The Proamphiregulin Cytoplasmic Domain Is Required for Basolateral Sorting, but Is Not Essential for Constitutive or Stimulus-induced Processing in Polarized Madin-Darby Canine Kidney Cells*

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In this study, the role of the amphiregulin precursor (pro-AR) cytoplasmic domain in the basolateral sorting and cell-surface processing of pro-AR in polarized epithelial cells has been investigated using Madin-Darby canine kidney cells stably expressing various human pro-AR forms. Our results demonstrate that newly synthesized wild-type pro-AR (50 kDa) is delivered directly to the basolateral membrane domain with >95% efficiency, where it is sequentially cleaved within the ectodomain to release several soluble amphiregulin (AR) forms. Analyses of a pro-AR cytoplasmic domain truncation mutant (ARTL27) and two pro-AR secretory mutants (ARsec184 and ARsec190) indicated that the pro-AR cytoplasmic domain is not required for efficient delivery to the plasma membrane, but does contain essential basolateral sorting information. We show that the pro-AR cytoplasmic domain truncation mutant (ARTL27) is not sorted in polarized Madin-Darby canine kidney cells, with ~65% of the newly synthesized protein delivered to the apical cell surface. Under base-line conditions, ARTL27 was preferentially cleaved from the basolateral surface with 4-fold greater efficiency compared with cleavage from the apical membrane domain. However, ARTL27 ectodomain cleavage could be stimulated equivalently from either membrane domain by a variety of different stimuli. The metalloprotease inhibitor BB-94 could inhibit both base-line and stimulus-induced ectodomain cleavage of wild-type pro-AR and ARTL27. These results indicate that the pro-AR cytoplasmic domain is required for basolateral sorting, but is not essential for ectodomain processing. Preferential constitutive cleavage of ARTL27 from the basolateral cell surface also suggests that the metalloprotease activity involved in base-line and stimulus-induced ARTL27 ectodomain cleavage may be regulated differently in the apical and basolateral membrane domains of polarized epithelial cells.

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sorting information. In contrast, pro-EGF is not sorted in polarized epithelial cells, and its delivery to the cell surface is independent of its cytoplasmic domain (18). Cytoplasmic domain sorting information not only defines spatial compartmentalization of different EGFR ligands in polarized epithelial cells, but can also determine ligand accessibility to basolateral EGFRs and modulate constitutive release at the different membrane domains (1, 17, 18). Other less well-characterized protein interactions with the pro-TGF-α cytoplasmic domain have been identified and have been postulated to be involved in ligand presentation and signaling (19). In addition, the cytoplasmic domains of several membrane proteins regulate ectodomain shedding (20–22), whereas for other membrane proteins, including pro-EGF (18), pro-TGF-α (14), and pro-HB-EGF (23), the cytoplasmic domain is not essential for ectodomain cleavage. However, recent studies using artificial membrane-anchored EGF ligands indicate that the membrane-anchoring domains can modulate constitutive and stimulated ligand release and endocytic trafficking as well as confer differential sensitivity to synthetic metalloprotease inhibitors (24).

AR belongs to a subset of EGF ligands that contain heparin-binding activity within their extracellular domains. For EGF-like growth factors capable of binding to heparin, ligand binding and subsequent activation of EGF-R can be modulated by cell-surface heparan sulfate proteoglycans or by addition of heparin (25). AR is a bifunctional modulator of cell growth, but in general acts as an autocrine growth factor for both normal and transformed cells (1, 25, 26). Interestingly, comparison of AR and EGF mitogenic signaling indicates that soluble AR may have distinct signaling properties associated with the EGFR cytoplasmic domain (27). Cell-surface pro-AR is sequentially cleaved, and phorbol esters and protein-tyrosine phosphatase inhibitors can stimulate ectodomain processing in a metalloprotease-dependent manner (28–30). In polarized colonic epithelial cells, cellular distribution and release of endogenous AR are restricted to the basolateral membrane domain, suggesting that AR signaling is spatially compartmentalized (1). The aim of this study was to examine the role of the pro-AR cytoplasmic domain in trafficking and sequential processing of pro-AR in polarized epithelial cells. We demonstrate that newly synthesized pro-AR is directly sorted to the basolateral membrane domain in polarized MDCK cells, where it is sequentially cleaved to release soluble AR forms into the basolateral conditioned medium. Analyses of pro-AR cytoplasmic domain truncation and secretory mutants show that the cytoplasmic domain is not required for efficient delivery of pro-AR to the plasma membrane, but is essential for basolateral sorting. A newly synthesized pro-AR cytoplasmic deletion mutant (ARTL27) is missorted and expressed in both the apical and basolateral membrane domains in polarized MDCK cells. We show that ARTL27 is preferentially cleaved from the basolateral membrane domain under constitutive conditions; however, efficient stimulated cleavage can occur from either membrane domain of polarized epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**Cell culture reagents were purchased from Life Technologies, Inc. Chemicals were purchased from Sigma unless otherwise stated. Tran35S-label was purchased from ICN Bio-medicals (Costa Mesa, CA). Sulfosuccinimidyl 6-(biotinamido)hexanoate and protein A-agarose were purchased from Pierce. All electrophoresis reagents were purchased from Bio-Rad. Rainbow markers and the ECL kit were purchased from Amersham Pharmacia Biotech. The long form of recombinant human AR was purchased from R&D Systems (Minneapolis, MN). The synthetic hydroxamate metalloprotease inhibitor BB94 (31) was kindly provided by Dr. Helen Mills (British Biotech, Oxford, United Kingdom). Phenylarsine oxide, A23187, ionomycin, calmidazolium chloride, and trifluoperazine dimaleate were obtained from Calbiochem. Pervanadate was freshly made for each experiment using 1 μM solutions of H2O2 and 1 M sodium vanadate-buffered saline (PBS) were prepared and then mixed to give a 0.5× solution of pervanadate. As pervanadate is unstable, this was diluted into the cell culture at the indicated concentration of 100 μM within 20 min of preparation.

Monoclonal antibodies to human AR (AR 6RIC2.4 (AR mAb) and AR 4.1.18) (mAb) used in this study (32, 33). Monoclonal antibody to human EGFR (mAb 528) was obtained from American Type Culture Collection (Manassas, VA). Affinity-purified rabbit antisera to mouse immunoglobulin was purchased from Cappel Laboratories (Durham, NC). Horseradish peroxidase-conjugated donkey anti-mouse IgG and horseradish peroxidase-conjugated streptavidin were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**Cells and Cell Culture—**MDCK strain II cells were obtained from Dr. Enrique Rodriguez-Boulan (Cornell University Medical College, New York). MDCK strain II cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) as previously described (17). For culture on Transwell filters (0.4-μm pore size; Costar Corp., Cambridge, MA), cells were seeded at 1 × 10^6 on 12- and 24-mm Transwell filters. For experiments, cells grown on Transwell filters, the medium was changed daily. The integrity of the cell monolayer was assessed using a Millicell electrical resistance system (Millipore Corp., Bedford, MA). Experiments were performed when transepithelial resistance was >200 ohms/cm^2.

**AR Constructs, Transfection, and Selection of Expressing Clones—**Human AR cDNAs encoding wild-type pro-AR and the AR cytoplasmic domain truncation mutant (ARTL27) were obtained from Dr. Greg Powlman (Sugen, Redwood City, CA) (29, 30, 32). The two AR secretory constructs (ARsec184) and (ARsec190) have been described previously (29). All AR cDNA constructs were subcloned into the pcB6-CMV expression vector and stably transfected into MDCK strain II cells by the calcium phosphate precipitation method as previously described (17). Transfected MDCK strain II cells were selected in medium containing 500 μg/ml G418. Individual clones were initially screened by indirect immunofluorescence. Subsequently, high expressing AR clones were identified by metabolic labeling and immunoprecipitation.

**Metabolic Labeling—**All cells were grown on 24-mm Transwell filters in the appropriate medium for 4–6 days. To increase AR expression in transfected cell lines, cells were treated with 5 mM sodium butyrate in the appropriate medium for 4–6 days before labeling. For pulse-chasing (2h), cells were rinsed twice with serum-free, L-cysteine/L-methionine-containing medium and incubated in PBS for 2 h at 37°C to chase endogenous AR. For direct delivery experiments, Tran35S-label labeling was combined with cell-surface biotinylation. After labeling, cells were chased at 37°C for various times as described and then cooled on ice and washed with ice-cold PBS. Labeled proteins that arrived at the cell surface were detected by domain-selective biotinylation as described above. Following biotinylation, cells were washed and lysed, and AR immunoprecipitation was performed as described below. To measure cell-surface biotinylated AR, AR immunoprecipitates were eluted with SDS buffer as previously described (17). Three-fourths of the eluted protein was used to determine total labeled protein.

**AR Immunoprecipitation—**All AR immunoprecipitation protocols were performed at 4°C or on ice as previously described (29). Immuno-
precipitates were analyzed under reducing conditions on 12.5% SDS-polyacrylamide gel unless otherwise specified. For metabolically labeled samples, gels were fixed, treated with Amplify (Amersham Pharmacia Biotech) for 30 min, dried, and fluorography was performed with BioMax MR film (Eastman Kodak Co.). For unlabeled biotinylated samples, proteins were electrophoretically transferred overnight at 30 V to nitrocellulose (0.2 μm; Bio-Rad). These membranes were subsequently rinsed with Tris-buffered saline containing 0.5% Tween 20 (TBS-T), blocked with PBS containing 3% BSA and 0.5% Tween 20. Samples were incubated for the indicated times with serum-free DMEM containing 100 μg/ml peroxidase-conjugated streptavidin. For membranes probed with 125I-labeled streptavidin or horseradish peroxidase-conjugated streptavidin. For membranes probed with 125I-labeled streptavidin, AR was detected by autoradiography using Kodak X-OMAT film. For membranes probed with horseradish peroxidase-conjugated streptavidin, AR was detected using an ECL Western blotting kit, and fluorography was performed using BioMax MR film.

**Immunofluorescence and Confocal Microscopy—** Immunofluorescent staining of MDCK-AR and MDCK-ARTL27 cells cultured on 12-mm Transwell filters was performed as previously described (17). All staining procedures were performed on ice or at 4 °C unless otherwise stated. For AR staining, cells were paraformaldehyde-fixed and stained with 2 μg/ml AR mAb. Specificity of AR staining was confirmed by preincubating this antibody overnight with 100 ng/ml recombinant AR prior to staining. Monoclonal antibody MOPC-21 (Sigma) was used as an isotopic antibody control. Laser scanning confocal microscopy was performed using a Zeiss LSM4 confocal microscope. Polarized cellular localization was visualized by z-scanning the images.

**AR ELISA—** Human AR sandwich ELISA was used to measure AR in conditioned media and cell lysates as described (26). Briefly, capture antibody (AR mAb) was absorbed to wells overnight (0.2 μg/ml) and blocked with PBS containing 3% BSA and 0.5% Tween 20. Samples were incubated for 1 h at 37 °C, and secondary antibody (0.1 μg/well biotinylated mAb AR 4.14.18) was added for 1 h at 37 °C, followed by incubation with 0.05 mg/ml peroxidase-conjugated streptavidin for 1 h at 37 °C. Substrate (0.5 mg/ml o-phenylenediamine dihydrochloride) was added for 5 min, and the reaction product was read in a microplate reader at 490 nm. The standard was recombinant human long form AR diluted in culture medium. To measure apical release of AR from cells grown on Transwell filters, AR levels in the apical conditioned medium were corrected for AR released from cells growing on the sides of Transwell filters as previously described (17). Analysis of AR release was also performed in the absence and presence of 20 nM mAb 528 (an EGFR-neutralizing antibody) (17).

**AR Ectodomain Cleavage Assay—** Seeded on 24-mm Transwell filters were biotinylated as described above. Following biotinylation, cells were washed twice with ice-cold PBS and once with serum-free DMEM. To measure processing of cell-surface biotinylated AR, cells were incubated for the indicated times with serum-free DMEM containing MmSO (control), 5 μM BB-94, a stimulus (1 μM phorbol 12-myristate 13-acetate, 100 μM pervanadate, 10 μM phenylarsine oxide, 25 μM A23187, 1 μM ionomycin, 5 μM calmidazolium chloride, or 50 μM trifluoroperazine dimaleate), or a combination of BB-94 and stimulus. After 30 min, the medium was collected, and cell lysates were prepared as previously described (29). Subsequently, AR immunoprecipitations were performed, and biotinylated AR was detected by Western blotting as previously described (29). To verify apical release of AR from cells grown on Transwells, either Trans35S-labeling combined with cell-surface biotinylation or unlabeled cell-surface biotinylation experiments were performed. After biotinylation, filters were excised from Transwell inserts, washed, and placed in fresh serum-free medium. Basolateral and apical stimulated AR cleavage assays with isolated filters were performed as described above. These studies confirmed that >80% of the apically released biotinylated AR was derived from the apical membrane domain of the intact cell monolayer (data not shown).

**Reproducibility of Results—** Unless otherwise stated, experiments were performed with two to three different clonal cell lines for each AR construct, and results are representative of at least three experiments with similar results.

**RESULTS**

**Multiple Pro-AR Forms Are Expressed on the Basolateral Cell Surface of Polarized MDCK Cells—** We have recently shown that endogenous pro-AR is expressed predominantly on the basolateral cell surface of two polarizing human colon cancer cell lines (1). To further examine the cell-surface localization of pro-AR in polarized epithelial cells, MDCK cells stably expressing human wild-type pro-AR (MDCK-AR) were grown on Transwell filters and examined by indirect immunofluorescence and confocal microscopy. As shown in Fig. 1A, pro-AR was localized to the basolateral and lateral surfaces of the MDCK cell monolayer. This localization is similar to that observed for endogenous human pro-AR in the polarizing human colon cancer cell lines (1), but is quite different from cell-surface localization of pro-EGF and pro-TGF-α in polarized MDCK cells. In MDCK cells, pro-EGF accumulates preferentially on the apical surface (18), whereas pro-TGF-α is distributed primarily on the lateral surface (17).

To biochemically characterize cell-surface distribution of pro-AR in MDCK-AR cells under steady-state conditions, unlabeled cell-surface biotinylation experiments were performed. Cells grown on Transwell filters were selectively biotinylated in either the apical or basolateral membrane domain. AR immunoprecipitates from total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin. Under steady-state conditions, several different molecular mass pro-AR species were detected predominantly in the basolateral membrane domain (Fig. 1B). Two major pro-AR forms were detected, one of 50 kDa and a 26/28-kDa doublet. In some experiments, an additional minor 16-kDa form that was detected in some experiments (data not shown).

**Pro-AR Is Released into the Basolateral Conditioned Medium of Polarized MDCK Cells—** To examine release of soluble AR from polarized MDCK-AR cells under steady-state conditions, cells grown on Transwell filters were grown in the presence or absence of EGFR-neutralizing antibody mAb 528 for 24 h prior to collection of the conditioned medium (17). In addition, AR release was examined under conditions of high AR expression induced by treatment with sodium butyrate (29). The level of
AR immunoreactivity in the apical and basolateral conditioned media was measured by human AR ELISA. Under both baseline and butyrate-induced conditions, >95% of the total AR immunoreactivity was detected in the basolateral conditioned medium (Fig. 2A). Surprisingly, treatment with EGFR-neutralizing antibody did not increase soluble AR levels in the conditioned medium, but rather slightly decreased AR levels. This result differs dramatically from the efficient EGFR-dependent consumption of soluble TGF-β from the basolateral membrane compartment in MDCK cells (17). One possible explanation for this observation is that soluble human AR cannot bind to canine EGFRs; however, we have observed similar results with several human cancer cell lines and primary cultures of normal human keratinocytes that express endogenous pro-AR (1).3

To further characterize cell-surface AR forms, experiments in which a pulse-chase was combined with cell-surface biotinylation were performed on MDCK-AR cells grown on Transwell filters. Cells were labeled with Tran35S-label for 2 h and either cell surface-biotinylated or chased for 2 h to release soluble AR forms into the conditioned medium. The 50-kDa, 26/28-kDa doublet, and minor 16-kDa forms were detected only at the basolateral cell surface (Fig. 2B). Consistent with the detection of multiple pro-AR species at the basolateral cell surface, several forms of soluble AR including a major 43-kDa form were efficiently released into the basolateral conditioned medium (Fig. 2B). The relative amounts of these forms were confirmed by metabolic labeling experiments using [35S]cysteine (data not shown) (29). Quantitation by scanning densitometry indicated that >95% of the 50-kDa pro-AR form and the 43-kDa soluble AR form were detected at the basolateral cell surface and in the basolateral conditioned medium, respectively. This result is in agreement with the steady-state expression and release of endogenous pro-AR from polarized human colon cancer cell lines (1).

Newly Synthesized Human Pro-AR Is Sorted Directly to the Basolateral Membrane Domain—To examine delivery of newly synthesized wild-type pro-AR to different cell-surface domains of polarized MDCK cells, a pulse-chase combined with domain-selective cell-surface biotinylation was performed. As shown in Fig. 2B, 50-kDa pro-AR was delivered directly to the basolateral cell surface. Quantitation of the arrival of 50-kDa pro-AR at the cell surface after 1 h of chase indicated that it is delivered in a polarized fashion with a basolateral/apical ratio of 95:5 (Fig. 3, A and D). The 50-kDa pro-AR form appeared at the basolateral cell surface after 20 min of chase and was cleaved sequentially at the cell surface to generate several different cellular and soluble AR forms (29). Several forms of soluble AR were released into the medium during the 2-h chase period (Fig. 3, B and E). The 43-kDa, 19/21-kDa doublet, and 9-kDa (data not shown) soluble AR forms were detected by 20 min of chase, with 43-kDa AR being the predominant form. These data indicate that the predominant AR forms are 50-kDa pro-AR and 43-kDa soluble AR and, in agreement with previous pulse-chase studies (29), suggest a direct relationship between the processing of cell-surface pro-AR forms and release of soluble AR forms. The relative amounts of these forms were confirmed by metabolic labeling using [35S]cysteine (data not shown). These results also demonstrate that newly synthesized pro-AR is delivered directly to the basolateral membrane domain and that sequential cleavage of pro-AR occurs at the basolateral cell surface (29). In addition, these studies show that the predominant pro-AR cleavage site is within the juxtamembrane region of the pro-AR ectodomain and that processing at this site releases a major 43-kDa soluble AR species into the basolateral conditioned medium.

Pro-AR Secretory Mutants (ARsec184 and ARsec190) Are Released in a Non-polarized Manner from MDCK Cells—To determine whether regions within the pro-AR transmembrane and cytoplasmic domains are involved in basolateral sorting, several different pro-AR C-terminal truncation mutants were expressed in MDCK cells (Fig. 4A). First, we examined two pro-AR secretory mutants (ARsec184 and ARsec190) stably expressed in MDCK cells (Fig. 4) (29). ARsec184 contains the first 184 amino acids of pro-AR and terminates at the C-termi-

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3 P. J. Dempsey, unpublished observations.
Fig. 3. Newly synthesized pro-AR is delivered directly to and sequentially cleaved from the basolateral membrane domain in polarized MDCK cells. MDCK-AR cells grown on Transwell filters were pulse-labeled with Tran"S-label for 20 min and then chased for various amounts of time (A). After the chase, the apical (Ap) and basolateral (Bl) conditioned media were collected, and cell-surface biotinylation was performed. AR immunoprecipitation was performed on total cell lysates (A) and the conditioned media (C). For cell-surface AR (B), AR immunoprecipitates from total cell lysates were eluted and re-immunoprecipitated with streptavidin-agarose. All immunoprecipitates were analyzed by SDS-PAGE and fluorography. Arrowheads indicate the various forms, and molecular masses are indicated in kilodaltons. The kinetics for delivery of 50-kDa pro-AR to the cell surface (D) and release of the major 43-kDa soluble AR species (E) are shown. Proteins were quantitated by scanning densitometry, and amounts of AR immunoreactivity were normalized to total pro-AR and are expressed in arbitrary units. The results of a representative experiment from three separate trials are presented.
conditioned media were measured using human AR ELISA. Under both base-line and butyrate-induced conditions, soluble AR immunoreactivity was detected equivalently (~50%) in the apical and basolateral conditioned media (Fig. 6A). This result indicates a complete loss of polarized release of AR in MDCK-ARTL27 cells. Similar to wild-type pro-AR, treatment with EGFR-neutralizing antibody did not increase soluble AR levels in the conditioned media, but rather slightly decreased AR levels.

To further characterize release of cell-surface ARTL27 forms, MDCK-ARTL27 cells grown on Transwell filters were examined using pulse-chase experiments in combination with cell-surface biotinylation (Fig. 6B). The results show that a 45-kDa form, a 22/24-kDa doublet, and a 12-kDa species were expressed on either cell surface. The intensities of the 45- and 12-kDa forms were consistently stronger in the apical membrane domain, whereas the 22/24-kDa doublet was expressed more intensely on the basolateral cell surface. After a 2-h chase, several different soluble AR forms were detected in the apical and basolateral conditioned media, and the profile of soluble AR forms correlated well with that of the ARTL27 forms detected at the cell surface. Similar to wild-type pro-AR, a 43-kDa AR species was the major form released into both the apical and basolateral conditioned media. The relative amounts of these forms were confirmed by metabolic labeling experiments using [35S]cysteine (data not shown). Quantitation by scanning densitometry indicated that the apical/basolateral ratios of 45-kDa ARTL27 at the cell surface and 43-kDa soluble AR in the media were 80:20 and 60:40, respectively. However, in comparison with the expression levels of 45-kDa cell-surface pro-AR in each membrane domain, the relative amounts of the 43-kDa soluble form released into the conditioned media were much greater at the basolateral surface.

Newly Synthesized ARTL27 Is Not Sorted, but Is Preferentially Cleaved from the Basolateral Cell Surface of Polarized MDCK Cells—To determine whether loss of polarized expression of ARTL27 in MDCK cells is due to missorting in the secretory pathway, direct delivery of newly synthesized ARTL27 to the different cell-surface domains of polarized MDCK cells was examined by pulse-chase experiments combined with domain-selective cell-surface biotinylation. A 45-kDa ARTL27 form, which appeared at the cell surface after 30 min of chase, was delivered equally to both the apical and basolateral membrane domains (Fig. 7, A and B). During the 2-h chase, the intensities of the 45-kDa band in the apical and basolateral membrane domains changed dramatically. The intensity of the 45-kDa band increased at the apical cell surface, whereas the intensity of this band was greatly diminished at the basolateral cell surface (Fig. 7B). As shown in Fig. 7 (B and C), 45-kDa cell-surface ARTL27 was sequentially cleaved from both membrane domains to generate different cellular and soluble AR forms. Several forms of soluble AR were released into the conditioned media during the 2-h chase period, including the 43-kDa, 19/21-kDa doublet, and 9-kDa soluble AR forms, with 43-kDa AR being the predominant soluble form. These data demonstrate that processing of the ectodomain of 45-kDa cell-surface ARTL27 at the distal cleavage site to release 43-kDa soluble AR can occur in both membrane domains under base-line conditions. Consistent with the dramatic loss of 45-kDa ARTL27 from the basolateral surface, the majority of 43-kDa soluble AR was released into the basolateral conditioned medium (Fig. 7C). Quantitative differences in the kinetics of delivery and stability of 45-kDa ARTL27 at the cell surface and release of 43-kDa soluble AR from the different membrane domains in polarized MDCK cells are represented in Fig. 7 (D and E, respectively). Further analysis of the release
of the 43-kDa soluble AR form after 1 h of chase indicated that cleavage of 45-kDa cell-surface ARTL27 was 3–4-fold greater from the basolateral membrane domain than at the apical cell surface under these base-line conditions. Importantly, these results show that the pro-AR cytoplasmic domain is not essential for the base-line rate of ARTL27 cleavage.

Preferential cleavage of ARTL27 from the basolateral membrane domain would explain the increased release of the 43-kDa soluble form into the basolateral conditioned medium and the stability of ARTL27 expression at the apical cell surface. Interestingly, the observation of preferential cleavage of ARTL27 from the basolateral cell surface suggests that regulation of pro-AR ectodomain cleavage may be spatially compartmentalized in polarized epithelial cells and is in agreement with previous findings on preferential cleavage of pro-EGF from the basolateral cell surface of polarized MDCK cells (18). Additionally, increased expression of the 22/24-kDa ARTL27 doublet at the basolateral cell surface during the chase (Fig. 7) suggests that enhanced N-terminal (or proximal) cleavage to remove the prodomain of ARTL27 occurs in the basolateral membrane domain.

Multiple Stimuli Can Activate Pro-AR Ectodomain Cleavage—Several recent studies have shown that a variety of different stimuli can activate cleavage of pro-HB-EGF and pro-TGF-α in a metalloprotease-dependent manner (7, 23, 35–40). We have also demonstrated that base-line and stimulus-in-
duced cleavage of wild-type pro-AR can be inhibited by the metalloprotease inhibitor BB-94 (29, 30). To further characterize stimulus-induced processing of wild-type pro-AR, unlabeled biotinylation chase experiments were performed (29). As shown in Fig. 8, three different stimuli, including phorbol esters (1 μM phorbol 12-myristate 13-acetate), tyrosine phosphatase inhibitors (100 μM pervanadate and 10 μM phenylarsine oxide (data not shown)), and calcium ionophores (25 μM A23187 and 1 μM ionomycin (data not shown)), all efficiently activated ectodomain cleavage of the 50-kDa and 26/28-kDa doublet cell-surface pro-AR forms. In addition, calmodulin antagonists (calmidazolium chloride (5 μM) and trifluoperazine diamelate (50 μM)) activated pro-AR processing (data not shown). The predominant soluble AR form detected in the conditioned medium was the 19/21-kDa doublet, which is consistent with increased expression of its corresponding 26/28-kDa doublet cell-surface pro-AR form under steady-state conditions in MDCK cells (29). The additional slower migrating band (marked by an asterisk in Fig. 8) probably represents the 50-kDa cell-surface pro-AR form, which is released from cells under both constitutive and stimulated conditions. For all stimuli tested, the metalloprotease inhibitor BB-94 blocked pro-AR processing (Fig. 8). These results are consistent with previous reports on the stimulus-induced metalloprotease-sensitive ectodomain cleavage of other EGFR ligands (26, 29, 41).

**Stimulus-induced Cleavage of ARTL27 Occurs Efficiently in Both the Apical and Basolateral Membrane Domains and Is Sensitive to BB-94.—** In a previous study, we showed that baseline and pervanadate-stimulated cleavage of ARTL27 from the basolateral membrane domain is sensitive to BB-94 (30). In the present study, our results suggest that ARTL27 is preferentially cleaved from the basolateral membrane domain of polarized MDCK cells, although efficient cleavage does occur at the apical cell surface under baseline conditions. This observation prompted further investigation of whether stimulus-induced ectodomain cleavage of ARTL27 can occur in the apical membrane domain and, if so, whether it can be inhibited by BB-94.

A potential problem with the unlabeled biotinylation cleavage assay is that it may not discriminate between ARTL27 expressed on the apical membrane domain of the polarized cell monolayer and ARTL27 expressed on the cell surface of non-polarized cells growing on the sides of the Transwell insert. For these studies, it was therefore important to verify that the unlabeled biotinylation cleavage assay faithfully represented cleavage at the apical cell surface. To eliminate this potential artifact, base-line and stimulated cleavage of ARTL27 was examined after the filter had been excised from the Transwell insert using several different experimental approaches (see "Experimental Procedures"). These studies confirmed that the majority of apically released AR in the standard cleavage assay was due to processing from the apical membrane domain (data not shown)).

**Experimental Procedures**—These studies confirmed that the majority of apically released AR in the standard cleavage assay was due to processing from the apical membrane domain (data not shown)).

**Fig. 7.** Newly synthesized ARTL27 is not sorted, but is preferentially cleaved from the basolateral membrane domain of polarized MDCK cells. MDCK-ARTL27 cells grown on Transwell filters were pulse-labeled with Tran35S-label for 30 min and chased for various amounts of time. After the chase, the apical (Ap) and basolateral (Bl) conditioned media was collected, and selective cell-surface biotinylation was performed. AR immunoprecipitation was performed on total cell lysates (A) and the conditioned media (C). For cell-surface AR (B), AR immunoprecipitates from total cell lysates were eluted and re-immunoprecipitated with streptavidin-agarose. All immunoprecipitates were analyzed by SDS-PAGE and fluorography. Arrowheads indicate the various forms, and molecular masses are indicated in kilodaltons. The asterisk designates an immature intracellular pro-AR form. The kinetics for delivery of 45-kDa ARTL27 to the cell surface (D) and release of the major 43-kDa soluble AR species (D) are shown. Proteins were quantitated by scanning densitometry, and amounts of AR immunoreactivity were normalized to total pro-AR and are expressed in arbitrary units. The results of a representative experiment from three separate trials are presented.

**Fig. 8.** Multiple stimuli activate pro-AR ectodomain cleavage, which can be inhibited by the metalloprotease inhibitor BB-94. MDCK-AR cells grown on Transwell filters were cell-surface-biotinylated and chased for 30 min in the presence of control, stimulus, BB-94, or stimulus and BB-94. The stimuli presented include phorbol ester (1 μM phorbol 12-myristate 13-acetate (PMA)), tyrosine phosphatase inhibitor (100 μM pervanadate (PV)), and calcium ionophore (25 μM A23187). Total cell lysates (data not shown) and the conditioned medium were immunoprecipitated with AR mAb. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin. AR immunoreactivity was detected using an ECL Western blotting kit in combination with fluorography. Only results for soluble AR released into the basolateral conditioned medium are shown. Arrowheads indicate the various AR forms, and molecular masses are indicated in kilodaltons. The asterisk designates 45-kDa cell-surface ARTL27 that is probably shed from cell membranes.
epithelial cells (43). For basolateral membrane proteins, sorting signals have been identified in their cytoplasmic domains and consist of motifs that can include critical tyrosine and dileucine residues as well as other unrelated sequences. Interaction of basolateral sorting motifs with adaptor proteins has been proposed as an essential step in recruitment of cargo to the basolateral transport pathway (43, 44). In polarized epithelial cells, EGFRs are localized predominantly to the basolateral membrane domain, and this localization is dependent on specific basolateral sorting information within the EGFR cytoplasmic domain (45). Basolateral distribution of EGFRs has important consequences for defining how functional and productive ligand-mediated receptor signaling can occur in polarized epithelial cells. This point is best illustrated in Caenorhabditis elegans, where correct basolateral localization of LET-23, which is homologous to mammalian EGFR, is essential for ligand-mediated vulval development (42). Likewise, spatial compartmentalization of different EGF-like ligands is also important for defining ligand accessibility to distinct EGFR populations. In polarized MDCK cells, we have previously shown that pro-TGF-α is sorted directly to the basolateral membrane, where it is released into the basolateral conditioned medium (17). Soluble mature TGF-α has direct access to and is rapidly taken up by basolateral EGFRs and is thought to act as a short-range autocrine factor. AR is also released from the basolateral surface, but does not appear to be taken up rapidly by EGFR (1, 29). These differences may contribute to the different phenotypes observed in transgenic mice with expression of TGF-α and AR targeted to the epidermis (46–48). In contrast, pro-EGF is not sorted, but is expressed on both membrane domains (18). Non-polarized release of EGF implies that under normal physiological conditions, basolateral EGF can act as an autocrine factor, whereas apical or luminal EGF can access distant receptor populations only in a paracrine manner.

In this study, we found that newly synthesized wild-type pro-AR was delivered directly to the basolateral membrane domain in polarized MDCK cells. Analysis of both pro-AR secretory mutants (ARsec184 and ARsec190) and the pro-AR cytoplasmic domain truncation mutant (ARTL27) indicated that the cytoplasmic domain contains essential basolateral sorting information. Removal of the pro-AR cytoplasmic domain did not result in apical sorting, but instead caused the loss of polarized sorting. For several membrane proteins, a similar loss of polarized sorting has been reported when the basolateral sorting motif(s) have been deleted (49). Sequence analysis of the pro-AR cytoplasmic domain showed that it is highly conserved between species and therefore may contain functional sorting information. Although further studies are required to identify the basolateral sorting motifs in the pro-AR cytoplasmic domain, a pro-TGF-α/AR chimera, which contains the pro-TGF-α ectodomain and transmembrane domains linked to the pro-AR cytoplasmic domain, is expressed on the basolateral cell surface of MDCK cells. This preliminary result suggests that the pro-AR cytoplasmic domain does contain dominant basolateral sorting information that can confer basolateral sorting upon another membrane protein.3

Additionally, analyses of the biosynthesis and trafficking of a pro-AR cytoplasmic domain truncation mutant (ARTL27) in the exocytic pathway showed that the cytoplasmic domain is not required for efficient delivery of pro-AR to the plasma membrane. Not only is ARTL27 delivered to the cell surface with kinetics similar to those of wild-type pro-AR, but the electrophoretic mobilities of the different ARTL27 forms display a profile similar to that found for wild-type pro-AR, suggesting that post-translational modifications of ARTL27 are also unaltered. Similarly, trafficking of pro-EGF (18) and pro-

**DISCUSSION**

The membrane-anchoring and cytoplasmic domains of several EGFR ligands have been reported to be important in regulating trafficking within the exocytic and endocytic pathways and in modulating ectodomain cleavage to release soluble ligand (14, 24). In polarized epithelial cells, spatial compartmentalization and cellular distribution of different EGF-like ligands in the apical and/or basolateral membrane domain are also critical factors in determining ligand release and accessibility to basolateral EGFRs (1, 17, 18, 42). In this study, we sought to define the trafficking and processing of pro-AR in polarized epithelial cells and to determine the role of the pro-AR cytoplasmic domain in these events.

Apical and basolateral sorting of membrane proteins is an important mechanism for the establishment and maintenance of domain-specific distribution of these proteins in polarized epithelial cells.  The data in Fig. 9 show that all stimuli that could activate wild-type pro-AR ectodomain cleavage could also stimulate cleavage of ARTL27 from both the apical and basolateral membrane domains. Unlike ARTL27 processing in the apical membrane domain under base-line conditions, all stimuli efficiently activated cleavage of the apical 45-kDa cell-surface ARTL27 form. Similar to wild-type pro-AR, base-line and stimulus-induced cleavage of ARTL27 from the apical and basolateral membrane domains of MDCK cells was inhibited by BB-94. Taken together, these results demonstrate that stimulus-induced cleavage of ARTL27 does not require the pro-AR cytoplasmic domain and can be activated by several different stimuli. In addition, ARTL27 can be efficiently processed from both the apical and basolateral membrane domains in a metalloprotease-sensitive manner. Finally, preferential processing of ARTL27 at the basolateral cell surface and the different characteristics of base-line and stimulated ectodomain cleavage of ARTL27 from the apical membrane domain suggest that the apical cytoplasmic domain may be regulated differently in apical and basolateral membrane domains in polarized epithelial cells.
HB-EGF (23) through the secretory pathway is also not affected by removal of their cytoplasmic domains. In contrast, studies of pro-TGF-α cytoplasmic domain truncation mutants have demonstrated that the pro-TGF-α C-terminal divaline residues are essential for correct maturation and transit of pro-TGF-α through the secretory pathway (13, 14). Although the exact nature of this defect in the secretory pathway is not known, sequence analysis has shown that the last 3 C-terminal amino acids (Thr-Val-Val) of the pro-TGF-α cytoplasmic domain compose a consensus binding motif for type 1 PDZ domains, and two hybrid analyses have identified several PDZ domain-containing proteins that bind to this motif (15, 16). A similar PDZ domain-binding motif has been identified within another membrane protein, membrane-type metalloprotease-1 (14). Interestingly, the three EGFR ligands (pro-EGF, pro-HB-EGF, and pro-AR), which do not appear to require the cytoplasmic domain for correct trafficking in the secretory pathway, also do not contain a consensus PDZ domain-binding motif at their C termini. This raises the possibility that transport of pro-TGF-α through the early secretory pathway may be regulated differently from these other EGF-like ligands.

Recent in vitro and in vivo studies have suggested that ectodomain cleavage of EGFR ligands and release of soluble mature growth factors may be essential for these ligands to achieve full biological activity (11, 26, 50). In vitro studies have demonstrated that broad-spectrum synthetic metalloprotease inhibitors such as BB-94 that block constitutive and stimulus-induced EGFR ligand processing (11, 18, 26, 29, 41, 51) can inhibit EGFR-dependent autocrine cell growth and migration in several different cell types (11, 26, 52, 53). A direct role for metalloproteases in ectodomain processing of different EGFR ligands has been confirmed by the recent identification of tumor necrosis factor-α-converting enzyme (TACE/ADAM17) as a disintegrin metalloprotease involved in constitutive processing of pro-TGF-α (50). Importantly, eyelid, hair, and skin defects found in mice lacking functional TACE (tace<sup>ΔZn</sup>/ΔZn) are very similar to those reported for TGF-α-null mice, suggesting that pro-TGF-α processing is required for functional signaling during development (50). The severity of developmental defects in tace<sup>ΔZn</sup>/ΔZn mice is similar to that observed in EGFR-null mice (54–56), and the comparable (although not identical) biochemical characteristics of processing of different EGF-like growth factor precursors suggest that TACE may also be involved in processing other EGF-like ligands. However, involvement of other metalloproteases, including matrix metalloprotease-3 and meltrin-Y/MDC9/ADAM9, in phorbol ester-activated processing of pro-HB-EGF (37, 57) together with the differential sensitivity of EGFR ligands to metalloprotease inhibitors (24) and the inability of phorbol esters to induce pro-EGF processing (18, 24) suggest that specificity and regulation of ectodomain processing of different EGF-like ligands may be more complex.

Ectodomain cleavage of EGF-like growth factor precursors can be activated by a variety of stimuli, including serum, phorbol esters, calcium ionophores, calmodulin antagonists, and GTPγS, through both protein kinase C-dependent and -independent pathways (18, 23, 29, 40, 41, 51, 58–60). In addition, the ability of several different physiological stimuli, including ligand-mediated activation of tyrosine kinase receptors (36, 39) and G protein-coupled receptors (52, 53), to stimulate release of EGF ligands further demonstrates that diverse and possibly overlapping signaling pathways can regulate EGF ligand release (61). Previous studies have shown that pro-AR is sequentially cleaved to produce multiple cellular and soluble forms in several cell types in a metalloprotease-dependent manner (28, 29, 34, 62). In addition, we have recently demonstrated that wild-type pro-AR processing can be activated by phorbol esters and the tyrosine phosphatase inhibitor pervanadate and that this processing is blocked by the metalloprotease inhibitor BB-94 (29, 30). In polarized MDCK cells, we have demonstrated that wild-type pro-AR is sequentially cleaved from the basolateral membrane domain under base-line conditions. The predominant soluble AR species released is a 43-kDa AR form, which is consistent with previous findings on the constitutive processing of pro-AR in polarizing human colon cancer cell lines (1, 29). Furthermore, we have shown that wild-type pro-AR processing at the basolateral cell surface can be activated by a variety of stimuli, including phorbol esters, pervanadate, calcium ionophores, and calmodulin antagonists, in a metalloprotease-dependent manner. These results indicate that the proteolytic machinery in the basolateral membrane domain of MDCK cells can faithfully reproduce constitutive and stimulus-induced pro-AR processing observed in other cell types.

The role of EGFR ligand membrane-anchoring and cytoplasmic domains in modifying ectodomain cleavage is not well understood. Earlier studies of pro-TGF-α processing had suggested that the C-terminal divaline residues within the cytoplasmic domain were essential for both base-line and regulated cleavage of the pro-TGF-α ectodomain (63); however, subsequent studies have shown that these pro-TGF-α cytoplasmic domain deletion mutants are defective in intracellular maturation (13, 14). Interestingly, the pro-TGF-α cytoplasmic domain truncation mutants that can reach the cell surface have a similar rate of cleavage compared with wild-type pro-TGF-α (14). Similarly, the cytoplasmic domain is not needed for base-line processing of pro-EGF (18) and for phorbol ester- and ionomycin-stimulated cleavage of pro-HB-EGF (23) in contrast, recent analysis of two artificial EGF ligand precursors that contained either the EGF or HB-EGF membrane-anchoring domain showed that these ligand precursors display different base-line and stimulated ligand release rates, different endocytic routing, and differential sensitivity to metalloprotease inhibitors (24). In addition, the cytoplasmic domain does appear to regulate shedding for other membrane proteins. In the case of L-selectin, binding of calmodulin to its cytoplasmic domain can inhibit processing (20), whereas distinct cytoplasmic domains of different neuregulin isoforms can modify their ectodomain cleavage (21, 22).

The importance of the pro-AR cytoplasmic domain in constitutive and stimulus-induced processing of pro-AR was examined by stable expression of ARTL27 in MDCK cells. Since ARTL27 was expressed on both the apical and basolateral membrane domains of polarized MDCK cells, a direct comparison of wild-type pro-AR and ARTL27 processing was valid only in the basolateral membrane domain. In agreement with previous findings with pro-EGF and pro-HB-EGF, the similar profiles of the kinetics, sequential cleavage, and metalloprotease sensitivity of ARTL27 processing compared with wild-type pro-AR processing indicate that the pro-AR cytoplasmic domain is not essential for constitutive or stimulus-induced ectodomain cleavage of pro-AR (29). Whether subtle differences in release rates can be detected between different constructs and with different stimuli awaits the development of more sensitive AR assays.

We have previously shown that pro-EGF is preferentially cleaved from the basolateral surface of polarized MDCK cells in a metalloprotease-dependent manner (18). Loss of polarized sorting of ARTL27 therefore provided an opportunity to compare regulation of constitutive and stimulated processing of pro-AR in the apical and basolateral membrane domains of...
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polarized MDCK cells. Under base-line conditions, we found that processing of ARTL27 occurs preferentially at the basolateral cell surface. Processing of the 45-kDa ARTL27 precursor was ~3–4-fold greater at the basolateral surface compared with the apical surface. This result is also consistent with differences in release rates of 43-kDa soluble AR in the two membrane domains and the enhanced expression of 45-kDa ARTL27 observed on the apical cell surface. Importantly, these results suggest that the distal cleavage site within the juxtaplasmalemma domain of pro-AR is preferentially regulated at the basolateral surface in polarized epithelial cells. Although we have shown that the pro-AR cytoplasmic domain is not essential for pro-AR processing, these data suggest that the cytoplasmic domain can indirectly modify constitutive processing by its ability to define the spatial compartmentalization of pro-AR in polarized epithelial cells. A similar model has been proposed for preferential constitutive processing of wild-type pro-EGF in the basolateral membrane domain in polarized MDCK cells (18). In direct contrast to constitutive ARTL27 processing, we found that all stimuli activated ARTL27 release equivalently at the apical and basolateral surfaces of MDCK cells. Even though the metalloprotease inhibitor BB-94 can block ARTL27 processing in either membrane domain, this result suggests that constitutive and stimulus-induced proteolytic activities may be differentially regulated in polarized MDCK cells.

One possible explanation for the preferential cleavage of pro-AR at the basolateral cell surface is that the metalloprotease(s) involved in base-line and activated processing events have different spatial compartmentalization in polarized epithelial cells. To date, trafficking and cellular distribution of disintegrin metalloproteases in polarized epithelial cells have not been defined, although in the case of other metalloproteases, polarized sorting has been demonstrated. For example, human meprina, an astacin-type metalloprotease, is apically sorted in MDCK cells and in normal intestinal epithelium (64, 65). Alternatively, the signaling pathways that regulate constitutive and activated ARTL27 processing could display a polarized distribution. Pro-TGF-α processing can be stimulated by ligand-mediated activation of EGFR signaling (36, 39). Pro-TGF-α processing induced by EGFR activation requires ERK MAPK activity, whereas constitutive processing of pro-TGF-α in cells lacking endogenous EGFRs requires p38 MAPK activity (39). Importantly, it was shown that these two MAPK signaling pathways can contribute additively to increase ligand release (39). MDCK cells express several endogenous EGF-like ligands and have endogenous basolateral EGFRs (17). In combination with overexpression of human pro-AR, these cells should have a significant base-line level of autocrine EGFR signaling, which could activate EGFR ligand release (36, 39). Although the effects of EGFR activation on sequential pro-AR processing have not been defined in detail, wild-type pro-AR processing can be stimulated >2-fold above base-line levels upon EGFR stimulation in a metalloprotease- and tyrosine kinase-dependent manner. Therefore, an alternative explanation for differences in constitutive rates of ARTL27 cleavage is that the faster basolateral rate is a combination of base-line p38 MAPK-dependent and EGFR-activated ERK MAPK-dependent processing, whereas the slower apical cleavage rate involves only the base-line p38 MAPK-dependent pathway. In this scenario, spatial compartmentalization of EGFIRs creates localized activation of the proteolytic machinery. Abilities of different pharmacological agents to activate apical processing could therefore be due to the fact that they act downstream within signaling cascades that no longer have a polarized distribution.

In conclusion, we have demonstrated that newly synthesized pro-AR is directly sorted to the basolateral membrane domain in polarized MDCK cells, where it is sequentially cleaved to release soluble AR forms into the basolateral conditioned medium. Analyses of two pro-AR secretory mutants (ARsec184 and ARsec190) and a pro-AR cytoplasmic domain truncation mutant (ARTL27) showed that the cytoplasmic domain is not required for efficient delivery of pro-AR to the plasma membrane, but is essential for basolateral sorting. ARTL27 is mis-sorted and expressed on both the apical and basolateral membrane domains in polarized MDCK cells. ARTL27 is preferentially cleaved from the basolateral membrane domain under base-line conditions, but stimulated cleavage can occur from either membrane domain of polarized epithelial cells. These results suggest that base-line and stimulus-induced cleavage of ARTL27 may be regulated differently in the apical and basolateral membrane domains.

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The Proamphiregulin Cytoplasmic Domain Is Required for Basolateral Sorting, but Is Not Essential for Constitutive or Stimulus-induced Processing in Polarized Madin-Darby Canine Kidney Cells
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