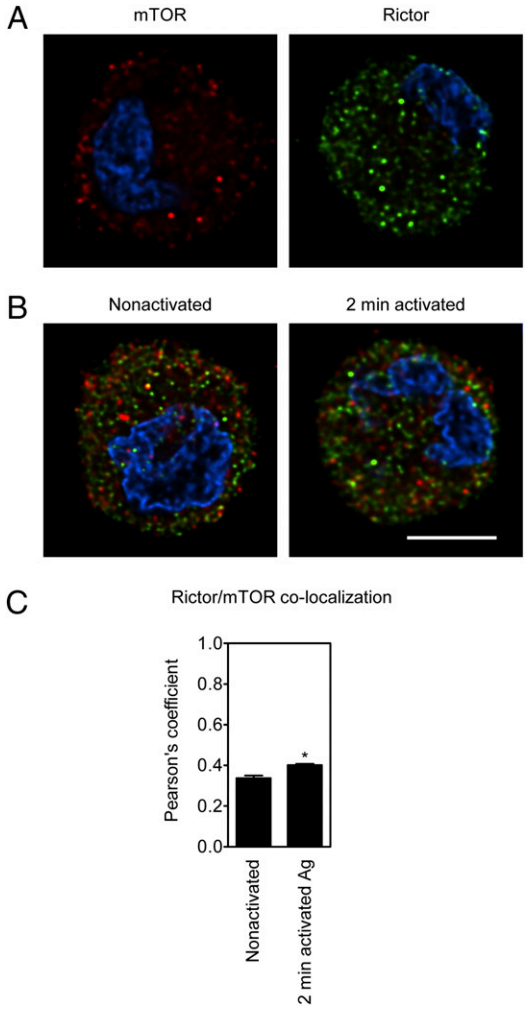

FIGURE 1. Localization of mTOR and rictor in resting and FcεRI-activated LAD2 cells. (**A**) Sensitized (biotin-huIgE) cells were stained with mouse-origin mTOR- or rabbit-origin rictor-specific Abs. The bound Abs were visualized with a mix of donkey-origin Cy3-labeled mouse and Alexa Fluor 488–labeled 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.

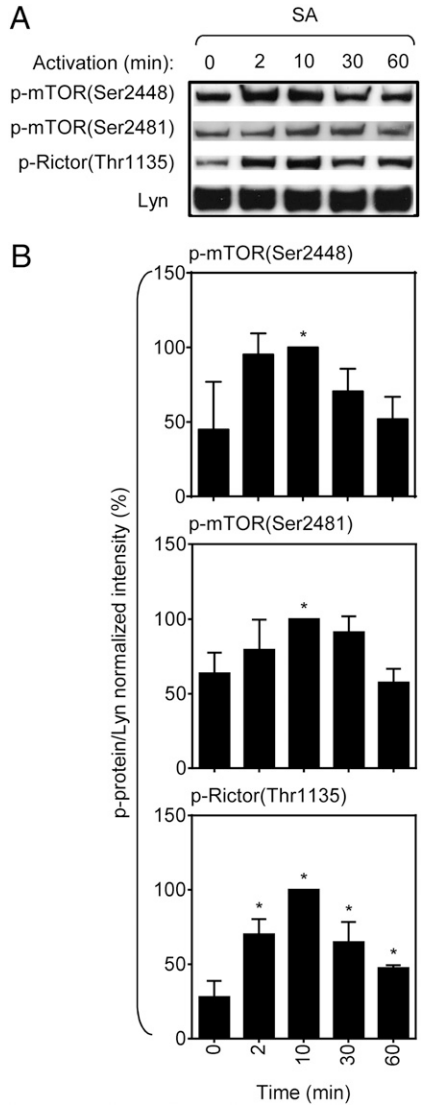

FIGURE 2. mTORC stimulation in FcεRI-activated LAD2 cells. (**A**) Sensitized cells were activated with SA (100 ng/ml) for time periods indicated. Cells were lysed and the indicated phosphoproteins analyzed by immunoblotting. Lyn content was used as a loading control. (**B**) Densitometric data from (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).

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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(Ser^{2481} and Ser^{2448}) and rictor(Thr^{1135}) 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

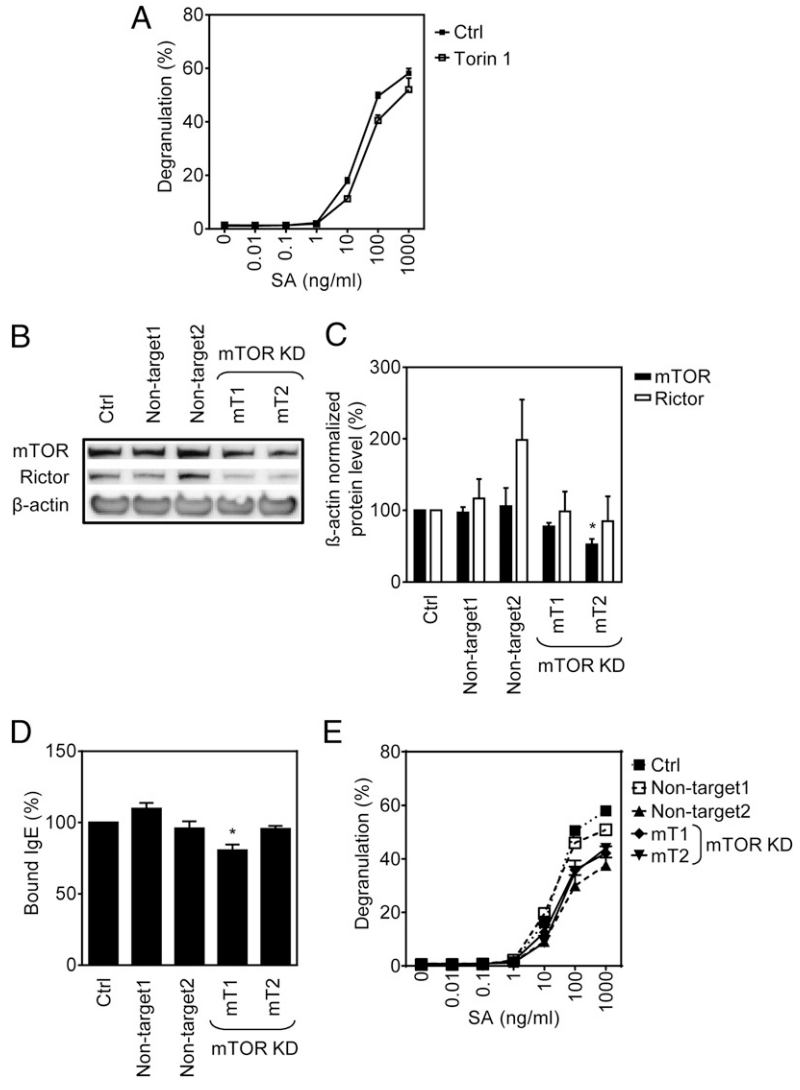

FIGURE 3. Role of mTOR in degranulation of FcεRI-activated LAD2 cells. (**A**) Sensitized cells were pretreated with Torin1 (200 nM) or vehicle alone (Ctrl) for 30 min. The cells were then activated with SA (0–1000 ng/ml) for 30 min and degranulation determined. (**B**) LAD2 cells were, or not (Ctrl), 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).

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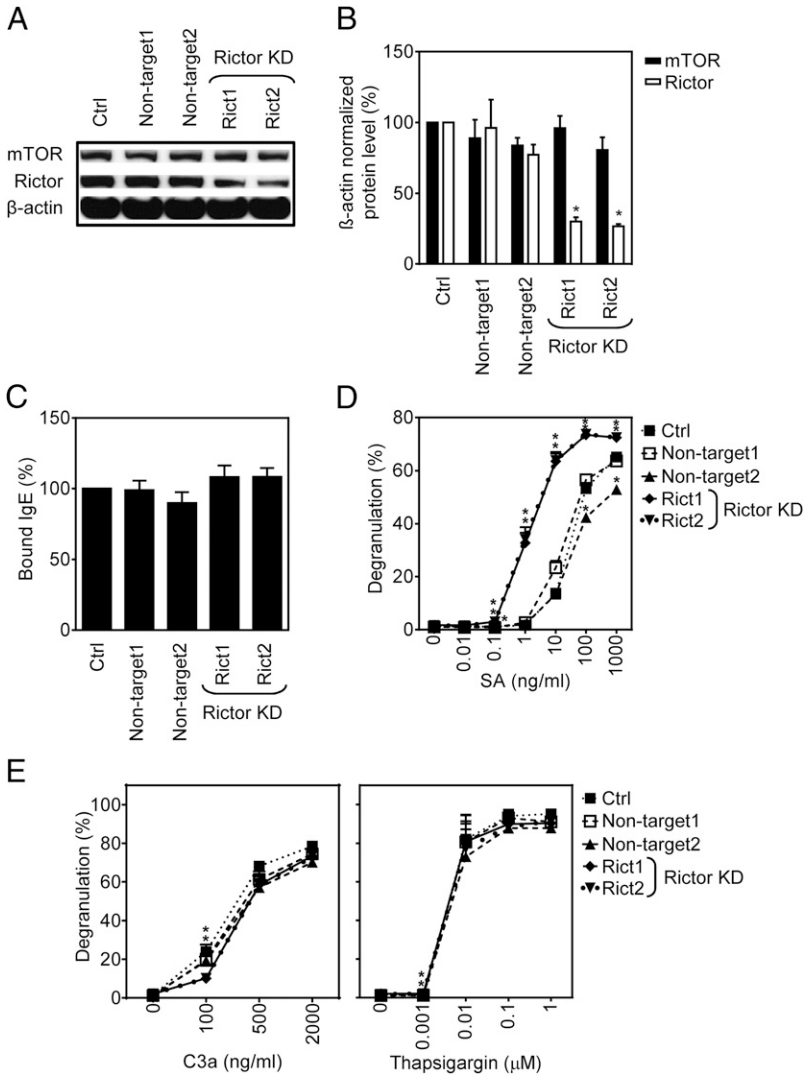

FIGURE 4. Role of rictor in degranulation of FcεRI-activated LAD2 cells. (**A** and **B**) LAD2 cells were or were not (Ctrl) transduced with the nontargeting or rictor-targeting shRNA plasmids as described in Fig. 3. Expression of mTOR and rictor was determined by immunoblotting. β-Actin content was 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 nontransduced 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).

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). 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 reorganization. As shown in Fig. 6A, stimulation with a suboptimal concentration of SA (10 ng/ml) induced a rapid and comparable elevation of F-actin levels in both the control and the rictor knockdown cells. However, in the control cells, the F-actin levels

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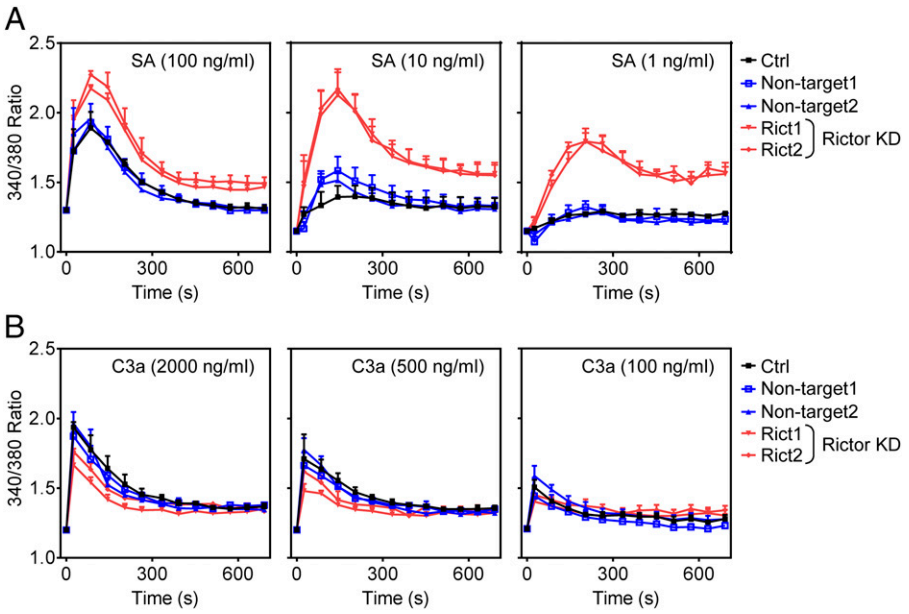

FIGURE 5. Role of rictor in the calcium response in FcεRI-activated LAD2 cells. LAD2 cells were transduced, or not (Ctrl), with nontargeting or rictor-targeting shRNA plasmids and sensitized overnight with biotin-huIgE as in previous figures. The sensitized cells were loaded with Fura-2 AM, stimulated 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).

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.

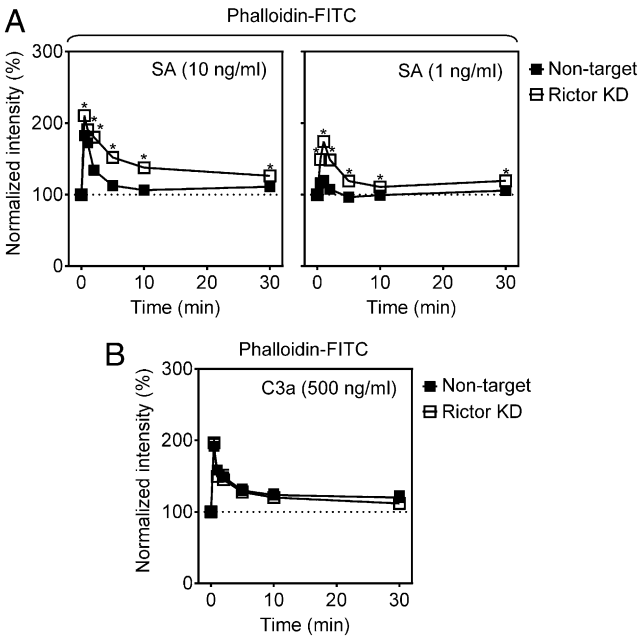

FIGURE 6. Role of rictor in F-actin depolymerization/repolymerization in FcεRI-activated LAD2 cells. (**A**) LAD2 cells were transduced with nontargeting (Non-target1; Non-target) or rictor-targeting (Rict1; Rictor KD) shRNA plasmids and sensitized. After activation with SA (10 and 1 ng/ml) for time 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).

Rictor negatively impacts early FcεRI-induced signaling events

We next focused 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 PLCγ_1. The ensuing release of intracellular calcium from the endoplasmic reticulum after production of inositol 1,4,5-trisphosphate by PLCγ_1 is coupled to influx of intracellular calcium and activation of phosphoinositide 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 PLCγ_1 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(Thr^{308}), a surrogate marker of PI3K activity (57), in rictor knockdown cells.

Although rictor deficiency clearly enhanced early FcεRI-induced signaling events, the effects on mTORC-mediated events after SA stimulation were less clear. A temporary suppression was observed after SA stimulation in the phosphorylation of Akt(Ser^{473}), a substrate of mTORC2 (9), 4E-BP1(Thr$^{37/46}$), a substrate of mTORC1 (28, 37), and S6RP(Ser$^{240/244}$) (58), a substrate for mTORC1-regulated p70 S6 kinase (59) (Fig. 7B). However, phosphorylation of S6RP(Ser$^{240/244}$) 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 activity and cell protein synthesis (60), and that rictor may inhibit phosphorylation of Akt(Thr^{308}) independently of its link to mTORC2-dependent phosphorylation of Akt(Ser^{473}).

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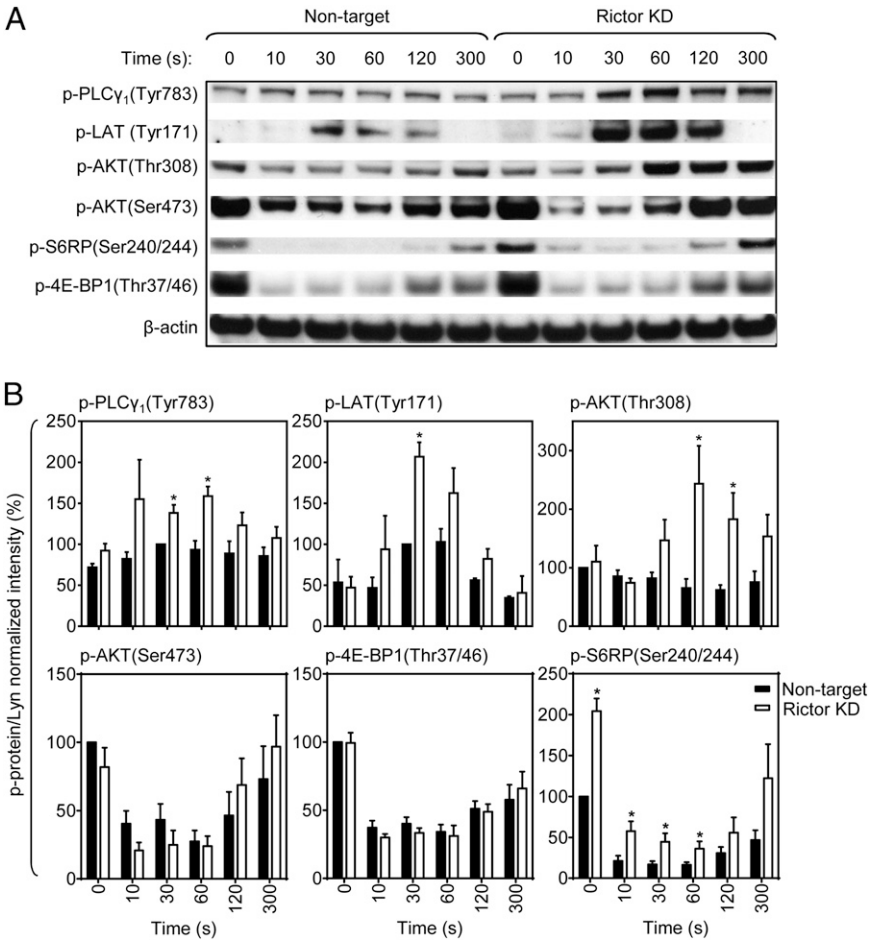

FIGURE 7. Role of rictor in regulation of early signaling events induced in FcεRI-activated LAD2 cells. LAD2 cells were transduced with the Non-target1 (Non-target) and Rict1 (Rictor KD) shRNA plasmids and sensitized overnight. Cells were then activated with SA (1 ng/ml) for the time periods indicated for immunoblotting. (**A**) Typical immunoblot and (**B**) densitometric data (mean and SEM) normalized to Non-target1 cells at maximal response (= 100) from four independent experiments are shown. Statistical significance between Non-target and Rictor KD cells is indicated (*$p < 0.05$, 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(Thr^{308}), whereas phosphorylation of Akt(Ser^{473}) 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(Thr^{308}) occurs independently of TORC2-dependent phosphorylation of Akt(Ser^{473}).

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 (Ser^{473}) (9) and the mTORC1/mTORC2 downstream signaling target 4E-BP1(Thr$^{37/46}$) (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

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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(Thr^{308}) without affecting mTORC2 signaling via phosphorylation of Akt(Ser^{473}).

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_1 that precede calcium mobilization and the activation of PI3K activity, as indicated by increased phosphorylation of Akt(Thr^{308}), 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(Thr^{308}), LAT, and PLCγ_1 after rictor knockdown, the increased constitutive phosphorylation of S6RP($Ser^{240/244}$) in resting rictor knockdown cells and its dephosphorylation during SA stimulation are paradoxical. The S6RP is a well-known substrate of p70 S6K (58), a recognized downstream signaling target of mTORC1 (59, 68) and a kinase that targets rictor(Thr^{1135}) (20) independently of mTORC2 (69). The p70 S6 kinase-mediated phosphorylation of rictor(Thr^{1135}) has been reported to be (19) or not to be (20) a negative regulator of mTORC2-mediated phosphorylation of Akt(Ser^{473}). 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.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Weber, J. D., and D. H. Gutmann. 2012. Deconvoluting mTOR biology. *Cell Cycle* 11: 236–248.
2. Foster, K. G., and D. C. Fingar. 2010. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *J. Biol. Chem.* 285: 14071–14077.
3. Sarbassov, D. D., S. M. Ali, D. H. Kim, D. A. Guertin, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* 14: 1296–1302.
4. Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110: 163–175.
5. Brunn, G. J., C. C. Hudson, A. Sekulić, J. M. Williams, H. Hosoi, P. J. Houghton, J. C. Lawrence, Jr., and R. T. Abraham. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* 277: 99–101.
6. Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. USA* 95: 1432–1437.
7. Hara, K., K. Yonezawa, M. T. Kozlowski, T. Sugimoto, K. Andrabi, Q. P. Weng, M. Kasuga, I. Nishimoto, and J. Avruch. 1997. Regulation of eIF-4E BP1 phosphorylation by mTOR. *J. Biol. Chem.* 272: 26457–26463.
8. Isotani, S., K. Hara, C. Tokunaga, H. Inoue, J. Avruch, and K. Yonezawa. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase α in vitro. *J. Biol. Chem.* 274: 34493–34498.
9. Sarbassov, D. D., D. A. Guertin, S. M. Ali, and D. M. Sabatini. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098–1101.
10. Guertin, D. A., D. M. Stevens, C. C. Thoreen, A. A. Burds, N. Y. Kalaany, J. Moffat, M. Brown, K. J. Fitzgerald, and D. M. Sabatini. 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev. Cell* 11: 859–871.
11. Jacinto, E., V. Facchinetti, D. Liu, N. Soto, S. Wei, S. Y. Jung, Q. Huang, J. Qin, and B. Su. 2006. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 127: 125–137.
12. Shiota, C., J. T. Woo, J. Lindner, K. D. Shelton, and M. A. Magnuson. 2006. Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev. Cell* 11: 583–589.
13. Masri, J., A. Bernath, J. Martin, O. D. Jo, R. Vartanian, A. Funk, and J. Gera. 2007. mTORC2 activity is elevated in gliomas and promotes growth and cell motility via overexpression of rictor. *Cancer Res.* 67: 11712–11720.

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14. Peterson, R. T., P. A. Beal, M. J. Comb, and S. L. Schreiber. 2000. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.* 275: 7416–7423.
15. Reynolds, T. H., IV, S. C. Bodine, and J. C. Lawrence, Jr. 2002. Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. *J. Biol. Chem.* 277: 17657–17662.
16. Sekulić, A., C. C. Hudson, J. L. Homme, P. Yin, D. M. Otterness, L. M. Karnitz, and R. T. Abraham. 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res.* 60: 3504–3513.
17. Copp, J., G. Manning, and T. Hunter. 2009. TORC-specific phosphorylation of mammalian target of rapamycin (mTOR): phospho-Ser2481 is a marker for intact mTOR signaling complex 2. *Cancer Res.* 69: 1821–1827.
18. Dibble, C. C., J. M. Asara, and B. D. Manning. 2009. Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. *Mol. Cell. Biol.* 29: 5657–5670.
19. Julien, L. A., A. Carriere, J. Moreau, and P. P. Roux. 2010. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. *Mol. Cell. Biol.* 30: 908–921.
20. Treins, C., P. H. Warne, M. A. Magnuson, M. Pende, and J. Downward. 2010. Rictor is a novel target of p70 S6 kinase-1. *Oncogene* 29: 1003–1016.
21. Gao, D., L. Wan, H. Inuzuka, A. H. Berg, A. Tseng, B. Zhai, S. Shaik, E. Bennett, A. E. Tron, J. A. Gasser, et al. 2010. Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction. *Mol. Cell* 39: 797–808.
22. Hagan, G. N., Y. Lin, M. A. Magnuson, J. Avruch, and M. P. Czech. 2008. A Rictor-Myo1c complex participates in dynamic cortical actin events in 3T3-L1 adipocytes. *Mol. Cell. Biol.* 28: 4215–4226.
23. Agarwal, N. K., C. H. Chen, H. Cho, D. R. Boulbès, E. Spooner, and D. D. Sarbassov. 2013. Rictor regulates cell migration by suppressing RhoGDI2. *Oncogene* 32: 2521–2526.
24. Guo, Z., Y. Zhou, B. M. Evers, and Q. Wang. 2012. Rictor regulates FBXW7-dependent c-Myc and cyclin E degradation in colorectal cancer cells. *Biochem. Biophys. Res. Commun.* 418: 426–432.
25. McDonald, P. C., A. Oloumi, J. Mills, I. Dobreva, M. Maidan, V. Gray, E. D. Wederell, M. B. Bally, L. J. Foster, and S. Dedhar. 2008. Rictor and integrin-linked kinase interact and regulate Akt phosphorylation and cancer cell survival. *Cancer Res.* 68: 1618–1624.
26. He, Y., D. Li, S. L. Cook, M. S. Yoon, A. Kapoor, C. V. Rao, P. J. Kenis, J. Chen, and F. Wang. 2013. Mammalian target of rapamycin and Rictor control neutrophil chemotaxis by regulating Rac/Cdc42 activity and the actin cytoskeleton. *Mol. Biol. Cell* 24: 3369–3380.
27. Kim, M. S., H. S. Kuehn, D. D. Metcalfe, and A. M. Gilfillan. 2008. Activation and function of the mTORC1 pathway in mast cells. *J. Immunol.* 180: 4586–4595.
28. Smrž, D., M. S. Kim, S. Zhang, B. A. Mock, S. Smrzová, W. DuBois, O. Simakova, I. Maric, T. M. Wilson, D. D. Metcalfe, and A. M. Gilfillan. 2011. mTORC1 and mTORC2 differentially regulate homeostasis of neoplastic and non-neoplastic human mast cells. *Blood* 118: 6803–6813.
29. Galli, S. J., and M. Tsai. 2010. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. *Eur. J. Immunol.* 40: 1843–1851.
30. Mekori, Y. A., and D. D. Metcalfe. 2000. Mast cells in innate immunity. *Immunol. Rev.* 173: 131–140.
31. Brown, J. M., T. M. Wilson, and D. D. Metcalfe. 2008. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clin. Exp. Allergy* 38: 4–18.
32. Kirshenbaum, A. S., J. P. Goff, T. Semere, B. Foster, L. M. Scott, and D. D. Metcalfe. 1999. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* 94: 2333–2342.
33. Metcalfe, D. D., D. Baram, and Y. A. Mekori. 1997. Mast cells. *Physiol. Rev.* 77: 1033–1079.
34. Okayama, Y., and T. Kawakami. 2006. Development, migration, and survival of mast cells. *Immunol. Res.* 34: 97–115.
35. Kraft, S., and J. P. Kinet. 2007. New developments in FcepsilonRI regulation, function and inhibition. *Nat. Rev. Immunol.* 7: 365–378.
36. Kuehn, H. S., M. Y. Jung, M. A. Beaven, D. D. Metcalfe, and A. M. Gilfillan. 2011. Prostaglandin E2 activates and utilizes mTORC2 as a central signaling locus for the regulation of mast cell chemotaxis and mediator release. *J. Biol. Chem.* 286: 391–402.
37. Thoreen, C. C., S. A. Kang, J. W. Chang, Q. Liu, J. Zhang, Y. Gao, L. J. Reichling, T. Sim, D. M. Sabatini, and N. S. Gray. 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J. Biol. Chem.* 284: 8023–8032.
38. Kirshenbaum, A. S., C. Akin, Y. Wu, M. Rottem, J. P. Goff, M. A. Beaven, V. K. Rao, and D. D. Metcalfe. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.* 27: 677–682.
39. Aimbetov, R., C. H. Chen, O. Bulgakova, D. Abetov, A. K. Bissenbaev, R. I. Bersimbaev, and D. D. Sarbassov. 2012. Integrity of mTORC2 is dependent on the rictor Gly-934 site. *Oncogene* 31: 2115–2120.
40. Cruse, G., M. A. Beaven, I. Ashmole, P. Bradding, A. M. Gilfillan, and D. D. Metcalfe. 2013. A truncated splice-variant of the FcεRIβ receptor subunit is critical for microtubule formation and degranulation in mast cells. *Immunity* 38: 906–917.
41. Smrž, D., S. Iwaki, D. W. McVicar, D. D. Metcalfe, and A. M. Gilfillan. 2010. TLR-mediated signaling pathways circumvent the requirement for DAP12 in mast cells for the induction of inflammatory mediator release. *Eur. J. Immunol.* 40: 3557–3569.
42. Kuehn, H. S., and A. M. Gilfillan. 2007. G protein-coupled receptors and the modification of FcepsilonRI-mediated mast cell activation. *Immunol. Lett.* 113: 59–69.
43. Kijima, Y., E. Ogunbunmi, and S. Fleischer. 1991. Drug action of thapsigargin on the Ca^{2+} pump protein of sarcoplasmic reticulum. *J. Biol. Chem.* 266: 22912–22918.
44. Lytton, J., M. Westlin, and M. R. Hanley. 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* 266: 17067–17071.
45. Choi, O. H., J. H. Lee, T. Kassessinoff, J. R. Cunha-Melo, S. V. P. Jones, and M. A. Beaven. 1993. Antigen and carbachol mobilize calcium by similar mechanisms in a transfected mast cell line (RBL-2H3 cells) that expresses ml muscarinic receptors. *J. Immunol.* 151: 5586–5595.
46. Tkaczyk, C., M. A. Beaven, S. M. Brachman, D. D. Metcalfe, and A. M. Gilfillan. 2003. The phospholipase C γ_1-dependent pathway of Fc ε RI-mediated mast cell activation is regulated independently of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 278: 48474–48484.
47. Smrž, D., G. Bandara, M. A. Beaven, D. D. Metcalfe, and A. M. Gilfillan. 2013. Prevention of F-actin assembly switches the response to SCF from chemotaxis to degranulation in human mast cells. *Eur. J. Immunol.* 43: 1873–1882.
48. Cohen, R., K. Corwith, D. Holowka, and B. Baird. 2012. Spatiotemporal resolution of mast cell granule exocytosis reveals correlation with Ca^{2+} wave initiation. *J. Cell Sci.* 125: 2986–2994.
49. Boulbés, D. R., T. Shaiken, and D. Sarbassov. 2011. Endoplasmic reticulum is a main localization site of mTORC2. *Biochem. Biophys. Res. Commun.* 413: 46–52.
50. Ma, H. T., and M. A. Beaven. 2009. Regulation of Ca^{2+} signaling with particular focus on mast cells. *Crit. Rev. Immunol.* 29: 155–186.
51. Pang, Z. P., and T. C. Südhof. 2010. Cell biology of Ca^{2+}-triggered exocytosis. *Curr. Opin. Cell Biol.* 22: 496–505.
52. Tolarová, H., L. Dráberová, P. Heneberg, and P. Dráber. 2004. Involvement of filamentous actin in setting the threshold for degranulation in mast cells. *Eur. J. Immunol.* 34: 1627–1636.
53. Pendleton, A., and A. Koffer. 2001. Effects of latrunculin reveal requirements for the actin cytoskeleton during secretion from mast cells. *Cell Motil. Cytoskeleton* 48: 37–51.
54. Nishida, K., S. Yamasaki, Y. Ito, K. Kabu, K. Hattori, T. Tezuka, H. Nishizumi, D. Kitamura, R. Goitsuka, R. S. Geha, et al. 2005. FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *J. Cell Biol.* 170: 115–126.
55. Deng, Z., T. Zink, H. Y. Chen, D. Walters, F. T. Liu, and G. Y. Liu. 2009. Impact of actin rearrangement and degranulation on the membrane structure of primary mast cells: a combined atomic force and laser scanning confocal microscopy investigation. *Biophys. J.* 96: 1629–1639.
56. Kim, M. S., M. Rådinger, and A. M. Gilfillan. 2008. The multiple roles of phosphoinositide 3-kinase in mast cell biology. *Trends Immunol.* 29: 493–501.
57. Hanada, M., J. Feng, and B. A. Hemmings. 2004. Structure, regulation and function of PKB/AKT—a major therapeutic target. *Biochim. Biophys. Acta* 1697: 3–16.
58. Ruvinsky, I., and O. Meyuhas. 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem. Sci.* 31: 342–348.
59. Magnuson, B., B. Ekim, and D. C. Fingar. 2012. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem. J.* 441: 1–21.
60. Liu, Y., D. Vertommen, M. H. Rider, and Y. C. Lai. 2013. Mammalian target of rapamycin-independent S6K1 and 4E-BP1 phosphorylation during contraction in rat skeletal muscle. *Cell. Signal.* 25: 1877–1886.
61. Oh, W. J., and E. Jacinto. 2011. mTOR complex 2 signaling and functions. *Cell Cycle* 10: 2305–2316.
62. Wander, S. A., B. T. Hennessy, and J. M. Slingerland. 2011. Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy. *J. Clin. Invest.* 121: 1231–1241.
63. Hresko, R. C., and M. Mueckler. 2005. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* 280: 40406–40416.
64. Paz, P. E., S. Wang, H. Clarke, X. Lu, D. Stokoe, and A. Abo. 2001. Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells. *Biochem. J.* 356: 461–471.
65. Saitoh, S., R. Arudchandran, T. S. Manetz, W. Zhang, C. L. Sommers, P. E. Love, J. Rivera, and L. E. Samelson. 2000. LAT is essential for Fc(ε)RI-mediated mast cell activation. *Immunity* 12: 525–535.
66. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Trible, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92: 83–92.
67. Su, Y. W., and H. Jumaa. 2003. LAT links the pre-BCR to calcium signaling. *Immunity* 19: 295–305.
68. Jacinto, E., and A. Lorberg. 2008. TOR regulation of AGC kinases in yeast and mammals. *Biochem. J.* 410: 19–37.
69. Boulbes, D., C. H. Chen, T. Shaikenov, N. K. Agarwal, T. R. Peterson, T. A. Addona, H. Keshishian, S. A. Carr, M. A. Magnuson, D. M. Sabatini, and D. Sarbassov. 2010. Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2. *Mol. Cancer Res.* 8: 896–906.

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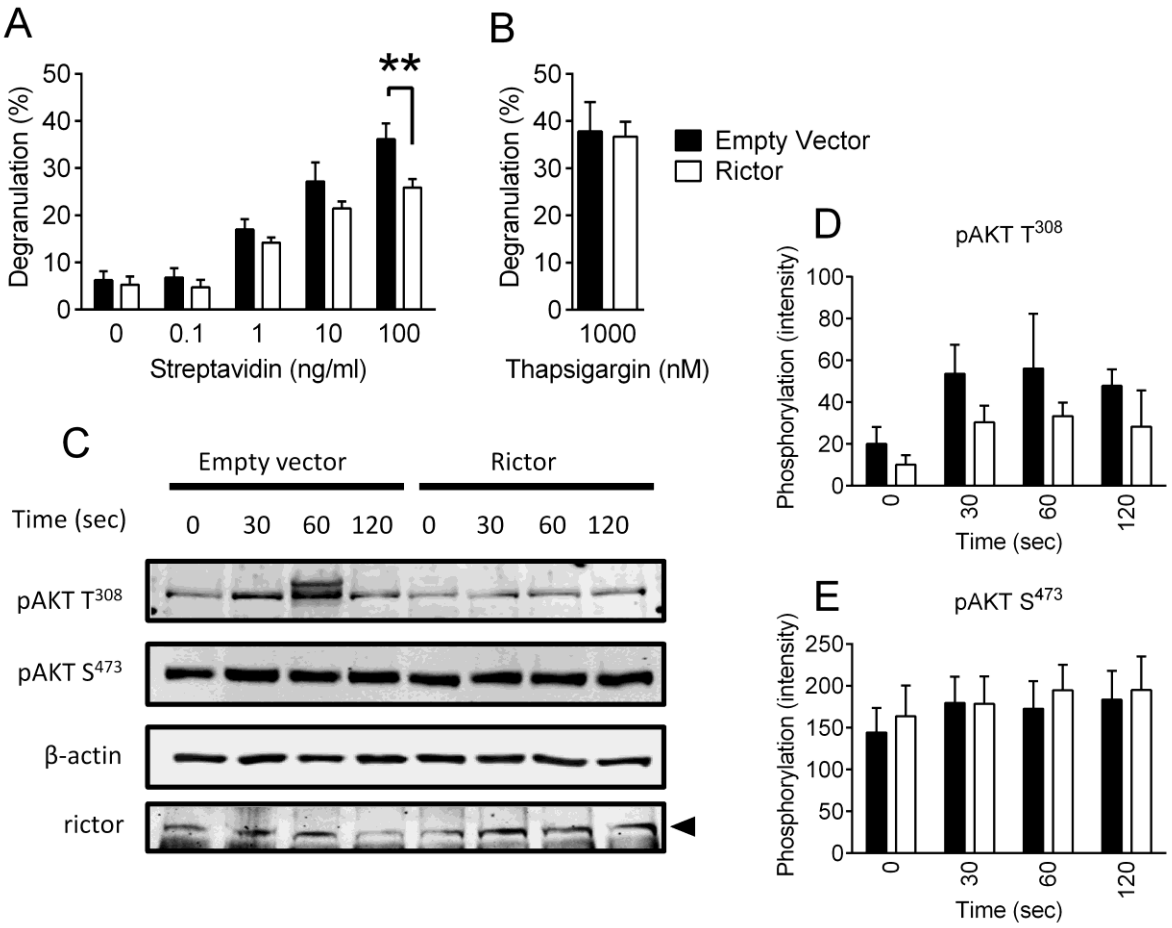

Supplementary Figure 1. Overexpression of rictor inhibits degranulation and Akt(T308) phosphorylation in LAD2 cells. A. Rictor overexpression (~25%) reduces FcεRI-dependent LAD2 cell degranulation, which is significant at 100 ng/ml SA (**p<0.01, n=4). B. There was no significant difference in LAD2 cell degranulation when thapsigargin was used as the stimulant (n=4). C. Immunoblots of LAD2 cell lysates stimulated with the same concentration (100 ng/ml) of SA for the indicated times using the phospho-specific or rictor-specific antibodies (Cell Signaling Technology, catalog No. 2140) for detection as described in the Methods section. β-actin was used as a

loading control. D. Quantification of phosphorylation as determined by fluorescence intensity (see Methods) after correction against β-actin loading control. There was a significant reduction in pAkt(T308) phosphorylation (B) ($p=0.0199$, 2 way ANOVA, rictor overexpression versus empty vector, $n=4$ data sets). No significant differences were observed for pAkt(S473) ($n=5$). Bars are the mean ± SEM. Closed bars = empty vector; open bars = rictor overexpression. Note that the transient decrease in phosphorylation of Akt with low concentration (1 ng/ml) of SA in Figure 7B was no longer apparent at 100 ng/ml SA as shown here.