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The Heparin-Binding Domain of Amphiregulin Necessitates the Precursor Pro-Region for Growth Factor Secretion

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The five members of the human epidermal growth factor (EGF) family (EGF, transforming growth factor α [TGF-α], heparin-binding EGF-like growth factor [HB-EGF], betacellulin, and amphiregulin [AR]) are synthesized as transmembrane proteins whose extracellular domains are proteolytically processed to release the biologically active mature growth factors. These factors all activate the EGF receptor, but in contrast to EGF and TGF-α, the mature forms of HB-EGF and AR are also glycosylated, heparin-binding proteins. We have constructed a series of mutants to examine the influence of the distinct precursor domains in the biosynthesis of AR. The transmembrane and cytoplasmic domains of the precursor are not required for secretion of bioactive AR from either COS or mammary epithelium-derived cells, although proteolytic removal of the N-terminal pro-region is less efficient in the absence of the membrane anchor. Deletion of the N-terminal pro-region, however, results in rapid intracellular degradation of the molecule with no detectable secretion of active growth factor. AR secretion is preserved by replacing the native pro-region with the corresponding domain of the HB-EGF precursor but not with that of the TGF-α precursor. In the absence of any N-terminal pro-region, secretion of the molecule is restored by deleting the N-terminal heparin-binding domain of mature AR. Both EGF and TGF-α, in contrast, can be secreted without their pro-regions. However, if the protein is fused with the AR heparin-binding domain, TGF-α secretion is inhibited unless the AR pro-region is also present. We propose that the heparin-binding domain of mature AR necessitates the presence of a specific structural motif in an N-terminal pro-region to permit proper folding, and thus secretion, of a bioactive molecule.

Many peptide growth regulators are synthesized as larger precursor proteins which are posttranslationally processed to the mature factor (reviewed in reference 38). Precursors for a number of growth factors, including transforming growth factor β (TGF-β)-, nerve growth factor-, and platelet-derived growth factor-related molecules, are soluble proteins containing the active peptide at the C terminus preceded by an N-terminal pro sequence (4, 15, 54, 71). Various functions have been ascribed to the pro-regions for this class of molecule. For example, both NGF and TGF-β require their pro sequences for secretion, PDGF is rendered inactive unless the pro sequence is removed, and the TGF-β pro-region is involved in latent complex formation (11, 21, 39–41, 61, 65). However, an increasing number of soluble growth factors are found to be cleaved from integral membrane proteins, suggesting the possibility that such precursors participate in cell-cell communication or juxtacrine interactions (18, 37). For example, colony-stimulating factor 1 and tumor necrosis factor alpha/cachexin can signal through their respective receptors as membrane-anchored molecules, and several lines of evidence indicate a biological function specific to the transmembrane form of Steel factor, the ligand for the c-Kit receptor (10, 20, 33, 47, 59, 64). A large family of growth factors initially synthesized as integral membrane precursors is composed of the epidermal growth factor (EGF)- and heregulin-related molecules.

The human genome encodes five known structurally related proteins that bind and activate the EGF receptor: EGF, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and amphiregulin (AR) (3, 16, 24, 48, 53, 57, 58). These proteins are all synthesized as part of larger precursors consisting of a signal peptide for localization to the secretory pathway, an N-terminal pro-region, and the mature peptide sequences, followed by a short spacer, a single transmembrane domain, and cytoplasmic tail. The bioactive domains are characterized by a six-cysteine consensus motif, $X_nCX_7CX_{4-5}CX_{10}CXCX_5GX_2CX_n$, and several additional conserved residues (12). Mature HB-EGF and AR also have N-terminal extensions, composed of predominantly basic residues, that are thought to confer their heparin-binding abilities. Outside the EGF-like region, these precursors show little sequence homology, and their biological function(s) remains unclear.

The N-terminal pro-regions vary considerably among the EGF family members: the pro-domain of EGF spans 950 residues and contains nine additional EGF-like sequences, whereas the pro-regions of AR (74 residues), HB-EGF (~54 residues), and TGF-α (17 to 18 residues) are considerably smaller (3, 7, 16, 25, 48). The betacellulin precursor has not yet been characterized but is predicted to contain a pro-region of only about seven residues (53, 57). In the case of EGF, the pro-region can be deleted without loss of protein expression or bioactivity, but the other molecules have not been similarly examined (17, 60). The presence of a transmembrane domain does suggest distinct functions for cell-associated and soluble forms of the factors. Although no physiological role has yet been attributed to the membrane-anchored factors, both EGF and TGF-α can bind and stimulate mitogenesis through the EGF receptor as transmembrane proteins, and the HB-EGF precursor has been implicated as the cellular receptor for diphtheria toxin (1, 6, 9, 43, 70).

Among the EGF family members, TGF-α has served as the primary model for studying precursor function and mechanisms of soluble factor production. Mature peptide is released from this precursor by independent processing at two flanking elastase-like cleavage sites (Ala-Val). Chinese hamster ovary

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(CHO) cells stably transfected with TGF-α efficiently cleave the N-terminal site but accumulate membrane-anchored factor as a result of inefficient cleavage at the C-terminal site (63). In contrast, retrovirally transformed rat embryo fibroblasts and hepatocarcinoma cells apparently process both sites at similar rates, resulting in the secretion of both *meso*-TGF-α (containing N-terminal pro-sequences) and the mature peptide (27, 36, 62). In certain cell types, cleavage at the C-terminal site is dramatically stimulated by activators of protein kinase C or elevated cytosolic calcium levels (44, 45). This regulated response is dependent on the cytoplasmic domain of the precursor (8).

While related to the other EGF family members, AR has several features which distinguish it from the TGF-α model. Precursor sequences flanking mature AR lack homology with the TGF-α cleavage sites, so processing may be mediated by different enzymes and thus subject to different regulating factors. Mature AR is a glycosylated heparin-binding peptide, and soluble heparin sulfate-like molecules inhibit its mitogenic activity. It also has a significantly lower affinity for the EGF receptor on A431 cells than do other family members (58). This reduction may be due to the absence of a leucine at position 42 in AR (as measured from the first cysteine of the EGF-like repeat), a residue critical for the activity of EGF and TGF-α and conserved in HB-EGF (19, 34, 50). However, AR may be a more specific and natural regulator of normal epithelial cells than the other factors, as evidenced by its discovery as an autocrine mitogen for cultured human keratinocytes, mammary epithelial cells (HMEC), and colorectal cells and by its diminished potency on mesenchymal cells (14, 31, 35, 58). Given the differences in the mature factors, the biologic relevance of the AR precursor may also differ from that of TGF-α.

In this study, we address the functions of AR precursor (ARP) domains. Using deletion analysis, our initial experiments examine the roles of the N-terminal pro-region, transmembrane, and cytoplasmic domains in the synthesis and secretion of bioactive peptide. This report demonstrates that both the N-terminal pro-region and the membrane anchor are important for the release of mature AR, with the pro-region being required for protein secretion while the transmembrane domain facilitates posttranslational removal of the pro-region.

MATERIALS AND METHODS

Cell lines. COS-1 (ATCC CRL 1650), 184A1N4-TH (13), and NRHer5 (5) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Normal HMEC (Clonetics Corp.) were cultured in mammary epithelium growth medium (Clonetics Corp.). All cells were maintained at 37°C in 5% CO_2.

Transient transfection assay. One day prior to transfection, COS cells were plated at 10^6 cells per 100-mm-diameter dish, and 184A1N4-TH cells were plated at twice that density. Cells were transfected with 30 μg of plasmid DNA by the DEAE-dextran-chloroquine method (55) and cultured for 2 days in DMEM–10% FBS. Cells were then washed twice and cultured for an additional 24 h in serum-free DMEM (5 ml/100-mm-diameter dish) before harvesting for Western blot (immunoblot) analysis.

For metabolic labeling experiments, cells were trypsinized 24 h after transfection, replated in parallel 35-mm-diameter dishes, and cultured for an additional 24 h. For pulse-chase experiments, cells were preincubated for 30 min in cysteine-free minimal essential medium and then labeled for 15 min in the same medium supplemented with 200 μCi of [^{35}S]cysteine (New England Nuclear) per ml. Plates were washed twice with DMEM and chased in 1 ml of DMEM–2% FBS at 37°C for the indicated lengths of time. For steady-state labeling experiments, cells were cultured for 24 h in cysteine-free minimal essential medium containing 2% dialyzed FBS, 1% complete DMEM, and 50 μCi of [^{35}S]Cys or [^{35}S]methionine-cysteine mix (EXPRE^{35}S^{35}S; New England Nuclear) per ml.

Construction of mutant AR. Human AR cDNA (48) was excised at *Hga*I and *Eco*RV sites and subcloned into pBluescript II SK(+) (Stratagene). Mutations were generated in this template by using one- or two-step PCR protocols and synthetic oligodeoxynucleotide primers (29). Secondary structure predictions of N-terminal pro-regions were based on the original method of Chou and Fasman, using the PeptideStructure program in the Genetics Computer Group sequence analysis software (Genetics Computer, Inc.) (30).

The ARTL construct terminates following arginine 225 in the ARP, which is immediately C terminal to its putative membrane-spanning region (...KIALAAIAAFMSAVILTAV AVITVQLRR^{225}). The PA and NPA constructs were truncated following threonine 188 in the precursor, seven residues C terminal to the final Cys of the EGF-like motif (...GERCGEKSMKT^{188}). Mature AR isolated from tetradecanoyl phorbol acetate-treated MCF7 cells was previously reported to terminate at lysine 184 in the precursor; however, sequence analysis of recombinant AR purified from CHO cells reveals equimolar amounts of peptide terminating at lysine 187 and threonine 188 (unpublished data). Mature 18-kDa AR produced endogenously by both cultured normal HMEC and normal human keratinocytes can be metabolically labeled with [^{35}S]methionine (unpublished data), indicating that the C terminus of native AR extends to at least methionine 186, since no other methionines are found within or immediately N terminal to the mature factor.

Constructs with N-terminal deletions (ARNP and NPA) were generated by fusing the signal sequence from the human TGF-β_1 precursor (MPPSGLRLLPLLLPLLWLLVLTPSRPA AG) (22) in frame with the N terminus of the predominant form of mature AR, beginning with valine 107 in the precursor (^{107}VVKPPQNK...). The domain swap mutants HB-AR and TGF-AR replaced the first 107 residues of the ARP with the first 72 and 38 residues of the human HB-EGF (24) and TGF-α (16) precursors, respectively. The human TGF-α cDNA used for this construct was isolated from A431 cells. Like one of the two forms of rat TGF-α message produced by exon sliding (7), this cDNA encodes a 159-residue primary translation product lacking alanine at position 32. The construct AR-NB contains a 43-residue internal deletion (from serine 101 to lysine 143) that leaves the N-terminal pro-region intact but removes the hydrophilic region of the mature factor just N terminal to the EGF-like motif (which begins at cysteine 146). The construct ARNP-NB contains the TGF-β signal sequence fused in frame to asparagine 144 (^{144}NPCNA EFQNFC...), deleting the first 143 residues of the ARP. Constructs ARNPΔN and ARNPΔC, created by using ARNP as the template for PCR priming, delete residues corresponding to amino acids 107 to 122 and 123 to 143, respectively, in the ARP (see Fig. 9A).

The TGF-α-based constructs (Pα, NPα, PAα, and NPAα) were derived from the human TGF-α cDNA described above (7, 16), and all were truncated at a position corresponding to the C terminus of the mature factor (...CEHADLLA^{88}). The N-terminal deletion mutant (NPα) was created by fusing the TGF-β_1 signal sequence in frame with the N terminus of mature TGF-α (^{39}VVSHFNDCPDSHTQFC...). The N-terminal regions of the AR–TGF-α chimeric constructs were de-

rived from the full-length ARP and pro-less ARNP constructs; PAα contains the first 143 residues of the ARP residues, and NPAα contains the TGF-β_1 signal with ARP residues 107 to 143. For both chimeras, these sequences were fused to the EGF-like domain of TGF-α, beginning at residue 45 in the TGF-α precursor (^{45}DCPDSHTQFC...).

All PCR products were sequence verified, and the final constructs were directionally subcloned into the cDM8 expression vector (Invitrogen Corp.) at *Hin*dIII and *Not*I sites. The vector drives expression from the cytomegalovirus promoter and contains the simian virus 40 (SV40) origin of replication to allow plasmid amplification in cells expressing the SV40 T antigen.

Western blot analysis. Culture supernatants were clarified and dialyzed against 0.1 M acetic acid, dried in a Speed Vac concentrator (Savant), and resuspended directly in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-Cl [pH 6.8], 2% SDS, 0.05% bromophenol blue, 10% glycerol, 2.5% 2-mercaptoethanol). Cell extracts were prepared as follows. Monolayers from 100-mm-diameter dishes were washed twice with phosphate-buffered saline (PBS) and scraped on ice in 1 ml of hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 1 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 μg of aprotinin per ml). Cells were then incubated for 30 min on ice and Dounce homogenized until ~95% appeared broken by visual inspection. Extracts were centrifuged 3 min at 300 × *g* at 4°C to sediment nuclei and unbroken cells, and the supernatants were separated into particulate and soluble fractions by 10 min of centrifugation at 160,000 × *g* (Beckman Optima TLX-120 tabletop ultracentrifuge). Pellets (particulate fraction) were resuspended directly in SDS sample buffer, while supernatants (soluble fraction) were first dried in a Speed Vac concentrator.

Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 15% acrylamide–0.5% bisacrylamide gels (52) and electrophoretically transferred to nitrocellulose. Nonspecific binding was blocked by preincubating blots in BLOTTO (PBS containing 5% [wt/vol] nonfat dried milk and 0.2% [vol/vol] Nonidet P-40 [Sigma]), and AR was detected by using rabbit antisera raised against synthetic peptides corresponding to either the N-terminal region of the mature factor (^{108}VKPPQNKTESENTSDKPKRKKKG^{130}; antibody [Ab] 035) or the N-terminal pro-region of the precursor (^{71}SSSEP SSGADYDYSEEYDNE^{90}; Ab 042) followed by ^{125}I-protein A. Bands were visualized with a Molecular Dynamics PhosphorImager.

Immunoprecipitations. Cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris-HCl [pH 8.0]) containing 1 mM PMSF and 20 μg of aprotinin per ml for 20 min on ice and clarified by 10 min of centrifugation at 15,000 × *g* at 4°C. To reduce nonspecific background in metabolically labeled cell extracts, RIPA extracts of unlabeled, nontransfected cells were added at fivefold excess. Medium samples were diluted with an equal volume RIPA buffer and used directly. All samples were preincubated 1 h at 4°C with either protein A-Sepharose or protein G-Sepharose and centrifuged for 10 min at 15,000 × *g* at 4°C to preclear prior to immunoprecipitation. AR was immunoprecipitated by overnight incubation at 4°C with constant mixing, using either affinity-purified Ab 035 and protein A-Sepharose or pooled hybridoma supernatants containing murine monoclonal Abs raised against mature AR (Abs 4.14, 12.33, 12.38, 14.3, 16.21, and 19.23) and protein G-Sepharose. Precipitated samples were washed on ice once each with high-salt buffer (0.5 M NaCl, 1.0% Nonidet P-40, 50 mM Tris-HCl [pH 8.0]), low-salt buffer (1.0% Nonidet P-40, 50 mM Tris-HCl [pH 8.0]), and RIPA buffer. Immunoprecipitates were then resuspended in SDS sample buffer and resolved by SDS-PAGE for either Western blotting or direct autoradiography with the PhosphorImager.

To affinity purify Ab 035 for immunoprecipitations, the antiserum was incubated for 3 h at room temperature with immunizing peptide coupled through primary amines to an Affi-Gel 10 support (Bio-Rad). The support beads were then transferred to a small column and washed with PBS, and the bound Abs were eluted with 50 mM glycine (pH 2.0). The eluate was immediately neutralized with 0.06 volume of 1 M Na_2HPO_4 and dialyzed against PBS, and 1% bovine serum albumin (BSA) and 0.02% NaN_3 were added for storage.

Endoglycosidase H digestion. Cell extracts and media were immunoprecipitated with affinity-purified Ab 035. At the final wash, each sample was divided into two tubes, and the immune complexes were resuspended in 10 μl of endoglycosidase H buffer (30 mM sodium acetate [pH 5.4], 0.1 M 2-mercaptoethanol, 0.5% Triton X-100, 1 mM PMSF, 0.2% SDS) and denatured 5 min at 95°C. To one tube, 1 mU of endoglycosidase H (Boehringer Mannheim) was added, and both tubes were incubated 16 h at 37°C. An equal volume of 2× SDS sample buffer was added directly to reactions, and samples were resolved by SDS-PAGE and analyzed by Western blotting.

Heparin binding assay. Heparin acrylic beads (Sigma) in PBS were mixed with medium samples from [^{35}S]cysteine metabolically labeled cells (15 μl of packed resin per 200 μl of medium) for 1 h at 4°C. The heparin beads were collected by centrifugation and washed twice with PBS. Supernatants were diluted with an equal volume of RIPA buffer and used directly for immunoprecipitations. AR was eluted from the heparin beads by incubation for 1 h at 4°C with 75 μl of 1 M NaCl in sodium phosphate buffer (pH 7.2). The eluate was diluted with 200 μl each of low-salt and RIPA buffers and then used for immunoprecipitations.

EGF receptor autophosphorylation assay. NRHer5 cells were seeded in 12-well plates at 5 × 10^4 cells per well and cultured for 24 h (cells achieved ~50% confluence). Culture medium was then replaced (0.5 ml per well sample) with standards diluted in DMEM containing 0.1% BSA and with conditioned media from transfected cells diluted with an equal volume of DMEM containing 0.1% BSA. Following a 10-min incubation at room temperature, medium was removed and cells were solubilized for 10 min on ice in PBS-TDS (10 mM sodium phosphate [pH 7.25], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% NaN_3, 1 mM NaF, 1 mM PMSF, 20 μg of aprotinin per ml), with occasional vortexing. Clarified extracts were incubated 1 h at 4°C with an anti-EGF receptor antibody (EGFR1; Amersham). Protein A-Sepharose was then added, and samples were incubated for an additional hour. Immune complexes were washed three times with PBS-TDS, resolved on a 7% polyacrylamide–0.23% bisacrylamide gel, and electrophoretically transferred to nitrocellulose. Phosphorylation of the receptor was assessed by Western blotting with an antiphosphotyrosine primary antibody (PY20; ICN Biochemicals) and ^{125}I-labeled sheep anti-mouse F(ab')$_2$ (Amersham). Bands were detected and quantitated on the PhosphorImager.

TGF-α ELISA. A TGF-α sandwich enzyme-linked immunosorbent assay (ELISA; Oncogene Science, Inc.) was used for quantitating TGF-α immunoreactivity as instructed by the manufacturer. Culture medium was diluted 40-fold with assay buffer and then analyzed directly.

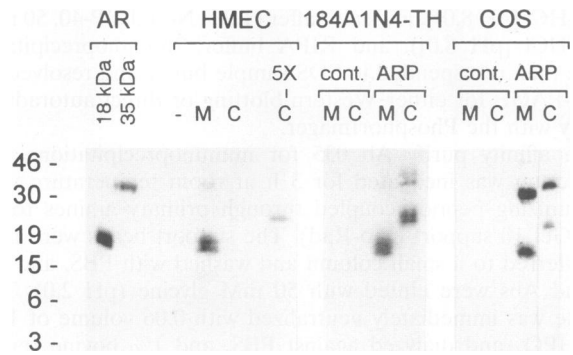

FIG. 1. Western blot analysis of HMEC, 184A1N4-TH, and COS cell AR. Conditioned media (M) and cell extracts (C) from normal HMEC and transfected COS and 184A1N4-TH cells were examined on SDS-polyacrylamide gels by immunoblotting with the AR-specific antiserum Ab 035. Cells were transfected with either control vector (cont.) or a construct containing the cDNA for the full-length ARP. Shown is the particulate fraction of Dounce-homogenized cells. The soluble fraction of cell extracts contained no detectable AR. The 18- and 35-kDa AR standards (50 ng of each) were purified from the conditioned medium of CHO cells transfected with a full-length precursor construct. HMEC samples correspond to 20% of a 100-mm-diameter dish of cells, except for the lane labeled 5×, which was immunoprecipitated from the extract of an entire 100-mm-diameter dish. Samples from 184A1N4-TH and COS cells correspond to 5% of a 100-mm-diameter dish. Sizes are indicated in kilodaltons.

RESULTS

Transient expression system for studying AR synthesis and secretion. To study the effects of precursor mutations on the synthesis and secretion of AR, we wished to have an expression system that lacked high levels of endogenous AR yet represented a cell lineage in which this factor is normally found. Normal proliferating HMEC express and secrete bioactive AR. However, transformed mammary epithelial cells typically lose AR expression, providing a potentially suitable background for studying mutants (14, 35). The HMEC line chosen for these studies, 184A1N4-TH, stably expresses the SV40 T antigen and is thus functionally similar to COS cells (13). Plasmids containing the SV40 origin are replicated to high copy number in such cells, allowing expression of foreign protein to be readily achieved in transient transfection assays.

To assess the validity of the model system, AR produced in COS and 184A1N4-TH cells transiently transfected with the full-length ARP cDNA was compared with that produced by normal HMEC. Two days after transfection, cells were changed to serum-free culture medium for an additional 24 h to collect secreted products. Cells were then harvested and fractionated into soluble and particulate fractions (see Materials and Methods). Secreted and cell-associated proteins were analyzed by Western blotting with a polyclonal antiserum directed against the N-terminal region of the mature peptide (Fig. 1). Normal HMEC secrete predominantly an 18-kDa form comigrating with mature AR, although a 35-kDa form comigrating with gp35 (soluble factor extended by the N-terminal pro-region from the precursor) was also detected. Relatively little AR was cell associated, with the predominant form migrating at 25 kDa, although a 42-kDa species was also detected. Both forms are consistent with the presence of a ~7-kDa transmembrane and cytoplasmic domain.

When transfected with vector alone, neither COS nor 184A1N4-TH cells produced detectable AR. However, cells transfected with the ARP construct secreted both 18- and 35-kDa forms. While COS cells produced similar levels of the two forms, the 184A1N4-TH cells predominantly secreted 18-kDa AR, similar to HMEC. Cell extracts of both lines had high levels of both the 25- and 42-kDa forms.

AR deletion mutants. To assess the role(s) of the membrane anchor and N-terminal pro-regions in the synthesis and secretion of bioactive AR, four deletion mutants were constructed (Fig. 2 and Materials and Methods): (i) ARTL (tail-less, deleting the cytoplasmic domain only leaving the membrane-spanning region intact), (ii) PA (soluble pro-AR, deleting both the transmembrane and cytoplasmic domains), (iii) ARNP (no-pro, deleting the N-terminal pro-region only), and (iv) NPA (soluble no-pro, deleting both N-terminal pro-region and transmembrane domains). Deletions were made in the cDNA for the full-length precursor by using PCR techniques, and all PCR products were confirmed by complete sequence analysis. Both the ARNP and NPA constructs utilized the N-terminal signal sequence from human TGF-β_1 to direct protein to the secretory pathway.

Native ARP and the four deletion mutants were transiently expressed in both COS and 184A1N4-TH cells. Secretion of bioactive material was assessed by testing conditioned media from transfected cells for the ability to stimulate ligand-induced EGF receptor autophosphorylation in intact cells (Fig. 3). Medium from cells transfected with vector alone exhibited no EGF-like activity, whereas cells transfected with the full-length precursor produced significant levels of receptor phosphorylation, corresponding to ~50 to 200 ng of AR activity per ml in the undiluted culture medium, depending on the transfection experiment. Deletion of either the cytoplasmic (ARTL) or transmembrane (PA) domain did not dramatically affect the secretion of bioactive material. However, no stimulation of EGF receptor autophosphorylation was observed with constructs lacking the N-terminal pro-region (ARNP and NPA).

Secreted and cell-associated protein from transfected cells was analyzed by Western blotting with Abs directed against either the mature peptide or the N-terminal pro-region (Fig. 4A and B). Data from 184A1N4-TH cells are shown, although similar results were found with COS cells. In agreement with the bioassay, AR immunoreactive protein was found in the media of cells transfected with the three constructs containing the N-terminal pro-region (ARP, ARTL, and PA) but not those lacking this domain (ARNP and NPA). Both the 18- and 35-kDa forms were secreted from transfected cells, with the 18-kDa peptide predominating for the two transmembrane precursors (ARP and ARTL) and the 35-kDa form predominating for the variant lacking a membrane anchor (PA). As before, the native precursor produced cell-associated forms migrating at 42 and 25 kDa. However two smaller proteins, migrating at 37 and 20 kDa, were found in extracts of ARTL-transfected cells, and the PA construct generated primarily a 35-kDa cell-associated form. These increased mobilities are consistent with precursors lacking either the cytoplasmic domain alone (ARTL) or both the transmembrane and cytoplasmic domains (PA). In comparison with either PA or the native ARP, a higher proportion of the ARTL-derived protein was typically cell associated, although the differences were not always dramatic (ARP and ARTL samples in Fig. 4A and C reflect the range of differences observed). No AR immunoreactivity was found in extracts of ARNP-transfected cells, although a 16-kDa form was found in cells expressing the NPA construct. In all cases where AR protein was detected, the higher-molecular-weight forms in both media and cell extracts also cross-reacted with the N-terminal pro-region-specific antiserum (Fig. 4B). Thus, the three mutant molecules

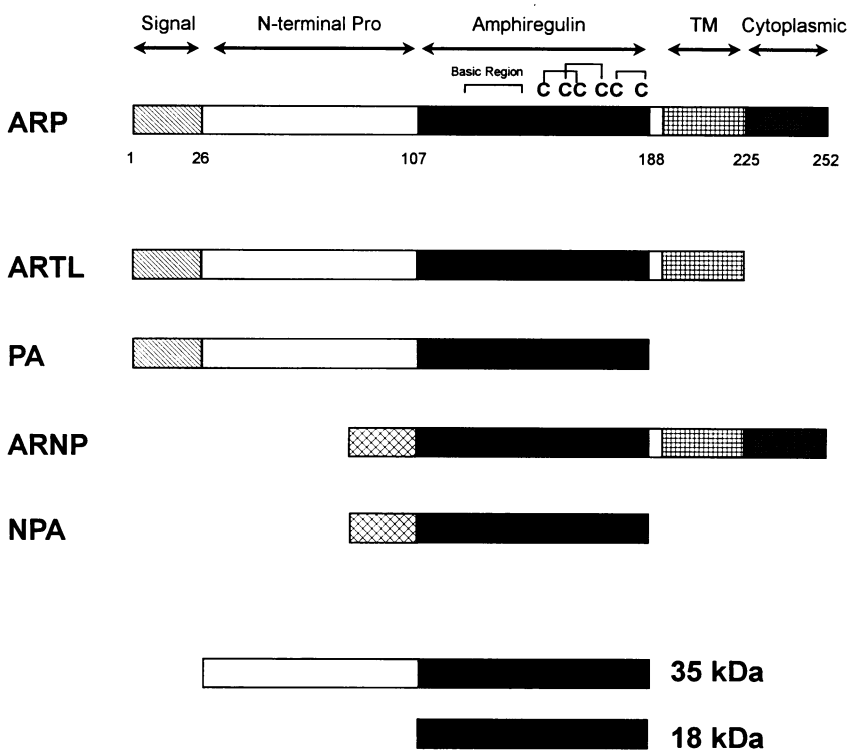

FIG. 2. AR deletion constructs. Functional domains and amino acid positions within the human ARP are indicated on the full-length construct, ARP. The EGF-like motif is represented by the six disulfide-linked cysteines (C). Compositions of the 35- and 18-kDa secreted products are also indicated. Signal, N-terminal signal sequence (▨, native AR signal; ▩, TGF-β signal); TM, transmembrane domain.

possessing the AR pro-region (ARP, ARTL, and PA) were secreted from transfected cells, while those lacking this domain (ARNP and NPA) were not.

Particulate and soluble fractions of cell extracts were next compared (Fig. 4C). Data from COS cells are shown, although 184A1N4-TH cells demonstrated similar results. Only the PA construct produced AR in the soluble fraction. However, approximately half of the intracellular PA and essentially all of the NPA was found in the particulate fraction, even though neither was predicted to possess a membrane anchor. To resolve this discrepancy, we examined the possibility that these forms were restricted to the endoplasmic reticulum, which would suggest insoluble aggregates of misfolded protein (see references 26, 46, and 51 for reviews).

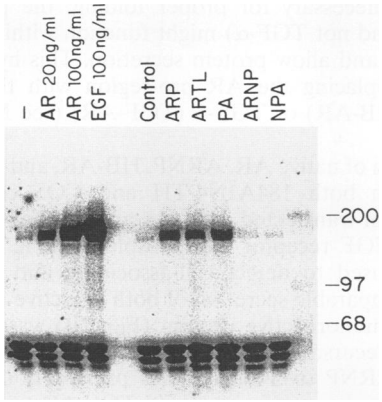

FIG. 3. Bioactivities of AR deletion mutants. NRHer5 cells were stimulated either with purified recombinant AR or EGF or with the conditioned medium from transfected COS cells. The 175-kDa EGF receptor was immunoprecipitated from solubilized NRHer5 cells, and autophosphorylation was assessed by antiphosphotyrosine Western blotting. The immunoprecipitating Ab appears in all lanes as a triplet migrating below the 68-kDa marker. Sizes are indicated in kilodaltons.

Since mature AR is a glycoprotein with N-linked oligosaccharides, the secreted and cell-associated proteins were examined for sensitivity to endoglycosidase H. This enzyme preferentially hydrolyzes high-mannose N-linked carbohydrates, such as intermediates found in the endoplasmic reticulum, and does not hydrolyze the complex carbohydrates produced during maturation in the Golgi complex. As shown in Fig. 5, AR synthesized from the PA construct and found in the particulate fraction of cell extracts was shifted to a single, faster-migrating band following digestion with endoglycosidase H. In contrast, the 25-kDa cell-associated form produced from the full-length precursor was endoglycosidase H insensitive. The secreted forms from both constructs were unaffected by this enzyme. These results suggest that despite high levels of PA released from cells, this membrane anchor-less molecule may have suboptimal folding and secretion efficiency.

AR from the NPA construct found in cell extracts was not affected by endoglycosidase H digestion, even immediately following a 15-min metabolic labeling with [^{35}S]cysteine, when all of the AR produced from the full-length precursor was endoglycosidase H sensitive (Fig. 5B). This observation suggests a lack of N-linked glycosylation in the NPA variant and correlates well with its migration on SDS-PAGE as a protein ~2 kDa smaller than the mature native factor. Protein misfolding and aggregation in the endoplasmic reticulum would be

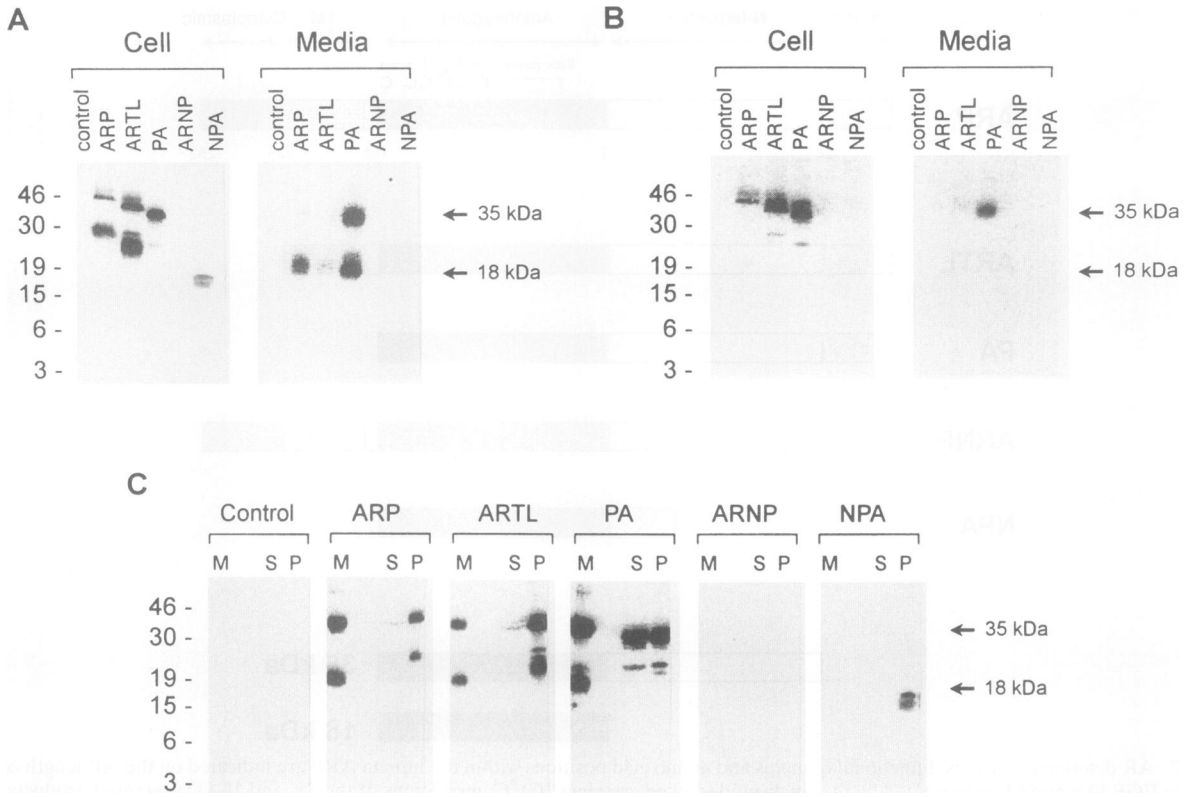

FIG. 4. Expression of AR protein from deletion mutant constructs. Cell extracts and conditioned media from transfected 184A1N4-TH cells (A and B) and COS cells (C) were examined on SDS-polyacrylamide gels by immunoblotting with antisera directed against either the mature peptide (Ab 035 [A and C]) or the N-terminal pro-region (Ab 042 [B]). All lanes correspond to 5% of a confluent 100-mm-diameter dish of cells. COS cell extracts were separated into soluble (S) and particulate (P) fractions. M, medium. Positions of molecular size markers (in kilodaltons) are shown on the left, and positions of migration of the 35- and 18-kDa forms of AR are shown on the right for reference.

consistent with intracellular accumulation and lack of normal glycosylation and secretion of the NPA construct.

Absence of the N-terminal pro-region results in rapid turnover of the AR precursor. The construct lacking the N-terminal pro-region (ARNP) was not detected in either the media or cell extracts, possibly because of either a deficiency in synthesis or rapid degradation of newly made protein. To distinguish between these possibilities, transfected 184A1N4-TH cells were metabolically labeled for 15 min with [^{35}S]cysteine and then chased for 0 min, 30 min, 1 h, or 4 h (Fig. 6). Immunoprecipitation with affinity-purified anti-AR serum revealed that a 22-kDa protein was synthesized but rapidly turned over ($t_{1/2}$, <30 min) in ARNP-transfected cells. In contrast, the native precursor (ARP) persisted in cell extracts for the entire 4 h and was partially converted from the 42-kDa to the 25-kDa cell-associated form, beginning by 1 h. Release into the media was minimal, even by 4 h. Like the more stable NPA product, the transiently observed ARNP migrated as a protein 2 to 3 kDa smaller than expected (~25 kDa) and was also endoglycosidase H insensitive (not shown).

These experiments indicate that the N-terminal pro-region of the ARP is required for AR secretion, presumably by directing proper folding of the molecule. We next addressed the possibility that the pro-regions of structurally related molecules can perform similar functions.

The N-terminal pro-region of HB-EGF can functionally replace the AR pro-region. While AR has a high degree of similarity to HB-EGF and TGF-α in the domain encompassed by the six cysteines (52 and 33%, respectively), minimal primary sequence homology can be found in the N-terminal pro-regions. Secondary structure predictions, however, suggest a similar distribution of hydrophilic stretches, preponderance of β turns, and lack of α-helical structure in the pro-regions of AR and HB-EGF but not TGF-α. If a conserved structural motif were necessary for proper folding, the pro-region of HB-EGF (and not TGF-α) might function within the context of the ARP and allow protein secretion. This hypothesis was tested by replacing the AR pro-region with that of either HB-EGF (HB-AR) or TGF-α (TGF-AR) (see Materials and Methods).

Expression of native AR, ARNP, HB-AR, and TGF-AR was compared in both 184A1N4-TH and COS cells (Fig. 7). Medium from transfected cells was assessed for bioactivity by measuring EGF receptor autophosphorylation, and Western blots were used to detect cell-associated and secreted AR protein. Comparable secretion of both bioactive material (Fig. 7A) and immunoreactive protein (Fig. 7B) was observed for the native precursor and HB-AR, but no protein was detected for either ARNP or TGF-AR. As previously observed with ARNP, pulse-chase experiments in TGF-AR-transfected cells demonstrated synthesis of an ~20-kDa protein that was turned over with a $t_{1/2}$ of <30 min (not shown).

Deletion of the basic domain abrogates the pro-region requirement. One structural feature shared by mature AR and HB-EGF, but not EGF or TGF-α, is a long (39 and ~34 residues, respectively), highly basic domain N terminal to the

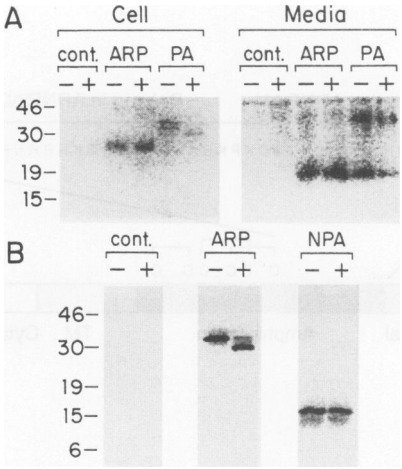

FIG. 5. Endoglycosidase H sensitivity of PA- and NPA-derived proteins. (A) Endoglycosidase H digestion of AR from 184A1N4-TH cells transfected with either control vector (cont.), full-length precursor (ARP), or the PA truncation mutant. Cells were transfected and harvested as for Western blots. Medium samples and the particulate fractions of cell extracts were then detergent solubilized and immunoprecipitated with affinity-purified anti-AR antiserum (Ab 035). Each immunoprecipitate was divided in half for incubation with either buffer alone (−) or endoglycosidase H (+) and then analyzed by Western blotting with the AR-specific antiserum Ab 035. (B) Endoglycosidase H digestion of metabolically labeled 184A1N4-TH cells transfected with either control vector (cont.), full-length precursor (ARP), or the NPA deletion mutant. Transfected cells were metabolically labeled 15 min with [^{35}S]cysteine and immediately detergent solubilized for immunoprecipitation with affinity-purified anti-AR antiserum. Each sample was divided, incubated both without (−) and with (+) endoglycosidase H, and resolved on an SDS-polyacrylamide gel for direct autoradiography. Sizes are indicated in kilodaltons.

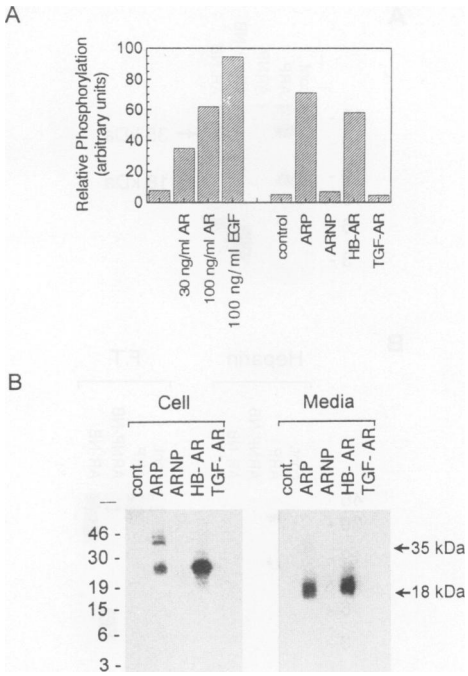

FIG. 7. Bioactivity and Western analysis of HB-AR and TGF-AR expressed in 184A1N4-TH cells. (A) EGF receptor autophosphorylation. NRHer5 cells were stimulated either with purified recombinant AR or EGF or with the conditioned media from transfected cells. EGF receptor was immunoprecipitated from solubilized NRHer5 cells, and autophosphorylation was assessed by antiphosphotyrosine Western blotting. Relative intensities of the 175-kDa phosphorylated receptor bands were determined by using a PhosphorImager and ImageQuant software. Similar results were also reproducibly observed with COS cells. (B) AR Western blot. Cell extracts and media from transfected 184A1N4-TH cells were analyzed by Western blotting with the anti-AR antiserum Ab 035 as described for Fig. 4. Only the particulate fraction of cell extracts is shown; no immunoreactivity was detected in the soluble fraction. Each lane corresponds to 5% of a confluent 100-mm-diameter dish. cont., control. COS cells produced similar results.

first cysteine of the EGF-like motif (24, 48). We hypothesized that the pro-regions of AR and HB-EGF might direct secretion by interacting with this basic domain to allow proper folding of the growth factor. To test this hypothesis, we generated a construct in which the entire N-terminal domain of mature AR was deleted (ARNP-NB [no-pro, no-basic]). A second construct retained the pro region (AR-NB [no-basic only]) to control for alternate mechanisms of pro-region function.

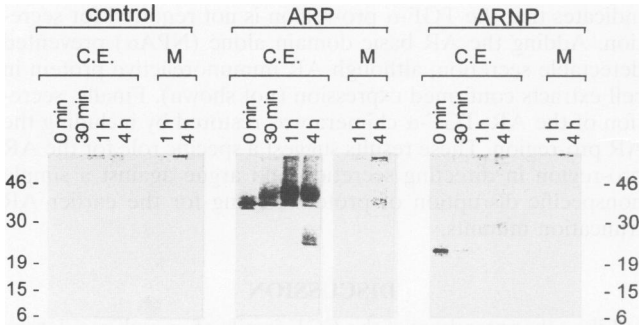

FIG. 6. Pulse-chase of ARNP-transfected 184A1N4-TH cells. Cells transfected with either control vector, full-length precursor (ARP), or the ARNP truncation mutant were metabolically labeled 15 min with [^{35}S]cysteine and then chased for either 0 min, 30 min, 1 h, or 4 h. AR was immunoprecipitated from media (M) and detergent-solubilized cell extracts (C.E.) with affinity-purified anti-AR antiserum Ab 035 and analyzed by direct autoradiography of an SDS-polyacrylamide gel.

Secretion of protein from transfected cells was assessed by immunoprecipitation of AR from the media of cultures metabolically labeled for 24 h with [^{35}S]cysteine. Pooled monoclonal Abs directed against the EGF-like domain were used in these experiments, since the no-basic constructs lack the epitope recognized by the rabbit antiserum previously used. Cells transfected with the ARNP-NB construct secreted a ~6-kDa protein (Fig. 8A), demonstrating that the pro-region is not required for secretion in the absence of the basic domain. Release of a ~30-kDa protein from AR-NB-transfected cells indicates that pro-region does not inhibit AR secretion in the absence of the basic domain. Unlike native AR, neither the 6-kDa ARNP-NB nor the 30-kDa AR-NB peptide was retained on heparin affinity resin (Fig. 8B), consistent with the colocalization of the heparin-binding domain to the basic N terminus of AR.

Closer inspection of the AR N terminus suggests that it may be divided into two subdomains, with most of the basic residues residing in the C-terminal half and the N-linked glycosylation sites residing in the N-terminal half (Fig. 9A). Deleting the C-terminal half (ARNPΔC) allowed secretion in

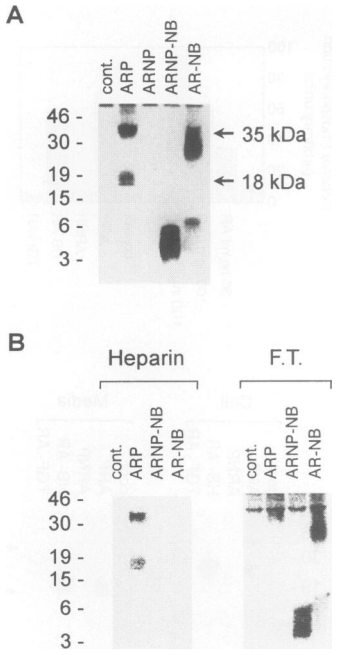

FIG. 8. Secretion of deletion mutants ARNP-NB and AR-NB from transfected 184A1N4-TH cells. (A) Metabolic labeling of 184A1N4-TH cells transfected with AR expression plasmids. Transfected cells were cultured 24 h in the presence of [^{35}S]cysteine, and secreted AR was immunoprecipitated with pooled monoclonal Abs raised against the mature factor. Samples were resolved on an SDS-polyacrylamide gel and visualized by direct autoradiography. cont., control. (B) Binding of metabolically labeled AR to immobilized heparin. Medium samples from metabolically labeled transfected cells were incubated with heparin acrylic beads and separated into bound and free fractions. AR on the heparin beads was eluted with high salt. The eluted samples (heparin) and unbound supernatant (flowthrough [F.T.]) were immunoprecipitated with the pooled anti-AR monoclonal Abs and analyzed by SDS-PAGE. cont., control. Sizes are indicated in kilodaltons.

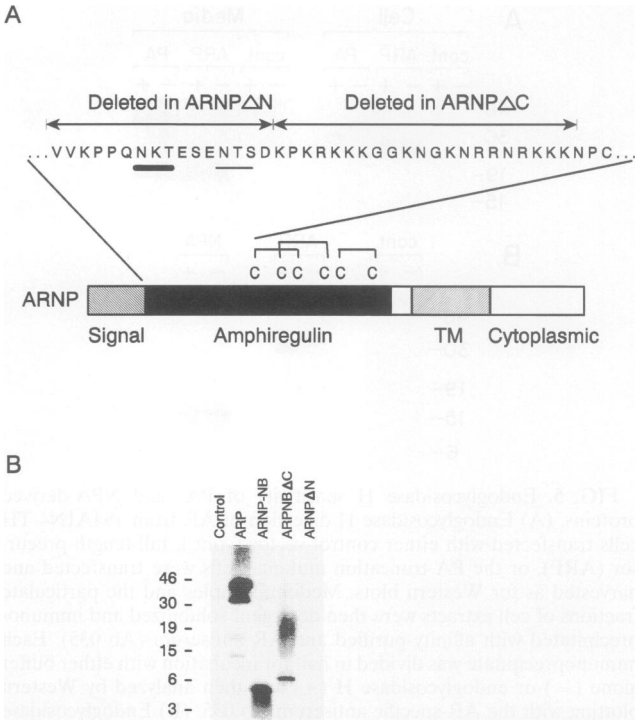

FIG. 9. Secretion of deletion mutants ARNPΔN and ARNPΔC from transfected COS cells. (A) Schematic of N-terminal domain deletions. Functional domains within the parent ARNP precursor are indicated, and the EGF-like motif within the mature factor is represented by the six disulfide-linked cysteines (C). Underlined residues indicate consensus sites for N-linked glycosylation. Thick underline indicates site known to be glycosylated in both MCF7 and CHO cells (unpublished data). Signal, N-terminal signal sequence from TGF-β; TM, transmembrane domain. (B) Metabolic labeling of COS cells transfected with AR expression plasmids. Transfected cells were cultured for 24 h in the presence of [^{35}S]methionine-cysteine, and secreted AR was immunoprecipitated with pooled monoclonal Abs raised against the mature factor. Samples were resolved on an SDS-polyacrylamide gel and visualized by direct autoradiography.

the absence of the pro-region (Fig. 9B). Deleting only the N-terminal half (ARNPΔN) was not effective, indicating that the inhibitory effect is specific to the basic region of mature AR.

The AR N terminus inhibits TGF-α secretion unless the AR pro-region is present. The foregoing results support the hypothesis that an N-terminal basic domain is inhibitory to folding and secretion of EGF-like molecules unless circumvented by a specific structural motif in the pro-region. However, an alternate interpretation is that deletion of the AR pro-region in certain contexts (ARNP and ARNPΔN but not ARNP-NB or ARNPΔC) nonspecifically disrupts the protein folding process. To distinguish between these possibilities, we studied the effect of the AR N terminus on secretion of TGF-α, an EGF-like molecule that lacks an analogous basic domain. Four constructs were designed, and to simplify secretion analysis, none contained the transmembrane or cytoplasmic domain of the TGF-α precursor (Fig. 10 and Materials and Methods): (i) Pα (soluble pro-TGF-α, analogous to the AR-derived PA construct), (ii) NPα (soluble no-pro TGF-α, analogous to NPA), (iii) PAα (chimera fusing the AR basic domain and pro-region to the EGF-like motif of TGF-α), and (iv) NPAα (chimera fusing the AR basic domain only to the EGF-like motif of TGF-α). Secretion of protein was assessed by analyzing the conditioned medium of transfected cells for EGF receptor-stimulating activity and for TGF-α or AR (basic domain) immunoreactivity (Table 1).

Consistent with the model, release of TGF-α bioactivity and immunoreactivity from both Pα- and NPα-transfected cells indicates that the TGF-α pro-region is not required for secretion. Adding the AR basic domain alone (NPAα) prevented detectable secretion, although AR immunoreactive protein in cell extracts confirmed expression (not shown). Finally, secretion of the AR–TGF-α chimera was restored by including the AR pro-region. These results suggest a specific role for the AR pro-region in directing secretion and argue against a simple nonspecific disruption of protein folding for the earlier AR truncation mutants.

DISCUSSION

Like other members of the EGF family of growth regulators, AR is initially synthesized as a transmembrane precursor, consisting of a signal sequence, an N-terminal pro-region, the EGF-like active factor, a small juxtamembrane and single transmembrane domain, and a cytoplasmic domain. Outside the EGF-like repeat, however, the precursors show little sequence homology. While membrane-anchored forms of EGF

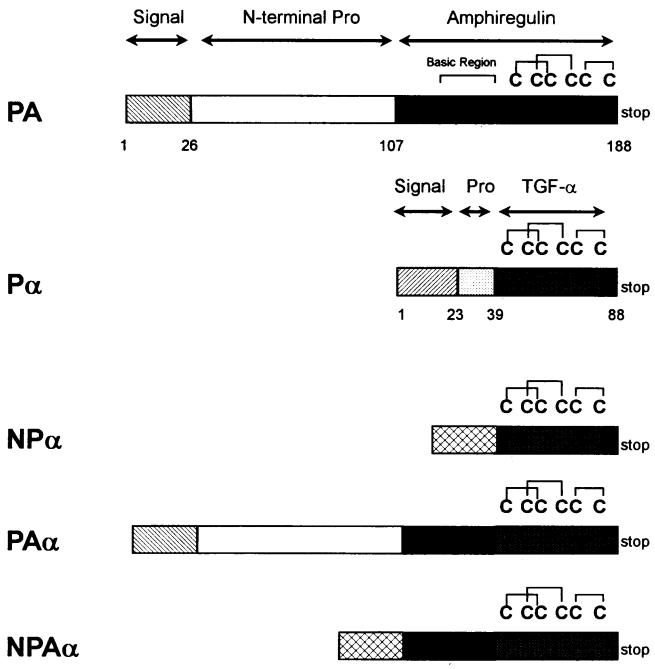

FIG. 10. AR–TGF-α chimeric constructs. Functional domains and amino acid positions within the human AR and TGF-α precursors are indicated in the full-length extracellular domain constructs (PA and Pα). All other deletion and domain swap constructs also truncate at the C terminus of the mature factors. The EGF-like motifs are represented by the six disulfide-linked cysteines (C). Signal sequences used: ▨, native AR signal; ▨, native TGF-α signal; ▨, TGF-β signal.

TABLE 1. Secretion of TGF-α derived chimeric molecules from transfected COS cells

Construct[a]	EGF receptor autophosphorylation[b]	TGF-α immunoreactivity[c]	AR immunoreactivity[d]
Vector	–	–	–
PA	+	–	+
Pα	+	+	–
NPα	+	+	–
NPAα	–	–	–[e]
PAα	+	+	+

[a] COS cells were transfected with each of the expression plasmids as described in Materials and Methods. Two days after transfection, cells were transferred to serum-free medium for an additional 24 h, when samples from the medium were collected for direct analysis.

[b] Ligand-induced EGF receptor autophosphorylation was assessed as in Fig. 3 and 7A. –, phosphorylation indistinguishable from that of unstimulated cells; +, phosphorylation levels >4-fold above background.

[c] Assayed by double-determinant ELISA. –, TGF-α immunoreactivity below detection levels (0.4 ng/ml); +, TGF-α immunoreactivity of ≥10 ng/ml in the conditioned medium.

[d] Determined by Western blotting with an Ab directed against the basic region of the mature factor. Medium samples of constructs with negative activity contained no specific AR bands, and the AR immunoreactive bands in positive samples were comparable in intensity to those observed for secreted forms in Fig. 4 and 7B.

[e] Although no AR immunoreactivity was detected in the conditioned medium, an ~16- to 18-kDa doublet was observed in the particulate fraction of NPAα-transfected cell extracts.

and TGF-α are mitogenic and have been observed in vivo and in cell culture, the biological function of the precursor molecules is still unclear (1, 6, 9, 17, 42, 49, 70). This study examines the role of each of the precursor domains in the secretion of bioactive AR.

AR deletion mutants were transiently expressed in COS and mammary epithelial cells, and conditioned medium was analyzed for secretion of active factor. Protein expression was examined by Western blotting. Active AR was synthesized and secreted by using a construct encoding the native precursor or constructs lacking the transmembrane and/or cytoplasmic domains. In contrast, deletion of the N-terminal pro-region completely abrogated AR release. Secretion was restored with a chimeric protein containing the HB-EGF pro-region but not with a chimera containing the pro-region from the TGF-α precursor. In the absence of a pro-region, secretion was restored by deleting the basic amino acid-rich region near the N terminus of the mature factor. Addition of this basic domain to the EGF-like motif of TGF-α (which itself does not require a pro-region) inhibited secretion of the chimeric molecule unless the AR pro-region was also present. These results demonstrate a role for the N-terminal pro-region in the synthesis and secretion of AR and indicate that a specific structural motif in this region is necessitated by the basic N terminus of the mature factor.

All experiments were performed in both fibroblast-like COS cells and human mammary epithelium-derived 184A1N4-TH cells. Both cell types processed the native precursor to two secreted and two cell-associated isoforms, similar to those produced endogenously by normal HMEC (Fig. 1). The transfected cell lines exhibited proportionately higher levels of the cell-associated AR than the normal cells did, but this may reflect the high rates of synthesis that occur when expression systems that amplify plasmid copy number are used. Importantly, the two expression systems produced similar results for the various mutant precursors. Thus, the observed effects on secretion and processing are probably inherent to the protein domains being examined and not due to tissue-specific factors. Compared with COS cells, however, HMEC did show more efficient removal the N-terminal pro-region from the membrane-anchored AR constructs (ARP and ARTL), which may indicate higher levels of processing enzyme activity in the more appropriate cell type.

The transmembrane and cytoplasmic domains of the precursor are not essential for AR production, since cells expressing just the extracellular sequences (PA construct) secreted bioactive protein at levels comparable to those found for the native precursor (Fig. 3 and 4). This result is consistent with reports that active recombinant EGF can be synthesized in the absence of a membrane anchor (60). However, the transmembrane domain apparently does play a role in the maturation of the ARP. Endoglycosidase H-sensitive PA precursor accumulated in the particulate fraction of cell extracts (Fig. 5), suggesting inefficient folding and transport out of the endoplasmic reticulum. More striking was the less efficient cleavage of the N-terminal pro-region, resulting in proportionately greater secretion of the 35-kDa form, gp35. For example, gp35 accounted for >50% of the AR secreted from PA-transfected 184A1N4-TH cells, compared to 10 to 20% for ARP (Fig. 4A).

Release of soluble TGF-α from its integral membrane precursor is thought to occur at the cell surface (63). Conceivably, similar AR processing by a cell-associated protease could explain the increase in gp35 secretion from PA-transfected cells, since absence of the membrane anchor would diminish the amount of AR in close proximity to the plasma membrane. Indeed, the lack of detectable 18-kDa AR in extracts of these cells indicates that propeptide cleavage must occur either late in the secretory pathway or extracellularly. Also consistent with this hypothesis is the long lag time before initiation of native

ARP processing. In transfected 184A1N4-TH cells, N-terminal cleavage begins approximately 1 h after synthesis, and C-terminal cleavage begins only by 4 h (Fig. 6).

In our experimental systems, deletion of the cytoplasmic domain alone did not prevent AR secretion. However, in comparison with the full-length precursor, this construct (ARTL) consistently produced a greater proportion of cell-associated material, although the relative differences were variable (Fig. 4A and C). The COS cell experiment depicted in Fig. 4C is a conservative example of this phenomenon; ~65% of the mutant protein was found in the cell, compared with ~40% for the native precursor. This observation is interesting in light of recent studies on pro-TGF-α, which demonstrate a role for the cytoplasmic domain in regulating release of mature factor from CHO and Ltk cells (8). Unstimulated cells retained most of the TGF-α on the membrane, but activators of protein kinase C or increases in cytosolic calcium levels lead to rapid and efficient cleavage of the ectodomain (44, 45). Critical for this response was the cytoplasmic carboxyl-terminal valine residue (8). Although it is tempting to speculate that the cytoplasmic domain of the ARP may function similarly, further characterization of the processing mechanism is required. An identical mechanism is unlikely, however, since the ARP terminates in Ala, a residue which was only 10% as effective in regulating TGF-α processing as Val, Leu, or Ile (8).

In contrast to the transmembrane and cytoplasmic domains of AR, the N-terminal pro-region was essential for protein secretion. Two constructs lacking this domain (ARNP and NPA, with and without a membrane anchor, respectively) were synthesized but not released into the media. The transmembrane ARNP was rapidly degraded ($t_{1/2}$, <30 min) (Fig. 6), although the nonanchored NPA accumulated within the cell (Fig. 4). Since a construct with the identical leader sequence in the same context (ARNPΔC [Fig. 9]) was efficiently secreted, the trivial explanation of a nonfunctional signal peptide was eliminated. Lack of secretion is frequently associated with protein misfolding, leading to either rapid turnover or retention in the endoplasmic reticulum, often in associations with proteins such as BiP or as large insoluble aggregates (see references 26, 46, and 51 for reviews). Indeed, the absence of normal N-linked glycosylation for the pro-less mutants suggests they may be structurally aberrant; neither molecule was subject to endoglycosidase H digestion shortly after synthesis, and both migrated as ~2 kDa smaller on SDS-PAGE than the corresponding native forms. The presence of anchor-less NPA in the particulate fraction of cell extracts would also be consistent with formation of insoluble aggregates. However, the reason for different intracellular fates of the anchored and nonanchored mutants is not known.

The requirement of the pro-region for AR secretion is in direct contrast with results for both TGF-α (Table 1) and EGF. Active EGF is readily synthesized and secreted from mammalian cells without its large N-terminal pro-domain, either in the presence or in the absence of a membrane anchor (17, 60). However, there are other examples in which a pro-domain is required for secretion or correct folding. For example, without their pro-sequences, NGF is unstable and is not secreted (61), activin and TGF-β accumulate intracellularly as insoluble aggregates (21), the interchain disulfide bonds necessary for functional von Willebrand factor oligomerization do not form (69), and many enzymes, such as subtilisin and carboxypeptidase Y, do not achieve an active conformation (28, 67). For activin, TGF-β, and von Willebrand factor, the pro-region need not be contiguous with the rest of the precursor to allow secretion or oligomerization but can function when coexpressed as a separate molecule (albeit inefficiently for TGF-β). In vitro, the isolated pro-region of subtilisin can direct proper folding of the denatured enzyme (72). In the case of AR, however, this domain apparently cannot complement the secretion defect when expressed in *trans* (unpublished data).

Substituting the AR pro-region with the comparable region of proHB-EGF, but not that of proTGF-α, restores protein secretion (Fig. 7). Minimal primary sequence homology in this domain suggests a conserved structural motif. Possibilities include (i) secondary structure, such as the preponderance of β turns and lack of α helices predicted for both AR and HB-EGF pro-regions; (ii) a surface patch, consistent with their similar distribution of hydrophilic stretches; or (iii) a posttranslational modification common to pro-AR and pro-HB-EGF but absent in pro-TGF-α. If the pro-region does indeed direct folding and thus exit from the endoplasmic reticulum, modifications occurring late in the secretory pathway (e.g., many types of carbohydrate maturation) are unlikely candidates for such a secretory motif.

AR secretion was also restored by simultaneous removal of both the pro-region and the heparin-binding N-terminal domain of the mature factor. Conversely, TGF-α secretion was inhibited by the AR N terminus and subsequently rescued by the presence of the AR pro-region. These observations suggest that the AR pro-region may function to prevent the heparin-binding sequences from interfering with folding of the EGF-like motif. This hypothesis could explain the different requirements for AR and EGF or TGF-α expression. By removing its basic N terminus, AR becomes structurally more similar to EGF or TGF-α and can then be expressed without additional precursor sequences. A pro-region could fulfill such a function either by directly facilitating correct folding or by masking an inhibitory region in the basic domain. Direct effects on folding have been documented for numerous pro-proteins, including α-lytic protease, in which case pro-sequences accelerate the rate-limiting step in the folding pathway by more than 10^7, and bovine pancreatic trypsin inhibitor, in which case a cysteine in the pro-region acts as a tethered thiol disulfide reagent to assist in native disulfide bond formation (2, 66). Masking inhibitory sequences implies a direct interaction between the pro-region and basic domains, which could also interfere with the heparin-binding function. In support of the second hypothesis, gp35 (containing pro-region sequences) does have a lower affinity for heparin than 18-kDa AR does (unpublished data).

The AR precursor plays a vital role in the production of bioactive factor. The N-terminal pro-region is required for export from the cell, while the transmembrane domain facilitates its subsequent removal. However, additional functions for these domains are still possible. Soluble and integral membrane forms of a growth factor may have distinct biological roles, as in the case of Steel factor. In addition to the phenotypic evidence provided by the steel dicky (Sl^d) mouse, the membrane-anchored factor is more effective at supporting survival of hematopoietic progenitor cells in vitro than is the soluble form (10, 20, 64). A variety of roles for N-terminal pro-regions have also been documented; they include providing sites for signal peptide recognition and cleavage or precursor processing, targeting the protein to specific compartments in the secretory pathway, and directing posttranslational modifications within the mature protein (23, 32, 56, 68). In the case of AR, a direct interaction between the N-terminal pro-region and the heparin-binding domain of the mature factor may modulate its activity. Important considerations for assessing physiological actions of AR should therefore include recognition of its heparin-binding properties and whether high-molecular-weight or transmembrane forms exhibit distinct biological functions.

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