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Stimulated release and functional activity of surface expressed metalloproteinase ADAM17 in exosomes

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ABSTRACT

By mediating proteolytic shedding on the cell surface the disintegrin and metalloproteinases ADAM10 and ADAM17 function as critical regulators of growth factors, cytokines and adhesion molecules. We here report that stimulation of lung epithelial A549 tumor cells with phorbol-12-myristate-13-acetate (PMA) leads to the downregulation of the surface expressed mature form of ADAM17 without affecting ADAM10 expression. This reduction could not be sufficiently explained by metalloproteinase-mediated degradation, dynamin-mediated internalization or microdomain redistribution of ADAM17. Instead, surface downregulation of ADAM17 was correlated with the presence of its mature form in exosomes. Exosomal ADAM17 release was also observed in monocytic and primary endothelial cells where it could be induced by stimulation with lipopolysaccharide. Antibody-mediated surface labelling of ADAM17 revealed that at least part of exosomal ADAM17 was oriented with the metalloproteinase domain outside and had been expressed on the cell surface. Suppression of iRHOM2-mediated ADAM17 maturation prevented surface expression and exosomal release of ADAM17. Further, deletion of the protease's C-terminus or cell treatment with a calcium chelator diminished exosomal release as well as surface downregulation of ADAM17, underlining that both processes are closely associated. Co-incubation of ADAM17 containing exosomes with cells expressing the ADAM17 substrates TGFα or amphiregulin lead to increased shedding of both substrates. This was prevented when exosomes were prepared from cells with shRNA-mediated ADAM17 knockdown. These data indicate that cell stimulation can downregulate expression of mature ADAM17 from the cell surface and induce release of exosomal ADAM17, which can then distribute and contribute to substrate shedding on more distant cells.

1. Introduction

Surface expressed proteases of the a disintegrin and metalloproteinase (ADAM)-family function as critical regulators of physiological and pathophysiological processes. ADAM10 and ADAM17 are the most prominent members of this family. Their importance has been evidenced recently by genetic data from humans as well as by various gene knockout studies. Rare loss of function mutations within the human ADAM17 gene have been described leading to disruption of the epithelial barrier and to recurrent infections [4]. Mice with genetically inactivated ADAM17 die rapidly after birth, and mice with ADAM10 deficiency die early during embryogenesis [18,29].

To a large extent the function of the proteases has been attributed to their ability to mediate proteolytic shedding of other surface molecules. The main substrates of ADAM17 are growth factors such as TGFα and the proinflammatory cytokine TNF while the most important ADAM10 substrate may be Notch controlling cell fate in development [18,29]. These substrates are expressed on the cell surface and their activity critically depends on their proteolytic cleavage. Thus, in many aspects the knockout of the protease resembles the phenotype of the substrate molecule knockout. However, many more cleavage events have been found to depend on ADAM10 or ADAM17 or both [8].

In vitro, several ADAM10 dependent cleavage events show a high degree of constitutive substrate release indicating that the protease is active under normal cell culture conditions in various cell types. By contrast, ADAM17 dependent shedding appears much lower in resting cells under normal culture conditions. However, the shedding can be rapidly increased upon cell stimulation. The phorbolester PMA is one of the most potent and effective inducers of ADAM17 activity leading to

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enhanced shedding of various substrates including TNF, TNF receptor, TGFα, amphiregulin, IL-6 receptor, L-selectin, the transmembrane chemokine CX3CL1, junctional adhesion molecule JAM-A, the proteoglycans syndecan-1 and −4 and many more [2,3,12,21,29,31]. Other inducers of ADAM17-mediated shedding include ligands of G-protein coupled receptors, cytokines and bacterial toxins such as lipopolysaccharide (LPS) [13,34].

Various mechanisms have been described to contribute to the regulation of ADAM17 activity [7]. Gene induction may occur in various cell types but this cannot explain the rapid enhancement of ADAM17 activity. The rhomboid family member 2 (rhbdf-2/iRhom2) has been demonstrated to mediate transport of the ADAM17 prodomain from the ER to the Golgi network where the proteases prodomain is removed via a proprotein convertase, e.g. furin [1,25]. However, this cleavage seems to occur to large degree in a constitutive manner after exit from the endoplasmic reticulum and during transit through the Golgi network and short term cell stimulation does not seem to enhance this conversion [37]. Possibly cell stimulation can affect the membrane localization as indicated by the finding that ADAM17 is localized to lipid rafts [43]. Membrane fluidity may therefore represent a critical factor for regulation of ADAM17 activity [33]. For ADAM10 shedding of the protease by ADAM9 and ADAM15 has been described as another mechanism by which the protease activity can be modulated [46]. Moreover, the protease can be shifted from the cell membrane into the extracellular compartment by the release of membrane vesicles, which has been observed for ADAM10 [40] and ADAM15 [22]. For ADAM10 it has been shown that the protease on microvesicles can still exert shedding functions [20]. These microvesicles have been identified as exosomes by means of their characteristic density (1.12–1.19 g/ml) and exosomal marker expression (e.g. CD9) [16,44]. Exosomes can be produced by numerous cell types in vitro and have been found in various body fluids including bronchoalveolar fluid of the lungs especially under pathologic conditions [47].

Structurally, different domains of ADAM17 may be involved in the regulation of the protease. Several phosphorylation sites were identified within the cytoplasmic tail of ADAM17 and removal of the phosphorylation sites has been found to suppress stimulation of ADAM17 activity [38]. Interestingly, deletion of the whole cytoplasmic tail of ADAM17 does not prevent activation of ADAM17 in response to stimulation with PMA [6,32]. Recently, also the extracellular domains of ADAM17 have been shown to undergo conformational changes that regulate the proteases activity [9,10]. Besides these activating mechanisms there also exist early reports that cell stimulation with PMA leads to degradation of cell expressed ADAM17 despite the fact that shedding events via the protease are enhanced [5,11].

We here report that stimulation of lung epithelial tumor cells with PMA leads to the loss of the mature ADAM17 variant but not ADAM10 on the cell surface. This could not be sufficiently explained by dynamin-mediated internalization or proteolytic degradation. Instead, mature ADAM17 was released in exosomes when cells were stimulated with PMA or LPS. Further, when exosomal ADAM17 release was suppressed no surface downregulation of ADAM17 was observed. Finally released exosomes showed ADAM17-dependent shedding activity. The data suggest that cell stimulation can shift the cell expressed mature form of ADAM17 into exosomes and by this ADAM17 activity can be distributed to reach more distant substrates.

2. Materials and Methods

2.1. Antibodies and reagents

For flow cytometry unconjugated and PE-coupled mouse monoclonal antibodies (mab) to ADAM17 ectodomain (# 111,633) and mab to ADAM10 ectodomain (# 163,003), mouse IgG_{2b}, IgG_1 and allophycocyanin (APC)-coupled IgG_1 isotype controls, and normal rabbit IgG were obtained from R&D Systems (Wiesbaden, Germany).

For western blotting mouse polyclonal antibody to ADAM8 (C-terminus), rabbit polyclonal antibodies to ADAM17 (C-terminus) and ADAM10 (C-terminus) were from Millipore (Darmstadt, Germany). Mouse mab to ADAM17 ectodomain used for detection of N-terminal fragments by Western blotting has been described previously. Rabbit polyclonal antibodies to HA-tag (Y11) and to RHBDF2 (N-terminal) were from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse mab to β-actin was from Abcam (Cambridge, USA). Rabbit polyclonal antibody to human dynamin I/II and rabbit mab to CD71 (D7G9X) were from Cell Signaling (Boston, USA). Mouse mab to flotillin-1 (Clon 18) was from BD Biosciences (Heidelberg, Germany). Rabbit polyclonal antibody to Hsp70 was from System Biosciences (California, USA). Mouse mab to CD9 (Ts9), CD63 (Ts63) and mouse mab to GAPDH (GA1R) were from Thermo Scientific (Waltham, MA USA). Peroxidase (POD)-conjugated secondary antibodies were from Jackson ImmunoResearch (Hamburg, Germany). The metalloproteinase inhibitor GI254023 was synthesized and characterized as preferential ADAM10 inhibitor [19,24]. Phorbol-12-myrastat-13-acetat (PMA), and lipopolysaccharide (LPS) were from Sigma-Aldrich (Steinheim, Germany). Recombinant human LPS binding protein (LBP) Protein was from R&D Systems (Wiesbaden, Germany). TAPI-1 was from Merck (Darmstadt, Germany). Dynasore and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) were from Tocris Bioscience, (Bristol, UK). The siRNA targeting dynamin was from Santa Cruz Biotechnology.

2.2. DNA constructs

Short hairpin RNA (shRNA) targeting ADAM10 or ADAM17 was inserted into the lentiviral expression vector pLVTHM as described [31]. shRNA targeting human iRhom2 was inserted into pLKO-1 system (Sigma Aldrich, Munich Germany, clones TRCN0000048687 and TRCN0000048685). The pcDNA3.0 expression vector for murine ADAM17 was from addgene plasmid repository (Cambridge, MA, USA). A sequence coding for a hemagglutinin tag followed by a stop codon was inserted into the mADAM17 cDNA at base pair position 2082 via site directed mutagenesis to generate a C-terminal truncation variant [23].

2.3. Cell culture and transfection

The alveolar lung carcinoma epithelial cell line A549, the epithelial breast cancer cell line MDA-MB231, the human embryonic kidney cell line HEK293 and the human monocytic leukemia cell line THP-1 were cultured in DMEM medium with 10% FCS and antibiotics as described [30,31]. The isolation and culture of human umbilical vein endothelial cells (HUVECs) were performed as described [21].

Transient transfection with siRNA or expression vector pcDNA3.0 was carried out with lipofectamine (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. For expression of shRNA recombinant lentiviruses were produced as described [31]. For transduction 1×10^5 A549 cells were seeded into six-wells, and concentrated lentivirus preparation (5 μl) was added after 24 h To enhance the transduction efficiency polybrene (8 μg/ml, Sigma) was added.

2.4. Cell stimulation

Cells were grown to confluence in fully supplemented medium for 48 h. For pretreatment with inhibitors cells were washed once with PBS and received serum-free medium with TAPI-1 (20 μM), GI254023 (10 μM), dynasore (100 μM), BAPTA-AM (30 μM) or 0.1% dimethylsulfoxide (DMSO). After 30 min, the cells were stimulated with PMA (200 ng/ml) or LPS (5 or 50 μg/ml) for the indicated periods of time. Conditioned media were harvested, supplemented with a protease inhibitor mixture (Complete, Roche, Manheim Germany) and