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Activation and Function of the mTORC1 Pathway in Mast Cells

Mi-Sun Kim, Hye Sun Kuehn, Dean D. Metcalfe, and Alasdair M. Gilfillan

Little is known about the signals downstream of PI3K which regulate mast cell homeostasis and function following FcεRI aggregation and Kit ligation. In this study, we investigated the role of the mammalian target of rapamycin complex 1 (mTORC1) pathway in these responses. In human and mouse mast cells, stimulation via FcεRI or Kit resulted in a marked PI3K-dependent activation of the mTORC1 pathway, as revealed by the wortmannin-sensitive sequential phosphorylation of tuberin, mTOR, p70S6 kinase (p70S6K), and 4E-BP1. In contrast, in human tumor mast cells, the mTORC1 pathway was constitutively activated and this was associated with markedly elevated levels of mTORC1 pathway components. Rapamycin, a specific inhibitor of mTORC1, selectively and completely blocked the FcεRI- and Kit-induced mTORC1-dependent p70S6K phosphorylation and partially blocked the 4E-BP1 phosphorylation. In parallel, although rapamycin had no effect on FcεRI-mediated degranulation or Kit-mediated adhesion, it inhibited cytokine production, and kit-mediated chemotaxis and cell survival. Furthermore, Rapamycin also blocked the constitutive activation of the mTORC1 pathway and inhibited cell survival of tumor mast cells. These data provide evidence that mTORC1 is a point of divergency for the PI3K-regulated downstream events of FcεRI and Kit for the selective regulation of mast cell functions. Specifically, the mTORC1 pathway may play a critical role in normal and dysregulated control of mast cell homeostasis.


Mast cells are major contributors to the initiation of allergic and nonallergic immune reactions (1, 2). These cells develop from CD34+/CD117+ pluripotent bone marrow progenitor cells (3) which migrate to the tissues where they undergo differentiation and maturation under the influence of stem cell factor (SCF), following binding to its receptor kit (4). Activating mutations in Kit have been linked to the dysregulated growth of mast cells associated with mast cell tumors and systemic mastocytosis (5). SCF, which is released from surrounding stromal cells, is also essential for the subsequent survival of mast cells (6). Furthermore, SCF may contribute to the homing of the mast cells to target tissues (7). In contrast, the high affinity IgE receptor (FcεRI) is primarily responsible for the activation of mature mast cells following occupancy by IgE and subsequent aggregation by binding of specific Ag to the receptor-bound IgE. Thus, these two receptors have a distinct set of responses. However, as Kit can enhance FcεRI-mediated mast cell activation (8–10), and as monomeric IgE has been reported to protect against mast cells apoptosis (11), some overlap in function exists.

Despite their divergent responses, the signaling cascades initiated by FcεRI and Kit share many common features. For example, the initiating event required for both receptors is receptor tyrosine phosphorylation. In the case of Kit, this follows activation of its inherent catalytic activity resulting in auto/trans-phosphorylation of specific tyrosine residues on its cytosolic tail (12). For FcεRI, this requires the Src kinase Lyn to phosphorylate tyrosines contained within the immuno-receptor tyrosine-based activation motifs (ITAMs) in the β- and γ-chain cytosolic domains (13). These early events lead to recruitment of other signaling molecules, ultimately forming a macromolecular receptor-signaling complex which regulates the compartmentalization and activation of downstream signaling enzymes crucial for the mast cell function (13, 14). The lipid kinase, PI3K is one such enzyme activated by both FcεRI and SCF in mast cells. PI3K induces the synthesis of membrane-associated PtdIns (3,4,5)-triphosphate (PIP3), which provides membrane docking sites for pleckstrin homology (PH) domain-containing signaling molecules such as PDK1, Akt, Btk, and phospholipase (PLC)γ1 and PLCγ2. Studies have provided evidence that PI3K is an essential enzyme for mast cell growth and survival, and many, if not all, of the responses attributable to Ag and SCF in mast cells (15–17). It is unclear, however, how this enzyme produces these diverse responses. The most likely explanation is differential requirements for specific signaling events downstream of PI3K.

One of the major signaling pathways described to be downstream of PI3K in a variety of cell types is the mammalian target of rapamycin (mTOR) pathway. mTOR (also known as RAFT1, FRAP, and RAPT) is a conserved Ser/Thr kinase (18) which exists in two distinct multimolecular complexes: mTOR complex 1 (mTORC1), which contains the interacting protein raptor and which is sensitive to rapamycin, and mTORC2, which contains the interacting protein rictor and which is insensitive to rapamycin (19). PI3K regulates the mTORC1 pathway via the activation of Akt, which directly phosphorylates the negative regulator of mTOR activation, tuberous sclerosis 2 (tuberin), thereby inactivating its inhibitory activity. This allows mTOR activation to proceed. The activation of mTORC1 directly results in the phosphorylation of two effector molecules, p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which leads to increased protein synthesis. Other downstream targets include the mammalian target of rapamycin (mTOR), which regulates the compartmentalization and activation of downstream signaling enzymes such as the PI3K, Akt, Btk, and phospholipase (PLC)γ1 and PLCγ2.
(4E-BP1). These events lead to mTOR-dependent gene transcription that regulates cell growth, protein synthesis, and metabolism in response to a variety of environmental stimuli. The mTORC1 pathway has been linked to abnormal cell division and survival associated with tumorogenesis. Based on the above background, we hypothesized that the PI3K-regulated activation of the mTORC1 pathway may play a role in mast cell homeostasis and may be selectively used by FceRI or Kit for the differential promotion of specific mast cell responses.

In this study, we show that both FceRI and Kit induce the PI3K-dependent activation of the mTORC1 cascade in human and mouse mast cells, and that this cascade is constitutively activated in human tumor mast cells. Furthermore, we provide evidence that mTORC1 is a point of divergence for the PI3K-regulated responses downstream of FceRI and Kit in mast cells. We finally propose that the mTORC1 pathway contributes to normal and dysregulated control of mast cell homeostasis.

Materials and Methods

**Mast cell culture and cell lines**

Primary human mast cells (HuMCs) were developed from CD34+ peripheral blood progenitor cells in StemPro-34 culture medium with supplement (Invitrogen Life Technologies) containing t-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), recombinant human IL-3 (30 ng/ml; first week only), IL-6 (100 ng/ml), and SCF (100 ng/ml) (PeproTech). The cells were used for experiments after 7–9 wk in culture. The human mast cell line, HMC-1 (21) was grown in IMDM medium supplemented with HEPES containing 0.04% BSA, before conducting the experiments. The resulting bone marrow-derived mast cells (BMMCs) were used between 4 and 6 wk in culture.

**Cell activation**

HuMCs were sensitized overnight in cytokine-free, supplemented StemPro medium containing human myeloma IgE (100 ng/ml, Calbiochem, EMD Biosciences) biotinylated as described (15). Following rinsing in HEPES buffer containing 0.04% BSA (23), the cells were activated in this buffer with streptavidin (SA, 100 ng/ml, Sigma-Aldrich) or SCF (30 ng/ml) for the indicated times in the figure legends. Mouse BMMCs were sensitized overnight with monoclonal mouse anti-dinitrophenyl (DNP)-IgE (clone SPE-7, 100 ng/ml, Sigma-Aldrich) in cytokine-free RPMI 1640 medium, processed as above, then triggered with DNP-human serum albumin, hereafter referred to as Ag (100 ng/ml, Sigma-Aldrich), or murine SCF (30 ng/ml, PeproTech). After sensitization, the cells were washed three times with HEPES containing 0.04% BSA, before conducting the experiments.

The cells were pretreated with inhibitors of mTORC1 (rapamycin, Calbiochem), PI3K (wortmannin, Calbiochem), and the p110α catalytic subunit isoform of PI3K (IC87114, obtained from the laboratory of Dr. Bart Vanhaesebroeck, University College London, U.K.) before SA, Ag, or SCF stimulation as indicated.

**Immunoblotting and immunoprecipitation**

Cell lysates were prepared as described (23) and loaded onto 4–12% NuPAGE Bis-Tris gels (Invitrogen Life Technologies). Proteins were separated by electrophoresis and electrotransferred onto nitrocellulose membranes. Following blocking with 3% nonfat dry milk in TBS containing 0.05% Tween 20, the membranes were probed with the following primary Abs: anti-phospho-LAT pAb (Tyr(P)-191), which also recognizes phospho-NTAL (Upstate Biotechnology), anti-phospho-Akt pAb (Ser(P)-473), anti-phospho-tubulin pAb (Thr(P)-1462), anti-phospho-mTOR pAb (Ser(P)-2448), anti-m-TOR pAb, anti-phospho-p70S6K pAb (Thr(P)-389), anti-p70S6K pAb, anti-phospho-4E-BP1 pAb (Thr(P)-37/46), anti-4E-BP1 pAb, anti-actin mAb (clone AC-15: Sigma-Aldrich), and anti-Syk pAb (N-19; Santa Cruz Biotechnology) for mouse samples, and anti-Syk mAb (4D10; UBI) for human samples. Unless specified otherwise, Abs were purchased from Cell Signaling Technology. Immunoreactive proteins were visualized by probing with HRP-conjugated secondary Abs and then by ECL (PerkinElmer). To quantitate changes in protein phosphorylation, the ECL films were scanned using an ImageQuant 5.0 scanner (Bio-Rad).

For immunoprecipitation experiments, the cells were lysed with buffer containing 0.3% CHAPS to preserve the integrity of the mTOR complexes (24, 25). Anti-raptor mAb (Cell Signaling Technology) was added to a 50% protein G-sepharose bead slurry and incubated with rotation at room temperature for 60 min. The cleared cellular lysates were added to Ab-bound protein G-sepharose and the incubation continued overnight at 4°C. Immunoprecipitates captured with protein G-sepharose were washed then following separation of immunoprecipitated proteins on 3–8% NuPAGE Tris-Acetate gels (Invitrogen Life Technologies), immunoblotted with indicated Abs.

**Degranulation**

Degranulation was monitored by β-hexosaminidase (β-hex) release as described (22). In brief, sensitized BMMCs were washed and triggered with Ag (0–100 ng/ml) for the indicated times in 96-well plates (4 × 10^4 cells per well, 100 µl final volume). For inhibitor studies, BMMCs or HuMCs (1 × 10^5 cells/well) were preincubated with rapamycin or wortmannin for 20 min before addition of Ag or SA, respectively. The reactions were terminated by centrifugation (3,000 rpm for 5 min) at 4°C and the supernatants were aliquoted to 96-well plates for further analysis. To quantitate changes in protein phosphorylation, the ECL (PerkinElmer). To quantitate changes in protein phosphorylation, the ECL films were scanned using an ImageQuant 5.0 scanner (Bio-Rad).

**Cytokeine secretion**

Sensitized BMMCs (5 × 10^5 cells/ml) were stimulated with Ag (100 ng/ml) or SCF (30 ng/ml) for the indicated times in the kinetic studies. For inhibitor studies, BMMCs were incubated with or without rapamycin or wortmannin for 20 min before stimulation, whereas sensitized HuMCs (1 × 10^5 cells/ml) were incubated with or without inhibitors before SA (100 ng/ml) stimulation. After 6 h incubation, the cell-free supernatants were harvested and stored at −80°C until required for cytokine assay. Secreted cytokine levels in the culture media were measured using a DuoSet ELISA system (R&D Systems) according to the manufacturer’s protocol.

**Cell adhesion**

BMMCs were cultured overnight in IL-3-free medium, resuspended in HEPES buffer containing 0.04% BSA and BMSCs (2.5 × 10^7/well) were seeded in a 96-well tissue culture plate (Falcon, BD Biosciences) precoated with 5 µg/ml fibronectin (Sigma-Aldrich). The cells were stimulated with SCF (30 ng/ml) for the indicated times. For inhibitor studies, BMMCs or HuMCs (1 × 10^5 cells/well) were incubated with or without rapamycin or wortmannin for 20 min before SCF stimulation. After 1 h stimulation, non-adherent cells were carefully removed and the wells were washed twice with PBS. Adherent cells were lysed with 0.1% Triton X-100 followed by β-hex determination to analyze the percentages of adherent cells. The adherent cells (% of input cells) were calculated as absorbance of sample/absorbance of total cell lysates × 100.

**Chemotaxis**

Chemotaxis assays were performed using Transwell polycarbonate membranes (Costar) (26). BMMCs (5 × 10^5 cells) and HuMCs (1 × 10^5 cells) were sensitized overnight in cytokine-free medium and then resuspended in HEPES buffer containing 0.5% BSA. The cell suspension was placed in the insert with filters with 5 µm pores for BMMCs and 8 µm pores for HuMCs and preincubated in wells containing 0.6 µl of HEPES/0.5% BSA at 37°C. After 30 min, the inserts were placed in wells with or without SCF (30 ng/ml). For kinetic studies, BMSCs were treated with SCF for the indicated times. For inhibitor studies, cells were preincubated with or without rapamycin or wortmannin for 20 min before SCF stimulation. After 4 h incubation at 37°C, cells migrating to the lower wells were collected and counted under microscopy.

**Cell survival**

To assess mast cell survival, BMMCs were incubated in cytokine-free RPMI 1640 medium for 24 h, and then the cells (1 × 10^5 cells) were seeded in 96-well plates and stimulated with SCF. After the indicated times, the percentage of viable cells was assessed with a MTT-based colorimetric assay (Sigma-Aldrich) according to the manufacturer’s protocol (27). For inhibitor studies, BMMCs or HuMCs were preincubated with or without rapamycin or wortmannin for 20 min before SCF stimulation.
After 48 h in culture, MTT solution was added, and the cells were incubated at 37°C for an additional 3.5 h. LAD 2 and HMC-1 cells were cultured in the absence or presence of rapamycin for 48 h, and then the MTT assay was conducted. The crystallized MTT was dissolved and the absorbance was measured at 570 nm.

**Statistical analysis**

The significance of difference between two independent groups was analyzed using the Mann-Whitney U test. For all tests, a p-value of <0.05 was considered statistically significant.

**Results**

The mTORC1 pathway is activated by FcεRI- or Kit-mediated mast cell stimulation

To examine whether the mTORC1 pathway is activated downstream of PI3K/Akt in activated mast cells, we first examined whether protein components of this pathway are phosphorylated in a PI3K-dependent manner following activation of FcεRI and Kit-dependent signaling in HuMCs. To establish that early signaling processes were activated as expected in these experiments, we also monitored the phosphorylation of LAT and NTAL, early events in FcεRI and Kit-mediated signaling. We furthermore established activation of the PI3K/Akt pathway by monitoring the phosphorylation of Akt, a surrogate marker for the activation of PI3K. As expected from previous studies (10) in HuMCs, SA-induced FcεRI aggregation resulted in a rapid increase in both LAT and NTAL phosphorylation (Fig. 1A) whereas SCF-induced Kit activation produced an increase in NTAL phosphorylation in the absence of detectable LAT phosphorylation (Fig. 1B). Similarly, both stimuli induced an increase in Akt phosphorylation, although this response was somewhat delayed compared with the phosphorylation of LAT and NTAL.

SA or SCF induced the activation of the mTORC1 pathway (Fig. 1) as revealed by the sequential phosphorylation of key elements of this pathway including tuberin (Thr1462), mTOR (Ser2448), and two downstream effectors of mTORC1, p70S6K (Thr389), and 4E-BP1 (Thr37/46). These events were delayed and took longer to reach their maximal responses than the phosphorylation of NTAL and LAT and the subsequent phosphorylation of Akt. In this respect, maximal phosphorylation of NTAL and LAT was observed between 1 and 2 min after cell activation, phosphorylation of Akt was maximal within 5 min, and the activation of mTORC1 pathway reached maximal levels at 10 min after FcεRI or Kit stimulation (Fig. 1, lower panels).

The mTORC1 pathway is elevated in human mast cell lines

There is increasing evidence that many cancer-promoting lesions are associated with the activation of the PI3K-mTORC1 pathway (28–30). Thus, we wished to compare the expression and phosphorylation status of key proteins of the mTORC1 pathway between primary cultured HuMCs and mast cell lines derived from mastocytosis patients. For these studies, two human mast cell lines were used: the LAD 2 cell line, which is an SCF-dependent human mast cell line, resembling CD34+-derived HuMCs, established from a patient with mastocytosis (20); and the HMC-1 cell line derived from a patient with mast cell leukemia, which is a growth factor-independent human mast cell line that has mutations of Kit (21). The HMC-1 cell line is divided into two subcell lines, HMC-1.1 and HMC-1.2 cells. HMC-1.1 contains a juxta-membrane domain mutation (V560G) and HMC-1.2 contains both the juxta-membrane and catalytic domain mutations (V560G and D816V).

As shown in Fig. 2, there was a marked constitutive phosphorylation of mTOR, p70S6K, and 4E-BP1 in all mast cell lines compared with primary cultured HuMCs. Interestingly, the phosphorylation of 4E-BP1 was substantially lower in the HMC-1.1 cells, which do not possess the D816V mutation in Kit. Furthermore, the levels of mTOR and two substrates, p70S6K and 4E-BP1, were also substantially higher in the human mast cell lines compared with cultured HuMCs. Again, the levels of 4E-BP1, and to a lesser extent p70S6K, in the HMC-1.1 cell line were lower than that in the HMC-1.2 cells. In contrast, there was no
difference in the expression of either actin or the key signaling protein Syk between all cell types. Thus, these data demonstrate that the activation and expression of key components of the mTORC1 cascade is up-regulated in human tumor mast cells.

The mTORC1 pathway is a downstream target of PI3K in activated mast cells

We next examined whether activation of the mTORC1 pathway in mast cells is regulated by PI3K. We elected to primarily conduct these and other signaling studies in mouse BMMCs, due to the greater number of BMMCs available. We therefore initially confirmed that Ag- or SCF-induced the activation of the mTORC1 pathway in mouse BMMCs is a similar manner (Fig. 3) to that observed in HuMCs (Fig. 1). To explore the role of PI3K in this response, we first assessed the effect of wortmannin, a specific PI3K inhibitor, on the activation of the mTORC1 pathway following Ag or SCF challenge. Pretreatment of BMMCs with wortmannin (100 nM) for 20 min completely blocked the FcεRI- or Kit-mediated phosphorylation of Akt, thus confirming the ability of wortmannin to block PI3K in BMMCs (Fig. 4A). In parallel, the phosphorylation of the mTORC1 pathway members, tuberin, mTOR, p70S6K, and 4E-BP1, was attenuated by wortmannin pretreatment. Similar results were obtained with another PI3K inhibitor, LY294002, in BMMCs. Furthermore, both wortmannin and LY294002 blocked the FcεRI- and Kit-induced activation of mTORC1 pathway in HuMCs (data not shown).

The p110δ subunit of PI3K is reported to be the major p110 isoform responsible for FcεRI- and Kit-mediated responses in mast cells (15). We therefore next examined whether the mTORC1 pathway was ablated in BMMCs derived from the bone marrow of p110δD910A/D910A mice which have a loss-of-function allele of p110δ. In p110δD910A/D910A BMMCs, the phosphorylation of Akt (Ser473) was clearly defected (Fig. 4B). Although mTOR was constitutively phosphorylated in p110δD910A/D910A BMMCs, this level was not further enhanced by Ag or SCF stimulation. However, the FcεRI- and Kit-induced activation of p70S6K (Thr389), and 4E-BP1 (Thr37/46) was dramatically diminished in p110δD910A/D910A BMMCs (Fig. 4B) in a similar manner to that observed following either wortmannin or rapamycin pretreatment in activated wild type BMMCs (Figs. 4A and 5A). To confirm the observations made in the p110δD910A/D910A BMMCs, we examined the ability of a specific inhibitor of p110δ (IC87114) (15) to block the phosphorylation of Akt, tuberin, mTOR, and downstream targets of mTORC1 following FcεRI- and Kit- induced activation of wild type BMMCs. As can be seen in Fig. 4C, IC87114 attenuated the phosphorylation of Akt, tuberin, mTOR, p70S6K, and 4E-BP1 in response to both Ag and SCF in a similar manner to that observed with wortmannin. Taken together, the above data support the conclusion that FcεRI- and Kit-mediated activation of the mTORC1 pathway is regulated by the activation of PI3K, particularly the p110δ isoform.
The mTORC1 complex is disrupted by rapamycin in activated mast cells

mTORC1 function is essential for early embryonic development, therefore, complete deletion or homozygous deletion of kinase domain of the mTOR gene results in a severe impact on embryonic development, and induces early stage lethality (31, 32). Similarly, mice lacking raptor die early in development (33). For these reasons, to determine how the mTORC1 pathway may regulate mast cell function, we opted to use rapamycin, a well established inhibitor of mTORC1. To investigate the role of the mTORC1 pathway on mast cell activation, we thus explored the ability of rapamycin to block the above responses.

The mTORC1 complex of rapamycin with its receptor FKBP12 binds directly to the FKBP12-rapamycin binding domain of mTOR and suppresses mTORC1-mediated phosphorylation of the substrates p70S6K and 4E-BP1 (34). It has been proposed the rapamycin may weaken the interaction between mTOR and raptor, again preventing the phosphorylation of downstream substrates (25). However, the exact mechanism by which rapamycin perturbs mTORC1 function is not fully understood.

We initially investigated the ability of rapamycin to block the phosphorylation of key components of the mTORC1 pathway and to disrupt the mTOR and raptor complex. When cells were pretreated with rapamycin (100 nM) for 20 min before Ag or SCF challenge, there was complete inhibition of the phosphorylation of p70S6K and partial inhibition of the phosphorylation of 4E-BP1 in response to both stimuli (Fig. 5A). In contrast, rapamycin did not inhibit the phosphorylation of mTOR in response to either Ag or SCF (Fig. 5A). As seen in Fig. 5, B and C, respectively, Ag- and SCF-induced p70S6K phosphorylation was inhibited in a dose-dependent manner by rapamycin (1–100 nM).
FIGURE 6. Effects of rapamycin on Ag-induced mast cell responses. A, Kinetics of (DNP-HSA, Ag 100 ng/ml)-induced degranulation (left panel). Sensitized BMMCs were treated with rapamycin (Rapa) or wortmannin (Wort) and then challenged with Ag for 30 min (right panel). Degranulation was assessed by monitoring β-hexosaminidase (β-hex) activity in the supernatant and in the whole cell lysates as described in Materials and Methods. B, Kinetics of Ag (100 ng/ml)-induced IL-6 release from mast cells (left panel). Sensitized BMMCs were treated with inhibitors and then incubated in the absence or presence of Ag for 6 h. IL-6 levels in cell-free supernatants were analyzed by ELISA (right panel). C, FcεRI-mediated degranulation and (D) IL-8 release were examined in the absence or presence of rapamycin (Rapa, 100 nM) in HuMCs. All data shown represent the mean ± SEM of four independent experiments conducted in duplicate. *, p < 0.05 vs vehicle pretreated Ag- or SA-stimulated cells.

nM) pretreatment with complete inhibition being observed with a concentration of 100 nM. The 4E-BP1 phosphorylation was similarly inhibited in a concentration-dependent manner. However, as before, only a partial inhibition was observed at concentrations up to 100 nM rapamycin. To confirm the specificity of rapamycin on the mTORC1 pathway, we examined the phosphorylation of other crucial signaling molecules including PLCγ2, Btk, LAT/NTAL, and ERK, which are phosphorylated by Ag or SCF in mast cells. Rapamycin (1–10^4 nM) did not affect the phosphorylation of these signaling molecules even at a 100-fold higher concentration than that used in this study (data not shown). These data would also require that upstream responses such as receptor phosphorylation and activation of Lyn, Syk, and Fyn would also be unaffected by rapamycin.

As rapamycin failed to inhibit mTOR phosphorylation, to investigate how rapamycin was blocking the phosphorylation of p70S6K and 4E-BP1, we next examined whether rapamycin blocked the mTOR and raptor interaction. When the cells were stimulated with Ag or SCF for 10 min, the binding between mTOR and raptor was dramatically increased compared with nonstimulated control cells (Fig. 5D). When cells were treated with rapamycin before challenge with Ag or SCF for 20 min, rapamycin (100 nM) completely blocked the association of mTOR with raptor in the Ag- or SCF-stimulated mast cells (Fig. 5D), but it had no effect on the total expression levels of mTOR and raptor. Taken together, these data support the conclusion that, under the conditions used in this series of experiments, rapamycin, inhibits mTORC1 mediated signaling in Ag- or SCF-stimulated mast cells by disrupting the mTOR and raptor complex, and hence mTOR activity, rather than by blocking the phosphorylation of mTOR. It is possible, however as discussed later, that the decreased phosphorylation of mTOR may contribute to its inhibition on long term exposure (>24 h) of rapamycin.

Rapamycin effects on FcεRI-mediated mast cell responses

Having confirmed that rapamycin blocked the mTORC1 pathway in activated BMMCs, to investigate the involvement of mTORC1 pathway in FcεRI-mediated mast cell responses, we next examined the effects of rapamycin on Ag-induced degranulation and cytokine production. As compared with the delayed activation of the mTORC1 cascade by Ag, degranulation in responses to Ag is an immediate response, maximizing between 5 and 10 min after cell activation (Fig. 6A, left panel). Furthermore, in contrast to the ability of rapamycin to block Ag-induced p70S6K and 4E-BP1 phosphorylation, rapamycin had no effect on Ag-mediated degranulation over an identical concentration range when examined for 30 min after Ag stimulation (Fig. 6A, right panel). The release of the cytokine IL-6 in response to Ag challenge was a much slower response than degranulation, maximizing ~6 h after Ag challenge (Fig. 6B, left panel). Again, rapamycin had only a marginal effect on this response (Fig. 6B, right panel). In contrast to rapamycin, wortmannin (100 nM) pretreatment significantly inhibited both responses. To confirm the effect of rapamycin on these responses in HuMCs, SA-induced degranulation and IL-8 production were analyzed in the absence or presence of rapamycin. Rapamycin (100 nM) had no effect on FcεRI-mediated degranulation (Fig. 6C), whereas rapamycin had significant inhibitory effects on FcεRI-mediated IL-8 production in HuMCs (Fig. 6D). The reason for the greater ability of rapamycin to inhibit cytokine production in the human mast cells compared with the mouse mast cells is unknown. However, taken together, the data indicate that the FcεRI-induced
and PI3K-mediated mast cell degranulation is largely independent of mTORC1 pathway whereas, cytokine production is partially regulated by the mTORC1 pathway.

**Rapamycin effects on Kit-mediated mast cell responses**

We next explored the ability of rapamycin to block specific SCF-mediated mast cell responses. First, we investigated SCF-mediated mast cell adhesion to fibronectin coated plates. This response was observed to be a relatively rapid response with maximal adhesion being observed within 15 min (Fig. 7A, left panel). However, as for the FceRI-mediated responses, rapamycin did not inhibit SCF-induced adhesion to fibronectin, whereas wortmannin reduced the response to basal levels (Fig. 7A, right panel). Similar results were obtained with rapamycin in SCF-stimulated HuMCs (data not shown).

We next explored the SCF-mediated chemotactic response. In comparison with the adhesion response, this was observed to be delayed with little evidence of chemotaxis being observed until after 30 min. Following 30 min incubation, chemotaxis proceeded in a linear manner for at least 4 h after SCF stimulation (Fig. 7B, left panel). Rapamycin significantly inhibited the cell migration toward SCF in a concentration-dependent manner and the chemotactic response was reduced by ~50% at 100 nM compared with non-rapamycin-treated cells. Wortmannin (100 nM), however, reduced the SCF-induced chemotactic response to basal level (Fig. 7B, right panel). In the HuMCs, rapamycin (100 nM) also significantly inhibited cell migration toward SCF compared with non-rapamycin-treated cells by greater than 50% (Fig. 7E).

Next, we examined the effect of rapamycin on SCF-mediated cytokine production. As was the case for Ag-mediated IL-6 production, this response was relatively slow with maximal release being observed after 6 h (Fig. 7C, left panel). In contrast to the Ag-mediated response, rapamycin significantly inhibited SCF-mediated IL-6 production by 56% at 100 nM treatment. This response was also inhibited by wortmannin (100 nM) but to a greater extent (Fig. 7C, right panel). Although several cytokines (IL-8, GM-CSF, and IL-13) were tested in HuMCs to confirm the result of mouse BMMCs, SCF failed to induce secretion of these cytokines from HuMCs under our condition.

We finally examined the ability of rapamycin to inhibit SCF-dependent cell survival. As shown in a Fig. 7D (left panel), SCF maintains cell survival in the absence of IL-3 in BMMC cultures. Rapamycin (1–100 nM) significantly inhibited this SCF-induced cell survival in a dose-dependent manner (Fig. 7D, right panel). In contrast to the aforementioned responses, although rapamycin did not completely block SCF-induced cell survival to basal level, the extent of attenuation was similar to that observed with wortmannin. Rapamycin (100 nM) also significantly inhibited SCF-induced cell survival in HuMCs (Fig. 7F). Thus, SCF-mediated cytokine production, chemotaxis, and cell survival appeared to be at least partly regulated by the mTORC1 pathway, a downstream of PI3K.

**The mTORC1 pathway controls cell survival and/or growth in human tumor mast cells**

As the above data suggest that the PI3K-mTORC1 pathway is important for mast cell survival and the expression and phosphorylation of molecular components of the mTORC1 pathway were up-regulated in human tumor mast cells (Fig. 2), we finally examined the ability of rapamycin to affect the phosphorylation of mTORC1 pathway and the viability of these cell lines. Short-term treatment with rapamycin for 20 min was not of sufficient duration to reverse the constitutive p70S6K phosphorylation in the human mast cell lines (data not shown). After treatment with rapamycin for 24 h, however, the constitutive phosphorylation of p70S6K was markedly reduced in LAD 2,
C

induced by SCF (Fig. 5) effects of rapamycin on the increase of 4E-BP1 phosphorylation by inhibition of mTOR phosphorylation. As was observed for the effects of acute exposure to rapamycin, the constitutive phosphorylation and expression level of mTORC1 pathway components. Where acute exposure of rapamycin inhibits mTOR activity following acute exposure to rapamycin, the constitutive phosphorylation of mTOR in the mast cell lines was also inhibited by rapamycin (100 nM) for 24 h, and then analyzed by Western blotting for the phosphorylation and expression level of mTORC1 pathway components.

D

FIGURE 8. Effects of rapamycin in human mast cell lines. A, All cell lines were cultured in the absence or presence of rapamycin (Rapa, 100 nM) for 24 h, and then analyzed by Western blotting for the phosphorylation and expression level of mTORC1 pathway components. B, After 48-h culture with or without rapamycin (Rapa, 100 nM), cell survival and/or proliferation was assessed. All data are presented as the mean ± SEM of two independent experiments conducted in triplicate. *, p < 0.05 vs vehicle pretreated control cells.

HMC-1.1, and HMC-1.2 cells (Fig. 8A). In contrast to the lack of inhibition of inducible mTOR phosphorylation observed following acute exposure to rapamycin, the constitutive phosphorylation of mTOR in the mast cell lines was also inhibited by long term exposure to rapamycin. These data suggest that, whereas acute exposure of rapamycin inhibits mTOR activity by primarily blocking the mTOR and raptor interaction, long term exposure to rapamycin may be also partially due to inhibition of mTOR phosphorylation. As was observed for the effects of rapamycin on the increase of 4E-BP1 phosphorylation induced by SCF (Fig. 5C), the phosphorylation of 4E-BP1 was also partially inhibited by rapamycin (Fig. 8A). When the LAD 2, HMC-1.1, and HMC-1.2 cells were cultured in the presence of rapamycin (100 nM), there was a similar significant reduction in the number of viable cells (Fig. 8B). Thus, these data demonstrate that the up-regulated mTORC1 activity and resulting dysregulated growth of these cells may be attenuated by inhibiting the mTORC1 pathway.

Discussion

In this study, we have demonstrated that both human and mouse mast cells express molecular components of the mTORC1 pathway and report for the first time that Ag and SCF induce the phosphorylation of key components of this pathway, tuberin, mTOR, and 4E-BP1 (Figs. 1 and 3). In addition, we demonstrate that both Ag and SCF induce the formation of a complex between mTOR and raptor, an interaction essential for the phosphorylation of p70S6K (Fig. 5D), and for specific mast cell responses including mast cell survival (Figs. 6 and 7). Finally, we report that elevated levels and constitutive activation of key molecular components of the mTORC1 pathway may be associated with the dysregulated growth of human tumor mast cells (Figs. 2 and 8).

The mTORC1 pathway is one of the major effector pathways regulated by PI3K in a variety of cell types (35). The use of knock-in mice and specific PI3K inhibitors including wortmannin and LY294002 have provided evidence that PI3K regulates multiple critical processes mediated by both FcεRI and Kit in mast cells (15, 22, 36). For example, PI3K is essential for SCF-mediated mast cell growth, survival, adhesion, and chemotaxis (15, 37, 38). Furthermore, FcεRI-mediated degranulation and cytokine production (15, 39–41), and the ability of Kit (15, 42) and adenosine (43) to amplify this response also requires activation of PI3K. Thus, our demonstration of the activation of the mTORC1 cascade in mast cells following Ag or SCF challenge is also dependent upon PI3K, and primarily the p110δ isofrom, (Fig. 4) suggested that this may pathway may contribute to the regulation of specific PI3K-regulated mast cell responses.

The ability of rapamycin to completely block the Ag- and SCF-induced assembly of mTOR and raptor and the phosphorylation of p70S6K, a downstream substrate, demonstrated that under the conditions used in this study, rapamycin produced selective and effective targeting of mTORC1 (Fig. 5). The inability of rapamycin to inhibit FcεRI-mediated degranulation (Fig. 6, A and C) is consistent with a previous report that rapamycin did not inhibit the [3H]-5HT release following FcεRI aggregation (44) and supports the conclusion that FcεRI-mediated degranulation does not require the activation of mTORC1 complex. Likewise, the contrasting ability of wortmannin, but inability of rapamycin, to block SCF-mediated mast cell adhesion (Fig. 7A) suggest that this response is also mTORC1-independent but PI3K-dependent. However, the ability of rapamycin to significantly inhibit cytokine production, chemotaxis, and cell survival supports a role for mTORC1 pathway in these responses (Figs. 6D and 7, B–F). The inhibition of these responses appears to more closely correlate with the inhibition of 4E-BP1 phosphorylation than the inhibition of p70S6K phosphorylation. In this respect, 4E-BP1 phosphorylation and cytokine production, chemotaxis and cell survival were all inhibited by ~50% whereas p70S6K phosphorylation was completely inhibited by rapamycin. The residual SCF-mediated 4E-BP1 phosphorylation, cytokine production, chemotactic response, and cell survival observed in mast cells treated with rapamycin suggest that mTORC1-independent signaling processes may also contribute to these responses. Based on the ability of wortmannin to block cytokine production and chemotaxis, however, such mTORC1-independent pathways leading to these responses would likely be PI3K-dependent. As Akt has also been demonstrated to be required for FcεRI-induced cytokine production (45), it is possible that mTORC2 or another pathway regulating Akt may account for the residual responses.

In light of the selectively enhanced expression and marked constitutive phosphorylation of mTORC1 pathway components in human tumor mast cells, the regulation of mast cell survival/growth, and hence homeostasis, was of particular interest. The mechanism responsible for these observations in the human tumor mast cells is unknown, however, it appears to be independent of the D816V Kit mutation as neither the LAD 2 cell line nor the HMC-1.1 cell line posses this mutation. Furthermore, rapamycin was observed to block the constitutive phosphorylation of mTORC1 components and cell survival not only in the HMC-1.2 cells which possesses D816V Kit mutation, but also in the LAD 2 and HMC-1.1 cell lines (Fig. 8). These observations contrast a recent study, which suggest that rapamycin-sensitive mTOR activity in a mast cell line was linked to the
D816V kit mutation (46). The role of the mTORC1 in mast cell survival may suggest a role for the observed increase in activation of the mTORC1 pathway following Ag-dependent FcεRI aggregation in mast cells. In this respect, recent data have suggested that both monomeric IgE (47) and Ag/IgE induced FcεRI aggregation (48) can promote mast cell survival. As with FcεRI-mediated mTORC1 activation, these responses are PI3K-dependent. Thus, it is possible that Ag-mediated, PI3K-dependent activation of the mTORC1 pathway may provide a mechanism whereby Ag/IgE may also support mast cell survival.

In conclusion, we have demonstrated that key components of the mTORC1 pathway are phosphorylated following FcεRI aggregation and Kit ligation in nontransformed mast cells. The mTORC1 pathway appears to contribute to the overall signaling cascade required for Ag- or SCF-induced cytokine production and SCF-mediated chemotaxis and cell survival in nontransformed mast cells. Thus, these data provide evidence that the mTOR and raptor complex is a point of divergence for the PI3K-regulated signaling downstream of FcεRI and Kit for the selective regulation of specific mast cell function. The demonstration that the mTORC1 pathway is important for mast cell survival/growth, and that components of this pathway are up-regulated and constitutively activated in human mast cell lines, suggests that the mTORC1 pathway may help drive the dysregulated proliferation of tumor mast cells. Thus, these data provide support for the concept of targeting the mTORC1 pathway for the treatment of mastocytosis and potentially other Kit-associated myeloproliferative disorders. Further studies in mast cells isolated from patients with mastocytosis would help verify that mTOR is a therapeutic target for the treatment of the disease.

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Disclosures

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References


