Gene 527 (2013) 123–130

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Evolution of the salivary apyrases of blood-feeding arthropods

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ARTICLE INFO

Article history:
Accepted 28 May 2013
Available online 18 June 2013

Keywords:
5′-Nucleotidase
Anti-coagulant
Apyrase
Arthropoda
Blood-feeding

ABSTRACT

Phylogenetic analyses of three families of arthropod apyrases were used to reconstruct the evolutionary relationships of salivary-expressed apyrases, which have an anti-coagulant function in blood-feeding arthropods. Members of the 5′nucleotidase family were recruited for salivary expression in blood-feeding species at least five separate times in the history of arthropods, while members of the *Cimex*-type apyrase family have been recruited at least twice. In spite of these independent events of recruitment for salivary function, neither of these families showed evidence of convergent amino acid sequence evolution in salivary-expressed members. On the contrary, in the 5′-nucleotide family, salivary-expressed proteins conserved ancestral amino acid residues to a significantly greater extent than related proteins without salivary function, implying parallel evolution by conservation of ancestral characters. This unusual pattern of sequence evolution suggests the hypothesis that purifying selection favoring conservation of ancestral residues is particularly strong in salivary-expressed members of the 5′-nucleotidase family of arthropods because of constraints arising from expression within the vertebrate host.

1. Introduction

Apyrases (or ATP-diphosophohydrolases) are a class of enzymes, found in all animals, which hydrolyze ADP and ATP to AMP (Meyerhoff, 1945; Plesner, 1995). Animal apyrases form three distinct families: (1) the 5′-nucleotidase family (Champagne et al., 1995); (2) a family first discovered in the bed bug *Cimex lectularius*, sometimes called the *Cimex*-type apyrases (Smith et al., 2002; Valenzuela et al., 1998); and (3) homologues of the human B cell antigen CD39, a cell-surface apyrase (Knowles, 2011). Apyrase activity has been detected in the saliva of mosquitoes, ticks, bed bugs and other blood-feeding arthropods, where this enzyme interferes with host blood coagulation by eliminating ADP which is required for platelet aggregation, thereby facilitating feeding by the arthropod (Cupp et al., 1994; Ribeiro and Garcia, 1980; Ribeiro et al., 1984, 1985; Sarkis et al., 1986). Arthropods with known salivary apyrase activity include the vectors of the organisms causing such major human infectious diseases as malaria, yellow fever, African sleeping sickness, Chagas' disease, leishmaniasis, and Lyme disease.

In several cases, distantly related arthropod species share salivary apyrases belonging to the same family. For example, salivary apyrases of the 5′-nucleotidase family have been reported from two different families in the insect order Diptera, Culicidae (Champagne et al., 1995; Lombardo et al., 2000) and Glossinidae (Alves-Silva et al., 2010; Van den Abbeele et al., 2007); from the insect order Hemiptera (Assumpção et al., 2008; Faudry et al., 2004); and from ticks (Stutzer et al., 2009). Similarly, *Cimex*-type apyrases have been reported not only from the saliva of bed bugs (Hemiptera) but also from the saliva of sand flies (Diptera: Psychodidae) belonging to the genera *Lutzomyia* and *Phlebotomus* (Anderson et al., 2006; Charlab et al., 1999; Hostomská et al., 2009). However, the phylogenetic relationships of arthropod apyrases have not been studied; thus, it is not known whether salivary-expressed apyrases of different blood-feeding arthropod taxa are orthologs. Here I address this question using phylogenetic methods and, by reconstructing ancestral sequences, test for parallel or convergent evolution of amino acid sequence motifs in salivary-expressed apyrases.

2. Methods

Sequences of Insecta (insects) and Acari (mites and ticks) representing the three apyrase families (5′-nucleotidase, *Cimex*-type, and CD39) were found in NCBI databases by BLASTP protein homology search. For purpose of phylogenetic analyses, sequences were used from 4 species of Acari and 31 species of insects, belonging to 6 orders and 16 families (Table 1). Sequences used in the analyses included available sequences from representative completely sequenced genomes and additional salivary apyrases reported from a number of blood-feeding species as well as species not feeding on blood (Table 1). Sequences with known salivary expression in blood-feeding arthropods were identified from the literature (Alves-Silva et al., 2010; Anderson et al., 2006; Assumpção et al., 2008; Champagne et al., 1995; Charlab et al., 1999; Faudry et al., 2004; Francischetti et al., 2000; Hostomská et

Abbreviations: ADP, adenosine diphosphate; MRCA, most recent common ancestor.

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http://dx.doi.org/10.1016/j.gene.2013.05.087

Table 1
Arthropod species from which apyrase sequences are analyzed.

Class	Infraclass	Order	Family	Species
Arachnida		Acari	Phytoseiidae	*Metaseiulus occidentalis*
			Ixodidae	*Amblyomma maculatum*[a]
				Ixodes scapularis[a]
				Ornithodoros savignyi[a]
Insecta	Exopterygota	Hemiptera	Aphididae	*Acyrthosiphon pisum*
			Pseudococcidae	*Maconellicoccus hirsutus*
			Reduviidae	*Triatoma infestans*[a]
				Triatoma matagrossensis[a]
			Cimicidae	*Cimex lectularius*[a]
		Phthiraptera	Pediculidae	*Pediculus humanus corporis*
	Endopterygota	Coleoptera	Tenebrionidae	*Tribolium castaneum*
		Lepidoptera	Bombycidae	*Bombyx mori*
			Nymphalidae	*Danaus plexippus*
			Papilionidae	*Papilio xuthus*
		Diptera	Culicidae	*Aedes aegypti*[a]
				Anopheles gambiae[a]
				Culex quinquefasciatus[a]
			Drosophilidae	*Drosophila melanogaster*
				Drosophila grimshawi
			Glossinidae	*Glossina morsitans morsitans*[a]
			Psychodidae	*Lutzomyia intermedia*[a]
				Lutzomyia longipalpis[a]
				Phlebotomus arabicus[a]
				Phlebotomus ariasi[a]
				Phlebotomus argentipes[a]
				Phlebotomus duboscqi[a]
				Phlebotomus papatasi[a]
				Phlebotomus perniciosus[a]
				Phlebotomus sergenti[a]
				Phlebotomus tobbi[a]
		Hymenoptera	Pteromalidae	*Nasonia vitripennis*
			Apidae	*Apis mellifera*
				Bombus terrestris
			Formicidae	*Camponotus floridanus*
				Harpegnathos saltator

[a] Blood-feeding species.

al., 2009; Karim et al., 2011; Kato et al., 2006; Lombardo et al., 2000; Ribeiro et al., 2006; Stutzer et al., 2009; Valenzuela et al., 1998, 2002; Van Den Abbeele et al., 2007). Salivary expression was not known in any species that was not blood-feeding. However, in the latter species, even if an apyrase is expressed in the saliva, it obviously does not naturally function as an anti-coagulant.

Sequences were aligned by the CLUSTALW algorithm in MEGA 5.05 (Tamura et al., 2011); and any site at which the alignment postulated a gap in any of a set of aligned sequences was excluded from analyses involving that set of sequences. Mammalian 5′ nucleotidases include a C-terminal hydrophobic domain serving to link to the cell membrane through a phosphatidylinositol lipid anchor; but Champagne et al. (1995) reported that this domain is absent in the salivary apyrase of the mosquito *Aedes aegypti*. Because such a domain was not present in all of the sequences analyzed here, it was not used in reconstructing the phylogenetic tree of 5′ nucleotidases. However, the presence of a C-terminal hydrophobic domain was plotted on the phylogenetic tree in order to understand the evolution of these domains.

Amino acid sequence evolution models were tested in MEGA 5.05 using the Bayes Information Criterion (Tamura et al., 2011). Maximum likelihood (ML) trees were constructed in MEGA 5.05. Reliability of branching patterns in the trees was tested by bootstrapping; 1000 bootstrap samples were used in each case. Ancestral amino acid sequences were reconstructed by ML in MEGA 5.05, assuming the same amino acid sequence model as was used for phylogenetic reconstruction.

3. Results

3.1. 5′-nucleotidase family phylogeny

A phylogenetic tree of 66 arthropod sequences from the 5′-nucleotidase family was inferred by the ML method based on the WAG + G + I model at 319 aligned amino acid sites and rooted with homologous vertebrate sequences (Fig. 1). There was a large, well-supported clade (designated clade A in Fig. 1) that included sequences from four orders of Endopterygota (or Holometabola; i.e., the insects with complete metamorphosis): Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Fig. 1 and Table 1). The internal branch supporting clade A was highly significant, receiving 99% bootstrap support (Fig. 1). Several additional sequences from Endopterygota fell outside clade A (Fig. 1). The latter included sequences from the orders Diptera, Lepidoptera, Coleoptera, and Hymenoptera; and, among the sequences from Diptera, there were several from mosquitoes (Fig. 1). Thus the phylogenetic tree provided strong support for the hypothesis that clade A arose by gene duplication prior to the MRCA of those four orders. The sequences from the order Hemiptera, traditionally classed in the Exopterygota (or Hemimetabola; i.e., insects with incomplete metamorphosis) but more recently classified within Paraneoptera, a sister clade to Exopterygota (Grimaldi and Engel, 2005; Kjer et al., 2006) formed a separate cluster, which received only weak bootstrap support (56%; Fig. 1).

Insect members of this family with known salivary expression were widely separated in the phylogenetic tree (Fig. 1). Salivary-expressed apyrases from mosquitoes (Culicidae) and tsetse flies (Glossinidae) formed part of clade A, but did not cluster together within that clade (Fig. 1). On the other hand, the salivary-expressed proteins from bugs of the genus *Triatoma* clustered with those of another member of Hemiptera, the aphid *Acyrthosiphon pisum* (Fig. 1). Thus, the phylogenetic tree suggested at least three separate events of recruitment of members of this family for salivary expression in blood-feeding insects.

The sequences from Acari formed a separate cluster, which received 94% bootstrap support (Fig. 1). There was weak support (54%) for clustering of the sequences from Acari with clade A of insects. If this pattern is reliable, it would imply that the ancestor of clade A arose by gene duplication prior to the most recent common ancestor (MRCA) of insects and Acari. Within the Acari, known salivary apyrases formed a cluster that received 100% bootstrap support (Fig. 1). Outside of this cluster fell a non-salivary sequence from the tick *Ixodes scapularis* and a sequence from the Western predatory mite *Metaseiulus occidentalis* (Fig. 1). Therefore, the phylogeny supported an independent event of recruitment of members of the 5′-nucleotidase family for salivary function in blood-feeding Acari.

Relationships were not well resolved within clade A of insect 5′-nucleotidase apyrases (Fig. 1). In order to provide further information regarding orthologous and paralogous relationships within that clade, a separate phylogenetic analysis was conducted with clade A