



Clarification of the C-terminal proteolytic processing site of human Amphiregulin

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ABSTRACT

Amphiregulin, like other ErbB ligands, is synthesized as a pro-protein which requires cleavage at the cell surface to release the active signaling domain. Prior studies using a variety of approaches have not yielded a consensus about the precise cleavage site. Here we report the purification and protein sequencing of the cell-associated human Amphiregulin stalk which remains following cleavage of the signaling domain. These data indicate that human Amphiregulin is cleaved at Lysine 187, a site homologous to the cleavage site reported in the mouse protein and distinct from the Lysine 184 site previously reported for the human protein.

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1. Introduction

Members of the ErbB ligand family, such as EGF and Amphiregulin, are synthesized as transmembrane precursors and are shed by cell-surface proteases of the ADAM family (in most cases either ADAM10 or ADAM17/TACE) so that they may act in an autocrine or paracrine fashion [1,2]. The Epidermal Growth Factor Receptor (EGFR) ligand, Amphiregulin, was initially identified as a secreted factor in the ER α -positive MCF7 breast cancer cell line [3]. Amphiregulin has recently been implicated as a required mediator of ER signaling during mouse mammary gland development [4] and plays many important roles in both development and disease in other tissues [5]. Amphiregulin is processed by ADAM17/TACE in the mouse mammary gland [6], in breast cancer cells [7] and in other tumor types, including head and neck squamous cell carcinoma [8,9]. This cleavage releases the EGF-like domain so that it can interact with its receptor and promote cell proliferation.

Since its original characterization by Shoyab and colleagues, there have been conflicting data on the amino acid sequence of the soluble EGFR-binding Amphiregulin signaling domain which is proteolytically released at the cell surface. Shoyab's study, which analyzed Amphiregulin purified from the conditioned medium of MCF7 cells treated with phorbol-12-myristate-13-acetate, reported that the C-terminus of the soluble signaling domain ended at Lysine-184 [10] (K184; in this report, all amino acids are numbered according to the reference sequence NP_001648). Surprisingly, experiments with a recombinant Amphiregulin protein based on this sequence demonstrated it had relatively weak bind-

ing affinity for EGFR and was weakly mitogenic in fibroblast proliferation assays [11]. These workers and others [12] reported that adding three to six amino acids from the full length Amphiregulin amino acid sequence to the C-terminus of the recombinant protein greatly increased its receptor binding and mitogenic activity, raising questions about why the previously characterized human protein would lack these critical residues.

In contrast, incubation of the recombinant ectodomain of mouse Amphiregulin in solution with recombinant human TACE resulted in a cleavage site three amino acids C-terminal to the reported cleavage site on the human protein, at a residue homologous to K187 of the human protein (Fig 1). If cleavage of the human protein occurred at K187, it would result in the production of one of the mitogenically active Amphiregulin proteins experimentally generated by Thomson and colleagues [11]. Collectively, these findings raise questions as to whether the originally reported cleavage site in human Amphiregulin is correct, although the differences between the experimental systems (mouse versus human Amphiregulin, and cell-generated versus purely *in vitro* cleavage) mean that a resolution from the existing data is not straightforward.

In this study, we have sought to reconcile the differences between these earlier divergent studies and determine the precise cleavage site in the extracellular juxtamembrane region of human Amphiregulin.

2. Materials and methods

2.1. Generation of the Amphiregulin-FLAG tagged construct

The Amphiregulin open reading frame without the stop codon was amplified from cDNA from MCF7 cells using the following

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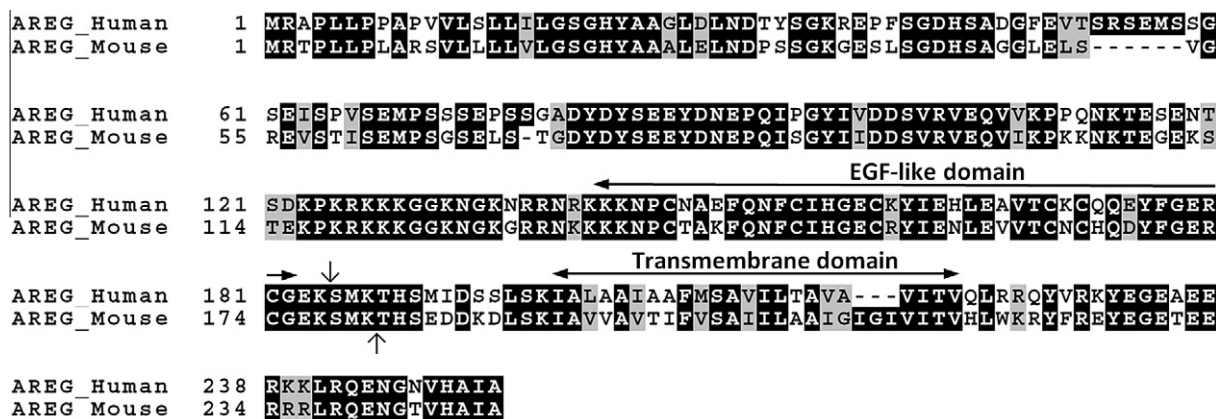


Fig. 1. Sequence alignment of human and mouse Amphiregulin proteins. The previously reported cleavage sites between the EGF-like and transmembrane domain at K184 (human, upper arrow) and the lysine residue homologous to K187 (mouse, lower arrow) are indicated.

primers: 5'-CCGCCATGAGAGCCCGCTGCTA-3' (Forward primer) and 5'-GGCTGCTATAGCATGTACATTTCCATTCTCTGTGCG-3' (Reverse primer), and cloned into the Stratagene pCMV-SC-CF vector (Stratagene).

2.2. Purification of Amphiregulin C-terminal fragment

The plasmid encoding AREG-FLAG was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Proteolytic processing of Amphiregulin was confirmed by western blot of total cell lysates using Anti-FLAG (Sigma). Lysates from transfected or control cells were incubated with anti-FLAG M2 affinity gel (Sigma) and washed four times with lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl and Halt Protease and Phosphatase Inhibitor Cocktail (Pierce)) before being eluted by boiling in SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS and 0.004% bromophenol blue) for 4 min. The eluate was resolved by SDS-PAGE on Tris-Tricine gels (16.5% Mini-Protean Tris-Tricine Precast Gel, Biorad), transferred to Immobilon-P^{5Q} PVDF membrane (Millipore) and visualized using Coomassie Blue (0.1% Coomassie Blue R-250 in 40% methanol/1%

acetic acid). Protein sequencing was performed using a Procise Protein Sequencing System (Applied Biosystems) at the Protein Chemistry Core Facility in Columbia University, New York.

3. Results and discussion

We generated an Amphiregulin expression vector encoding three C-terminal FLAG epitopes (AREG-FLAG) and expressed it in HEK293 cells which express ADAM17/TACE endogenously [13] and efficiently process Amphiregulin. As previously reported [14,15], Amphiregulin is extensively proteolytically processed. Western blot analysis of total cell lysate showing the fragments retaining the C-terminal flag peptide is shown (Fig 2A). An arrow indicates the lowest molecular weight form of AREG-FLAG detected, which we predicted would correspond to the fragment resulting from the most C-terminal cleavage and would contain the residual extracellular, transmembrane and cytosolic domains.

1000 µg of total cell lysate from the HEK293 cells expressing the AREG-FLAG construct was incubated with anti-FLAG M2 affinity gel. After washing, the eluate was fractionated by SDS-PAGE in Tris-Tricine gels, transferred to a PVDF membrane and stained with Coomassie Blue. A band (arrow) of similar molecular weight to the band observed in the previous western blot was excised and subjected to N-terminal protein sequencing by Edman degradation.

Unambiguous sequencing data was obtained for six of the first seven residues (1, 2, 3, 4, 6 & 7) of the fragment resulting in a sequence of THSMIDS. Based on the known protein sequence (Fig 1), we infer that the N-terminus of the smallest cell-associated fragment is THSMIDS. These data indicate that this human protein is cleaved at K187, three amino acids more C-terminal than the site reported by Shoyab [10] and homologous to the site identified in the mouse protein by Hinkle [14]. Based on the studies of Thompson [11], who generated a recombinant protein with this three amino acid extension, the protein produced by this cleavage would have superior EGFR-binding and mitogenic activity.

The reasons for the disparity between our data and the previous report [10] are unclear. In the previous work, conditioned medium was harvested from MCF7 cells over a period of several days, and an extensive multi-step purification protocol was followed [3] to obtain the endogenously produced soluble EGF-domain containing fragment. Our focus was on purifying the fragment left behind upon release of this soluble domain, and our use of the FLAG-epitope allowed us to simply and rapidly purify an amount of the C-terminal Amphiregulin fragment sufficient for protein sequencing. Although we differ with Shoyab et al. [10] on the identity of the cleavage site on human Amphiregulin, our data are consistent with

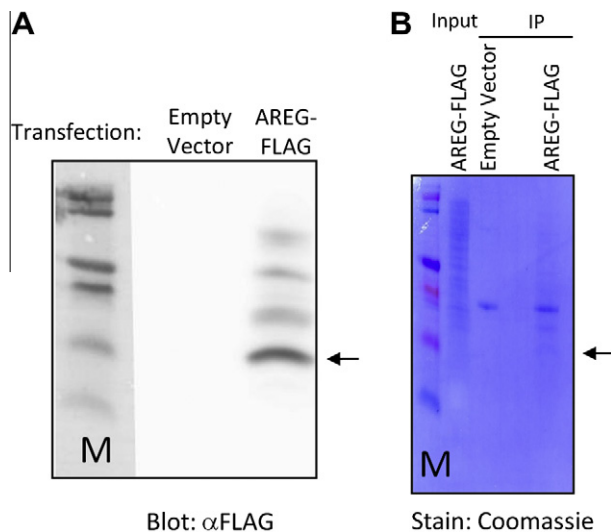


Fig. 2. Isolation of the smallest cell-associated Amphiregulin fragment. (A) Western blot of total cell lysate from cells transfected with C-terminally Flag-tagged AREG. The arrow indicates the smallest fragment. (B) Coomassie blue stained membrane containing immunopurified Flag-tagged Amphiregulin protein. The arrow indicates band selected for protein sequencing.

the reports about the cleavage site of the homologous mouse protein [14]. From our data, we cannot fully exclude the possibility that human Amphiregulin may be cleaved at both K184 and K187, however it is noteworthy that a short 12 amino acid peptide containing 6 residues on each side of the predicted K184 cleavage site reported by Shoyab (ERCGEK₁₈₄SMKTHS) was not cleaved by recombinant TACE under conditions that similar peptides representing four other bona fide TACE substrates (TGF α , betacellulin, epiregulin and HB-EGF were cleaved) [15]. Taken with the data from Thompson that the 3 amino acids between K184 and K187 are very important for receptor binding and mitogenic function [11], we consider it most likely that the K187 site identified here is the physiologically relevant C-terminal cleavage site of human Amphiregulin.

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