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Expression, purification and insights into structure and folding of the ADAM22 pro domain

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ABSTRACT

The ADAMs (a disintegrin and metalloproteases) are an important class of enzymes in the regulation of human disease. The pro domains of ADAMs are responsible for the latency and secretion of mature enzymes. Unlike other metzincins, ADAM pro domains remain bound to the mature enzyme after secretion. To understand the functions of human ADAM pro domains and to determine three-dimensional structures, we have screened promising targets for expression and purification properties when using *Escherichia coli* as the host. The pro domain of ADAM22 (ADAM22-P) expressed in *E. coli* was folded, as determined by CD and NMR spectroscopy. An ADAM22-P fragment encoding residues 26–199 could be expressed in high amounts, remained soluble above 1 mM, and was suitable for structural studies by NMR spectroscopy. CD spectroscopy and predictions suggest that the secondary structure in ADAM22-P consists of β-strands. Furthermore, our data indicate that the pro domains of ADAMs are expressed as two subdomains. The most N-terminal subdomain (ADAM22-P_N) was found to be susceptible to proteolysis and was required for folding stability of the second subdomain (ADAM22-P_C).

Introduction

Metalloproteases in the plasma membrane and extracellular matrix are key molecules mediating the response of cells to their microenvironment. Among the metalloproteases are the ADAMs (a disintegrin and metalloproteases).[1] This family of closely related proteins play a critical role in human biology and pathology, including in severe diseases, such as cancer [1,2].

The human genome encodes at least 23 different ADAMs [3]. They share a homologous modular architecture with pro, catalytic, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane, and cytoplasmic domains [4,5]. ADAMs are metzincins, which are characterized by the methionine found in the conserved met-turn motif [6,7]. The *HExxHxxGxxG* zinc-coordinating consensus motif located upstream from the met-turn is indispensable for ADAM substrate hydrolysis. Consequently, human ADAMs that lack this motif are considered inactive [8]. ADAMs mediate ectodomain shedding through the catalytic domain [1,9,10], and the non-catalytic ancillary disintegrin and cysteine-rich domains exhibit cell-binding properties [11,12]. Ectodomain shedding is a process where a membrane-bound protease releases extracellular ligands from membrane-bound inactive precursors. These ligands include growth factors and cytokines [13]. Timely and precise regulation of ectodomain shedding is essential, and dysregulation is a plausible explanation for the role of ADAMs in cancer [14]. The pro domain is thought to keep the enzyme in a latent state and to aid folding and maturation [15–17]. Processing of the pro domain is mediated by proprotein convertases during transit in the *trans*-Golgi network [18]. Following this step of maturation, the enzyme reaches its biological active conformation [19,20]. However, the pro domain of ADAM12 remains associated with the mature enzyme after secretion [21]. Endogenous inhibitors of ADAMs are their own pro domains [16,22,23] and the tissue inhibitors of metalloproteases (TIMPs) [24–26].

ADAM proteases are an important group of potential therapeutic targets [27]. Clinical targeting of ADAMs by newly developed compounds depends on an accurate understanding of their exact function *in vivo* [28]. The lesson learned from disappointing clinical trials with inhibitors of matrix metalloproteases (MMPs) was that specificity is a crucial factor [29–31]. In this light, it is exceptionally important to understand the interplay between the pro and catalytic domains of ADAMs. The mechanisms that still remain to be

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[1] *Abbreviations used:* ADAM, a disintegrin and metalloprotease; EGF, epidermal growth factor; TIMP, tissue inhibitor of metalloproteases; MMP, matrix metalloproteases; DC, disintegrin–cysteine-rich; VAP, vascular apoptosis-inducing protein; P, pro domain; C, catalytic domain; PC, pro-catalytic domains; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; IMAC, immobilized metal affinity chromatography; HSQC, heteronuclear single quantum coherence; TNFR2, tumor necrosis factor receptor 2; ADAMTS-9, ADAM with thrombospondin motifs 9.

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fully understood include the activation and maturation of ADAMs by proteolytic processing and removal of the pro domain [32], the cysteine-switch mechanism [23,28,33], inhibition of the proteolytic activity by pro domains [16,23], and the chaperone effects of ADAM pro domains [17]. A complete structural and functional elucidation of the interaction between ADAM pro and catalytic domains is essential to advance the field with respect to therapeutic targeting of ADAMs. Our current structural insight into the pro domains are informed from a number of proMMP structures, including proMMP-1 [34]. However, based on the high sequence divergence, ADAM pro domains probably exhibit a fold different from the characteristic triple-helical bundle found in MMP pro domains [34].

Previous studies have revealed the structures of the catalytic domains of ADAM17 and ADAM33 and the DC (disintegrin–cysteine-rich) domains of ADAM10 [35–37]. Structures of the catalytic-disintegrin–cysteine-rich domains for the snake venom metalloproteases vascular apoptosis-inducing protein (VAP)-1 and VAP2-B have also recently been solved [38,39]. A major limitation of such structural studies is the synthesis and purification of correctly folded protein in sufficient quantities for crystallization and data acquisition by X-ray crystallography and NMR.

The ADAM17, ADAM33, and ADAM10-DC homologous structures described above were solved by X-ray crystallography using protein heterologously expressed by insect or mammalian cells or from snake venom [38–41]. Structural studies by NMR spectroscopy require millimolar concentrations (0.5–1 mM) of protein labeled by isotopes (^{15}N and ^{13}C) [42]. Isotope labeling is expensive and protein is preferably produced in *Escherichia coli* [42,43]. Human proteins often have characteristics that lead to poor expression or insoluble aggregates when recombinantly expressed in *E. coli*. In addition, the codon preference in *E. coli* is different from that of human cells and in some cases leads to low protein expression. Furthermore, a high number of paired cysteines are characteristic of ADAM proteins. Disulfide bond formation represents a major obstacle to structural analyses, because the cytoplasm of *E. coli* is a reduced environment. In general, misfolding is a recognized outcome when expressing ADAM-derived proteins in *E. coli* [44].

Here, we evaluate the expression and purification of pro domains of human ADAMs from *E. coli* cells. We specifically show that the ADAM22 pro domain is suitable for soluble expression in *E. coli*. An ^{15}N-labeled deletion mutant representing residues 26–199 of the ADAM22 pro domain was produced in millimolar concentrations in a folded conformation. Biochemical characterization and CD indicate that the ADAM22 pro domain contains two subdomains primarily composed of β-strands. Our data show that the first subdomain (ADAM22-P_N) is highly protease susceptible. We suggest that ADAM22-P_N mediates the folding of the second subdomain (ADAM22-P_C).

Materials and methods

Cloning

ADAM pro (P) domains were all cloned from cDNAs into the expression vectors pET3a or pET44a (both from Novagen) using restriction enzymes (Supplementary Table 1 lists restriction sites, oligos, and cDNA clones used).

Small-scale protein expression

The media used for protein expression was 2× TY (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) or M9 minimal media. Carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml) were included. Protein expression for small-scale batch purification trials was conducted in 250-ml baffled flasks containing 50 ml of media. The Rosetta (DE3) strain of *E. coli* from Novagen was used for all experiments. Bacteria transformed by the different expression constructs were inoculated to an optical density at 600 nm (OD_{600}) of approximately 0.05 and cultivated at 37 °C with shaking at 220 rpm. Protein expression was induced by adding 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) when OD_{600} reached 0.8. When expression was conducted at 20 °C or 30 °C, cultures were adjusted to the desired temperature 30 min prior to induction. All cultivation was in 2× TY until 30 min before induction. Cells where harvested 30 min before induction and resuspended in fresh media (2× TY or M9 minimal media). Expression was continued for 5, 7, or 14 h at 37 °C, 30 °C, and 20 °C, respectively.

Small-scale batch purification trials

Batch purifications were conducted to test the expression of C-terminally histidine-tagged ADAM pro domains. Cells from 50 ml of media were harvested by centrifugation and resuspended in 800 μl of 25 mM Tris–HCl, pH 8.5, 25 mM imidazole, 500 mM NaCl, 0.5 mM 2-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) (buffer A). Resuspended cells were lysed by sonication and centrifuged at 15,000*g* for 10 min. Samples taken before and after centrifugation represented the total (T) and soluble (S) protein fractions, respectively. Chelating Sepharose FF beads (GE-Healthcare) charged with nickel and equilibrated in buffer A (50 μl beads) were incubated with 600 μl of soluble fraction for 30 min. Following incubation, beads were washed four times in 1 ml of buffer A and eluted using 3× 100 μl buffer B (25 mM Tris–HCl, pH 8.5, 250 mM imidazole, 500 mM NaCl, 0.5 mM 2-mercaptoethanol, 0.1 mM PMSF). The eluates represent the fraction bound and eluted from nickel-charged beads (E).

Western immunoblotting

Western immunoblotting was conducted by standard methods using the HRP-anti-his (C-term) antibody from Invitrogen.

Expression and purification of ADAM22-P (26–222) and ADAM22-P (26–199)

ADAM22-P (26–222) and ADAM22-P (26–199) were purified from M9 minimal media using the following procedure. Cells were cultivated in 5-l baffled flasks containing 1 l of media and treated essentially as described above for small-scale protein expression. Labeling was achieved using [^{15}N]ammonium sulfate in M9 minimal media. Induction for both proteins was performed at 20 °C for 14 h. Harvested cells were resuspended in 50 ml of buffer A per liter of culture, lysed by sonication, and centrifuged for 20 min at 20,000 rpm in a SS34 rotor (Sorvall). The supernatant was loaded on a 1-ml nickel-charged chelating Sepharose FF column equilibrated in buffer A and eluted using a linear gradient from buffer A to buffer B. Fractions containing the target protein were dialyzed extensively against buffer C (18.75 mM potassium phosphate, pH 7.4, 750 mM ammonium sulfate, 3.75% 2-propanol, 1 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, 0.1 mM PMSF). The dialyzed sample was loaded on a 5-ml phenyl–Sepharose HP column and eluted by a linear gradient from buffer C to buffer D (15% 2-propanol, 0.5 mM 2-mercaptoethanol, 1 mM $CaCl_2$, 0.1 mM PMSF). Fractions containing the target protein were concentrated to approximately 1.5 ml using a Vivaspin 20 filter device (Sartorius) with a 5 kDa cut-off, then loaded on a 150-ml Superdex 75 prepgrade gel filtration column preequilibrated in buffer E (25 mM potassium phosphate pH 7.4, 150 mM NaCl, 0.1 mM PMSF, 0.5 mM 2-mercaptoethanol). The eluted protein was concentrated using a Vivaspin 20 filter device (previously washed extensively in water) and the buffer was changed to 15 mM potassium phosphate, pH 7.2, 0.03% sodium azide. The final protein concentration was 0.5–1 mM as determined by measuring the absorbance at 280 nm.