Tumor Necrosis Factor-α-converting Enzyme Controls Surface Expression of c-Kit and Survival of Embryonic Stem Cell-derived Mast Cells*

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Transmembrane metalloproteinasises of the disintegrin and metalloproteinase (ADAM) family control cell signaling interactions via hydrolysis of protein extracellular domains. Prior work has shown that the receptor tyrosine kinase, c-Kit (CD117), is essential for mast cell survival and that serum levels of c-Kit increase in proliferative mast cell disorders, suggesting the existence of c-Kit shedding pathways in mast cells. In the present work, we report that tumor necrosis factor α-converting enzyme (TACE; ADAM-17) mediates shedding of c-Kit. Stimulation of transfected cells with phorbol 12-myristate 13-acetate (PMA) induced metalloproteinase-mediated release of c-Kit ectodomain, which increased further upon TACE overexpression. By contrast, TACE-deficient fibroblasts did not demonstrate inducible release, thus identifying TACE as the metalloproteinase primarily responsible for PMA-induced c-Kit shedding. Surface expression of c-Kit by the human mast cell-1 line decreased upon phorbol-induced shedding, which involved metalloproteinase activity susceptible to inhibition by tissue inhibitor of metalloproteinase (TIMP)-3.

To further explore the role of TACE in shedding of c-Kit from mast cells, we compared the behavior of mast cells derived from murine embryonic stem cells. In these studies, PMA decreased surface c-Kit levels on mast cells expressing wild-type (+/+) TACE but not on those expressing an inactive mutant (ΔZn/ΔZn), confirming the role of TACE in PMA-induced c-Kit shedding. Compared with TACE−/+ cells, TACEΔZnΔZn mast cells also demonstrated decreased constitutive shedding and increased basal surface expression of c-Kit, with diminished apoptosis in response to c-Kit ligand deprivation. These data suggest that TACE controls mast cell survival by regulating shedding and surface expression of c-Kit.

Proteolytic shedding of cell surface proteins by transmembrane metalloproteinasises of the α disintegrin and metalloprotease (ADAM) family releases receptors, adhesion molecules, and growth factors that participate in a variety of disease pathways (1). Although many ADAMs participate in ectodomain shedding, murine phenotypes resulting from inactivation of tumor necrosis factor α-converting enzyme (TACE; ADAM-17) predicted its broad participation in the release of membrane-anchored proteins, including TNF-α, transforming growth factor-α, p55 and p75 TNF receptors, type II interleukin-1 receptor, VCAM-1, fractalkine, and amyloid precursor protein (2–7). Shedding mediated by TACE occurs at either low, constitutive levels or in a regulated fashion induced by phorbol stimulation (8), which may be differentiated using cells derived from TACE null mice (5). TACE expression occurs in a ubiquitous fashion in human tissues (6, 9), but the full complement of susceptible substrates, the cell specificity of TACE-mediated surface protein shedding, and its relevance in homeostatic or pathophysiologic mechanisms remain unclear.

Receptor shedding may program biological responses by diminishing surface ligand binding sites and solubilizing receptor extracellular domains (ectodomains) whose competitive binding to free ligand may antagonize transduction of incoming signals to the cell (10, 11). Serum levels of c-Kit (CD117) ectodomain increase in infiltrative mast cell disorders and correlate with clinical severity, suggesting that receptor shedding occurs as a response of activated mast cells (12, 13). c-Kit is a 145-kDa glycosylated transmembrane protein whose extracellular domain contains Ig-like domains that bind c-Kit ligand (KL; stem cell factor) to initiate signaling that programs mast cell proliferation, differentiation, migration, and survival (14–16). Soluble c-Kit isolated from human serum or supernatants of murine bone marrow-derived mast cells migrates at 98–100 kDa, which suggests that focalized hydrolysis occurs in the extracellular juxtamembrane region at critical sequences in the fifth Ig-like domain (17–20). In addition to cleavage at preferred sites, shedding may also depend on variations in the stalk length, which is defined as the distance between the scissile bond and the transmembrane domain, due to mutations in the juxtamembrane region (11, 21, 22). Divalent metal chelators attenuate release of c-Kit ectodomain from bone marrow-derived mast cells, thus identifying metalloproteinasises as

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The abbreviations used are: ADAM, a disintegrin and metalloprotease; ES/MC, embryonic stem cell-derived mast cell; KL, c-Kit ligand; MMP, matrix metalloproteinase(s); TACE, tumor necrosis factor α-converting enzyme; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; ES, embryonic stem; HEK, human embryonic kidney; FBS, fetal bovine serum; PMMA, phorbol 12-myristate 13-acetate; PP2, protein phosphatase 2; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; Ab, antibody; FITC, fluorescein isothiocyanate; PI, propidium iodide.
candidates for the c-Kit sheddase(s) (17, 18). Therefore, metalloproteolytic ectodomain shedding may regulate critical mast cell signaling interactions involving c-Kit and KL.

In this work, we used in vitro cell-based systems to investigate the role of TACE in mechanisms regulating c-Kit shedding and to clarify its relevance to mast cell biology. Comparative analysis of transfected murine embryonic fibroblasts expressing wild-type or inactive protease identified TACE as the metalloprotease responsible for phorbol-inducible c-Kit shedding. We show that cultured murine mast cells (ADAM-9 (meltrin-γ), ADAM-10 (kuzbanian), and TACE), which substantiates their candidacy as sheddases of mast cell surface proteins. To demonstrate that TACE activity regulates shedding of c-Kit by mast cells, we differentiated murine embryonic stem (ES) cells to generate mast cells expressing wild-type (c-Kit by mast cells, we differentiated murine embryonic stem (ES) cells to generate mast cells expressing wild-type (+/+ ) or inactive (ΔZn/ΔZn) TACE. TACE ΔZnΔZn ES-derived mast cells demonstrated decreased c-Kit shedding, increased surface receptor expression, and decreased apoptosis upon growth factor deprivation, showing that TACE-mediated metalloproteolytic shedding controls mast cell survival.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Murine fibroblasts derived from mice expressing wild-type (+/+ ) or inactive TACE, taceΔZnΔZn (5, 23), were maintained in medium comprised of 50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 medium containing 10% FBS, HMC-1 cells, a human mast cell line (obtained from J. Butterfield (Mayo Clinic, Rochester, MN)), were maintained at a concentration of ~1 × 10⁶ cells/ml in Iscove's modified Dulbecco's medium with 10% FBS, as previously described (24). Cells were incubated alone or with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma), 100 ng/ml recombinant human c-Kit ligand (Peprotech, Rocky Hill, NJ), mammalian (serine, cysteine, and aspartic) protease inhibitor mixture (Sigma) with 2 mM 1,10-phenanthroline, or 25 μM PP2 (Src kinase inhibitor) (Calbiochem) at 37 °C for various periods of time. To determine the effect of metalloproteinases, cells were preincubated with various concentrations of GM6001 (Calbiochem), TNP-α protease inhibitor-2 (IC-3) (Peptides International, Louisville, KY) (23), or tissue inhibitor of metalloproteinase (TIMP)-1 or -3 (Triple Point Biologics, Portland, OR) at 37 °C for various time intervals.

Measurement of Soluble c-Kit Ectodomain—Concentrations of the soluble extracellular domain of c-Kit released into culture media were measured by sandwich enzyme-linked immunoabsorbent assays in 96-well microtiter plates using the human c-CD117 ELISA Kit (sensitivity range from 0.312 to 10 ng/ml) (Diaclone, Besançon, France), according to the manufacturer's protocols. Levels of soluble c-Kit ectodomain (range of 1.0–6.0 ng/ml) in conditioned media were expressed as a percentage relative to the experimental control.

Plasmid Construction—To express c-Kit containing V5 and His₆ epitopes (KitV5His), PCR-amplified full-length human c-Kit cDNA (gift of J. Longley (University of Wisconsin, Madison, WI)) was subcloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen) containing a COOH-terminal V5 epitope and polyhistidine region. To express ADAM-17 with a COOH-terminal FLAG epitope, human ADAM-17 cDNA obtained by reverse transcription of HEK-293 mRNA was amplified by PCR and cloned into the KpnI site of pFLAG-CMV-5a. Reverse transcription of HEK-293 mRNA was amplified by PCR and cloned into the KpnI site of pFLAG-CMV-5a. Primers modified ADAM-9 (meltrin-γ), ADAM-10 (kuzbanian), and TACE, which substantiates their candidacy as sheddases of mast cell surface proteins. To demonstrate that TACE activity regulates shedding of c-Kit by mast cells, we differentiated murine embryonic stem (ES) cells to generate mast cells expressing wild-type (+/+ ) or inactive (ΔZn/ΔZn) TACE. TACE ΔZnΔZn ES-derived mast cells demonstrated decreased c-Kit shedding, increased surface receptor expression, and decreased apoptosis upon growth factor deprivation, showing that TACE-mediated metalloproteolytic shedding controls mast cell survival.

Flow Cytometry—Cell surface c-Kit expression on tace+/+ and taceΔZnΔZn fibroblasts was identified after transfection by flow cytometric analysis of cells stained with phycoerythrin-conjugated antibody to c-Kit (clone K45) (Neomarkers, Fremont, CA), human ADAM-9 or -17 (R&D Systems, Minneapolis, MN), or herbipolonal antibody to the N-terminus of human ADAM-10 (Triple Point Biologics) at 4 °C for 45 min. To control for variable transfection efficiency and protein expression, c-Kit-positive fibroblasts were sorted on a triple-laser FACSVantage SE flow cytometer based on mean fluorescence intensity, with data analyzed by CellQuest Pro 4.1 software (Becton Dickinson, San Jose, CA). To detect cell surface protein expression, cells were washed three times with culture media containing 1% FBS prior to incubation with mouse monoclonal Ab against human c-Kit (clone K45) (Neomarkers, Fremont, CA), human ADAM-9 or -17 (R&D Systems, Minneapolis, MN), or rabbit polyclonal antibody to the N-terminal of human ADAM-10 (Triple Point Biologics) at 4 °C for 30 min. Incubation of cells with secondary Ab alone was performed to exclude nonspecific cross-reactivity. Cells were examined on a FACSFlow Cytometer with data analyzed using CELLQuest Pro 4.1 software (Becton Dickinson). Iso-type control experiments were performed using either mouse IgG1, IgG2b (eBioscience, San Diego, CA), or IgG2a (Southern Biotech, Birmingham, AL) or rabbit IgG (Zymed Laboratories, San Francisco, CA). Flow cytometric analysis of ESMC c-Kit expression was performed using FITC-conjugated rat anti-mouse c-Kit (clone 2B8) (BD Pharmingen, San Jose, CA).

FACS-based apoptosis assays using Hoechst 33342 (25) and annexin V (26) were used to detect mast cell apoptosis. Incorporation of propidium iodide (PI) was used to identify necrotic cells. Induction of apoptosis was performed using 1 μM staurosporine (Sigma) (data not shown) and either partial or complete withdrawal of KL (15). Cells were incubated in serum-free culture media for 24 h prior to the addition of 0, 10, or 100 ng/ml KL (Peprotech) at 37 °C for various time intervals. After incubation and equilibration of cells to room temperature, flow cytometry was performed on cells treated with 2 μg/ml PI and 6 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) at 4 °C for detection on a triple laser FACSVantage SE flow cytometer. To detect bound annexin V, cells were incubated with FITC annexin V in binding buffer (Molecular Probes), according to the manufacturer's instructions and analyzed similarly. Survival was determined by identifying the population of nonapoptotic and necrotic cells. Gates were established using control populations of cells (prior to induction of apoptosis) that exclude PI and either demonstrate only background levels of annexin V binding (left lower quadrant; see Fig. 7) or stain positive for Hoechst 33342 and negative for PI (right lower quadrant). Apoptotic cells were identified by decreased Hoechst 33342 and increased PI staining or by positive annexin V staining.

Flow fraction precipitation—To determine the c-Kit shedding of metalloproteinases on c-Kit ectodomain shedding, 20 × 10⁶ HMC-1 cells were incubated in 300 μl of serum-free medium in the absence or presence of 50 μM IC-3 at 37 °C for 15 min, followed by incubation with 100 ng/ml PI at 37 °C for 2 h. Aliquots of normal human serum (Sigma) or cell supernatants were equilibrated with an equal volume of PBS and incubated with monoclonal anti-c-Kit receptor Ab that recognizes the second Ig-like domain (clone K45) at 4 °C for 2 h. Antibody-antigen complexes were incubated with protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 16 h and washed three times in 25 mM Tris buffer (pH 7.4) containing 135 mM NaCl and 2.6 mM KCl (TBS). To elute proteins, beads were resuspended in reducing running buffer prior to SDS-PAGE. Western blot analysis was performed using anti-mouse c-Kit receptor Ab that recognizes the second Ig-like domain (clone K45) at 4 °C for 2 h. Antibody-antigen complexes were incubated with protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 16 h and washed three times in 25 mM Tris buffer (pH 7.4) containing 135 mM NaCl and 2.6 mM KCl (TBS). To elute proteins, beads were resuspended in reducing running buffer prior to SDS-PAGE. Western blot analysis was performed using anti-mouse c-Kit receptor Ab that recognizes the second Ig-like domain (clone K45) at 4 °C for 2 h. Antibody-antigen complexes were incubated with protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 16 h and washed three times in 25 mM Tris buffer (pH 7.4) containing 135 mM NaCl and 2.6 mM KCl (TBS). To elute proteins, beads were resuspended in reducing running buffer prior to SDS-PAGE. Western blot analysis was performed using anti-mouse c-Kit receptor Ab that recognizes the second Ig-like domain (clone K45) at 4 °C for 2 h.
The absence or presence of PMA. Cell supernatants were analyzed by phoresis and immunoblotting. 10 min. Ice-cold TBS was added to the detergent phase prior to electrophoresis. HEK-293 cells expressing c-Kit were pretreated with 25 amol of TACE to attenuate c-Kit shedding. HEK-293 cells expressing c-Kit receptor were incubated in the absence or presence of metalloprotease inhibitors, GM6001 or IC-3, for 30 min prior to incubation in culture medium containing 1% FBS. Conditioned medium was analyzed by ELISA for soluble c-Kit ectodomain. Values represent the mean ± S.E. of soluble c-Kit ectodomain analyzed in triplicate (*, p < 0.01 compared with control; **, p < 0.005 compared with control). PMA was incubated under various conditions were assayed by ELISA. Murine embryonic stem (ES) cells expressing wild-type TACE (TACE+/+) or homozygous for an inactivating mutation in the zinc-binding domain (TACEzincas/τ236H) (5, 6) were differentiated into mast cells, using previously described protocols (29, 30). Briefly, ES cells were cultured on plates coated with 0.1% gelatin (Sigma) in Iscove’s modified Dulbecco’s medium containing 15% FBS, 100 ng/ml leukemia inhibitory factor (Stem Cell Technologies, Vancouver, Canada), and 1.5 × 10⁻⁴ M β-mercaptoethanol (Sigma) at 37 °C for 2–3 days. Cells were then cultured in differentiation media for 8–10 days to develop embryoid bodies, which were dissociated in 0.25% trypsin at 37 °C for 5 min, disrupted mechanically, and cultured in maintenance media containing 2 ng/ml murine interleukin-3 and 100 ng/ml murine IL-13 (Peprotech). Embryonic stem cell-derived mast cells (ESMCs) were used between weeks 6 and 12. To identify mast cell phenotypes, cytopsin cells were incubated with 1% toluidine blue (which yields metachromatic staining in mast cells or basophils) (31) or 2 µg/ml Texas Red avidin (Molecular Probes) (which binds specifically to mast cell granules) (32, 33). Chymase activity was identified by enzyme histochemistry using naphthol AS-D chloroacetate and Fast Garnet GBC (Sigma), which yields reddish brown cytoplasmic granules (34). Expression of ADAM-9, ADAM-10, and TACE by TACE+/+ or TACE−/− ES cells was determined by PCR analysis of cDNA reverse transcribed from mRNA isolated from the murine ADAMs (GenBankTM accession numbers NM007404, NM001110, and NM009815) (data not shown). To block c-Kit receptor internalization, cells were incubated with 25 µM PP2 (Calbiochem) at 37 °C for 30 min prior to induction of c-Kit shedding by PMA, as previously described.

Statistical Analysis—Differences with a p value of <0.05 using Student’s two-tailed t test were considered significant.

RESULTS

TACE Mediates c-Kit Ectodomain Shedding—To investigate metalloprotease-dependent ectodomain shedding, soluble c-Kit levels in supernatants of HEK-293 cells stably expressing wild-type c-Kit incubated under various conditions were assayed by ELISA. As seen in Fig. 1B, HEK-293 cells constitutively released c-Kit ectodomain, whose levels increased by 2-fold upon phorbol stimulation. Incubation of cells with metalloprotease inhibitors, GM6001 or IC-3, decreased levels of soluble c-Kit, demonstrating a dependence of c-Kit ectodomain shedding on metalloprotease activity. Expression of c-Kit containing a Gly-Asn-Asn-Lys (GNNK) insertion, a common variant in the c-Kit juxtamembrane region (36), did not alter constitutive or phorbol-inducible shedding, since soluble levels of both the GNNK (−) and GNNK (+) isoforms were similar (Fig. 1C). Therefore, c-Kit ectodomain shedding occurred independently of the GNNK sequence, despite altering the sequence and length of the juxtamembrane region. As shown in Fig. 1D, overexpression of TACE-FLAG in c-Kit-expressing...
HEK-293 cells increased constitutive ectodomain release by 2-fold, demonstrating that TACE mediates shedding of c-Kit. Compared with unstimulated cells, incubation of TACE-transfected cells with phorbol further increased release of soluble c-Kit, substantiating a role for TACE activity in phorbol-inducible c-Kit shedding.

**Phorbol-inducible c-Kit Shedding Requires TACE**—To further investigate the dependence of ectodomain shedding on TACE activity, c-Kit receptor was transiently expressed in wild type (tace+/+) or tace<sup>Δzv/Δzv</sup> fibroblasts, which express an inactive form of TACE due to a mutation in the Zn<sup>2+</sup>-containing active site. Transfected fibroblasts expressing high surface levels of c-Kit were isolated by flow cytometry after staining with phycoerythrin-conjugated anti-c-Kit Ab. Following sorting, purity of c-Kit-positive cells exceeded 94% for both tace<sup>+/+</sup> and tace<sup>Δzv/Δzv</sup> fibroblasts, which demonstrated similar c-Kit surface expression (data not shown). As seen in Fig. 2, supernatants of unstimulated tace<sup>+/+</sup> and tace<sup>Δzv/Δzv</sup> fibroblasts demonstrated similar levels of soluble c-Kit by ELISA, suggesting that constitutive shedding of c-Kit occurred largely independently of TACE in these cells. Phorbol stimulation of tace<sup>+/+</sup> fibroblasts increased soluble c-Kit levels by 2-fold but had no effect on shedding by tace<sup>Δzv/Δzv</sup> fibroblasts. Therefore, these data substantiate a role for TACE in phorbol-induced c-Kit ectodomain shedding. It is important to note that the sensitivity of constitutive tace<sup>Δzv/Δzv</sup> fibroblast c-Kit shedding to inhibition by either GM6001 or IC-3 also suggests the potential involvement of other non-TACE metalloproteinases in c-Kit ectodomain cleavage in these cells.

**Mast Cell c-Kit Ectodomain Shedding**—Ectodomain shedding using fibroblasts identified TACE as a membrane-anchored ADAM that regulates c-Kit shedding. Therefore, we investigated metalloproteinase-dependent c-Kit ectodomain shedding in the human mast cell (HMC)-1 line, which natively expresses the receptor. Binding of KL regulates proliferation and survival of HMC-1 cells, which phenotypically resemble human mast cells in their expression of proteases (27, 37). As shown in Fig. 3A, incubation with KL or phorbol decreased HMC-1 cell surface expression of c-Kit. Whereas phorbol increased c-Kit ectodomain levels in media conditioned by HMC-1 cells by ~2.5-fold, KL decreased levels to half of those due to constitutive release (Fig. 3B). As shown in Fig. 3C, immunoprecipitation of cell supernatants identified an ~100-kDa protein immunoreactive for Ab detecting extracellular Ig-like domains whose levels increased after phorbol stimulation. To investigate the role of receptor internalization, cells were incubated with PP2, a Src family kinase inhibitor. Phorbol-induced c-Kit ectodomain shedding occurred to the same magnitude upon incubation of cells with PP2, suggesting that receptor internalization does not account for the observed decrease in cell surface c-Kit expression (Fig. 3D) (35). By contrast, incubation of cells with protease inhibitors maintained cell surface c-Kit expression, implicating proteolysis as the mechanism for the decrease induced by phorbol. Cell surface c-Kit levels also remained unchanged upon incubation of cells with both PP2 and protease inhibitors, suggesting no phorbol-induced receptor trafficking between intracellular pools and the cell surface. Thus,
phorbol induces metalloproteinase-dependent shedding of HMC-1 cell c-Kit ectodomain.

Metalloproteinase Inhibitors Attenuate c-Kit Ectodomain Shedding—We next examined the sensitivity of mast cell c-Kit shedding to metalloproteinase inhibitors known to block the activity of ADAMs. As shown in Fig. 4A, co-incubation of HMC-1 cells with GM6001 or IC-3 attenuated loss of cell surface c-Kit expression induced by phorbol. Incubation of HMC-1 cells under basal conditions or upon phorbol stimulation in the presence of GM6001 or IC-3 decreased amounts of c-Kit ectodomain released in cell supernatants in a dose-dependent manner (Fig. 4B). Similarly, incubation with TIMP-1 or -3 also decreased levels of soluble c-Kit in a concentration-dependent fashion (Fig. 4C). Attenuation of soluble c-Kit levels upon incubation with TIMP-3 implicates TACE in HMC-1 c-Kit ectodomain cleavage, but its lack of specificity and the ability of both TIMP-1 and -3 to inhibit ADAM-10 (38) also suggests potential involvement of other ADAM or non-ADAM metalloproteinases. These data demonstrate that proteases susceptible to inhibition by structurally diverse metalloproteinase inhibitors regulate constitutive and phorbol-inducible HMC-1 c-Kit ectodomain shedding.

Mast Cell ADAM Protease Expression—Our results suggest that a metalloproteinase-mediated event regulates c-Kit shedding by mast cells. To explore mast cell expression of membrane-anchored ADAM metalloproteinases implicated in ectodomain shedding, HMC-1 cell surface proteins were isolated by Triton X-114 detergent extraction and analyzed by immunoblotting. As seen in Fig. 5A, HMC-1 cells expressed proteins immunoreactive for ADAM-9, ADAM-10, and TACE, which demonstrated the electrophoretic profiles of the mature forms of the enzymes. Flow cytometric analysis of HMC-1 cells confirmed constitutive cell surface expression of ADAM-9, ADAM-10, and TACE (Fig. 5B). These data demonstrate that mast cells also express ADAM metalloproteinases, which may participate in membrane protein shedding events.

TACE Regulates Mast Cell c-Kit Shedding—To determine whether TACE mediates mast cell c-Kit ectodomain cleavage, cell surface c-Kit expression was explored in mast cells derived from murine ES cells expressing wild type (TACE⁺/⁺) or inactive TACE (TACEΔZn/ΔZn), TACE⁺/⁻ or TACEΔZn/ΔZn murine ESMCs generated by culturing and differentiating ES cells in the presence of interleukin-3 and KL demonstrated cellular metachromasia, granular morphology, and chymase activity, as seen in Fig. 6A, which are typical morphological and protease phenotypes of murine mast cells. To examine the dependence of regulated c-Kit shedding on TACE, ESMCs were stimulated with phorbol and analyzed by flow cytometry. As seen in Fig. 6B, phorbol stimulation decreased cell surface c-Kit expression on TACE⁺/⁺ ESMCs, whereas that on TACEΔZn/ΔZn ESMCs remained unchanged. Flow cytometric analysis of c-Kit with inhibition of receptor internalization by PP2 demonstrated that the selective effect of phorbol on TACE⁺/⁺ ESMCs resulted from TACE-dependent c-Kit ectodomain shedding. In-
TACE Regulates Mast Cell Survival—The results showing differential c-Kit expression on TACE\textsuperscript{+/+} and TACE\textsuperscript{−/−} ESMCs predicted a possible cell type-specific response to stress. In cultured murine mast cells, withdrawal of serum and critical growth factors such as KL induces apoptosis in a temporal manner (15, 39, 40). We hypothesized that increased surface c-Kit expression on TACE\textsuperscript{−/−} ESMCs would be protective from induction of apoptosis. Flow cytometric analysis differentiated viable cells from those undergoing stages of apoptosis detected either by Hoechst 33342 staining or annexin V binding (40, 41). As seen in Fig. 7, both TACE\textsuperscript{+/+} and TACE\textsuperscript{−/−} ESMCs demonstrated similar survival when incubated in the presence of 100 ng/ml KL, a concentration used during differentiation or maintenance of cultured murine mast cells. TACE\textsuperscript{+/+} ESMCs deprived completely of KL yielded survival of only 13–15% at 84 h. Therefore, mast cells derived from murine ES cells undergo apoptosis induced by growth factor depletion in a temporal manner similar to that observed for mouse bone marrow-derived mast cells at 72–96 h (39, 40). By contrast, 57–63% of TACE\textsuperscript{−/−} ESMCs remained viable in the absence of KL. Partial growth factor withdrawal by incubation in 10 ng/ml KL, a log-fold lower concentration, yielded ~33% survival of TACE\textsuperscript{+/+} ESMCs, compared with 72% viability for TACE\textsuperscript{−/−} ESMCs. Flow cytometric analysis using either Hoechst 33342 or annexin V yielded similar results. TACE\textsuperscript{−/−} ESMCs maintained a dependence upon KL for survival, since inclusion of the growth factor in media increased survival by 11–17%. TACE\textsuperscript{−/−} ESMCs also demonstrated a ~50% increase in survival compared with TACE\textsuperscript{+/+} ESMCs even in the absence of KL. These data suggest that TACE controls mast cell responses to external stimuli by regulating cell surface c-Kit expression. The proteolytic shedding of c-Kit decreases surface receptor expression and availability of KL binding sites, thus diminishing reception of incoming survival signals via the c-Kit-KL dyad.

**DISCUSSION**

Correlation of c-Kit receptor ectodomain serum levels with disease activity in systemic mastocytosis and atopic dermatitis suggests a mechanistic link between proteolytic shedding and disorders of mast cell activation and infiltration (12, 13). By binding with high affinity to KL, soluble c-Kit blocks its mast cell-stimulatory effects, thus implicating controlled shedding of Ig-like KL-binding domains as a critical regulatory pathway in mast cell biology (10). Attenuation of soluble c-Kit levels in the supernatants of mast cells incubated with chelators of divalent cations implicated metalloproteinases in c-Kit ectodomain shedding (17, 18). Although proteases of diverse classes hydrolyze transmembrane proteins, the broad substrate specificity of membrane-anchored metalloenzymes of the ADAM family suggests their candidacy as c-Kit sheddases (42). This work demonstrates that TACE-mediated c-Kit receptor shedding not only regulates its dynamic surface display but also programs mast cell survival.

Ubiquitous TACE expression in organ tissues and diverse TACE null mouse phenotypes suggest a broad array of potential substrates for the protease in addition to TNF-α (5, 6, 9). Reconstitution of the membrane shedding event in stably transfected HEK-293 cells in studies using metalloproteinase inhibitors and TACE overexpression demonstrate that TACE activity mediates c-Kit receptor shedding. Lack of inducible shedding in transfectants TACE\textsuperscript{+/+} fibroblasts substantiates susceptibility of c-Kit to TACE-mediated ectodomain shedding. Experiments using TACE\textsuperscript{+/+} and TACE\textsuperscript{−/−} ES-derived mast cells further demonstrate that constitutive or inducible shedding and cell surface receptor levels depend on TACE activity.

Phorbol stimulation of cells expressing wild-type or inactive enzyme permits assignment of constitutive or inducible shedding activity to TACE. Since transfected fibroblasts expressing wild-type or inactive TACE demonstrate similar levels of constitutive c-Kit shedding that is sensitive to GM6001 or IC-3 inhibition, basal sheddase activity in these cells probably involves metalloproteinases other than TACE and possibly pro-
teases of other classes. By contrast, soluble c-Kit levels remain unchanged in supernatants of tace\textsuperscript{Zn/Zn} fibroblasts after phorbol treatment, demonstrating that TACE is responsible for virtually all of the induced ectodomain shedding observed in wild-type cells. Analysis using ESMCs substantiates the inducibility of c-Kit shedding as demonstrated by a decrease in cell surface receptor levels on phorbol-stimulated TACE\textsuperscript{-/-} ESMCs, which does not occur in similarly treated TACE\textsuperscript{Zn/Zn} ESMCs. Furthermore, the higher basal surface c-Kit receptor levels on TACE\textsuperscript{Zn/Zn} ESMCs in concert with decreased supernatant levels of c-Kit ectodomain demonstrate that TACE also participates in constitutive shedding in mast cells. Whether this apparent cell-specific difference in constitutive shedding results from regulatory mechanisms unique to mast cells remains a focus of investigation. The phorbol induction of c-Kit shedding is similar to that observed for other TACE substrates and thus implies up-regulation of TACE activity (rather than changes in surface levels or dependence on its cytoplasmic domain) (8) and potential involvement of mitogen-activated protein kinase cascades (11) in pathways regulating the dynamic surface expression of c-Kit. Stimuli such as lipopolysaccharide, growth factors, and G-protein-coupled receptor ligands induce shedding (11), but the physiologic inducer(s) of c-Kit shedding remain unidentified.

Mast cell c-Kit receptor ectodomain shedding illustrates an intersection of complex pathways involving proteases and signaling cascades that defines a unique mechanism in which metalloproteolysis programs mast cell survival. Although known as rich sources of serine and cysteine proteases, mast cells also express a variety of secreted matrix metalloproteinases (MMP) including collagenase (MMP-1), stromelysin (MMP-3), and gelatinases A (MMP-2) and B (MMP-9), which may be complexed with inhibitors such as TIMP-1 (27). Expression of TACE, ADAM-9, and ADAM-10 by HMC-1 cells and ESMCs demonstrates that cultured mast cells also express membrane-anchored metalloproteinases and further expands the potential diversity of mast cell protease phenotypes. Derivation of mast cells from ES cells expressing an inactive protease permits attribution of an observed phenotype in a cell-specific manner that may be precluded by the embryonic lethality of a given mutation (29). TACE inactivation results in increased surface levels of c-Kit on TACE\textsuperscript{Zn/Zn} ESMCs and decreased shedding. Differential responses of TACE\textsuperscript{-/-} and TACE\textsuperscript{Zn/Zn} ESMCs to apoptotic signaling induced by KL deprivation highlight the functional significance of TACE-mediated c-Kit shedding as a novel survival phenotype. A log-fold decrease in KL concentration elicits selective apoptotic responses that are either not apparent at the higher concentration or exaggerated upon complete withdrawal. TACE activity may program mast cell survival responses by several different mechanisms. Solubilization of the extracellular domains of transmembrane receptors releases growth factor binding domains, which retain a high ligand affinity. TACE-mediated shedding may decrease the surface c-Kit receptor density and desensitize cells to incoming survival signals transmitted by KL. TACE may also control binding of KL to c-Kit by releasing soluble ectodomains whose competitive binding to KL decreases its availability to transmembrane c-Kit (10, 11). Furthermore, its broad substrate specificity suggests that TACE may shed other as yet unidentified transmembrane substrates that balance survival and apoptotic pathways in mast cells.

Since tissue microenvironments dictate mast cell protease phenotypes (43), physiologic regulation of TACE-mediated c-Kit ectodomain shedding probably depends upon programmed responses to signaling induced during injury or inflammation. The transmembrane domains of TACE and c-Kit may define enzyme-substrate interactions in a focalized fashion imposed by steric restrictions that somehow permit cleavage in the

Fig. 6. TACE regulates cell surface c-Kit expression in murine embryonic stem cell-derived mast cells. A, cytospin mast cells derived from TACE \textsuperscript{+/+} or ΔZn/ΔZn ES cells were stained with toluidine blue (a and d) or Texas Red avidin (b and e) to detect metachromatic and granular phenotypes typical of mast cells. Enzyme histochemistry detected mast cell chymotryptic activity (c and f). B, FACS analysis was performed on ESMCs stained with FITC-conjugated antibody to murine c-Kit. Cells were pretreated in the absence or presence of 25 μM PP2 (blue) prior to stimulation with 100 ng/ml PMA (red) for 2 h. Unstained cells (black) are also shown. C, immunoprecipitation of murine c-Kit. Medium conditioned by TACE\textsuperscript{-/-} and TACE\textsuperscript{Zn/Zn} ESMCs for 16 h at 37 °C was subjected to immunoprecipitation and immunoblotting using antibody to murine c-Kit. Data shown are representative of experiments performed in triplicate.
extracellular juxtamembrane region, as predicted by the size of the isolated soluble ectodomain (17, 19). The absence of any detectable difference in the shedding of c-Kit containing the GNNK insertion suggests that neither loss of the Lys-Glu bond nor a 4-residue change in the stalk region impairs shedding. P1 and P1' preferences defined for TACE include short side chains and aliphatic residues, respectively, with Ala-Val identified as the favored bond in peptides mimicking the scissile bond of TNF-α (11). Although the c-Kit juxtamembrane region contains favored P1 Ala residues, aromatic residues occupy the corresponding P1' positions. Since TACE also cleaves at bonds containing other residues at the P1 and P1' positions (11), it remains difficult to predict the scissile bond(s) cleaved during c-Kit ectodomain shedding. Indirect involvement of TACE as an intermediate protease in shedding events remains a possibility and may also complicate the prediction and interpretation of cleavage data (11). Furthermore, a requirement for interactions distal to the cleavage site due to the membrane anchoring of TACE and the target substrate may also regulate shedding, as exemplified by the requirement for the disintegrin/cysteine-rich domain in TACE's shedding of the type II interleukin-1 receptor (3). Mechanisms controlling membrane co-localization of TACE and its substrates, facilitation of sheddase-substrate interactions by adaptor proteins, and binding of TACE inhibitors such as TIMP-3 (11) may also play potential regulatory roles in TACE-mediated mast cell c-Kit ectodomain shedding.

In summary, our data demonstrate that TACE programs unique survival responses of mast cells through its role in regulating the shedding and surface expression of c-Kit receptor. The critical role of tissue microenvironments in defining the protease phenotypes of mast cell subpopulations suggests that further investigation of pathways regulating the activity of TACE and other membrane-anchored or secreted metalloproteinases, in concert with their binding to TIMPs, may provide critical insights into the contributions of mast cells to extracellular matrix signaling and remodeling.

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