Developmental and Comparative Immunology 47 (2014) 129–139

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

The Lepidopteran endoribonuclease-U domain protein P102 displays dramatically reduced enzymatic activity and forms functional amyloids

Mariarosa Pascale [a], Simona Laurino [a], Heiko Vogel [b], Annalisa Grimaldi [c], Magnus Monné [a], Lea Riviello [a], Gianluca Tettamanti [c], Patrizia Falabella [a,*]

[a] Università della Basilicata, Dipartimento di Scienze, via dell'Ateneo Lucano 10, 85100 Potenza, Italy
[b] Department of Entomology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, 07745 Jena, Germany
[c] Università dell'Insubria, Dipartimento di Biotecnologie e Scienze della Vita, via Dunant 3, 21100 Varese, Italy

ARTICLE INFO

Article history:
Received 5 June 2014
Revised 8 July 2014
Accepted 9 July 2014
Available online 18 July 2014

Keywords:
Endoribonuclease-U
Lepidoptera
Amyloids
Heliothis virescens
Trichoplusia ni
Immunity

ABSTRACT

Hemocytes of *Heliothis virescens* (F.) (Lepidoptera, Noctuidae) larvae produce a protein, P102, with a putative endoribonuclease-U domain. In previous works we have shown that P102 is involved in Lepidopteran immune response by forming amyloid fibrils, which catalyze and localize melanin deposition around non-self intruders during encapsulation, preventing harmful systemic spreading. Here we demonstrate that P102 belongs to a new class of proteins that, at least in Lepidoptera, has a diminished endoribonuclease-U activity probably due to the lack of two out of five catalytically essential residues. We show that the P102 homolog from *Trichoplusia ni* (Lepidoptera, Noctuidae) displays catalytic site residues identical to P102, a residual endoribonuclease-U activity and the ability to form functional amyloids. On the basis of these results as well as sequence and structural analyses, we hypothesize that all the Lepidoptera endoribonuclease-U orthologs with catalytic site residues identical to P102 form a subfamily with similar function.

1. Introduction

P102, the amyloidogenic protein produced by hemocytes of *Heliothis virescens* (F.) (Lepidoptera, Noctuidae) larvae (Falabella et al., 2012) is a member of the novel XendoU protein family (Renzi et al., 2006) that includes several proteins annotated in distantly related organisms ranging from viruses to humans (Renzi et al., 2006; Snijder et al., 2003; Caffarelli et al., 1994). Members of the XendoU family are characterized by RNA binding and processing (hydrolytic) activity. They are Mn^{2+}-dependent nucleases that cleave U-stretches of RNA and produce 2′, 3′-cyclic-phosphate termini products, which is a unique characteristic for this class of RNases (Laneve et al., 2008). The amphibian XendoU, the human PP11 and the genetic marker of Nidovirales NendoU (Nsp 15) are three well-characterized proteins belonging to this family. They all share conserved regions containing a proposed active site with five amino acid residues that are crucial for the catalytic activity (Renzi et al., 2006; Gioia et al., 2005). Despite their similarities these proteins are involved in distinct RNA processing pathways in different organisms (Bohn and Winckler, 1980; Bohn et al., 1981; Inaba et al., 1980a,b, 1981, 1982; Laneve et al., 2003, 2008; Ivanov et al., 2004; Ulferts and Ziebuhr, 2011). Other XendoU family members were frequently, but erroneously annotated as serine protease-like enzymes on the basis of their homology with the human placental protein 11 (PP11), that previously was thought to be a protease (Grundmann et al., 1990; Laneve et al., 2008). However, the highly conserved active site found in a wide range of orthologs (Gioia et al., 2005; Renzi et al., 2006; Laneve et al., 2008) may suggest that they all have endoribonucleolytic activity, although they are involved in different RNA-processing pathways.

In previous work, (Falabella et al., 2012) we identified and characterized the 102 gene (EBL-Bank ID: FR751090) in larval hemocytes of the Lepidopteran *H. virescens* (F.) (Lepidoptera, Noctuidae), encoding a predicted protein (P102), that showed 86% sequence identity with a venom protein from *Lonomia obliqua* (Lepidoptera, Saturnidae) larvae (Veiga et al., 2005), and possesses a putative endoribonuclease-U (XendoU) domain. We demonstrated that P102 is involved in insect cellular defenses in hemocytes of *H. virescens* larvae by forming amyloid fibrils in the reticulum endoplasmic cisternae. Upon immune challenge, these amyloid fibrils are released and form a fibrillar scaffold around the non-self intruder, promoting melanin synthesis directly on this scaffold (Falabella et al., 2012; Grimaldi et al., 2012). To our knowledge this was the first model that describes the mechanism of melanin deposition during encapsulation in a Lepidopteran,

* Corresponding author. Tel.: +39 0971 205501; fax: +39 0971 205503.
E-mail address: patrizia.falabella@unibas.it (P. Falabella).

http://dx.doi.org/10.1016/j.dci.2014.07.009
0145-305X/

shedding new light on an aspect of insect cellular immune response previously unknown.

Here, we investigate the putative enzymatic activity of P102, belonging to the novel poly(U) specific endoribonuclease protein family (Laneve et al., 2003; Renzi et al., 2006), trying to understand if it could have a catalytic function in RNA cleavage and fragmentation apart from its roles in amyloidogenesis (Falabella et al., 2012) and hemocytes capsule melanin deposition. The endoribonuclease assay shows only a slight residual activity for P102, which could be explained by the fact that two of the conserved residues crucial for catalysis (Gioia et al., 2005) are missing in P102. These results most likely rule out any direct involvement of P102 in RNA fragmentation. Sequence analysis of homologous proteins of P102 from other insects leads us to hypothesize that P102 belongs to a XendoU subfamily, characterized by a conserved set of alternative residues in the catalytic site, that is restricted to Lepidoptera. This protein subfamily is distinct from other insect XendoU sequences that generally belong to groups roughly according to species phylogeny. In addition, the *in vitro* functional characterization of the P102 homolog from *Trichoplusia ni* (Hübner) (Lepidoptera, Noctuidae) corroborates our hypothesis by demonstrating that the P102 characteristics extend to other members of this subfamily. Thus our results suggest that the amyloid-mediated melanin synthesis in the defense against intruders is a conserved mechanism within the order Lepidoptera, and that there could be an association between the loss of ancestral enzymatic RNA cleavage activity and the ability of the Lepidopteran alternative XendoU-like proteins to form amyloids.

2. Material and methods

2.1. Sequence analysis and molecular modeling

Database searches were performed using the nucleotide and the amino acid sequences of P102 (GenBank IDs: FR751090.1 and CBY85302.1, respectively) and the BLAST algorithm available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). In particular, the complete P102 sequence was compared to the NCBI non-redundant protein database, the NCBI non-redundant nucleotide and EST databases and our extensive in-house transcriptome databases (H. Vogel, MPI Jena). The conserved domain architecture in protein sequences was identified searching the Conserved Domain Database (CDD) at NCBI. The translation into amino acid residue sequences and analysis of the physico-chemical parameters of proteins were carried out using on-line tools available at the Expasy SIB Bioinformatics Resource Portal (http://expasy.org/). Occurrence and position of signal peptides, glycosylation and phosphorylation sites in protein sequences were predicted using the CBS prediction server (http://www.cbs.dtu.dk/services/).

Multiple amino acid sequence alignments were performed using the default options of the Uniprot program (http://www.uniprot.org). Conserved residues in the alignments were highlighted with Multiple Align show (http://www.bioinformatics.org/sms/multi_align.html).

Phylogenetic analyses based on MAFFT (E-INS-I parameter set; Katoh et al., 2005) alignments were made using deduced amino acid sequences from insect endoribonuclease-U (XendoU) transcripts retrieved from NCBI and the other sources described above. The phylogenetic tree was inferred by the maximum likelihood (ML) method using PhyML (Dereeper et al., 2008) available at LIRMM (http://www.phylogeny.fr/) and displayed and edited with FigTree (http://tree.bio.ed.ac.uk/software/figtree). Additionally, a Bayesian analysis implemented in Mr. Bayes 3.2.2 (Ronquist and Huelsenbeck, 2003) was performed, using the *Xenopus laevis* XendoU sequence as outgroup. The Markov Chain Monte Carlo runs were carried out for 1,000,000 generations after which log likelihood values showed that equilibrium had been reached after the first 400 generations in all cases, and those data were discarded from each run and considered as 'burnin'. Two runs were conducted per dataset showing agreement in topology and likelihood scores. The maximum likelihood and the Bayesian tree topologies including their general subfamily relationships were generally in good agreement.

Three-dimensional models of P102 and P102*Tni* were made with Modeller (Eswar et al., 2006) and analyzed with PyMol (http://www.pymol.org).

2.2. Production of recombinant proteins in Escherichia coli

The cDNA coding for the 102 ORF of *H. virescens* was cloned into pET32 Ek/LIC vector (Novagen, San Diego, California, USA) for bacterial expression, as previously described by Falabella et al., 2012. The cDNA coding for the mature *T. ni* P102 homolog lacking the signal peptide was amplified by PCR using the following primers:

Tn102 pET32 forw 5′ GACGACGACAAGATGGACAACCTAGCCAAC GCA 3′
Tn102 pET32 rev 5′ GAGGAGAAGCCCGGTTAGGAGAAGGGGGTG GG 3′

The primers were designed by adding appropriate extensions (underlined) to allow direct cloning of the fragment into the expression vector pET32 Ek/LIC, in frame with Trx, His and S tags (a total of 17 kDa) according to the manufacturer's instructions (Ek/LIC Cloning Kits Novagen, San Diego, California, USA). The obtained construct was sequenced and used to transform *E. coli* Rosetta-gami 2 (DE3) cells (Novagen, San Diego, California, USA) according to the manufacturer's protocol.

The expression in bacterial cells and the purification of P102 and *T. ni* P102 (named P102*Tni*) recombinant proteins were performed as described by Falabella et al., 2012. Briefly, the recombinant proteins were expressed by growing the transformed Rosetta-gami 2 (DE3) cells at 37 °C and induced (OD_{600} 0.6) with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 2 h at room temperature. The recombinant proteins were subsequently purified by nickel-nitrilotriacetic acid-agarose affinity chromatography under native condition on Protino Ni-TED 1000 packed columns (Macherey–Nagel, Düren, Germany). The purified proteins were dialyzed to eliminate imidazole and digested with 1 U of enterokinase (Novagen, San Diego, California, USA) for 16 h at room temperature in order to eliminate the fusion tag. Purified proteins were quantified by using the Bio-Rad protein Assay kit (Bio-Rad, Hercules, California, USA).

2.3. Expression of P102, P102Tni and XendoU in insect cells

The expression of *H. virescens* cDNA 102 in *Drosophila* Schneider 2 (S2) cells (Life Technologies, Carlsbad, California, USA) was performed as described by Falabella et al., 2012. The cDNA coding for P102*Tni* and the cDNA coding for XendoU (kindly provided by Dr. P. Laneve) were used to amplify the coding region by PCR using specific primers containing restriction sites for Xba I and Sac II (underlined sequences), respectively:

Tn102 pIZT forw 5′ CTAGTCTAGAATGAAGATTGCCATTGTG 3′
Tn102 pIZT rev 5′ TCCCCGCGGGTATAGAGGGTAGG 3′
XendoU pIZT forw 5′ CTAGTCTAGAATGGCGAGTAACAGGGGGCA 3′
XendoU pIZT rev 5′ TCCCCGCGGCAATAACCCGGATCTGTAC 3′

The PCR products were both cloned into the pCR2.1 TOPO vector (Life Technologies, Carlsbad, California, USA) following the manufacturer's protocol and the sequence identity was verified by sequencing. The inserts were then digested with Xba I and Sac