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Review

Posttranslational arginylation as a global biological regulator

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ABSTRACT

Posttranslational modifications constitute a major field of emerging biological significance as mounting evidence demonstrates their key role in multiple physiological processes. Following in the footsteps of protein phosphorylation studies, new modifications are being shown to regulate protein properties and functions in vivo. Among such modifications, an important role belongs to protein arginylation — posttranslational tRNA-mediated addition of arginine, to proteins by arginyltransferase, ATE1. Recent studies show that arginylation is essential for embryogenesis in many organisms and that it regulates such important processes as heart development, angiogenesis, and tissue morphogenesis in mammals. This review summarizes the key data in the protein arginylation field since its original discovery to date.

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Contents

Introduction

Since the discovery of protein phosphorylation, posttranslational modifications have been emerging as highly important biological regulators, critical for many physiological processes. During the past decades numerous studies continue to unveil their complexity and the intricate networks of the metabolic pathways whose proper function depends on tight posttranslational control. Many of these regulatory modifications are coming into focus only now. Among them, a prominent role belongs to arginylation — tRNA-dependent posttranslational addition of Arg onto proteins. In this short review, we summarize the historical development as well as the most recent findings in the field of protein arginylation. For recent reviews with the emphasis on the relation between arginylation and protein ubiquitination see Dougan et al. (2010); Hershko et al. (2000); Ravid and Hochstrasser (2008); Varshavsky (2006).

Arginylation is mediated by arginyltransferase (ATE1), an enzyme present in all eukaryotic cells. A similar modification also exists in prokaryotes, where a homologous enzyme L/F transferase modifies proteins by addition of Leu and Phe. Thus, tRNA-dependent posttranslational addition of amino acids to proteins is highly conserved in evolution.

Every organism from yeast to human contains an Ate1 gene, which encodes a single protein in lower eukaryotes, and multiple isoforms in higher species (Hu et al., 2006; Kwon et al., 1999; Rai and Kashina, 2005). Recent projects involving global gene knockout screens in different organisms reveal that while in lower eukaryotes, from yeast

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to *C. elegans*, Ate1 gene is non-essential (i.e., not required for the organism's viability), starting with Drosophila knockout of Ate1 results in embryonic lethality (Table 1). Thus, it is clear that ATE1 is highly important physiologically, and that its function in advanced developmental processes is required for survival.

Over the years, many research groups attempted characterization of the ATE1 enzyme, its biological targets, and mechanisms of function, obtaining puzzling and often controversial results. The recent breakthroughs in genome sequencing, mass spectrometry, and mouse transgenesis enabled more targeted studies, finally elucidating some of the older mysteries that surrounded arginylation since its original discovery. This review will provide a comprehensive overview of all the available data on arginylation, its mechanisms, molecular targets, and physiological role in various developmental pathways.

Identification of arginylation enzymes and insights into the chemistry of arginylation

In 1963, a group of researchers discovered that in cell-free extracts depleted of the components for conventional protein synthesis (such as ribosome, mRNA, GTP and other factors) proteins can incorporate radioactive amino acids by mechanisms dependent on tRNA but independent of conventional translation. This phenomenon, observed both in prokaryotes (Kaji et al., 1963a, 1965a,b; Momose and Kaji, 1966) and in mammalian systems (Kaji, 1968; Kaji et al., 1963b) was surprising, since it was assumed that tRNA-dependent amino acid incorporation into proteins must be coupled to de novo formation of protein chains by ribosomes. However, it soon became clear that the new phenomenon is not an alternative translation process, but constitutes a previously unknown posttranslational modification, which is restricted to highly specific amino acids (Kaji et al., 1963a,b, 1965a,b; Momose and Kaji, 1966). To date, only tRNA-dependent addition of Arg in eukaryotic cells and Leu and Phe in bacteria has been observed. Interestingly, an independent group of enzymes have also been shown to modify proteins by addition of amino acids, including glycylation, glutamylation, and tyrosination (reviewed in (Bulinski, 2009; Hammond et al., 2008). However, these enzymes display a number of important differences from arginyltransferases and bacterial L/F transferases. First, they act by direct transfer of amino acids onto proteins rather than via aminoacyl-tRNA. Second, they have only one major intracellular target, tubulin (which is exclusive for tyrosination and predominant for glycylation and glutamylation), while R- and L/F-transferases have a much broader range of substrates (see following discussion).

Follow-up studies further characterized the arginyl transfer reaction (Kaji, 1968; Kemper and Habener, 1974; Soffer, 1968) and identified the enzymes that mediate Arg transfer in plants (Manahan and App, 1973), in guinea-pig hair follicles (Lock et al., 1976) and yeast (Balzi et al., 1990), and Leu/Phe transfer in E. coli (Kaji et al., 1963a, 1965a,b; Leibowitz and Soffer, 1969, 1970; Momose and Kaji, 1966). It has been determined that these enzymes, similarly to the translation machinery, utilize aminoacyl tRNA to conduct the amino acid transfer to protein substrates, and thus depend on the presence of Arg-tRNA synthetase (Ciechanover et al., 1988). It was also found that the bacterial Leu/Phe (L/F) transferase prefers N-terminally exposed Arg as an acceptor site, while Arg-transferase (named ATE1, for Arginine Transfer Enzyme 1) shows a preference for N-terminally exposed Asp and Glu (Leibowitz and Soffer, 1970, 1971; Soffer, 1970a,b, 1971b, 1973a,b; Soffer and Horinishi, 1969).

Identification of Ate1 in multiple species from yeast to human (Balzi et al., 1990; Kwon et al., 1999; Manahan and App, 1973; Rai and Kashina, 2005) enabled more in-depth characterization of these enzymes. It was found that evolutionarily higher organisms have multiple ATE1 isoforms generated by alternative splicing, that differ by activity, tissue specificity, and intracellular localization (Hu et al., 2006; Kwon et al., 1999; Rai and Kashina, 2005).

Based on the Arg-tRNA dependence of the reaction and on the fact that it apparently occurs only on the residue(s) with an N-terminally exposed amino group, it has been postulated that the arginylation reaction results in the formation of a peptide bond between the alpha amino group of the N-terminal residue of a protein and the carboxy group of the added Arg (Kaji, 1968; Soffer and Horinishi, 1969). In agreement with this, added Arg on proteins could be identified by N-terminal Edman sequencing (Kaji, 1968; Kwon et al., 1999; Rai and Kashina, 2005). However, recent studies showed that Arg can also be added to a mid-chain Glu residue in the biological regulatory peptide neurotensin (Eriste et al., 2005) — a modification that can only happen if the amino group of Arg reacted with the carboxy group of the Glu side chain, suggesting that the chemistry of arginylation may be more complex than originally believed and/or additional enzyme(s) may be involved in this novel side chain arginylation. It was also found that proteins could be modified not only at the N-terminus but also at the internal sites in the amino acid chain (Rai et al., 2008; Wong et al., 2007), presumably via transiently exposed alpha amino groups of the mid-chain residues, but potentially also via linkages to the amino acid side chains in the protein back bone. These new data suggest that the arginylation reaction may be more complex than originally believed

Table 1
Ate1 knockout phenotypes in different organisms.

Organism	Phenotype	Screen	References	Related information
Saccharomyces cerevisiae	Viable, no observable defects.	Yeast deletion project	http://www-deletion.stanford.edu/YDPM/ Balzi et al. (1990)	
C. elegans	No observable defects.		http://www.wormbase.org/db/gene/gene?name=WBGene00010615;class=Gene	*The following phenotypes were not observed in Ate1 knockout*: larval lethality, slow growth, sterile progeny, organism morphology variant, lethal, larval arrest, postembrynic development variant, embryonic lethal, maternal sterile.
Drosophila melanogaster	Lethal	P element screen	Spradling et al. (1999); Mutsuddi et al., (2004)	Overexpression of human SCA8, a noncoding RNA involved in neurodegeneration, causes rough eye phenotype which is enhanced in the Ate1 knockout background.
Mus musculus	Embryonic lethal	Targeted gene knockout	Kwon et al. (2002)	Heart development, and angiogenesis defects
Arabidopsis thaliana	Delayed leaf senescence. Abnormal shoot and leaf development and defects in seed germination.		Yoshida et al. (2002) Holman et al. (2009)	

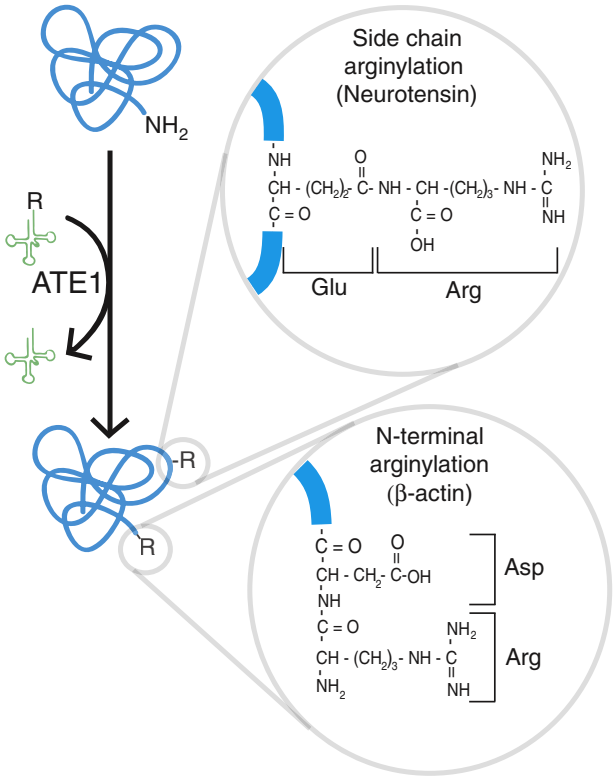

Fig. 1. Summary of the arginylation reaction. According to the conventional theory, ATE1 transfers Arg from tRNA onto the N-terminally exposed amino group of the acceptor protein, forming a peptide bond (circled on the bottom of the diagram). Recent finding also demonstrates an example of Arg addition to the side chain of the Glu residue of neurotensin (circled on the side of the diagram). If mediated by ATE1, this reaction constitutes an additional or alternative mechanism of protein arginylation.

and possibly occurs by more than one mechanism. The currently known forms of chemical linkage of Arg to proteins are summarized in Fig. 1.

Over the years, attempts have been made to reconstitute the arginylation reaction in vitro and determine the essential components in this reaction. Several groups have reported success with partially purified ATE1 fractions, yielding insights into the molecular requirements for enzymatic Arg transfer (Ciechanover et al., 1988; Horinishi et al., 1976; Kato and Nozawa, 1984; Soffer, 1970b). However, only very recently have the sufficient purity in an in vitro arginylation system been achieved, enabling direct testing of the molecular requirements and components utilized by ATE1 (Wang et al., 2011). Using this system, it has been found that ATE1 is a self-sufficient enzyme that does not require ATP or the presence of any protein components besides the substrate to conduct the Arg-transfer reaction. It has also been determined that different ATE1 isoforms possess different activity and substrate specificity and are differentially modulated by intracellular factors, suggesting that they play distinct physiological roles. Moreover, it has been found that ATE1 can also undergo self-arginylation, pointing to another level of potential self-regulation of this enigmatic enzyme (Wang et al., 2011).

Arginylation and ubiquitin-dependent protein degradation

Earlier studies demonstrated that knockout of Ate1 in yeast (Balzi et al., 1990) and L/F transferase in E. coli (Deutch et al., 1977) do not visibly perturb their growth, deeming these enzymes non-essential. It has been found, however, that engineered proteins containing N-terminal Arg can be metabolically unstable in yeast (Bachmair et al., 1986; Gonda et al., 1989) and that addition of N-terminal Arg can target proteins for ubiquitination (Elias and Ciechanover, 1990), suggesting that N-terminal arginylation might play a role in ubiquitin-dependent protein regulation and turnover. In support of this, researchers observed degradation of arginylated proteins in the cytosol of various cells (Bohley et al., 1988a,b, 1991), and arginylation-dependent targeting of proteins with acidic N-termini for ubiquitination and degradation (Ciechanover et al., 1988). It was shown that ubiquitin associates with aggregates of arginylated proteins in injured nerves (Jack et al., 1992) and that arginylation and ubiquitin-dependent proteolysis are involved in nerve regeneration (Chakraborty and Ingoglia, 1993; Zanakis et al., 1984).

A recent study demonstrated that in some cases protein arginylation and subsequent ubiquitination may occur co-translationally (rather than posttranslationally), and that the metabolic fate of such co-translationally arginylated proteins may differ depending on the rate of their translation and folding (Zhang et al., 2010). By this mechanism, proteins that translate and fold slowly become targets for ubiquitination and rapid degradation, while those fast-synthesized proteins that bypass this co-translational 'checkpoint' are able to exist in vivo in an arginylated state, which likely regulates their structure and functions in the cell. This mechanism appears to be one of arginylation's regulatory mechanisms; it ensures that only those proteins that can fold, traffic, and function properly once arginylated will persist in vivo.

Oxidized proteins were also shown to be targets for arginylation (Zhang et al., 1998). Follow-up studies demonstrated that in specialized cases involving proteins with N-terminal Cys, arginylation can happen only after nitric oxide-dependent Cys oxidation and that such oxidation-dependent arginylation likely targets proteins for degradation, suggesting that arginylation can play a role in nitric oxide signaling and oxygen sensing (Davydov and Varshavsky, 2000; Hu et al., 2005). However, other studies suggested that Cys can also be arginylated in an unmodified form, and that many proteins do not become metabolically unstable upon arginylation (Wong et al., 2007).

Overall, while protein degradation appears to play a selective role in determining the intracellular stability of some arginylated proteins, global effects of arginylation likely extend beyond the regulation of the proteins' metabolic fate and constitute a general regulatory mechanism that affects protein structure, molecular interactions, and in vivo functions.

Proteins arginylated in vivo

Growing evidence demonstrates that a multitude of proteins in different organisms, cell types, and subcellular fractions can serve as posttranslational acceptors of Arg. It has been found that secreted proteins, including BSA and alpha-lactalbumin, can be arginylated after the removal of signal peptides in the presence of crude preparations of ATE1 (Ciechanover et al., 1988). Other secreted proteins, such as bovine thyroglobulin (Soffer, 1971a), were also found to be targets for arginylation, raising a possibility that ATE1 activity might be coupled to the endoplasmic reticulum and protein transport through the membrane. In support of this, it was also found that some regulatory peptides and hormones that exist in the plasma and extracellular liquids can serve as substrates in the arginylation reaction, including neurotensin (Eriste et al., 2005), beta-melanocyte stimulating hormone (Soffer, 1975), insulin (Zhang et al., 1998) and angiotensin II (Soffer, 1975), and that in the case of angiotensin arginylation appeared to alter its ability to induce cellular responses (Soffer, 1975), suggesting that this posttranslational modification may constitute a novel regulatory mechanism not only for proteins but also for biological peptides. Human erythrocyte membranes that consist largely of alpha or beta lipoproteins whose N-termini are aspartic or glutamic acid were found to incorporate arginine (Kaji and Rao, 1976). While at present it is unclear how ATE1 can exert its activity on the secreted proteins and plasma peptides, it is possible that arginylation constitutes a step in their intracellular processing prior to secretion, or that ATE1 itself might be secreted in complex with arginylated tRNA, which is essential for the Arg-transfer reaction.

Other arginylated proteins have been identified in multiple studies, include chromosomal proteins (Kaji, 1968, 1976), ornithine decarboxylase (Bohley et al., 1988a,b; Kopitz et al., 1990), as well as at least 25 different additional cytosol proteins in hepatocytes and at least 15 different proteins in Dictyostelium discoideum (Bohley et al., 1991). It was found that 16 soluble proteins can incorporate labeled Arg in rat brain extracts (Hallak et al., 1985), and multiple soluble Arg acceptor proteins have been observed in different regions of the rat tissues (Lamon and Kaji, 1980; Takao and Samejima, 1999), brain (Hallak et al., 1991), bovine lens (Wagner and Margolis, 1991), and cultured cells (Fissolo et al., 2000; Rao and Kaji, 1977a). Studies measuring the correlation between protein's in vivo stability and its arginylation state have identified several members of the regulator of G-protein signaling (RGS) family as targets for arginylation-dependent degradation in vitro (Davydov and Varshavsky, 2000) and in vivo (Lee et al., 2005). It was found that arginylation of beta amyloid peptide increases the probability of alpha-helix formation, suggesting that arginylation may play a role in preventing neurodegeneration (Bongiovanni et al., 1995). It was also found that arginylation of calreticulin induces its association with the stress granules (Carpio et al., 2010; Decca et al., 2007), suggesting an additional role of arginylation in intracellular stress responses and the ER function.

Recent development of high precision mass spectrometry and its applications to posttranslational modifications enabled higher throughput screens for identification of proteins arginylated in vivo (Wong et al., 2007; Xu et al., 2009). Using such screens, additional 43 proteins arginylated on highly specific sites in mouse embryonic and adult tissues have been identified (Wong et al., 2007). The functions of these proteins range from structural to metabolic, and include transcription factors, tumor-related genes, and glycolytic enzymes. A prominent cytoskeleton protein – non-muscle beta actin – was found to be dependent on arginylation in performing its role at the cell leading edge (Karakozova et al., 2006) via mechanisms that affect its interaction with other proteins and regulate actin intracellular polymer level (Saha et al., 2010), apparently independent of its metabolic stability. For many proteins it has been found that arginylation occurs on the surface sites, exposed after the completion of folding and the assembly of the protein's tertiary structure (Wong et al., 2007), suggesting that arginylation of these proteins may regulate their properties and protein-protein interactions at the arginylated sites. Many of these proteins are highly abundant and stable, suggesting that their regulation by arginylation is independent of their metabolic stability.

Thus, in vivo functions of arginylation appear to be highly diverse and include regulation of large numbers of different proteins and a wide range of physiological processes. Fig. 2 summarizes some of the intra- and extracellular processes regulated by arginylation.

Developmental events and physiological pathways regulated by arginylation

Studies through the years have reported that changes in intracellular arginylation levels correlate with such fundamental physiological processes as aging (Kaji et al., 1980; Lamon and Kaji, 1980), stress (Lamon et al., 1980), rat liver regeneration (Tanaka and Kaji, 1974), temperature sensitivity and heat shock (Bongiovanni et al., 1999; Rao and Kaji, 1977b), nerve regeneration (Chakraborty and Ingoglia, 1993; Wang and Ingoglia, 1997; Xu et al., 1993), and protein degradation in skeletal muscle (Solomon et al., 1998). A breakthrough in these studies came with the identification of arginyltransferases in multiple species

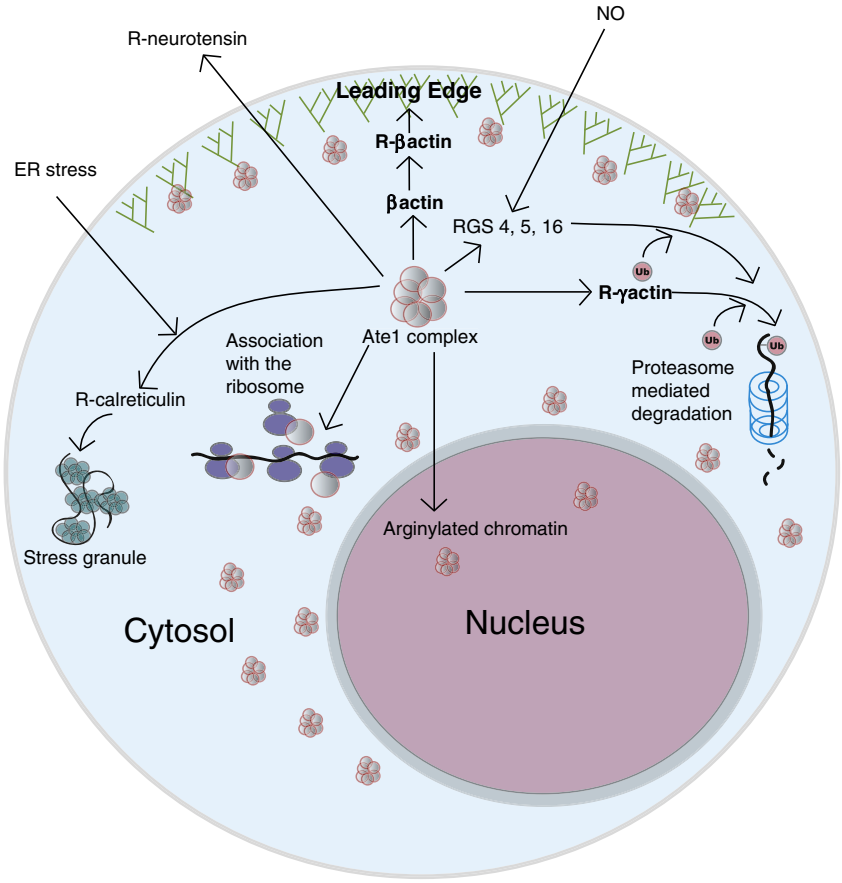

Fig. 2. Biological processes regulated by arginylation. ATE1 forms different complexes in vivo with roles in various intracellular processes that occur in the cytosol, nucleus, and the extracellular space.

(Kwon et al., 1999) and characterization of the mouse Ate1 gene (Kwon et al., 1999, 2002). It was found that mouse Ate1 knockout results in embryonic lethality and defects in cardiovascular development and angiogenesis — a discovery that showed for the first time that arginylation plays an essential physiological role. Follow-up studies demonstrated that mammalian ATE1 can serve as a nitric oxide sensor that controls the levels of multiple biological regulators (Hu et al., 2005, 2008), and that arginylation regulates neural crest morphogenesis (Kurosaka et al., 2010), cardiac contractility (Rai et al., 2008), and gametogenesis (Leu et al., 2009). Postnatal deletion of Ate1 results in loss of fat and increased metabolic rates and affects spermatogenesis and the nervous system (Brower and Varshavsky, 2009). Thus, it became evident that arginylation is not only essential, but also takes part in a number of highly diverse physiological pathways. The currently known developmental processes regulated by arginylation in mice are summarized in Figs. 3 and 4.

Studies in other organisms have shown that the essential role of arginylation is not confined to mammals. In *Arabidopsis thaliana* arginylation knockout causes delayed leaf senescence (Lim et al., 2007; Yoshida et al., 2002), abnormal shoot and leaf development (Graciet et al., 2009) and defects in seed germination (Holman et al., 2009). In Drosophila, high throughput genomic studies demonstrated that Ate1 knockout results in embryonic lethality (Table 1). Interestingly, such studies also demonstrated that Ate1 gene is non-essential for survival in *C. elegans* (Table 1), suggesting that the evolutionary boundary defining the global importance of arginyltransferases for an organism's viability manifests only in species evolutionarily higher than worms.

Several of the identified ATE1 substrates have been implicated in exerting these biological effects. Proteins of the RGS family, which become metabolically unstable upon arginylation (Davydov and Varshavsky, 2000; Lee et al., 2005), are known regulators of cardiovascular development and are implicated in cell motility and related responses. Conceivably, lack of arginylation can lead to accumulation of these proteins, facilitating some of the developmental defects seen in Ate1 knockout mice. Another striking example concerns arginylation of non-muscle beta actin and actin cytoskeleton proteins (Karakozova et al., 2006; Wong et al., 2007), which have been functionally linked to impaired lamella formation (Karakozova et al., 2006), defective cell adhesion, and a dramatic reduction of actin polymer level in cells (Saha et al., 2010) — the effects that likely underlie the impairments in cell migration and adhesion seen in vivo. Given the large list of identified arginylation targets, it is only a matter of time before the researchers uncover more molecular links and identify the role of arginylation in regulation of the specific functions of individual proteins.

In summary, multiple studies show that arginylation is a global physiological regulator that modifies many proteins in vivo to modulate their functions in many critical pathways during embryogenesis and adulthood.

Fig. 3. Mouse Ate1 knockout phenotypes. Top two panels, complete deletion of Ate1 results in embryonic lethality at and after E12.5, with severe defects in cardiovascular development and angiogenesis (Kwon et al., 2002) (embryos shown at E12.5). Middle panel, deletion of Ate1 in Wnt1-expressing migratory neural crest cells results in perinatal lethality accompanied by general retardation and defects in craniofacial morphogenesis (Kurosaka et al., 2010) (pups shown at postnatal day P6). Bottom panel, deletion of Ate1 in premeiotic germ cells driven by Tek promoter results in early post-implantation lethality (littermate control and mutant embryos at E12.5 are shown in bottom and top rows, respectively) (Leu et al., 2009). Arrows in all panels indicate mutant embryos/pups. Photos courtesy of N. A. Leu (University of Pennsylvania).

Physiological regulators of protein arginylation

While mounting evidence shows that arginylation is apparently a global regulatory mechanism, very little is known about how arginylation itself is regulated in vivo. It has been shown that the levels of arginyltransferase expression rise and fall during embryonic development (Kwon et al., 1999) and aging (Lamon and Kaji, 1980) and vary between different tissues in the embryos and adult organisms (Rai and Kashina, 2005), however deletion of one copy of the Ate1 gene in mouse (that could potentially reduce the protein level by ~50%) does not produce detectable physiological effects (Kwon et al., 2002). Numerous studies have shown that various proteins, drugs, and physiological compounds can inhibit in vivo arginylation levels, including hydrocortisone (Lamon et al., 1982), heparin (Kato, 1983), arsenite (Berleth et al., 1992; Klemperer and Pickart, 1989; Li and Pickart, 1995a,b), tripeptide L-Glutamyl-L-Valyl-L-Phenylalanine (Bohley et al., 1991), RNase, canavanine, hemoglobin, and hemin (Goz and Voytek, 1972). A peptide that inhibits arginylation in rat brain has been reported (Yu et al., 1993). It was also shown that serine protease inhibitors reduce arginylation levels (Yu et al., 1994), and that the increase of arginylation observed during nerve regeneration can be influenced by exogenously added Arg, S-adenosylmethioninie, and polyamines (Cestaro, 1994). While the role of some of these compounds in the arginylation reaction can be explained based on the available data — including RNase, which likely destroys Arg-tRNA and thus makes it unavailable for the reaction, or Glu-Val-Phe tripeptide, which could conceivably act as a competitor

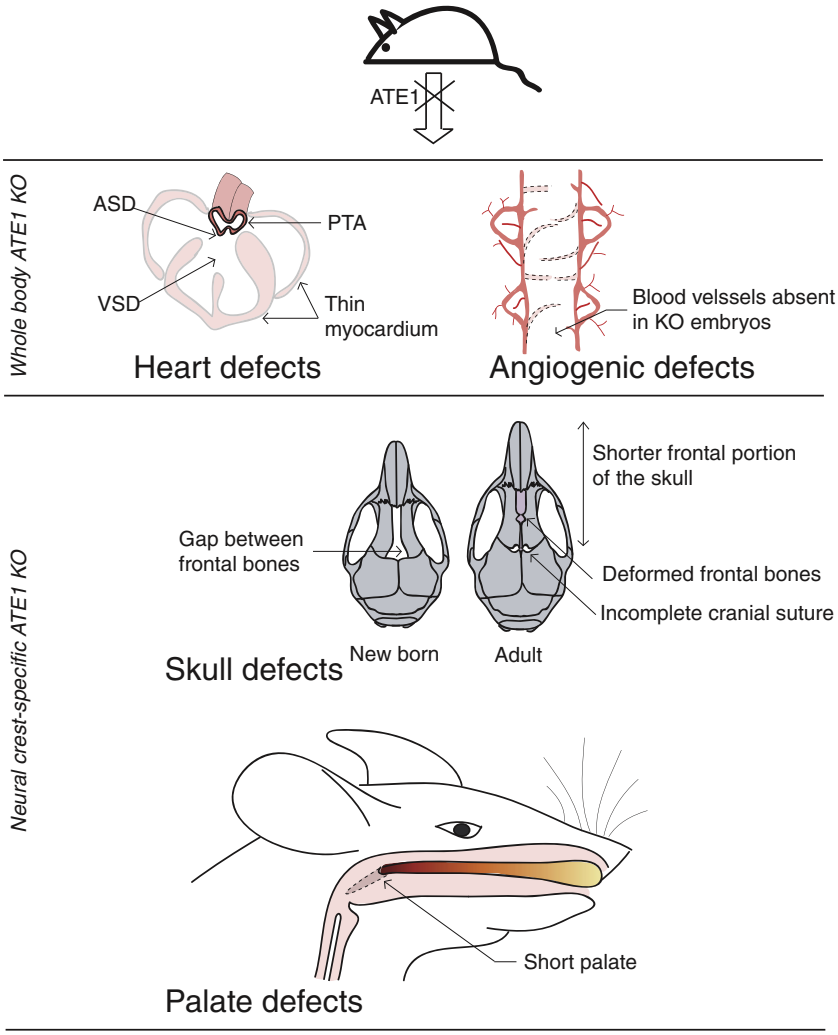

Fig. 4. Schematic representation of the major known organogenic defects resulting from Ate1 knockout in mice.

to other ATE1 substrates — the role of other compounds in inhibiting arginylation reaction remains to be elucidated. Some of these compounds (such as hemoglobin, a demonstrated arginylation substrate (Wong et al., 2007)) could also act by competition with other proteins arginylated by ATE1 for the enzyme recognition and availability, others could potentially act as the inhibitors of the enzyme itself or its molecular complex, and might be involved in the regulation of arginylation in vivo.

Recent work suggests extensive parallels between protein arginylation and such major regulatory modifications as phosphorylation (Kaji and Kaji, 2011; Wong et al., 2007). Understanding of the biological role of arginylation, its involvement in different physiological pathways, and the mechanisms of its regulation, constitute exciting directions of further studies.

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