The mature secreted form of the epidermal growth factor (EGF) receptor ligand amphiregulin (AR) is reported to be an 84-amino acid residue polypeptide, which is generated by proteolytic processing of a 252-amino acid precursor. This form of recombinant AR (rAR84) and two forms with COOH-terminal extensions corresponding to sequences from the AR precursor (rAR87 and rAR92) were expressed at high levels in Escherichia coli, oxidized to the correct disulfide arrangement, and purified to homogeneity. rAR84 competed poorly for binding of radiolabeled EGF to the EGF receptor and had little ability to stimulate growth of Balb/c3T3 cells. In striking contrast, rAR87 and rAR92 possessed 42- and 20-fold greater receptor binding activity and 55- and 14-fold greater bioactivity, respectively. Furthermore, addition of the COOH-terminal four amino acids from transforming growth factor α to the COOH terminus of rAR84 improved the activity of rAR84 by 100- and 1000-fold, respectively, in these assays. rAR87 was found to have –32% of the specific activity of natural AR from MCF-7 cells when compared in two different bioassays. These findings strongly suggest that the 84-amino acid sequence is not the correct structure of the naturally occurring secreted form of AR and that natural AR contains additional amino acid residues at the COOH-terminal end.

Cell growth and differentiation are controlled in part by growth factors and their receptors. The interaction between a receptor and its ligand results in activation of intracellular signals and the cellular response. One such example is the epidermal growth factor receptor (EGFR) and its family of ligands, which includes EGF (1), transforming growth factor α (TGF-α) (2), amphiregulin (AR) (3), heparin-binding EGF-like growth factor (4), betacellulin (5), and epiregulin (6). AR is a potent mitogen for fibroblasts (7), keratinocytes (7, 8), and both normal and malignant epithelial cells (7, 9–16). AR functions by binding to the extracellular domain of the EGFR, which results in rapid tyrosine phosphorylation of the EGFR, activation of the EGFR tyrosine kinase, and tyrosine phosphorylation of a number of cellular substrates, including the EGFR-like tyrosine kinase erbB2 (11). Unlike EGF and TGF-α, but similar to heparin-binding EGF-like growth factor (17, 18), heparan sulfate proteoglycan is essential to the ability of AR to activate the EGFR (13, 19).

AR mRNA is expressed in vivo by numerous normal human tissues (20), and AR protein has been localized by immunohistochemistry to the epithelium of the colon (10, 21), stomach (22), pancreas (23), breast (24), and placenta (15). AR expression can be induced by phorbol ester (7, 25), estrogen (25, 26), androsten (27), and other EGFR ligands, such as EGF, TGF-α, and heparin-binding EGF-like growth factor (22, 23, 28). Over-expression of AR is often observed in human cancers of the breast (24), colon (10, 21), stomach (22, 29, 30), and pancreas (16, 23). Furthermore, in human pancreatic cancer AR expression correlates with decreased patient survival (16). In vitro, AR functions as an autocrine growth stimulator to drive the proliferation of colon carcinoma cells (10), normal (31, 32) and oncogene-transformed mammary epithelial cells (14), cervical carcinoma cells (33), prostate cancer cells (27), and keratinocytes (8). AR also appears to play an important role in normal physiological processes, such as development of the human placenta (15) and murine mammary gland (34).

Secreted AR is proteolytically processed from a 252-amino acid residue transmembrane precursor (20). The mature AR contains an EGF-like motif consisting of six cysteine residues, which form disulfide bonds in a specific arrangement (3). This prototypical form of secreted AR is an 18-22 kDa polypeptide, which contains N- and O-linked carbohydrate and a Lys/Arg-rich region possessing putative nuclear localization signals and is reported to be 84 amino acids in length (3, 7, 12). The NH2-terminal region of AR containing the glycosylation sites is not necessary for AR bioactivity (12), whereas the very basic NH2-terminal region of AR is probably required for the requisite interaction with heparan sulfate proteoglycan (13). In addition to the prototypical 18-22 kDa form of AR, numerous structurally distinct forms of AR, which range in size from 9.5 to 55–60 kDa (25), have been identified in the conditioned medium of human breast cancer cells. Recently, it has been demonstrated that extension of the COOH-terminal end of AR by six amino acids from the predicted coding sequence of the AR precursor improved bioactivity relative to the prototypical 84-amino acid form (35). However, the true physiological relevance of this observation is not clear. In this work we demonstrate that extension of the COOH terminus by three amino acids generates a recombinant form of AR with biological activity that is very close to that of natural AR purified from MCF-7 conditioned media.
**EXPERIMENTAL PROCEDURES**

Materials—Balb/c3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). The ITS+ culture supplement was purchased from Collaborative Biomedical Products (Bedford, MA). Waymouth's media was purchased from Life Technologies, Inc. All other cell culture media, supplements, and fetal bovine serum were purchased from BioWhittaker (Walkersville, MD). [methyl-^3^H]Thymidine (2 Ci/mmol) was purchased from DuPont NEN. NR-6 WT cells (36) and the anti-AR monoclonal antibody AR1 (8) were generously provided by Dr. Alan Wells (University of Alabama at Birmingham) and Dr. Mohammed Shoeyab (Bristol-Myers Squibb Pharmaceutical Research Institute), respectively.

**Plasmic Construction and Expression**—The DNA sequence coding the 84-amino-acid form of AR was cloned by PCR of PolyA+ RNA isolated from phorbol 12-myristate 13-acetate-treated MCF-7 cells. The coding sequence was cloned into the expression vector pETBl22 fused in-frame with the TrpE peptide. This consists of the first 15 amino acids of the E gene of the tryptophan operon of Escherichia coli plus an EcoRI restriction site at the 3'-coding end. A methionine residue was inserted between the TrpE peptide and the AR coding sequences to allow for cyanogen bromide cleavage of the expressed protein. The enterokinase cleavage site (Asp-Pro-Asp-Lys) was used in place of the methionine residue for two of the mutant proteins that had methionine residues in the coding sequence of AR.

The expression of the TrpE-AR fusion was under the control of the λ-pl promoter. Expression of the TrpE-AR fusion was accomplished in the E. coli strain K12 N4830, which carries the temperature-sensitive λ repressor gene cI857, necessary for regulation of protein expression from the λ-pl promoter. Recombinant proteins were grown in LB broth containing 150 μg/ml ampicillin. Expression was induced at midlog phase by a temperature shift to 42°C for 20 min. Incubation was continued at 37°C for a minimum of 6 h. The cells were harvested by centrifugation. The cell pellet was resuspended in 20 mM Tris-HCl, pH 8.5, 10 μg/ml lysozyme, and 1 mM dithiothreitol and ruptured by sonication. The inclusion bodies were collected by centrifugation. The different forms of rAR were prepared by a polymeric chain reaction procedure using mutagenic oligonucleotide primers. All forms of the protein were expressed and purified in the same manner.

**Purification of Recombinant AR**—The inclusion bodies were resuspended in 8 M urea (4°C) and centrifuged to clarify the solution. The solubilized inclusion bodies were loaded onto an S-Sepharose column equilibrated with 20 mM sodium phosphate, pH 7.5, 5 mM EDTA, 0.1 M NaCl, and 6 M urea. The column was re-equilibrated in the same buffer without urea, and the bound TrpE-AR was eluted in a stepwise manner with 0.2 M and 1 M NaCl. The 1 M NaCl elute was adjusted to 40 mM Tris-HCl, pH 8.5, 0.1 mM Na2SO3, and 0.2 mM Na2S4O6 and incubated for a minimum of 4 h to generate the S-sulfonated form of TrpE-AR. The reaction mixture was applied to an S-Sepharose column equilibrated in 20 mM Tris-HCl, pH 7.5, and 0.1 M NaCl and washed in the same buffer. The S-sulfonate form of TrpE-AR was eluted with 1 M NaCl.

The reaction was rerolled overnight by incubating a 0.2 mg/ml solution of the protein in 0.1 M sodium borate, pH 9, 5 mM EDTA, and 2 mM cysteine at 4°C. The refolded protein was purified by RPHPLC on a Vydac C4 column eluting with a gradient of 15–35% acetonitrile with 0.1% trifluoroacetic acid over 40 min. The purified fusion protein was treated with cyanogen bromide (Sigma) or enterokinase (Kodak Scientific Imaging Systems) to produce the final product. For the CNBr cleavages a protein solution was adjusted to 0.1 M HCl, and a 3000-fold molar excess of CNBr was added. The reaction mixture was incubated overnight. The reaction was stopped by desalting on a Sephadex G-10 column with PBS. For the CNBr mutants that contained the enterokinase cleavage site, a 1 mg/ml solution of rAR was prepared in 10 mM Tris-HCl, pH 8.5, and 10 mM CaCl2, and enterokinase was added in a ratio of 1 unit:250 μg of rAR. The enzymatic digest was incubated at 37°C and complete in 4 h. In both cases, final purification was accomplished by RPHPLC as before. Successful removal of the fusion partner was confirmed by NH2-terminal sequence analysis and electrospray mass spectrometry.

**Peptide Mapping—Protein (25–300 μg) was dissolved in 0.1 M pyridinium acetate, pH 6.5, at a final concentration of 1 mg/ml. Thermolysin (Boehringer Mannheim) was added to achieve a final enzyme-to-protein ratio of 1:25 (w/w). The mixture was incubated at 37°C overnight. The digest was chromatographed on a Vydac C18 column (0.2 × 25 cm) at a flow rate of 1 ml/min and eluted over 60 min with a gradient of 0–40% acetonitrile with 0.1% trifluoroacetic acid. Absorbance was monitored at 214 nm. The absorbance peaks were collected, and peptide sequence analysis was performed on an ABI 477 protein sequencer.

**Receptor Binding Assay—** Microtiter Removewall strips (Dynatech) were precoated with A431 cell membranes in PBS (1.5 μg/ml total protein, 100 μl/well) and placed in a 37°C dry oven overnight, allowing the wells to dry completely. Immediately before use, the wells were washed twice with 200 μl/well binding buffer (Dulbecco’s modified Eagle’s medium containing 50 mM sodium phosphate, 0.1% bovine serum albumin, 0.1% sodium azide, and 0.4 μg/ml 125I-mouse-EGF (Amersham Corp.) in binding buffer were added to triplicate wells. Following incubation for 2 h at room temperature, the wells were aspirated to remove the media and washed twice with binding buffer, and the counts per minute were determined using the Isolato 500 series gamma counter. The percentage of bound 125I was plotted versus ars amphiregulin concentration, and the IC50 values were determined using nonlinear curve-fitting software.

**Purification of Natural AR**—Natural AR was purified to homogeneity from the conditioned medium of phorbol 12-myristate 13-acetate treated MCF-7 human breast carcinoma cells by sequential heparin affinity, immunopurification, and reverse phase high performance chromatography, as described by Johnson et al. (12). This procedure isolates the 38–22-kDa form of the protein.

**Enzyme-linked Immunosorbent Assay (ELISA)—** AR was quantified using the AR1 monoclonal antibody directed against the EGF-like core of mature AR (residues 44–84) (8). Serial dilutions (2×) of solutions containing either the purified rAR or purified natural AR were adhered to the bottom of 96-well ELISA plates in PBS, 0.1% sodium chloride, and 0.03 sodium citrate. After an overnight incubation at 4°C, the wells were blocked for 2 h at room temperature with 3% bovine serum albumin in phosphate-buffered saline (bovine serum albumin/PBS). The AR1 antibody was added as a 50-fold dilution of hybridoma supernatant in bovine serum albumin/PBS, and the plates were incubated for 2 h at room temperature. After thorough washing of the wells, 2 μg/ml biotinylated anti-mouse IgM and IgG (Vector Labs) in bovine serum albumin/PBS was added to each well, and the plates were incubated for 1 h at room temperature. Bound AR1 antibody was detected using the Vector avidin-biotin complex alkaline phosphatase reagent and paratriphenyl phosphate as the substrate. Color development was quantified by reading the absorbance at 405 nm.

**Peptide Mapping—** Balb/c3T3 cells were grown in growth media containing Dulbecco’s modified Eagle’s medium supplemented with 4.5% dextrose, 10% fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, and 1% L-glutamine at 37°C, in a humidified atmosphere containing 10% CO2. Cells were seeded at 2 × 105 cells/well in 24-well plates with 2 ml of growth media until they became 95–100% confluent. The cells were then switched to assay media (1:1 Dulbecco’s modified Eagle’s medium containing Waymouth’s 1% L-glutamine and 1% ITS+) and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24 h. Cells were treated with assay samples supplemented with antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamycin) at various concentrations for 16 h. Cells were pulsed with 5 μCi/ml methyl-3H thymidine and incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO2. The radioactive media were aspirated, and 1 ml/well trypsin-EDTA was added to detach the cells. The contents of the well plus 1 ml of PBS were collected onto a UniFilter-24 plate using a Packard harvester (Packard Instrument Co.). Three 1-ml aliquots of 1× PBS, four 1-ml aliquots of 10% trichloroacetic acid, and three 1-ml aliquots of 95% ethanol, all chilled on ice, were passed through the UniFilter-24 plate. To each well of the UniFilter-24 plate, 125 μl of MicroScint-20 was added. The radioactivities were counted using a Packard Topcounter. DNA synthesis assays on MCF-10A and NR6-WT cells were performed as described previously (12, 13).

**RESULTS AND DISCUSSION**

**Expression and Purification of Recombinant Amphiregulin—**

The reported 84-amino-acid form of the protein was selected for expression (rAR84). This begins with residue 101 of the translated gene product and continues through residue 184. Purification of the protein was accomplished by the procedure described under "Experimental Procedures." The solubilized inclusion bodies were determined to be 40–50% amphiregulin by scanning densitometry of Coomassie Blue-stained SDS-polyacrylamide gels. Partial purification was accomplished by S-Sepharose chromatography, which increased the purity to 75% (Fig. 1a). The protein was reoxidized essentially by the procedure of Spear and Sliwkowski (37), whereby the protein was first converted to the thiosulfonate, followed by refolding in the
presence of cysteine. This proceeded to completion as determined by RPHPLC analysis (Fig. 1, b and c). Prior to removal of the TrpE fusion partner, the protein was purified by RPHPLC. Cleavage at the lone methionine residue to release the final form of the protein was determined to be most effective when the cyanogen bromide was used in a 3000-fold molar excess (Fig. 1 d). Final purification was again accomplished by RPHPLC. On completion of the purification procedure, the pure protein yielded a single band on SDS-polyacrylamide gel electrophoresis and a single peak on RPHPLC, and electrospray mass spectrometry indicated a mass of 9765.1 (± 0.5) kDa, in agreement with the expected value.

All proteins containing EGF-like motifs consist of six cysteine residues in disulfide bonds, with a specific arrangement, that is, cysteine 1 is paired to cysteine 3, cysteine 2 to cysteine 4, and cysteine 5 to cysteine 6. To verify that this pattern had been established in the refolding process, the protein was proteolytically digested with thermolysin, and the peptide fragments were separated by RPHPLC. Fragments containing disulfide bonds were identified by comparison of the digest before and after reduction with dithiothreitol. Those peaks that changed retention time on reduction were analyzed by peptide sequence analysis. Three peaks were identified in this manner, (see Fig. 2). Peak 1 contained two sequences including the first and third cysteines, confirming the presence of a disulfide bond between them. Peak 2 consisted of three sequences including the remaining four cysteines. To decipher the disulfide arrangement, we relied on the appearance of the phenylthiodantoin-derivative cystine in the sequencing chromatogram. This appears only after both half-cystines have undergone the Edman degradation and elutes at the position of phenylthiodantoin-derivative tyrosine on the ABI 477A protein sequencer. This approach to determining cysteine arrangement has been used previously (38–40). Sequence analysis of peak 2 demonstrated that the second and fourth cysteines were linked, and the fifth and sixth cysteines were linked, confirming the expected disulfide arrangement. Peak 3 represented an incomplete digestion of peak 2.

Receptor Binding and Mitogenic Activity of Recombinant Amphiregulin—To determine the biological activity of the rAR84, we first assessed its ability to compete with 125I-EGF for binding to the EGF receptor in an in vitro receptor binding assay, and the results are shown in Table I. In this assay, EGF competed with 125I-EGF for receptor binding, with an IC50 of 0.24 nM, in agreement with the reported value (41). Natural amphiregulin from MCF-7 cells has been reported to bind the EGF receptor with lower affinity than EGF by about 1 order of magnitude (3). Surprisingly, our rAR84 displaced the label from the receptor, with an IC50 of 850 nM. Therefore, our
recombinant protein had less than 1% of the expected receptor binding activity.

EGF and natural AR have been reported to be equipotent when assayed for stimulation of proliferation of murine keratinocytes (3). We found that recombinant human EGF stimulated DNA synthesis in Balb/c/3T3 cells with an EC₅₀ of 30–60 pM, whereas rAR84 had an EC₅₀ of 10–12 nM, less than 1% of the activity of the EGF standard.

**COOH-terminal Extended Forms of Recombinant Amphiregulin**—All known ligands for the EGF receptor have a conserved leucine in the COOH-terminal region of the protein, which has been shown to be required for full biological activity. The conspicuous absence of this conserved residue suggested that the biological activity of rAR may be improved by incorporation of this leucine residue. If the product of the AR gene were extended from the mature COOH terminus, residue 86 would align with the conserved leucine of TGF-α and EGF. The AR gene product incorporates a methionine at this position. Since methionine represents a conservative amino acid change from leucine, we investigated whether extension of the COOH-terminal end of rAR84 to include this residue would improve biological activity. Furthermore, we added the four amino acids (DLLA) from the COOH-terminal end of TGF-α as an alternative method of including the conserved leucine residue.

Two forms of the protein were prepared representing COOH-terminal extensions of the COOH-terminal processing site by three and eight amino acids, resulting in 87- and 92-amino acid forms of rAR and referred to as rAR87 and rAR92, respectively (Fig. 3). Both of these extended forms of AR include the methionine residue at position 86. Inclusion of a methionine residue in the sequence of the protein prevented the use of cyanogen bromide for the cleavage of the TrpE fusion peptide. Therefore an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys) replaced the methionine site used for the rAR and rAR-DLLA forms. Otherwise, all four proteins were purified and refolded by the same procedure.

In our assays, all three COOH-terminal extended forms of rAR had significantly more biological activity than the 84-amino acid form of the protein (Table I). When assayed for the ability to compete with ¹²⁵I-EGF for receptor binding, rAR87 displayed an IC₅₀ of 20 nM, more than 40-fold higher affinity than rAR84 (850 nM). In the mitogenesis assay, rAR87 showed an EC₅₀ of 0.2 nM, which is 50-fold more potent than rAR84. These data demonstrate that with the addition of three amino acids to the COOH-terminal end of rAR84, the activity of the protein was significantly improved. In both the receptor binding assay and the mitogenesis assay, rAR92 was found to be slightly less active than rAR87, with IC₅₀ value of 43 nM for EGF receptor competition and an EC₅₀ of 0.8 nM for mitogenesis. This suggests that the residues beyond Lys-87 are not critical for receptor binding or mitogenesis, although it does not rule out the possibility that a sequence longer than rAR87, but shorter than rAR92, may further improve biological activity. When assayed for receptor binding on A431 membranes, rAR-DLLA had an IC₅₀ of 4 nM, approximately 200-fold higher affinity than rAR84. Furthermore, in the mitogenesis assay, rAR-DLLA was only 2-fold less active than recombinant human EGF, with an EC₅₀ of 0.8 nM.

In these assays, we found complete competition of ¹²⁵I-EGF with rAR-DLLA and rAR87, in contrast with the results previously reported for MCF-7-derived AR which would only compete with 50% of the radiolabel from A431 membranes although with a comparable IC₅₀ of 5.7 nM (3).

**Comparison of the Specific Activities of rAR87 and Natural AR from MCF-7 Conditioned Media**—Since rAR87 represented the most active form of recombinant AR, the bioactivity of rAR87 was compared with that of prototypical natural AR from MCF-7 conditioned media (7, 12). Natural amphiregulin was purified to homogeneity from phorbol ester-treated MCF-7 cell conditioned media by heparin affinity chromatography, immunoaffinity chromatography, and reverse phase HPLC and represents the secreted 18–22-kDa form of the protein (12). Solutions containing purified rAR87 or natural AR were normalized for AR protein content by ELISA using the AR1 monoclonal antibody (8) generated against the EGF-like core of AR (8). The concentrations of AR used in the DNA synthesis assays have been expressed as AR equivalents/ml. Serial dilutions (2×) of the AR solutions were assayed for DNA synthesis activity on MCF-10A mammary epithelial cells (lower panel) and NR6-WT fibroblasts (upper panel). DNA synthesis assays were performed as described previously (12, 13). Data points represent the mean ± S.D. (bars) of experiments performed in triplicate.

![Fig. 3. Sequence alignments of the AR precursor and four forms of rAR. A 25-residue portion of the 252-amino acid amphiregulin gene product is shown beginning with the fifth cysteine of the EGF-like motif (20). An arrow points to the methionine residue, which aligns with the conserved leucine in EGF and TGF-α. The COOH-terminal ends of the four recombinantly expressed forms of AR are shown with the COOH-terminal end extensions underlined.](https://example.com/fig3.png)

![Fig. 4. Comparison of the specific activities of rAR87 and natural AR from MCF-7 conditioned media. Stock solutions of purified recombinant AR87 and natural AR were quantified by ELISA as described under "Experimental Procedures" using the AR-specific monoclonal antibody AR1 generated against the EGF-like core of AR (8). The concentrations of AR used in the DNA synthesis assays have been expressed as AR equivalents/ml. Serial dilutions (2×) of the AR solutions were assayed for DNA synthesis activity on MCF-10A mammary epithelial cells (lower panel) and NR6-WT fibroblasts (upper panel). DNA synthesis assays were performed as described previously (12, 13). Data points represent the mean ± S.D. (bars) of experiments performed in triplicate.](https://example.com/fig4.png)
COOH-terminal Extended Forms of Amphiregulin

stability to induce DNA synthesis in MCF-10A human mammary epithelial cells (12) and NR6-WT murine fibroblasts (36) (Fig. 4). DNA synthesis assays in MCF-10A cells (Fig. 4, lower panel) and NR6-WT cells (Fig. 4, upper panel) revealed that rAR87 possessed ~31 and 33% of the specific bioactivity of natural AR, respectively. These findings demonstrate that rAR87 has biological activity comparable with natural human AR from MCF-7 conditioned media.

The data presented here demonstrate that the reported 84-amino acid form of amphiregulin expressed in E. coli does not have significant activity. This was unexpected, since all other EGF receptor ligands have been successfully expressed in E. coli. The lack of glycosylation of the recombinant protein may account for the difference, but this seems very unlikely, since the presence of N-linked carbohydrate is not required for the activity of natural AR (7, 12). We observed that the activity of AR is dramatically improved by the addition to the COOH-terminal end of either of two amino acid sequences, DLLA and SMK. The former sequence represents the four COOH-terminal residues from TGF-αR is dramatically improved by the addition to the COOH-terminal end processing site of the AR precursor. Therefore, we conclude that the COOH-terminal end of natural AR extending to include this methionine, although we do not have direct evidence to support this. Therefore, we conclude that the COOH-terminal end of natural secreted EGF and TGF-αR possessed biological activity comparable with natural human AR, respectively. These findings demonstrate that the COOH-terminal end processing site of the AR precursor. Although this sequence does not include a leucine, it does include a methionine residue, which aligns with the conserved leucine. This suggests that the biological activity of the protein may be significantly improved by inclusion of a hydrophobic residue at that position. Although AR was originally reported to be 84 amino acids in length, our data suggest this to be incorrect. It may be that the COOH-terminal end of the mature form of the natural secreted protein extends to include this methionine, although we do not have direct evidence to support this. Therefore, we conclude that the COOH-terminal end of natural AR from MCF-7 conditioned media has not been conclusively established. It is interesting that the COOH-terminal end of the EGF-like domain of heregulin β also has a methionine residue that aligns with the conserved leucine of the erbB-1 family of ligands (42). Perhaps this is another example of a conserved leucine-to-methionine substitution in this family of growth factors.

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