Molecular dissection of arginyltransferases guided by similarity to bacterial peptidoglycan synthases

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Post-translational protein arginylation is essential for cardiovascular development and angiogenesis in mice and is mediated by arginyl-transfer RNA-protein transferases Ate1—a functionally conserved but poorly understood class of enzymes. Here, we used sequence analysis to detect the evolutionary relationship between the Ate1 family and bacterial FemABX family of aminoacyl-tRNA-peptide transferases, and to predict the functionally important residues in arginyltransferases, which were then used to construct a panel of mutants for further molecular dissection of mouse Ate1. Point mutations of the residues in the predicted regions of functional importance resulted in changes in enzymatic activity, including complete inactivation of mouse Ate1; other mutations altered its substrate specificity. Our results provide the first insights into the mechanisms of Ate1-mediated arginyl transfer reaction and substrate recognition, and define a new protein superfamily called Dupli-GNAT to reflect its origin by the duplication of the GNAT acetyltransferase domain.

Keywords: arginyltransferase; Ate1; acetyltransferase

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INTRODUCTION

Arginyltransferases (R-transferases or Ate1; EC 2.3.2.8) are found in the eukaryotes and in many bacteria (Balzi *et al*, 1990; Kwon *et al*, 1999; Rai & Kashina, 2005; supplementary Fig 1 online). These enzymes transfer arginine from charged transfer RNA-Arg to amino-terminally exposed residues of their protein substrates (Gonda *et al*, 1989). Arginylation decreases the stability of some proteins and induces their degradation by the N-end rule pathway

(Bachmair *et al*, 1986). R-transferase gene *Ate1* is non-essential in yeast *Saccharomyces cerevisiae*, whereas its mammalian orthologue is essential for cardiovascular development and angiogenesis in mice (Kwon *et al*, 2002). Unlike the intronless yeast transcript, the product of *Ate1* in the mouse undergoes alternative splicing to produce four protein forms with different activity, substrate specificity, tissue expression and intracellular localization (Kwon *et al*, 1999; Rai & Kashina, 2005).

In addition to R-transferases, there are other known classes of enzymes with aminoacyl-tRNA-protein transferase activity. One such class is the Leu/Phe-tRNA-protein transferases (L/F-transferases; Leibowitz & Soffer, 1970). Yet another class of aminoacyl-tRNA-protein transferases includes the bacterial low-affinity penicillin-binding proteins, also known as methicillin resistance proteins. These enzymes use aminoacyl-tRNA cofactor, typically charged with Gly or Ala, to form interchain peptide linkers and to attach them to L-lysine in the linear pentapeptide-disaccharide chains of peptidoglycan (Mallorqui-Fernandez *et al*, 2004). Crystal structures of several enzymes of this family, including FemA from *Staphylococcus aureus* and FemX from *Weissella viridescens*, have been determined (Benson *et al*, 2002; Biarrotte-Sorin *et al*, 2004). The enzyme consists of two domains with similar three-dimensional folding, which bears striking resemblance to acetyltransferases from the GNAT family (Neuwald & Landsman, 1997). The N-acetyltransferase reaction catalysed by GNATs is mechanistically similar to attaching an amino acid to the N terminus of a peptide, although, as a rule, GNATs use acyl-coenzyme A, rather than aminoacylated tRNA, as the acyl group donor.

Unlike bacterial FemABX, R-transferases are found in all eukaryotes and in many bacteria (supplementary Fig 1 online). Although all R-transferases share a set of conserved sequence elements, there is substantial sequence divergence within the family, as well as insertions in several regions of the protein, especially in eukaryotic homologues (see below). Three Cys residues, located close to the N terminus, are important for yeast Ate1 activity (Li & Pickart, 1995) and deletion of the portion of Ate1 encoded by the first exon of the mouse gene results in the loss of its ability to induce degradation of model substrates in yeast (Kwon *et al*, 1999). The alternative splicing of the N-terminal exon results in marked differences in substrate specificity: mouse Ate1

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Fig 1 Multiple alignment of the conserved carboxy-terminal domain of the Dupli-GNAT superfamily. Yellow indicates hydrophobic residues (FILMVWY), green indicates small residues (AGS), red indicates aromatic residues (FWY) and blue indicates the conserved lysine. Only those residues that are conserved in both Ate1 and FemABX family are highlighted. Residues that were mutated are designated by #. α -Helices, β -strands and loop regions are indicated by the letters h, s and l respectively. See the supplementary information online for the abbreviations of the species names. Numbers before species names are GenBank or Protein Data Bank identifiers; numbers after the species names indicate distance, in amino acids, from the amino termini of proteins and between blocks of high sequence conservation respectively.

Examination of the known three-dimensional structures of *Wajira virescens* FemX protein in free form and in complex with

To identify relatives of Ate1, we searched the non-redundant protein sequence database at NCBI using sensitive sequence comparison methods. We found that the R-transferase (Ate1) and L/F-transferase (Aat) families, previously thought to be unrelated to each other, are evolutionarily related and form a superfamily with methicillin resistance proteins FemABX and many uncharacterized proteins, mostly from bacteria. All these proteins contain carboxy-terminal domain with GNAT-like acetyltransferase fold, which seems to bear most of the determinants of the catalytic activity (see below). Several proteins with established acetyltransferase activity and GNAT fold were also retrieved in our search

the substrate UDP-*N*-acetyl muramic acid (UDP-MurNAc) pentapeptide (Biarrotte-Sorin *et al*, 2004) provides functional interpretation to these conserved sequence features and a better prediction of the Ate1 activity determinants. The UDP-MurNAc pentapeptide is bound to the *W. virescens* FemX in a groove between the N- and C-terminal domains, and most of the direct contacts between the protein and the substrate are provided by the solvent-exposed surface of the N-terminus. The only place on the molecule that would be able to accommodate the aminoacylated 3' end of tRNA is the cleft produced by a bulge in the middle of two parallel strands in the C-terminal GNAT fold that pushes the distal ends of these strands apart from each other and from the adjoining α -layer.

The crystal structure of L/F-transferase (Aat) from *Escherichia coli* has been deposited in the PDB (PDB ID 2CXA). In full agreement with the conservation between C-terminal halves of the Aat and Ate1 family, the C-terminal half of Aat matches the GNAT-like folds with high significance (see supplementary information online). The N-terminal portion of Aat, however, has an abbreviated $\alpha + \beta$ structure, with only one layer of helices and with shorter strands. Thus, a trend in the evolution of tRNA-dependent aminoacyl transferase structure seems to be the duplication of the GNAT domain followed by constrained evolution of the catalytic C-terminal copy and a more rapid divergence of the substrate-binding N-terminal copy.

Identification of targets for site-directed mutagenesis

To probe the putative cofactor-binding site more directly, we used the arginylated CCA end of a tRNA and the high-resolution structure of FemX (PDB ID 1P4N) to perform computational docking experiments, seeking the most likely structure of a putative complex between FemX and CCA-Arg. Although this fragment represents a non-cognate substrate of FemX, the analysis of the complex provided further insights into the structural features required for the activity of FemABX and Ate1 enzymes (Fig 2).

The minimum free energy was given by the CCA-Arg moiety docked to the crevice in the C-terminal domain, as has been also predicted for the cognate cofactor (Biarrotte-Sorin *et al*, 2004). The depth of the crevice in FemX is more than sufficient to accommodate the large side chain of the non-cognate arginine, but the tRNA seems to be stopped by steric hindrance, and possible repulsion, between the base of the adenine and the aromatic ring of F304 (Y416 in mouse Ate1). Another highly conserved aromatic residue, Y254 (F313 in mouse Ate1), seems to be in direct contact with the α -carbon atom of Arg in our model. These two residues tentatively identify the determinants of tRNA positioning in the active centre. The side chain of the only universally conserved, positively charged residue in the whole alignment, K307/K417, points in the direction of the peptide substrate. We suggest that this residue has a direct role in catalysis. As a control, we have also mutagenized a nearby lysine residue, K413 of mouse Ate1, which is likely to point into the solution and is replaced by tyrosine in FemX.

Several features of the multiple alignment attracted our attention for a complementary reason, especially notable patterns of family-specific or isoform-specific conservation. One such feature at the rim of the putative cofactor-binding crevice is conserved in the Ate1 but not in FemABX enzymes: at the end of

Fig 2 | The 3' end of arginylated tRNA docked on the high-resolution structure of FemX (Protein Data Bank ID 1P4N) shown in overview (top) and in detail (bottom). Two terminal nucleotides in tRNA are shown in the enmeshed form. Conjugated Arg residue is shown in green; the main chain of the peptide moiety of the muramylpeptide, indicating the predicted position of the protein substrate in the homologous structure of Ate1, is shown in orange; conserved F304 (Y416 in mouse Ate1) is in yellow; and predicted catalytic residue K305 (K417 in mouse Ate1) is in red.

strand 10, Ate1 family has a highly conserved proline residue (P370), which is expected to produce a tight kink at the entrance to the putative active centre of the enzyme and is the closest residue to the conjugated 2'OH atom of the last adenine in the 3' end of tRNA; this 'proline shoulder' is missing in FemX.

In the N-terminal domain of Ate1, sequence conservation patterns are complicated by the alternative exon usage. Among the four mammalian Ate1 forms, mouse Ate1-1 and Ate1-2 are able to arginylate all known types of substrate, whereas mouse Ate1-3 and Ate1-4 are apparently specific for the N-terminal Cys

22002033 ATE1-2p (HOM)	SPAVVDYFPEDFYRCGYCKNESGSRSN
3806094 ATE1-1p (HOM)	FAASVDYFPEDFYRCGYCKNESGSRSN
3806096 ATE1-2p (HOM)	FAASVDYFPEDFYRCGYCKNESGSRSN
55732465 (PON)	SPSLVDYFPEDFYRCGYCKNESGSRSN
3806090 ATE1-1/2 (MUS)	SPSLVEYFESQTSFQCGYCKNKLSRSY
62641384 (RAT)	SPSLVEYFESQTSFQCGYCKSNFQSRSH
76655326 (BOS)	SPSLVEYFESQTSFQCGYCKNESGSRSN
57107671 (CAN)	SPSLVEYFESQTSFQCGYCKNESGSRSN
60098515 (GAL)	SPSLVEYFESQTSFQCGYCKNESGSRSH
Consensus_form_1/100%	.tshh-YF.upt.apCGYCKst.Gt.S.
55962466 (HOM)	MAFVVEYKGLRAGYHCGYCKDSKGGKS
55728544 (PON)	MAFVVEYKGLRAGYHCGYCKDSKGGKS
69145650 ATE1-3/4 (MUS)	MAFVVEYKGLRAGYHCGYCKDSKGGKS
62641384 (RAT)	MAFVVEYKGLRAGYHCGYCKDSKGGKS
76655318 (BOS)	MAFVVEYKGLRAGYHCGYCKDSKGGKS
50749877 (GAL)	RRFVVEYKGLRAGYHCGYCKDSKGGKS
Consensus_form_2/100%	.tshh-Y+GhptGa.CGyCtstpGtsSs

Fig 3 | First exons of the two Ate1 forms with different specificity. Experimentally characterized Ate1 forms are indicated. Positions that are most significantly different in two forms are designated by red asterisks. See the supplementary information online for further details.

(Rai & Kashina, 2005). These forms differ by a homologous N-terminal stretch of ~30 amino-acid residues, suggesting that the determinants that confer this difference in substrate specificity may be located within this stretch. Comparison of Ate1 forms from warm-blooded vertebrates (Fig 3) indicates that the site with the largest degree of chemical dissimilarity between the broad-specificity and narrow-specificity groups is the stretch of amino acids 8–10 (as counted in the mouse Ate1-3 sequence). We speculated that these residues might be potential determinants of substrate specificity.

Analysis of the targeted substitutions in Ate1 *in vivo*

To test the predictions described above, we performed point mutagenesis of mouse Ate1-1—the most active form of mouse Ate1, which has universal substrate specificity. The standard *in vivo* assay exploits the effect of Ate1 on the rate of protein degradation of test substrates in yeast. Proteins containing N-terminal Arg are extremely short-lived in *S. cerevisiae*, and Ate1 can therefore induce rapid degradation of a test substrate (β-galactosidase or β-Gal) containing arginylatable N-terminal residues. Mammalian Ate1 forms are able to complement *ate1Δ* strains of *S. cerevisiae* and induce degradation of β-Gal derivatives containing N-terminally exposed Asp or Glu, which are otherwise stable in this strain of yeast (Bachmair *et al*, 1986). We applied this complementation assay to the mammalian Ate1-1 derivatives containing point mutations. The results of this experiment are presented in Fig 4 and Table 1.

In agreement with our prediction, the K417A mutation reduced mouse Ate1-1 activity to the levels undetectable by our assay. This result indicates that the universally conserved, positively charged residue in that position is crucial for Ate1 activity.

Interestingly, other mutants had various changes in arginylation activity and, in some cases, differential effects on Asp- versus Glu-containing substrates. F313A mutation slightly decreased mouse Ate1 activity, which suggested that despite the conservation of the aromatic residue, this site is at best a secondary determinant of cofactor recognition. In contrast, changing the main-chain-bending amino acid P370 into more flexible A residue, which should result in the relaxation of the proline shoulder, enhanced

Fig 4 | Point mutagenesis of mouse Ate1. Liquid assays of β-Gal substrate activity in *ate1Δ Saccharomyces cerevisiae* strain in the presence of wild-type and mutant mouse Ate1 forms. (A) Point mutagenesis of mouse Ate1-1. (B) Point mutagenesis of mouse Ate1-3. M, D, E and R correspond to the amino-acid residue at the amino terminus of each β-Gal substrate. Bars represent levels of β-Gal activity calculated as a percentage of Met-β-Gal, which serves as a control in each data set. Error bars represent standard deviation for each data set, which consisted of 7–15 independent measurements. Colours correspond to the measurements for each mouse Ate1 mutant as noted to the right of the graph. Activity changes for mouse Ate1 mutants are reported in Table 1 as percentages of the β-Gal activity in the presence of wild-type or mutant Ate1-1 and Ate1-3 and difference between the inversed percentages for mutant Ate1 and the corresponding wild-type controls.

mouse Ate1 activity, especially on the Asp-containing substrate. This gain of activity, however, was reversed by replacing two aspartic acid residues flanking P370 and, in addition, the activity of the DPD→AAA mutant towards the glutamate-containing substrate was significantly reduced compared with the control. Thus, negative charge at the rim of the putative cofactor-binding crevice seems to be required for enzymatic activity. Replacement of Y416A in the depth of the cofactor-binding crevice had virtually no effect on the degradation of Asp-containing substrate, but affected the Glu-containing substrate (Fig 4A; Table 1).

Substitution of the three-amino-acid stretch in the positions 8–10 (KGL) of Cys-specific mouse Ate1-3 with the FEG stretch found in the corresponding positions of Ate1-1 (positions 15–17) resulted in Ate1-3 gaining the ability to induce degradation of Asp- and Glu-containing substrates (Fig 4B; Table 1), indicating that this amino-acid stretch, in agreement with predictions, is indeed important for the differential substrate specificity of Ate1 forms.

Table 1 | Point mutagenesis of mouse Ate1

Residue and mutation (Ate1-1 background unless otherwise indicated)	Predicted role of the amino acid (see text)	Percentage of β -Gal activity (compared with M- β -Gal)	Mutant activity change versus control
Wild-type Ate1-1	NA	D: 25.8 ± 2.2 E: 32.3 ± 7	NA NA
Wild-type Ate1-3	NA	D: 97.9 ± 24.4 E: 96.8 ± 12.9	NA NA
KGL8-10 to FEG; Ate1-3 background	Substrate specificity	D: 4.4 ± 1.4 E: 37.0 ± 10.0	93.5%, increase 59.8%, increase
F313 to A	Interaction with amino acid on tRNA	D: 4.4 ± 7.2 E: 37.0 ± 4.9	21.4%, increase 0, unchanged
P370 to A	Kink, structural role	D: 5.2 ± 2.2 E: 25.7 ± 4.6	20.6%, increase 6.6%, slight increase
DPD369–371 to AAA	Kink, structural role	D: 35.5 ± 4.2 E: 67.1 ± 15	–9.7%, decrease –34.8%, decrease
K413 to A	Control	D: 39.6 ± 4.2 E: 71.3 ± 10.5	–13.8%, decrease –39%, decrease
K417 to A	Catalytic	D: 101.7 ± 15.7 E: 93.8 ± 10.3	–75.9%, decrease –68%, decrease
Y416 to A	Interaction with 3' adenosine of tRNA	D: 29.8 ± 7.8 E: 16.1 ± 0.3	0, unchanged 16.2%, increase

Mutant activity change (%) was calculated by subtraction of averaged inverse percentages of β -Gal activity (that directly reflects Ate1 activity) for control Ate1 from the corresponding mutant values. Statistically insignificant change was denoted as 0. NA, not applicable.

DISCUSSION

This study demonstrates sequence and structural similarity between R-transferases, L/F-transferases and bacterial FemABX tRNA-dependent aminoacyl-peptide transferases. A mechanistic analogy between non-ribosomal peptide synthases involved in methicillin resistance and aminoacyl-tRNA-protein transferases has been suggested before (Hegde & Shrader, 2001), but no sequence similarity between these enzymes has been previously found. All the identified Ate1 homologues consist of two domains, apparently evolved by duplication and subsequent divergence of the GNAT-like acetyltransferase domain. The C-terminal, catalytic domain, is predicted to bind the charged tRNA. The N-terminal domain, while maintaining characteristic GNAT-like fold, has lost its cofactor-binding role and, in the case of the L/F-transferase, has been structurally remodelled. We propose to call this newly identified superfamily of proteins Dupli-GNAT, to reflect their likely evolutionary emergence by the duplication of the GNAT-like domain.

In the course of sequence database searches, we observed other significant sequence similarities between members of the Dupli-GNAT superfamily and proteins with other known or unknown activities (supplementary Fig 2 online). Many of these proteins were found in fusions with other domains. The emerging picture of these and other protein fusions indicates that non-ribosomal transfer of an amino acid from charged tRNA to various

substrates by Dupli-GNAT proteins might be a widespread strategy of biosynthesis and regulation in bacteria and eukaryotes. In R-transferases, which are the subject of this study, the basic Dupli-GNAT architecture is observed in the bacterial representatives, but is typically elaborated by insertions in the eukaryotic Ate1 homologues. Most of the eukaryotic Ate1 sequences are predicted gene products, but some of the extra sequences are conserved across many eukaryotes and we believe that they represent genuine insertions (supplementary Fig 1 online).

Dupli-GNAT proteins are widespread among bacteria, are mostly represented by Ate1 family in eukaryotes and are missing from the Archaea. This is compatible with an evolutionary model in which an ancestral enzyme has emerged by duplication of the ancient GNAT fold and was adapted to using tRNA instead of acetyl-CoA as the donor of an acyl group. The ancestral function of Dupli-GNATs was probably in membrane modification and peptidoglycan biosynthesis. Diversification of this fold produced R-transferases and L/F-transferases—most probably in the Proteobacteria, in which both these families are widespread today. From Proteobacteria, R-transferases may have been disseminated by horizontal transfer into a few other bacteria and by symbiogenesis to eukaryotes.

Guided by sequence comparison and structural modelling, we mutated several putative determinants of Ate1 structure and function. In an *in vivo* complementation assay, most of our

mutants had interesting molecular phenotypes. First, we identified the universally conserved lysine residue that is crucial for Ate1 role in destabilization of a model protein bearing either aspartic or glutamic acid at its exposed N terminus. This lysine (K417) resides in a putative tRNA-binding cleft in the C-terminal domain of Ate1 and is directed towards the interdomain interface, the predicted site of interaction with the protein substrate. It is likely that this amino acid has a direct role in catalysis. Second, we identified a three-amino-acid stretch that is apparently responsible for the differential substrate specificity of different mouse Ate1 forms, FEG15-17 in Ate1-1/2, the absence of which in the corresponding positions (8–10) of Ate1-3/4 leads to inability to arginylate N-terminal Asp and Glu. This stretch probably represents an important determinant of substrate specificity in the Ate1 molecule. Most of the other mutated residues seem to have further roles in differentiating between aspartate-bearing and glutamate-bearing substrates (Table 1; Fig 4). Together with the fact that some of these mutants show increased activity, this strongly argues that the point mutations did not cause general destabilization of the protein conformation. The same observations, together with earlier results on differential substrate specificity of the alternatively spliced mouse Ate1 forms (Rai & Kashina, 2005), also suggest that the activity and substrate specificity of the wild-type Ate1 are in an exquisite balance, which is enabled by many molecular determinants dispersed across the molecule.

The phenotypes of the mutants suggest several lines of future investigation. Subsets of targets of Ate1 may now be isolated using the mutants that show preference towards different residues at the N terminus of the substrate protein. Mutant forms with increased or reduced activity and specificity should be useful for structural studies of R-transferase activity. Finally, our results on the identification of specific functionally important amino-acid residues based on the homology between Ate1 and FemABX should provide better understanding of the catalytic mechanism of bacterial enzymes of peptidoglycan biosynthesis, which are promising targets of new antibiotic development.

METHODS

Sequence analysis. Sequence similarity searches were performed with the BLAST family of programs (Altschul *et al*, 1997) and using the HHSearch server (Soding, 2005); homologous sequences were aligned using MUSCLE (Edgar, 2004). Docking was performed using the GRAMM program v 1.03 (Vakser, 1996). The results of the computational analysis are described in more detail in the supplementary information online.

Ate1 yeast complementation assay. Yeast wild type and *ate1Δ* mutants were co-transformed with plasmids carrying mouse Ate1-1, Ate1-3, or their mutant derivatives (see the supplementary information online for mutant construction details), and Ub-X-βGal protein substrates in high copy number pUB23-X plasmids expressing Ub-X-βGal proteins from PGAL promoter as described by Bachmair *et al* (1986), where X represents the N-terminal

amino acid of the β-Gal substrate. In this study, we used plasmids encoding Ub-M, D, E, R-βGal. Protein expression was induced by 1% galactose and β-Gal activity was measured using ONPG liquid assay as described by Kwon *et al* (1999).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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