A Basolateral Sorting Signal Directs ADAM10 to Adherens Junctions and Is Required for Its Function in Cell Migration*

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ADAM10 (a disintegrin and metalloprotease) initiates regulated intramembrane proteolysis by shedding the ectodomain of a number of different substrates. Shedding is followed by subsequent intramembrane proteolysis leading to the liberation of intracellular domains capable of nuclear signaling. ADAM10 substrates have been found at cell-cell contacts and are apparently involved in cell-cell interaction and cell migration. Here we have investigated the cellular mechanism that guides ADAM10 to substrates at cell-cell contacts. We demonstrate that intracellular trafficking of ADAM10 critically requires a novel sorting signal within its cytoplasmic domain. Sequential deletion of the cytoplasmic domain and site-directed mutagenesis suggest that a potential Src homology 3-binding domain is essential for ADAM10 sorting. In a polarized epithelial cell line this motif not only targets ADAM10 to adherens junctions but is also strictly required for ADAM10 function in E-cadherin processing and cell migration.

The ADAM³ (a disintegrin and metalloprotease) family of metalloproteases is involved in ectodomain shedding of membrane-bound proteins such as cytokines, growth factors, and adhesion molecules that play a role in cell-cell contact formation, cell migration, and neurite outgrowth (1–4). The release of the extracellular domain of cell adhesion proteins, which contains the homophilic binding sites, disengages cell-cell contact and supports cell migration and proliferation. The inhibition of ectodomain shedding promotes fibroblast adhesion through stabilization of focal adhesion contacts, accompanied by increased cell surface levels of N-cadherin (5). Furthermore, for several ADAM substrates not only is the release of the ectodomain functionally relevant but the resulting membrane-bound C-terminal fragments are substrates for regulated intramembrane proteolysis (6, 7). In some cases these intracellular fragments are involved in signal transduction as has been shown for Notch1–4, N-cadherin, CD44, ErbB4, and the receptor protein tyrosine phosphatase κ (8–10). Regulation of intramembrane proteolysis is thought to occur at least in part at the level of ectodomain shedding, because shedding is known to be a prerequisite for the subsequent intramembranous cut (11, 12).

Specifically, ADAM10, which represents a major α-secretase activity (13), plays an important role in shedding of Alzheimer disease β-amyloid precursor protein (βAPP). Alzheimer disease is characterized by excessive deposition of amyloid β-peptide (Aβ) in the brain parenchyma. Aβ is generated from βAPP by proteolytic processing involving β- and γ-secretase (6). The competitive non-amyloidogenic pathway, where βAPP is initially cleaved by α-secretase within the amyloid domain, prevents Aβ generation (14). Overexpression of ADAM10 prevents amyloid pathology, whereas expression of a dominant negative variant enhances Aβ deposition in a transgenic mouse model (15). Thus ADAM10 is not only a major sheddase involved in signaling cascades but also an important therapeutical target in Alzheimer disease.

In previous work we have shown that β-secretase and βAPP are differentially sorted in polarized Madin-Darby canine kidney (MDCK) cells. Whereas β-secretase is targeted to the apical domain (16), βAPP is restricted to the basolateral plasma membrane (17). We now investigated the cellular mechanism allowing ADAM10 to interact with its substrates such as βAPP (13), E-cadherin (18), and CD44 (19), which are all located at the basolateral plasma membrane (17, 20, 21).

MATERIALS AND METHODS

cDNA Constructs—cDNA constructs encoding bovine ADAM10 were amplified by PCR and subcloned into the XhoI/HindIII site of the pcDNA3.1/Hygro(-) expression vector (Invitrogen). The C-terminal deletions of ADAM10 were created by the insertion of a stop codon after the indicated position using PCR. The HA tag was introduced by the reverse primers, and point mutations were introduced by site-directed mutagenesis (Stratagene).

Cell Culture—MDCK cells (strain II) were maintained as described (16). Stable transfections of MDCK cells were performed with Lipofectamine 2000 (Invitrogen). Cell lines expressing ADAM10 were selected with 400 µg ml⁻¹ Hygromycin. Cell lines with moderate expression levels were selected for the study. To obtain polarized monolayers of surface biotinylated cells, plates were plated and grown to confluence on 24-mm polycarbonate Transwell filter (Corning Costar Corp.). To

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³The abbreviations used are: ADAM, a disintegrin and metalloprotease; MDCK, Madin-Darby canine kidney; SH3, Src homology 3; βAPP, β-amyloid precursor protein; WT, wild type; HA, hemagglutinin.

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increase expression levels, cells were treated overnight with 10 mM butyrate. Similar results were obtained with untreated and treated cells.

Antibodies—The polyclonal antibody H-6908 (Sigma) was used for recognition of HA-tagged ADAM10 C-terminal deletion variants, whereas the monoclonal antibody HA11 (clone 16B12; Covance) was used for detection of HA-tagged ADAM10 WT and amino acid exchange variants. The polyclonal antibody directed to the C terminus of ADAM10 was purchased from Calbiochem. The monoclonal antibodies against β-catenin (clone 14) and the cytoplasmic domain of E-cadherin (C20820) were purchased from BD Transduction Laboratories. The anti-ZO-1 (rat monoclonal) was obtained from the Hybridoma Bank.

Immunocytochemistry and Confocal Imaging—MDCK cells grown on filters were washed with phosphate-buffered saline (PBS) containing 1 mM CaCl₂, 0.5 mM MgCl₂ (PBS⁻). For ADAM10 staining, cells were fixed in methanol (−20°C) for 5 min at −20°C. After extensive washing in PBS⁻ and blocking with 0.2% bovine serum albumin/PBS⁻, cells were incubated with the indicated first antibodies for 1 h at 37°C and then, after additional washing, with the fluorochrome-conjugated secondary antibody (Alexa 488, Alexa 555; Invitrogen). After extensive washing, filters were cut out and mounted in Mowiol. Confocal images were obtained with a Zeiss confocal laser scanning microscope.

Surface Biotinylation—Surface biotinylation was performed as described (16). Cells were lysed in 1% SDS containing lysis buffer and diluted to 0.1% SDS before precipitation with streptavidin-Sepharose (Amersham Biosciences). The precipitated, biotinylated proteins were separated on SDS gels and transferred to polyvinylidene difluoride membranes. For detection the indicated HA antibody was used. Specifically bound antibodies were visualized by a horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence technique (Amersham Biosciences). For quantification the chemiluminescence signals of three independent experiments were measured with a CD camera-based imaging system (Alpha Innotech).
Wound Healing Assay—Untransfected MDCK cells or cells stably transfected with ADAM10 WT or ADAM10 ΔC were seeded in six-well plates (Sarstedt) and cultured until cells just reached confluence (48 h). Three linear wounds were scratched in each dish of cells with a pipette tip (p200; Sarstedt). To avoid proliferation, cells were treated with 100 mM hydroxyurea for 24 h (Sigma-Aldrich). Cell motility was monitored at 6-h intervals by using an inverted phase-contrast microscope (Zeiss). The width of each wound was measured at three positions for the indicated time points.

RESULTS

To investigate the localization of endogenous ADAM10 we used a polarized cell line with distinct cell-cell contacts. MDCK cells form an epithelial-like monolayer when grown on a filter support. Apical and basolateral membrane domains are separated by tight junctions that are located close to the apical surface area of the cells. By immunofluorescence endogenous ADAM10 can be detected at the basolateral plasma membrane, primarily at cell-cell contacts (Fig. 1). Costaining for junction marker proteins revealed that ADAM10 predominantly localizes at adherens junctions (Fig. 1). Double stainings in cross-sections of confocal images show an overlap of ADAM10 with β-catenin but almost none with the tight junction marker ZO-1 (Fig. 1).

Next the domain required for basolateral targeting of ADAM10 was determined. Because basolateral sorting motifs are generally located within the cytoplasmic tail of transmembrane proteins, an ADAM10 cDNA construct missing the cytoplasmic domain was generated (Fig. 2A, ADAM10 ΔC). ADAM10 wild type (WT) and ADAM10 ΔC were stably expressed in MDCK cells, and single cell clones were analyzed. Surface biotinylation of polarized MDCK cells revealed a predominant basolateral distribution of ADAM10 WT, whereas ADAM10 ΔC was missorted to the apical surface (Fig. 2B). Basolateral sorting of ADAM10 WT and apical missorting of ADAM10 ΔC were confirmed by immunofluorescence. Consistent with apical missorting of ADAM10 ΔC, almost no colocalization with β-catenin was detected (Fig. 2C).

To identify the basolateral sorting signal, the cytoplasmic domain of ADAM10 was sequentially truncated (Fig. 3A). The deletion constructs of ADAM10 (Δ13, Δ24, Δ28, Δ33, Δ37) were stably transfected into MDCK cells, and surface biotinylation experiments were performed with single cell clones. Up to 28 amino acids of the cytoplasmic tail can be removed without affecting basolateral sorting (Fig. 3B). However, removal of five additional amino acids results in apical missorting of ADAM10 Δ33 (Fig. 3B). Thus, correct sorting of ADAM10 requires at least some amino acids within the LPGTL domain. We first investigated a putative function of the LPGTL sequence in basolateral sorting. Single amino acids of the LPGTL sequence were exchanged to alanine (Fig. 3C). The resulting constructs ADAM10-APGTL, -LAGTL, -LPATL, and -LPGAL were stably transfected into MDCK cells and cell surface biotinylation assay of MDCK cells expressing HA-tagged ADAM10 point mutants (ADAM10-APGTL, -LAGTL, -LPATL, and -LPGAL). Biotinylated proteins were recovered with streptavidin precipitation and detected by immunoblotting. Graphs below show the mean result of quantifications of at least three independent experiments ± S.D.

FIGURE 3. ADAM10 sorting requires two hydrophobic amino acids. A, schematic representation of ADAM10 C-terminal deletion constructs showing their amino acid sequence after the transmembrane domain. B, domain-selective cell surface biotinylation assay of MDCK cells. Stably transfected MDCK cells expressing ADAM10 deletion mutants were biotinylated from either the apical (api) or the basolateral (baso) side. Biotinylated proteins were recovered with streptavidin precipitation and after SDS-PAGE detected by immunoblotting using ant-HA antibodies. Graphs below show the mean result of quantifications of at least three independent experiments ± S.D. C, schematic representation of ADAM10 point mutations. The to-alanine mutated residue is underlined. D, domain-selective cell surface biotinylation assay of stably transfected MDCK cells expressing HA-tagged ADAM10 point mutants (ADAM10-APGTL, -LAGTL, -LPATL, and -LPGAL). Biotinylated proteins were recovered with streptavidin precipitation and detected by immunoblotting. Graphs below show the mean result of quantifications of at least three independent experiments ± S.D.
cells, and the expressed ADAM10 variants were analyzed for their cell surface distribution. Strikingly, the exchange of the first two amino acids of the analyzed stretch led to missorting of ADAM10 (Fig. 3D), whereas the exchange of other amino acids further C-terminal did not affect sorting of ADAM10 (Fig. 3D, ADAM10-LPGTA data not shown). Therefore, the LP motif is essential for basolateral sorting of ADAM10. This may suggest a di-hydrophobic amino acid sequence motif required for basolateral sorting as described previously for other proteins (21, 22). However, a potential Src homology 3 (SH3)-binding domain is adjacent N-terminal to the amino acid stretch analyzed (residues 708–715) (Fig. 4A). SH3-binding domains are defined by a proline core PXXP motif, accompanied by additional prolines or other hydrophobic residues (23). We therefore investigated the influence of the putative SH3-binding domain on polarized sorting of ADAM10. Single proline residues at the N terminus (P708A), within the center (P712A), and the C terminus (P715A) of the predicted SH3 binding region were exchanged to alanine as indicated in Fig. 4A. In addition, a double mutant (P708/712A) was investigated (Fig. 4A). Surface biotinylation experiments revealed that Pro708 and Pro715 are required for the basolateral sorting of ADAM10, but not Pro712 (Fig. 4B). ADAM10 containing the double substitution of P708/712A is apically missorted to the similar extent as the variant containing the single proline exchange at position 708 (Fig. 4B). Because the first and the last residue of the potential SH3-binding domain is required for basolateral transport of ADAM10, our data suggest a critical function of the SH3-binding domain in basolateral transport of ADAM10.

We next investigated the physiological importance of basolateral sorting of ADAM10. Wound healing in epithelia requires coordinated cell migration and proliferation regulated by signaling mechanisms that are poorly understood. Recently it has been shown that ADAM10 regulates cell-cell adhesion and migration in a human keratinocyte cell line (18). To investigate the role of the ADAM10 cytoplasmic domain for cell migration, we applied a classical wound healing assay. For this assay non-transfected MDCK cells and MDCK cells stably expressing ADAM10 WT or ADAM10 ΔC were used. Linear wounds were scratched in confluent monolayers, and wound closing was measured after 6 and 12 h (Fig. 5, A and B). Representative photographs of wound closure were taken (Fig. 5A), and the widths of the open wounds were measured (Fig. 5B). Expression of ADAM10 WT accelerated wound healing (Fig. 5, A and B). In contrast, the apically missorted variant ADAM10 ΔC did not affect wound healing (Fig. 5, A and B). Because it has been shown that ADAM10 is involved in E-cadherin processing (18) and E-cadherin is localized at cell-cell junctions, we next analyzed the effect of the ADAM10 ΔC sorting mutant on E-cadherin processing. Stable overexpression of ADAM10 WT enhances processing of E-cadherin as measured by the increased C-terminal E-cadherin fragments in cell lysates (Fig. 5C), whereas the expression of ADAM10 ΔC does not influence E-cadherin processing (Fig. 5C). Thus, these data support a central role of sorting of ADAM10 for its function in substrate processing and cell migration.

**DISCUSSION**

We have shown that ADAM10 is basolaterally sorted in polarized epithelial cells. Further, we identified within the cytoplasmic domain of ADAM10 certain proline residues of a putative SH3-binding domain (residues 708–715) that are essential for basolateral sorting. In addition, adjacent leucine and proline residues are critically required for the basolateral transport. ADAM10, like many members of the ADAM family, contains two putative SH3-binding domains (Fig. 4A). Sequential deletion of the cytoplasmic domain revealed that the more C-terminal located SH3-binding domain did not influence the basolateral localization of ADAM10. Site-directed mutation identified that proline residues within the juxtamembrane SH3-binding domain affected basolateral expression of ADAM10.

The first (Pro708) and the last (Pro715) prolines of the SH3-binding domain are critically required for correct sorting of ADAM10, whereas Pro712 is not. Proline-rich SH3 binding motifs contain a PXXP core and a positively charged amino acid. The positively charged amino acid could be located at either side of the PXXP core and determines the orientation of the ligand toward the SH3 protein (23). The two possible orientations of ligands are classified as class I (N terminus to C
Our findings are consistent with a role of ADAM10 in cell migration (18). ADAM10-induced cell migration might be mediated by enhanced E-cadherin shedding (18) as well as by shedding of other ADAM10 substrates that are also important for cell migration, such as CD44 or ephrins (19, 25, 26). Strikingly, expression of ADAM10 ΔC (as well as the ADAM10-LAGTL mutation; data not shown) failed to increase cell migration. In addition, we observed increased E-cadherin shedding only upon overexpression of ADAM10 WT but not upon expression of an ADAM10 sorting mutant.

Therefore, our data demonstrate that the motif is required for targeting of ADAM10 to adherens junctions and for the interaction with at least one of its substrates. For several ADAMs a role in modulation of cell-cell contacts and cell-matrix interaction has been suggested (2, 27). Of particular interest is the recent finding that ADAM10 is able to cleave substrates located on the membrane of opposing cells (26).

Consistent with these reports, we find that ADAM10 colocalizes with its basolaterally sorted substrates βAPP, CD44, and E-cadherin (17, 20, 21). ADAM10 colocalizes predominantly with proteins associated with adherens junctions but only poorly with the tight junction marker ZO-1.

ADAMs participate in signaling pathways through specific ectodomain shedding. In particular, ADAM10 cleaves Notch (28), N-cadherin (29), and the Eph receptor (30), which are all known to undergo subsequent intramembrane proteolysis followed by nuclear signaling. Furthermore, βAPP, CD44, and E-cadherin all undergo intramembrane proteolysis as well (6). Thus, sorting and transport of ADAM10 to adherens junctions may be required to initiate signaling pathways dependent on cell-cell contacts. This may also have consequences for Alzheimer disease therapy. Therapeutic strategies aiming to increase α-secretase activity should take enhanced cell migration and reduced cell-cell contacts into consideration. To circumvent problems with cell adhesion one may selectively enhance APP shedding on the plasma membrane without affecting substrates located at adherens junctions.

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