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Subolesin/akirin orthologs from *Ornithodoros* spp. soft ticks: Cloning, RNAi gene silencing and protective effect of the recombinant proteins

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ABSTRACT

Subolesin/akirin is a well characterized protective antigen highly conserved across vector species and thus potentially useful for the development of a broad-spectrum vaccine for the control of arthropod infestations including hard ticks, mosquitoes, sand flies and the poultry red mite *Dermanyssus gallinae*. Soft ticks could be also targeted by this vaccine if proved that the soft tick subolesin orthologs are conserved and induce protective immune responses too. However, to date no soft tick subolesin orthologs have been fully characterized nor tested as recombinant antigens in vaccination trials. The objectives of the present work were to clone and characterize the subolesin orthologs from two important vector species of soft ticks as *Ornithodoros erraticus* and *O. moubata*, to evaluate the effect of subolesin gene silencing by RNAi, and to test the protective value of the recombinant antigens in vaccination trials. The obtained results demonstrate that both soft tick subolesins are highly conserved showing more than 69% and 74% identity with those of hard ticks in their nucleotide and amino acid sequences, respectively. Additionally, we demonstrate that both soft ticks possess fully operative RNAi machinery, and that subolesin gene silencing by dsRNA injection inhibits oviposition indicating the involvement of subolesin in tick reproduction. Finally, vaccination with the recombinant soft tick subolesins induced a partial protective effect resulting in the reduction of the oviposition rate. These preliminary results encourage further studies on the use of recombinant subolesins as vaccines for the control of soft tick infestations, either alone or in combination with other specific molecules.

1. Introduction

Mosquitoes and ticks are, respectively, the first and the second most important arthropod vectors of pathogens to humans and, further, ticks are the most important vectors of diseases affecting cattle worldwide (Peter et al., 2005; de la Fuente et al., 2008).

The argasid ticks *Ornithodoros erraticus* and *O. moubata* have great medical and veterinary importance as vectors of the African swine fever virus and several species of human relapsing fever borreliae (Piesman and Gage, 2004; Basto et al., 2006; Cutler, 2006; Oleaga et al., 2007). *O. erraticus* is distributed in the Mediterranean basin, and in southern Europe it lives in close association with swine on free-range pig farms, hidden in holes and fissures inside and around pig-pens (Oleaga-Pérez et al., 1990; Manzano-Román et al., 2007). *O. moubata* is distributed throughout South and East Africa and Madagascar, where it colonises wild and domestic habitats and feeds on warthogs, domestic swine and humans (Vial, 2009). Elimination of these two ticks,

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especially from synanthropic environments, would greatly improve the control of the diseases they transmit.

Tick control has been primarily based on the use of acaricides, but these chemicals have serious drawbacks, including the selection of tick resistant strains, environmental pollution and contamination of food products (George et al., 2008). Among the alternative approaches for tick control, anti-tick vaccines have proven to be a feasible, cost-effective and environmental friendly method (de la Fuente et al., 2011). However, since the first release of commercial recombinant anti-tick vaccines in 1994, the progress in development of new or more effective vaccines has been more slow than expected, being the identification of tick protective antigens a major limiting step (Willadsen, 2006, 2008). In the search for new protective antigens, expression library immunization (ELI) and RNA interference (RNAi) have been applied to hard ticks for structured screening of tick antigens, thereby identifying new promising antigenic targets as was the case of the subolesin protein (Almazán et al., 2003; de la Fuente et al., 2005). Subolesin is a tick-protective antigen first discovered in *Ixodes scapularis* and later cloned from at least ten hard tick species, showing between 60% and 98% identity in their amino acid sequences (de la Fuente et al., 2006). Subolesin gene silencing by RNAi and immunization trials using recombinant tick subolesins have demonstrated to protect hosts against ticks infestations, reduce tick survival and reproduction, cause degeneration of gut, salivary gland, reproductive tissues and embryos, and reduce *I. scapularis* vector capacity for *Anaplasma marginale* and *A. phagocytophylum* (Canales et al., 2009; Prudencio et al., 2010; de la Fuente et al., 2011).

Tick subolesin is an intracellular protein and a structural and functional ortholog of insect and vertebrate akirins, which are evolutionarily conserved proteins that function as transcription factors in the regulation of gene expression, and thus affecting multiple cellular processes such as the innate immune response, digestion, reproduction and development (de la Fuente et al., 2006, 2011; Galindo et al., 2009). Vaccination trials with the recombinant *I. scapularis* and/or *Aedes albopictus* mosquito subolesin/akirin ortholog antigens induced reduction of fertility and survival not only in hard ticks and mosquitoes but also in other haematophagous arthropods such as sand flies and the poultry red mite *Dermanyssus gallinae* (Harrington et al., 2009; Canales et al., 2009; Moreno-Cid et al., 2011).

These experiments suggest that subolesin/akirin could be used as antigen target for development of a vaccine for the broad control of multiple arthropod infestations, and for the reduction of vector capacity to transmit pathogens that affect human and animal health (de la Fuente et al., 2011). Such a vaccine could be extended to target soft ticks if proved that the soft tick subolesin/akirin orthologs are conserved and induce protective immune responses too. To date, no soft tick subolesin orthologs have been fully characterized nor tested as recombinant antigens in vaccination trials.

The objectives of the present work were to clone and characterize the subolesin orthologs of *O. erraticus* and *O. moubata*, to evaluate the effect of subolesin gene silencing by RNAi technology, and to test the protective value of recombinant soft tick subolesin orthologous antigens in vaccination trials.

2. Materials and methods

2.1. Ticks and tick material

Colonies of both *O. moubata* and *O. erraticus* ticks are maintained in the laboratory of Animal Parasitology (IRNASA-CSIC, Spain). The colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain, and the colony of *O. moubata* was established from specimens submitted from the Institute for Animal Health, Pirbright, Surrey, UK. Ticks are fed regularly on rabbits and kept in a culture chamber at 28 °C, 85% relative humidity and a 12 h light–dark cycle.

Tick saliva and salivary gland extracts (SGE) from adult unfed female ticks were obtained from both *Ornithodoros* species as described in Baranda et al. (1997) and Oleaga et al. (2007), respectively. The protein concentrations of the SGE and saliva samples were measured with the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were stored at −20 °C.

Total RNA was purified from the whole internal organ content and, separately, from the salivary glands and midguts of unfed females of both tick species. The purification of RNA was done using the NucleoSpin RNA II kit (Macherey-Nagel), following the manufacturer's instruction and preserved at −80 °C.

2.2. RT-PCR amplification, cloning and sequencing of subolesin orthologs

Total RNA isolated from whole internal content was used as starting material. Reverse transcription was performed using the First Strand cDNA Synthesis kit (Roche). PCR amplification was done on cDNA using a pair of degenerate oligonucleotide primers designed on an alignment of the primers already published for the amplification of different hard ticks subolesin orthologs (de la Fuente et al., 2006), (dSub5, 5′-ATGGCTTGYGCRACATTAAAGCGRAC; idSub3, 5′-TTTGGTCGTASGTAAAYTTRACAAATGTG). PCR was performed in 35 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min 30 s. The PCR products were electrophoresed in 1% agarose gels and the corresponding bands were purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene), cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene), and sequenced on both strands. At least three different clones from each cloned PCR product were sequenced.

The deduced amino acid sequences were used to seek for orthologs in the non-redundant Genbank protein sequence database by BLASTP analysis. The retrieved sequences from hard ticks (*E*-values less than 10^{-50}) and diptera (*E*-values between 10^{-34} and 10^{-27}) were aligned using ClustalW. Using this alignment, neighbour-joining analysis was performed using the Mega4 package (Tamura et al., 2007). Diptera sequences were used as outgroups. Gaps were treated as pairwise deletions, amino acid distances were calculated using Poisson model, and branch