Tyrosine phosphorylation directs TACE into extracellular vesicles via unconventional secretion

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Synopsis: Src family tyrosine kinases, in particular Hck, can induce an unconventional protein secretion (UPS) pathway leading to Golgi-bypass secretion of a transmembrane protein into extracellular vesicles (EV). These results reveal an iRhom-independent alternative secretion route for the proinflammatory protease TACE, and have general implications for understanding the regulation of UPS and EV biogenesis. ER = Endoplasmic reticulum. MVE = Multivesicular endosome. pTyr = phosphotyrosine.

ABSTRACT

When studying how HIV-1 Nef can promote packaging of the proinflammatory transmembrane protease TACE into extracellular vesicles (EV) we have revealed a novel tyrosine kinase-regulated unconventional protein secretion (UPS) pathway for TACE. When TACE was expressed without its trafficking co-factor iRhom allosteric Hck activation by Nef triggered translocation of TACE into EVs. This process was insensitive to blocking of classical secretion by inhibiting ER to Golgi transport, and involved a distinct form of TACE devoid of normal glycosylation and incompletely processed for prodomain removal. Like most other examples of UPS this process was GRASP-dependent but was not associated with ER stress. These data indicate that Hck-activated UPS provides an alternative pathway for TACE secretion that can bypass iRhom-dependent ER to Golgi transfer, and suggest that tyrosine phosphorylation might have a more general role in regulating UPS.

Key words: ADAM17 / exosome / GRASP / Hck / TACE

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INTRODUCTION

Extracellular vesicles (EV) are a heterogeneous group of membraneous particles, including exosomes and shedding vesicles, and have recently emerged as important mediators of intercellular communication[1,2]. The biogenesis, composition, and functions of different classes of EVs differ greatly, but remain incompletely understood. EVs isolated from blood and other biofluids are considered as valuable sources of biomarkers for diagnosis and monitoring of various diseases, such as cancer.

It is likely that EVs also serve as pathogenic mediators in many different types of disease[3,4]. EVs containing inflammation promoting factors can be found in the blood of HIV-infected individuals, and correlate with disease progression and poor recovery of the immune system following antiviral therapy[5]. A prominent component of circulating EVs collected from HIV-infected individuals is tumor necrosis factor-α converting enzyme (TACE), also known as a disintegrin and metalloproteinase 17 (ADAM17)[6,7]. Uploading of TACE into EVs is promoted by the HIV-1 pathogenicity factor Nef via its interactions with cellular protein kinases[8]. In addition to its role in TNF release, TACE is responsible for proteolytic maturation of other cytokines, cytokine receptors, and extracellular matrix components involved in inflammation[6,7]. Thus, EV-associated TACE could be a key player in the chronic immune activation that drives AIDS pathogenesis and persistent viral replication.

EVs have also been implicated in stimulated secretion of interleukin-1β (IL-1β), a potent proinflammatory cytokine[9] produced upon inflammasome activation by bacterial and viral infections, or endogenous danger signals like ATP and urate[10]. IL-1β lacks a signal sequence for targeting it to the conventional ER-Golgi secretion route, and instead represents a prominent example of a protein released via unconventional protein secretion (UPS)[11].

Several different types of UPS pathways exist and can be utilized as an alternative Golgi-bypass secretion mechanism also by proteins having a canonical signal sequence[12-14]. A well-characterized example of this is the disease-causing variant ΔF508-CFTR of cystic fibrosis transmembrane conductance regulator, which is functional as an ion channel, but is defective in its trafficking to the plasma membrane[15]. Recently Gee and coworkers showed that surface expression and thereby function of ΔF508-CFTR could be rescued *in vitro* as well as in a mouse model of cystic fibrosis by directing an immature core-glycosylated form of ΔF508-CFTR to the plasma membrane via an unconventional GRASP-dependent secretion route[16].

The Golgi reassembly stacking proteins GRASP55 and GRASP65 were originally identified as factors required for the stacking and integrity of Golgi cisternae[17]. However, GRASPs are largely dispensable for the conventional anterograde protein transport[18,19], but instead have recently emerged as key factors in unconventional protein secretion that bypasses the Golgi[20,21].

In this study we have examined the role of Src family kinases in regulating uploading of TACE into EVs, which led us to identify a tyrosine phosphorylation-regulated and GRASP-dependent unconventional secretion pathway that enables vesicle secretion of TACE independently of its otherwise essential ER-Golgi trafficking co-factor iRhom2.

RESULTS

Src family tyrosine kinases can trigger TACE secretion into extracellular vesicles

We have previously shown that Lck and Hck kinases are involved in HIV Nef-induced secretion of TACE into extracellular vesicles[8,22]. To test if this is a general property of Src family tyrosine kinases (SFK) we transfected TACE into human HEK293 cells alone or together with activated (Y-to-F change in the C-terminal Csk phosphorylation site) variants of human Lck, Hck, Lyn, Fgr, or Fyn. In order to monitor SFK catalytic activity in these cells a vector expressing paxillin, a 68 kDa adaptor protein and SFK substrate[23], was

also co-transfected and detected with regular or phospho-Y31-specific antibodies.

As shown in Figure 1A, TACE was found in EV preparations from supernatants of all SFK-transfected cells, but not from cells transfected with TACE in the absence of a SFK (correlating with the lack of paxillin Y31 phosphorylation). Thus, at least in a tyrosine kinase overexpression model, triggering EV secretion of TACE is a property shared by different SFK members, and does not require ectopic expression of additional cellular or viral signaling factors.

Among the SFK family Hck is potently and preferentially activated by HIV-1 Nef[24,25]. To generate a cell line model for studying TACE EV secretion caused by Nef-induced natural allosteric SFK activation, we transduced HEK293 cells with a lentiviral vector expressing wild-type human p59Hck. Individual clones were selected that expressed Hck at levels that were readily detectable but still subjected to efficient suppression of kinase activity by conformational autoinhibition, as indicated by the failure to phosphorylate residue Y31 of paxillin (Figure 1B).

Similar to mock-transduced cells (puromycin selection marker only) these stably Hck-expressing cells did not show any EV secretion of transfected TACE. However, when TACE was transfected together with Nef, robust paxillin-Y31 phosphorylation and EV secretion of TACE could be observed in Hck-transduced but not in mock-transduced cells (Figure 1B). Thus, similar to transient over-expression of SFK activity (Figure 1A), conformational activation of Hck mimicking the effects of Nef in HIV-infected cells can also efficiently activate EV secretion of TACE.

Hck isoforms differ in their capacity to induce EV secretion of TACE

In addition to the myristoylated and palmitoylated p59Hck an alternative p61Hck isoform exists, whose translation starts at a CTG codon upstream of the p59 ATG start site. p61Hck is also myristoylated but lacks the palmitoylation signal. These differing acylation signals have been shown to direct p59 and p61 preferentially to the plasma membrane and lysosomes, respectively[26].

We compared the capacity of the p59 and p61 isoforms to activate secretion of TACE into extracellular vesicles. As shown in Figure 1C, only p59Hck activated TACE secretion, although both isoforms were expressed at comparable levels and similarly phosphorylated paxillin. An N-terminally epitope-tagged version of p59Hck lacking both acylation sites was also unable to induce TACE secretion, but could mediate low-level paxillin Y31 phosphorylation. Catalytically inactive Hck mutant K269N failed to activate TACE secretion, and as expected, was also completely deficient in paxillin phosphorylation. Thus, mere overexpression of Hck together with TACE does not induce secretion of TACE into EVs, and instead catalytic activity and proper membrane association domain is required for Hck to accomplish this.

Inducible EV secretion involves hypoglycosylated TACE

Although TACE was detected both in cell lysates and in EVs as a predominant doublet of bands in Western blotting we noted that the electrophoretic mobility of these two protein doublets were clearly different (approximately 130 and 105 kDa in cell lysates vs. 105 and 80 kDa in EVs; see Figure 2A). The apparent molecular weight of TACE can vary depending on its glycosylation status [27] and whether it has undergone proteolytic activation by furin-mediated removal of the N-terminal prodomain[28,29]. Therefore, we tested antibodies against the C-terminus or the N-terminus (prodomain) of TACE, and studied the effect of enzymatic deglycosylation on these TACE protein species. The 130 kDa species ("band A") that was found only in the cell lysates, as well as the 105 kDa species ("band B") that was present in the lysates and in the EVs could be detected either with an antibody targeting the TACE C-terminus or an antibody against the N-terminal prodomain, indicating that they had not been subjected to proteolytic activation. By contrast, the 80 kDa band ("band C") that was found only in the EVs

could not be detected with the anti-prodomain antibody, indicating that it represented a mature form of TACE lacking the autoinhibitory prodomain.

When the cell lysates were treated with the glycosidases PNGase F or Endo H, the 130 kDa band disappeared, whereas the intensity of the 105 kDa species increased, indicating that bands A and B represented differentially glycosylated forms of the same polypeptide, *i.e.* prodomain-containing full-length TACE. By contrast, deglycosidase treatment had no effect on the mobility of the 105 kDa and 80 kDa TACE bands detected in the EVs, indicating that they represented hypoglycosylated forms of TACE with and without the prodomain, respectively.

Both PNGase F and Endo H can remove high-mannose glycan modifications introduced in the ER, but following their further modification in the Golgi they become resistant to Endo H and can be cleaved only by PNGase F[30]. Whereas no mature TACE (lacking the prodomain) was seen in untreated cell lysates, a faint signal for the 80 kDa band ("band C") could be detected after treatment with PNGase F (but not with Endo H). This indicated that a small amount proteolytically activated TACE was also present in the cells, but was not evident in the untreated lysates because it was glycosylated and therefore co-migrated with prodomain-containing, hypoglycosylated TACE species (band B). Thus, the prodomain-containing 130 kDa TACE species in cell lysates was glycosylated only in an ER-specific manner, whereas the scarcely present prodomain-lacking form of TACE ("band-B*" as illustrated in Figure 2D) had undergone further glycosylation in the Golgi.

However, the most important finding of these experiments was that neither PNGase F nor Endo H treatment changed the mobility of the prodomain-containing 105 kDa species or the proteolytically matured 80 kDa species of TACE in the EVs, thus showing that TACE secreted into extracellular vesicles is hypoglycosylated.

To confirm that the 80 kDa TACE species in the EVs was indeed a product of prodomain cleavage at the furin target site we created a mutant form of TACE (TACE-FM) in which this furin cleavage site was disrupted by a KR^{214} to NG substitution[28]. Confirming our assumption, when EVs from cells expressing TACE-FM were purified and analyzed the mature 80 kDa isoform (band C) was absent and only the 105 kDa precursor form of TACE (band B) could be detected (Figure 2B).

To study if the protease activity of TACE itself was relevant for EV secretion we also generated an inactive TACE mutant (TACE-CD) carrying a previously described E406A substitution[31] that disrupts its catalytic site. No difference in Hck-induced EV secretion was observed between this mutant and wild-type TACE (Figure 2B).

Vesicle secretion of TACE is not mediated by iRhom2

The catalytically inactive members of the rhomboid family of proteases, iRhom1 and iRhom2, have been established as essential regulators of TACE maturation[27,32,33] required for ER-to-Golgi trafficking and subsequent cleavage of the prodomain of TACE. More recently, it was shown that iRhom2 regulates TACE throughout its lifecycle, and remains stably associated with TACE all the way through the secretory pathway[34]. Thus, overexpression of TACE alone in transfected HEK293 cells could be expected to result in a relative iRhom deficiency and a failure of the majority of cellular TACE to traffic and mature normally.

To further substantiate the idea that Hck-activated EV export of TACE was not iRhom-regulated we examined the effects of RNAi-mediated knock-down of iRhom2 expression as well as overexpression of iRhom2 together with TACE. We found that despite a potent suppression of iRhom2 expression in shRNA-transduced cells compared to the parental HEK293 cells, secretion of TACE into EVs was equally robustly activated by Hck in both cells (Figure 3A).

To overexpress iRhom2 we co-transfected the HEK293 cell with a vector for iRhom2-eGFP. Of note, this vector has been previously shown to encode a functional iRhom2

protein[35], and it is known that addition of multiple epitopes tags at the C-terminus of iRhom2 does not affect its function as a transport/maturation co-factor and regulator of TACE[34].

Co-transfection of the iRhom2-GFP vector led to robust iRhom2 overexpression, but instead of promoting export of TACE into EVs, this resulted in a striking loss of TACE from the EVs (Figure 3B). Overexpression of iRhom2 caused no apparent change in the proteolytic maturation or glycosylation pattern (*i.e.* electrophoretic mobility) of TACE, suggesting that proper trafficking of TACE through the trans-Golgi network might require additional co-factors whose expression in HEK293 cells become limiting in this setting.

In any case, we conclude that tyrosine phosphorylation-regulated uploading of TACE into vesicles is active when TACE is transfected alone, whereas co-expression of iRhom2 directed TACE to a different fate. On the other hand, export of TACE into EVs is not affected by potent knock-down of iRhom2 expression. Thus, Hck-activated EV secretion provides an iRhom-independent, alternative exocytosis pathway for TACE.

EV secretion of TACE is independent of anterograde transport from the ER to the Golgi

The hypoglycosylated status of TACE uploaded into the EVs, and the independence of this process from the TACE trafficking co-factor iRhom suggested involvement of an unconventional secretion pathway. To test this idea, we treated the cells with brefeldin A (BFA), an inhibitor of the guanine nucleotide exchange factor GBF1[36] that has been widely used to inhibit protein transport from ER to Golgi.

We found that BFA treatment failed to prevent EV secretion of TACE triggered by Nef-mediated Hck activation in stably Hck-expressing cells (Figure 4A), although it efficiently inhibited trafficking of the multivesicular body (MVB)-derived exosome marker LAMP-1 into vesicles (Figure 4B). Of note, EV secretion of LAMP-1 was constitutive, and not positively or negatively regulated by Hck activation.

To ensure that the failure of BFA to block export of TACE into EVs was not due to an incomplete pharmacological effect we also tested a 10-fold higher concentration (2 μg/ml) of BFA and obtained identical results (Figure S1A). In addition, we verified the continued effectiveness of 200 ng/ml BFA treatment during the entire EV harvesting period by using confocal fluorescent microscopy to confirm a complete absence of normal Golgi morphology in these cells (Figure S1B).

Secretion of LAMP-1 into extracellular vesicles was also blocked by tunicamycin (an inhibitor of N-glycosylation) and significantly reduced by thapsigargin (an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase), two agents that disturb ER function and induce ER stress[37] (Figure 4B). By contrast, Hck-activated EV secretion of TACE was not affected by treatment of the cells with tunicamycin or thapsigargin (Figure 4A). Thus, we conclude that secretion of TACE into extracellular vesicles is mediated by an unconventional pathway that is not affected by ER dysfunction and does not involve anterograde vesicular transport of proteins from ER to Golgi, a key step in canonical protein secretion.

Of note, whereas blocking of conventional ER to Golgi-mediated secretion did not abolish vesicle secretion of TACE, it resulted in a specific disappearance of the proteolytically matured 80 kDa TACE species from the EVs (Figure 4A). This indicates that in order for unconventionally secreted TACE to be activated by prodomain removal it must meet at some point along its way with furin or a furin-like protease that is trafficked via the conventional secretion pathway.

Inducible EV secretion of TACE is not mediated by ER stress

A feature shared by many different types of unconventional protein secretion is that these pathways are activated by ER stress[13]. Therefore, whereas ER malfunction/stress did not prevent tyrosine kinase-activated EV

secretion of TACE, it is also important to note that in the absence of Hck activation (when TACE was transfected without Nef) BFA, tunicamycin, or thapsigargin treatment alone did not induce secretion of TACE into vesicles without Hck activation (Figure 4A).

To confirm that treatment with these compounds did indeed induce ER stress we monitored changes in XBP1 mRNA splicing in these cells. As shown in Figure 4C, BFA and thapsigargin activated XBP1 mRNA splicing, an established outcome and reporter of ER stress[37]. By contrast co-transfection of TACE together with Hck did not result in a change in XBP1 mRNA splicing. Thus, we conclude that induction of ER stress does not trigger unconventional secretion of TACE, and conversely, tyrosine phosphorylation-induced EV secretion of TACE was not associated with ER stress.

Hck-induced EV secretion of TACE is GRASP55-dependent

Many unconventional protein secretion pathways have been found to involve the GRASP-family proteins[21]. We therefore used the CRISPR/Cas9 technology to knock out GRASP55 expression in HEK293 cells. In contrast to control cells the GRASP55-KO cells failed to secrete TACE into EVs upon overexpression of Hck activity (Figure 5A). The failure to upload TACE into EVs was not associated with obvious abnormalities in the subcellular localization, as a very similar TACE immunostaining pattern with an extensive co-localization with a fluorescent ER marker protein was seen both in wild-type and GRASP55-KO HEK293 cells (Figure S2).

Endogenous GRASP65 protein was abundantly expressed both in control and GRASP55-KO cells, indicating that these two GRASP proteins serve non-redundant roles regarding inducible protein secretion into EVs. Indeed, when similar knock-out experiments were carried out to examine the role of GRASP65, only a very modest or no reduction in Hck-activated EV export of TACE was observed (Figure 5B).

In addition, we found that overexpression of GRASP55 but not GRASP65 could efficiently trigger EV secretion of TACE also in the absence of Hck activation (Figure 5C). Thus, while the mechanistic role of GRASP proteins in this process remains to be further elucidated, we conclude that similar to previously reported other unconventional secretion pathways[21], tyrosine kinase-activated secretion of TACE into extracellular vesicles requires GRASP55.

TACE-containing EVs are produced by an nSmase2-dependent exosome pathway

The two principal classes of EVs are named exosomes and ectosomes. Exosomes are derived from intraluminal vesicles of multi-vesicular endosomes (MVE) following fusion of the MVE with the plasma membrane, whereas ectosomes are shedding vesicles that assemble and bud at the plasma membrane[38]. Ceramide is involved in MVE formation, and since the original study by Trajkovic *et al*[39] the inhibition of neutral sphingomyelinase-2 (nSmase2) to block ceramide synthesis has been widely used to interfere with exosome production. By contrast, nSmase2 inhibition does not prevent and can instead enhance the production of ectosomes[40].

In order to characterize the origin of the EVs involved in Hck-activated vesicle secretion of TACE we generated knock-down derivatives of HEK293 cells in which nSmase2 expression had been suppressed by lentivirally delivered shRNAs. We found that similar to the spontaneous EV secretion of LAMP-1, Hck-activated packaging of TACE into EVs was almost completely blocked as a consequence of the Smase2 knock-down (Figure 6). By contrast, the EV association of HIV-1 Nef, a myristoylated membrane-targeted protein, was not at all affected by nSmase2 knock-down, suggesting that it was mainly associated with ectosomes.

Thus, we conclude that Hck-activates packaging of TACE into exosome-like vesicles that are derived from intraluminal vesicles of MVE rather than by budding directly from the plasma membrane.

DISCUSSION

Signal peptide-containing proteins are traditionally thought to reach their final destinations by trafficking via the classical ER to Golgi early secretory pathway[41]. However, secretion of transmembrane proteins via a Golgi-bypass mechanism has also been described, which together with pathways for secretion of cytoplasmic proteins is known as unconventional protein secretion (UPS)[12-14,42].

Our interest in understanding how HIV-1 Nef can stimulate packaging of the immune regulator TACE into extracellular vesicles has now revealed a novel tyrosine kinase-regulated UPS mechanism for TACE secretion. Characteristic of UPS, Hck-triggered delivery of TACE into EVs could not be blocked by brefeldin A, whereas this inhibitor of ER-to-Golgi transport effectively blocked incorporation of LAMP-1 into EVs. Also, compared to the cellular pool of TACE EV-resident TACE was distinctly hypoglycosylated, and did not change its electrophoretic mobility by a deglycosidase treatment.

Normal trafficking and proteolytic maturation of TACE depend on iRhom1/2, catalytically inactive members of the rhomboid-like superfamily that serve as chaperones or regulatory subunits controlling ER-to-Golgi transport as well as subsequent fate of TACE[34,43]. Thus, tyrosine phosphorylation-regulated UPS can provide an alternative, iRhom-independent secretion route for TACE. It is interesting to note that also previous examples of transmembrane proteins identified as clients of UPS are similarly restricted in moving from ER to Golgi because of the lack of a relevant binding partner or a mutation that interferes with folding. Specifically, under certain conditions *Drosophila* α-integrins are secreted via a Golgi-bypass mechanism without heterodimerization with a β subunit[44,45]. On the other hand, the most common cystic fibrosis-causing mutation ΔF508-CFTR results in ER accumulation because of impaired folding[15], which can be circumvented by activating trafficking to the plasma membrane via UPS[16]. Similarly, the predominant H723R mutation of pendrin (causing hearing loss and Pendred syndrome) leads to defects in folding and cell-surface expression of pendrin, which can be rescued by HSP70 co-chaperone DNAJC14-dependent UPS[46]. It should be noted, however, that certain transmembrane proteins may also use UPS as their regular mode of trafficking route[12,47,48].

While overall UPS-mediated trafficking of hypoglycosylated TACE into EVs was not prevented by brefeldin A treatment, such inhibition of ER to Golgi transfer caused a specific loss of proteolytically matured TACE species from the EVs. Removal of the TACE prodomain is catalyzed by the proprotein convertase furin[28,29], which is acting in the trans-Golgi network (TGN) and subsequent endosomal compartments[49]. Thus, in order for the prodomain cleavage to occur the Golgi-bypass UPS pathway utilized by TACE must converge with trafficking of furin in a post-Golgi MVB-like endosomal compartment where the extracellular vesicles containing proteolytically matured but hypoglycosylated TACE originate from. It would be interesting to examine whether this compartment is related to the syntaxin 13-containing endosomes involved in unconventional plasma membrane trafficking of CFTR described by Yoo *et al*[50]. Regardless of the identity of this vesicular compartment, the fact that furin can recognize and remove the prodomain of the hypoglycosylated TACE suggests that it has been properly folded. This is in agreement with the work of Adrain *et al.* who found that inactive TACE immunopurified from cells lacking iRhom could be converted to a fully active TNF-processing enzyme by removal of its prodomain with recombinant furin[27].

Similar to most[21] but not all[51] UPS pathways tyrosine kinase triggered EV secretion of TACE was found to be GRASP-dependent. TACE secretion was not observed in cells where GRASP55 was inactivated, and interestingly, could also be elicited in the absence of tyrosine kinase activation by forced GRASP55 overexpression. However, in contrast to most reported forms of UPS, including Golgi-bypass secretion of ΔF508-CFTR UPS[16], tyrosine phosphorylation-regulated routing of TACE from ER into EVs

was not triggered by ER stress. The effect of Hck activation could not be mimicked by compounds that induce ER stress by various mechanisms, and no ER stress could be observed in cells with activated Hck. Thus, Src family tyrosine kinase activation can trigger a distinct type of UPS response with similarities as well as differences compared to the previously characterized unconventional secretion pathways.

It will be interesting and informative to identify the specific substrate(s) of tyrosine phosphorylation that activate this novel UPS pathway. Our preliminary results indicate that the cargo, i.e. TACE itself, is not tyrosine phosphorylated (ZZ, unpublished). It has been previously shown that Hck activation can modulate Golgi function and affect glycosylation and trafficking of colony stimulating factor-1 receptor (M-CSFR/Fms), which was associated with phosphorylation of Erk1 at Tyr204 and GRASP65 at Ser277[52]. Src kinases can stimulate the MAPK pathway in several ways, including phosphorylation of residues Y340 and Y341 of Raf-1[53], which leads MEK1/2-mediated activation of Erk1/2[54]. On the other hand, Erk kinases are known to regulate Golgi dynamics via GRASP serine phosphorylation[55-57]. Thus, Erk kinases, especially the Golgi-associated Erk1c isoform[58], seem like good candidates for downstream mediators of Hck in activating EV secretion of TACE. In support of this possibility we observed that this process can be blocked using the specific MEK1/2 inhibitor U0126 (ZZ, unpublished).

Further molecular characterization and comparison of UPS triggered by Src family tyrosine kinases with other unconventional secretion pathways, as well as identification of other proteins secreted to EVs, plasma membrane, or extracellular space via phosphotyrosine-regulated UPS are all areas that would need additional clarification. The importance of TACE in regulating inflammation and immunity, the ubiquitous involvement of Src family kinases in the development of cancer, as well as the emerging roles of extracellular vesicles in normal physiology and different pathological conditions suggest that such studies would be worthwhile topics for future research.

MATERIALS AND METHODS

Reagents and antibodies

The details and vendors of the reagents and antibodies used in this study were the following. 1,10-phenanthroline, tunicamycin, thapsigargin and brefeldin A (Sigma Aldrich); Endoglycosidase H (Endo H) and Peptide-N-Glycosidase F (PNGase F) (New England Biolabs); protease and phosphatase inhibitor (Thermo Fisher Scientific). Mouse anti-GAPDH (MAB374, EMD Millipore), mouse anti-Myc (SC-40, Santa Cruz Biotechnology), mouse anti-TACE cyto-domain (MAB2129, R&D systems), rabbit anti-TACE pro-domain (ab39161, Abcam), rabbit anti-paxillin (SC-5574, Santa Cruz Biotechnology), mouse anti-pY31 paxillin (BD Biosciences), rabbit anti-Hck (SC-72, Santa Cruz Biotechnology), mouse anti-α-tubulin (T9026, Sigma-Aldrich), rabbit anti-GRASP65 (ab174834, Abcam), rabbit anti-GRASP55 (ab209338, Abcam), sheep anti-Nef (Targeted Affinity Oy, Helsinki, Finland), rabbit anti iRhom2 Antibody (PA5-48602, Invitrogen). Streptavidin IRDye680CW, Streptavidin IRDye800CW, IRDye680CW goat anti-mouse IgG and IRDye800CW goat anti-rabbit IgG were from LI-COR Biotechnology. Alexa Fluor 488 secondary antibody and ProLong™ Glass Antifade Mountant with NucBlue™ Stain were from Invitrogen.

Plasmids

pRK5M-TACE was from Rik Derynck (Addgene plasmid # 31714), and TACE K213N/R214G, E406A mutations were created to the same vector background. pmCherry-N1-GalT was from from Lei Lu (Addgene plasmid # 87327). HIV-1(SF2) Nef (P03407) coding sequence was cloned into pEF vector (Invitrogen). Mouse iRhom2, human GRASP55, GRASP65, and LAMP-1 cDNAs were obtained from Genome Biology Unit core facility (University of Helsinki, Finland). iRhom2 was cloned into pEGFP-N1 vector; GRASP55, GRASP65, LAMP-1 and paxillin cDNAs were cloned into pEBB vector[59] with a C-terminal myc-tag. To create the pWPI-puro vector we replaced EGFP in pWPI-EGFP (from Didier Trono; Addgene plasmid # 12254) with the puromycin resistance gene. Human Hck p59 cDNA (isoform b / NP_001165604) was cloned into pEBB and pWPI-puro vectors. We added an N-terminal HA-tag with Hck, created an Hck K269N mutation or added a longer N-terminus corresponding to Hck p61 (isoform a / NP_002101). Constitutively active mutants of human Hck p59, Lyn, Lck, Fyn and Fgr were generated by changing their C-terminal Csk target tyrosine residues (corresponding to Y530 in c-Src) into a phenylalanine, and the mutant forms were cloned into pEBB together with a 123 aa biotin acceptor domain fused to their C-termini[60]. gRNA expression plasmids for GRASP55 and GRASP65 knock-out, and shRNA expression plasmids for iRhom2 and nSMase2 knock-down were from Biomedicum Functional Genomics Unit (University of Helsinki, Finland) and CAG-Cas9-T2A-EGFP-ires-puro was from Timo Otonkoski (Addgene plasmid # 78311). The ER marker mCherry-KDEL was generated by replacing the BFP of Addgene plasmid #49150 from Gia Voeltz with mCherry. All mutants were created by standard overlap PCR mutagenesis. All plasmid constructs were verified by DNA sequencing or restriction enzyme digestion.

GRASP knock-out cells

HEK293 cells were maintained in high-glucose DMEM (Sigma), supplemented with 10% FBS, 0.05 mg/ml penicillin and 0.05 mg/ml streptomycin (cell culture media). Cells were plated in 6 well plates for around 80% confluence after 24 hours. Cells were co-transfected with 0.5 µg Cas9 plasmid and 1.5 µg pKLV-puro plasmid containing gRNA 5'-CCTTTCTTTGATTTTATTGTTTC-3' targeting the second exon of the human GRASP55 gene (NM_015530) or gRNA (5'-GAGAAATTCCCGACACGTCCAGG-3') expression pKLV-puro plasmid targeting the eighth exon of human GRASP65 gene (NM_031899) in Opti-MEM medium (Gibco) with 6 µg polyethylenimine (PEI). In general, µg DNA:µg PEI was 1:3 in total 50 µl of Opti-MEM per well. 48 hours post-transfection, cells were selected with 6 µg/ml puromycin for 2 days, then diluted and seeded in 96-well plates for single cell selection.

Lentiviral transduction of HEK293

HEK293 cells were cultured in 6 well plates and co-transfected with 2.5 µg pDelta8.9, 1.5 µg VSV-G, 3 µg pWPI-puro plasmid containing human Hck-p59, iRhom2 or nSMase2 shRNA expression pLKO.1 plasmid in Opti-MEM medium with 12 µg PEI. After 5 hours, medium was refreshed with cell culture media. After 48 hours, medium was collected and purified via 0.22 µm filter, then added to fresh HEK293 cells. After 24 hours, transduced cells were selected with 6 µg/ml puromycin for 2 days.

Deglycosylation analyses

HEK293 cells were collected and lysed on ice for 10 minutes in lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.4; 1% NP40, containing 10 mM 1, 10-phenanthroline, protease and phosphatase inhibitor cocktail), and centrifuged at 16,000g at 4°C to obtain post-nuclear supernatants. Subsequently the lysates were denatured at 90°C and treated with Endo H or PNGase F according to the manufacturer's instructions.

Isolation of EVs

48 hours post-transfection, 12 ml supernatants were collected and centrifuged for 10 min at 1,000 g, 30 min at 10,000 g and ultra-centrifuged for 90 min at 100,000 g. Pellets were resuspended in 100 µl 1 x SDS PAGE sample buffer as EV preparations. For N-glycosylation study, EV pellets were resuspended in 10 ml PBS and ultra-centrifuged again at 100,000 g for 90 min, and solubilized in 30 µl lysis buffer for further de-glycosylation analysis.

XBP1 splicing

Total cellular RNA was isolated using the RNeasy Mini kit (Qiagen), including DNase digestion (RNase free DNase kit; Qiagen). 500 ng of total RNA was transcribed to cDNA using the TaqMan reverse transcriptase kit (Applied Biosystems). Subsequently, 1µl of diluted cDNA template (20 ng) was mixed with 200 *µM* of each dNTP, 300 n*M* forward (5´-TTA CGA GAG AAA ACT CAT GGC C-3´) and reverse (5´- GGG TCC AAG TTG TCC AGA ATG C -3´) primers, 5 *µ*l 10 × optimized DyNAzyme buffer (F-511, Thermo), 1 *µl* DyNAzyme II DNA polymerase (Thermo), and nuclease-free water to a total volume of 50 *µl.* The PCR cycle starts with a 1 min incubation at 94 °C, then 35 cycles for 1 min at 94°C, 1 min at 54 °C, and 1 min at 72 °C; this is followed by a final incubation at 72 °C for 10 min. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by Midori Green Advance DNA staining (Nippon Genetics Europe).

Immunoblots

SDS gel electrophoresis and Western blotting were carried out following standard procedures and using IRDye-labeled detection reagents. The blots were visualized and the signals quantified using the Odyssey infrared imaging system and Image Studio v3.1. software (LI-COR Biosciences, Lincoln, NE, USA). All protein blotting results shown are representative of at least three comparable independent experiments.

Microscopy

Cells were plated on 13mm #1.5 coverslips 24h before transfection. After indicated times the cells were fixed with 4% PFA for 15min, washed two times with PBS+0.2% BSA and permeabilized with 0.2% for 10min followed by washing two times with PBS+0.2% BSA. The cells were stained with c-Myc Antibody (sc-40) for TACE in PBS containing 0.2% BSA at 37°C for 45min. Cells were washed and stained with secondary antibody Alexa Fluor 488 (Invitrogen) as described above. After washing the cells, the coverslips were mounted on a microscope slides with ProLong™ Glass Antifade Mountant with NucBlue™ Stain (Invitrogen). The cells were visualized under Zeiss LSM 780 Confocal microscope.

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FIGURE LEGENDS

Figure 1. SFK activation induces TACE secretion into EVs. (A.) HEK293 cells were transiently transfected with TACE, paxillin, dominantly active Hck, Lyn, Lck, Fyn or Fgr. Lysates of the transfected cells (Cell) and EV preparations (EV) isolated from their culture supernatants were subjected to Western blotting using the indicated antibodies. The TACE antibody used was against the C-terminal tail. The SFKs were fusion proteins containing a biotin acceptor domain and were detected using labeled streptavidin. Even loading of EV preparations was demonstrated by probing the blots with an anti-tubulin antibody. **(B.)** Mock-transduced control cells (Stable Hck: -) or HEK293 cells stably expressing native Hck-p59 (Stable Hck: +) were transfected with TACE, Nef, and paxillin as indicated, and analyzed as in A. **(C.)** HEK293 cells were transfected with TACE and Hck-p59, catalytically inactive mutant of Hck-p59 (KD), N-terminally HA-tagged Hck-p59 (N-HA), or Hck-p61, and analyzed as in A. Quantification of the relative intensities of the specific protein signals are indicated under the blots.

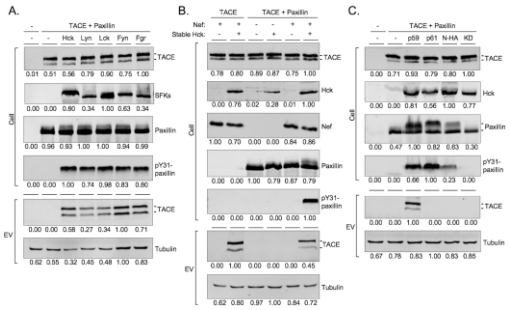

Figure 2. Characteristics of TACE species found in cell lysates and in EVs. (A.) Cell lysates and EVs preparations from cells co-transfected with TACE and Hck-p59 were deglycosylated with Endo H (H), PNGase F (F) or left untreated (c) and examined by Western blot analysis using antibodies targeting the C-terminal tail (α-C-term.) or the N-terminal prodomain (α-prodom.) of TACE. The deduced prodomain composition and glycosylation status of the observed 130 kDa, 105 kDa, and 80 kDa species of TACE (indicated by arrows A, B, and C, respectively) are illustrated on the right. Of note, unglycosylated prodomain-containing TACE ("Species B") and mature TACE (glycosylated in a Golgi-specific manner and lacking the prodomain; "Species B*") co-migrate within the band indicated by arrow B. **(B.)** Cell lysates and EVs preparations from cells transfected with Hck-p59 alone (-) or together with wild-type (WT) or K213N/R214G (FM) or E406A (CD) mutants of TACE were examined by Western blot analysis using antibodies against the C-terminus or the prodomain of TACE. Even loading of cell lysates and vesicle preparations was demonstrated by probing the blots with an anti-tubulin antibody. Quantification of the relative intensities of the specific protein signals are indicated under the blots.

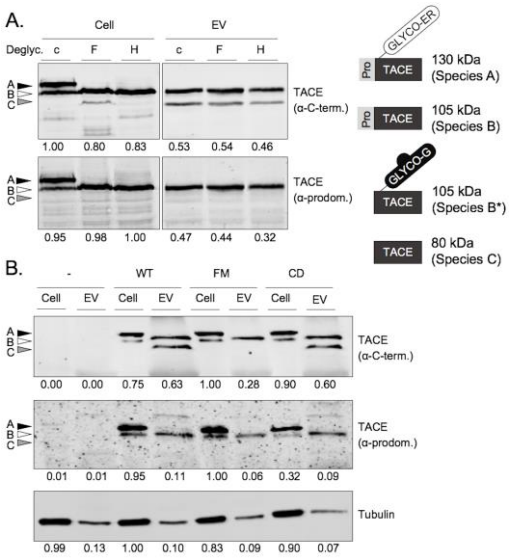

Figure 3. TACE vesicle secretion is independent of ER to Golgi trafficking and ER stress. (A.) HEK293 cells stably expressing native Hck-p59 were transfected with TACE alone or in combination with HIV-1 Nef, a potent allosteric activator or Hck. After 5 h the transfected cultures were treated with 4 μg/ml tunicamycin (Tun), 200 ng/ml brefeldin

A (BFA), 500nM thapsigargin (TG) for 36 h or left untreated (-), followed by Western blotting analysis of TACE and Nef expression in lysates of these cells, and TACE in EVs in their culture supernatants. **(B.)** Stably Hck-p59 expressing cells were transfected and examined exactly as in Figure 3A, except that instead of TACE now LAMP-1 was transfected and detected by Western blotting. **(C.)** HEK293 cells were transfected with TACE and/or Hck, and starting at 5 h post-transfection treated for 24 h with brefeldin A or thapsigargin, or left untreated before lysates of these cells were analyzed by Western blotting for the expression of transfected TACE and Hck, and endogenous GAPDH proteins, and examined by RT-PCR for ER stress-associated alternative splicing of a short intron in XBP1 mRNA, which is indicated by the appearance of a 255 bp RT-PCR product (black arrow) and disappearance of the 257 bp product (white arrow). Quantification of the relative intensities of the specific protein signals are indicated under the blots.

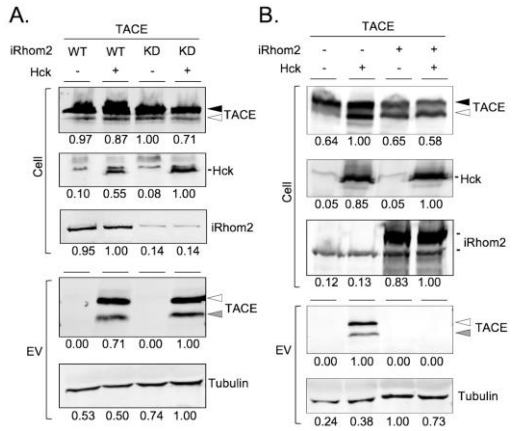

Figure 4. Hck-induced TACE secretion into EVs involves GRASP55. (A. and B.) TACE and/or Hck-p59 were transfected into wild-type (WT), GRASP55 knock-out (KO) or GRASP65 knock-out (KO) HEK293 cells as indicated. Lysates of these cells were analyzed by Western blotting for the expression of endogenous GRASP55 and GRASP65 and for transfected TACE and Hck proteins. EVs secreted by these cells were examined for their TACE content. **(C.)** TACE was transfected into HEK293 alone (-) or together with Myc-tagged GRASP55 or GRASP65. Lysates of these cells were analyzed for the expression of the transfected TACE, Hck, and GRASP65/55 proteins, and the EV preparations from these cultures were probed with antibodies against TACE and tubulin. Quantification of the relative intensities of the specific protein signals are indicated under the blots.

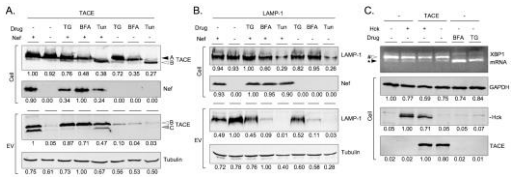

Figure 5. Hck-activated export of TACE into EVs is not mediated by iRhom2. (A.) TACE was transfected alone (-) or together with Hck (+) into wild-type (WT) or iRhom2 knock-down (KD) HEK 293 cells as indicated. Lysates of these cells were analyzed by Western blotting for the expression of endogenous iRhom2 and for the transfected TACE and Hck proteins. EVs secreted by these cells were examined for their TACE content. **(B.)** TACE was transfected alone or in combination with Hck and/or iRhom2 as indicated. Lysates of the transfected cells were examined by Western blotting to confirm proper TACE, Hck, and iRhom2 expression. EV preparations from these cells were blotted with antibodies against TACE (anti-C-terminus) or tubulin as a vesicle protein loading control. Quantification of the relative intensities of the specific protein signals are indicated under the blots.

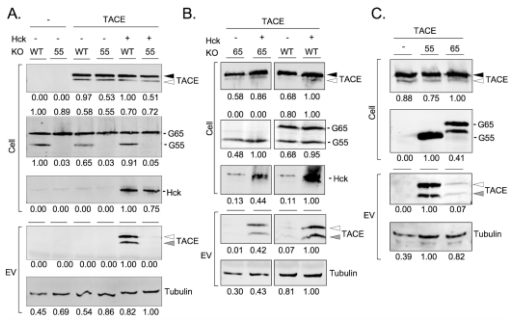

Figure 6. Production of TACE-containing vesicles requires nSmase2 activity. Wild-type (WT) and nSMase2 knock-down (KD)

HEK293 cells were transfected with TACE plus Hck, myc-tagged LAMP-1, or GFP-tagged Nef as indicated. Lysates of these cells were analyzed by Western blotting for expression of the transfected proteins. EV preparations from these were examined for their TACE, LAMP-1, or Nef content, and for tubulin as a vesicle protein loading control. Quantification of the relative intensities of the specific protein signals are indicated under the blots.

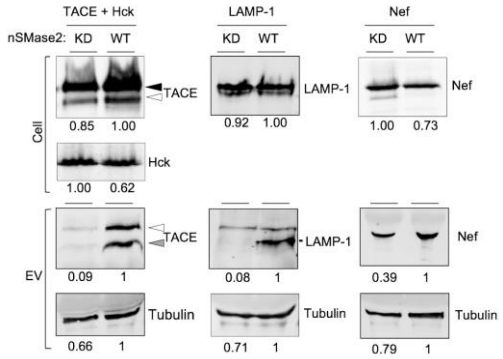

Figure S1. BFA-resistance of Hck-activated export of TACE is not due to an incomplete pharmacological effect of BFA. (A.) TACE and/or Hck-p59 were transfected into wild-type HEK293 cells as indicated. After 5 h the transfected cultures were supplemented with 0.2 µg/ml or 2 µg/ml BFA for 24 h or left untreated (-), followed by Western blotting analysis of TACE and Hck expression in these cells, and TACE in the EVs in their culture supernatants. Quantification of the relative intensities of the specific protein signals are indicated under the blots. (B.) HEK293 cells were cotransfected with TACE and a vector encoding a red fluorescent Golgi marker (β1,4-galactosyltransferase fused to mCherry; GalT-mCherry). After incubation for 24 h with 0.2 µg/ml of BFA (or no treatment) the Golgi morphology in the transfected cells was examined by confocal immunofluorescence microscopy imaging (red signal). The nuclei were visualized using NucBlue stain (blue signal). A scale bar of 10 µm is shown.

Figure S2. TACE is localized predominantly in the ER both in wild-type and in GRASP55-deficient HEK293 cells. Parental (WT) and GRASP55 knock-out (KO) HEK293 cells were transfected with the ER marker mCherry-KDEL and with Myc-tagged TACE. 24h post-transfection the cells were fixed and examined by confocal fluorescence microscopy imaging for TACE immunostaining (anti-TACE; green) and ER marker fluorescence (mCherry-KDEL; red). An overlay of these signals is shown in the bottom panel (Merge; yellow). A scale bar of 5 µm is shown.