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Molecular cloning and characterization of Ras- and Raf-homologues from the fox-tapeworm *Echinococcus multilocularis*☆

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Abstract

To better understand growth regulation in the human parasitic cestode *Echinococcus multilocularis*, we have cloned and characterized the parasite's orthologues of the key regulatory factors Ras and Raf. Using a degenerative PCR approach a gene, *emras*, was identified whose gene product, EmRas, showed high homology (79% identical residues) to human Ras and contained all amino acid residues which are characteristic for this subfamily of small GTPases at the corresponding positions. Recombinantly expressed EmRas bound GTP and was farnesylated, but not geranyl-geranylated, by *Echinococcus* lysate in an in vitro prenylation assay. Furthermore, upon expression in yeast, *emras* was able to functionally complement the *Saccharomyces* orthologue *RAS2* in an invasive growth assay. In Western blot analyses using an anti-EmRas antibody, the *Echinococcus* factor could be detected in lysates of the larval stages metacestode and protoscolex. By immune-histochemistry, EmRas was shown to localize to the germinal layer of the metacestode and to tegumental structures of the protoscolex, particularly around the rostellum and the sucker regions.

In addition, we fully characterized the gene *emraf* whose product, EmRaf, displayed considerable homology to mammalian Raf-kinases and orthologous factors from *Drosophila* and *Caenorhabditis elegans*. *emraf* was co-expressed with *emras* in the larval stages metacestode and protoscolex during in vitro cultivation and during an infection of the intermediate host as assessed by RT-PCR experiments. The *emraf* gene was composed of nine exons and eight introns and shared four highly conserved exon–intron boundaries with the human gene encoding Raf-1, suggesting that both genes derived from a common evolutionary ancestor. Southern blot hybridizations demonstrated that *emraf* is a single copy gene. Using the yeast two-hybrid system, EmRaf was shown to interact with EmRas, but not with EmRal, a previously characterized orthologue of mammalian Ral GTPases. This is the first characterization of a Ras orthologue from a cestode and the first report on a Raf-like kinase from a platyhelminth. The data presented herein will form a solid basis for further investigations on *Echinococcus* signaling systems that are involved in growth control and development of the parasite.

Keywords: *Echinococcus*; MAP kinase cascade; Raf; Ras; Signaling; Parasite

1. Introduction

Echinococcus multilocularis is a human parasitic cestode whose larval stage causes alveolar echinococcosis, one of the most dangerous zoonoses of the northern hemisphere [1]. During infection of the intermediate host (small rodents and, occasionally, humans), the parasite undergoes several developmental transitions involving the larval stages oncosphere, metacestode and protoscolex [1,2]. Although the molecular mechanisms of *Echinococcus* development and its interaction

Abbreviations: bp, base pairs; CRD, cysteine-rich domain; CR, conserved region; EGF, epidermal growth factor; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; MAP, mitogen activated protein; RACE, rapid amplification of cDNA ends; RBD, Ras-binding domain

☆ *Note*: Nucleotide sequence data reported in this paper are available in the EMBL, GenBank™ and DDJB databases under the accession numbers AJ785001, and AJ785002.

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with the host are not yet understood, these issues can be experimentally addressed using in vitro cultivation systems for the parasite's larval stages which we [3] and others [4] have established during recent years. Using one of these in vitro systems, we have previously shown that an *Echinococcus* gene, *egfd*, encoding a mitogen of the epidermal growth factor (EGF)-family, is significantly upregulated in the metacestode stage upon incubation with host hepatocytes, thus indicating an important role of EGF-like signaling in *Echinococcus* development during an infection [5]. Furthermore, we were able to characterize a possible receptor molecule for Egfd, the *E. multilocularis* surface receptor tyrosine kinase EmER, which is an orthologue of the human EGF receptor [6]. Besides the characterization of EGF-signaling components in *Echinococcus*, we also demonstrated that the parasite's larval stage expresses a surface tyrosine kinase of the insulin receptor family, EmIR, which can interact with mammalian insulin in vitro and which constitutes an *Echinococcus* candidate receptor for the detection of host signaling molecules [7]. To obtain a broader basis for our analyses on these signaling mechanisms, and to gain insight into intracellular signal transduction events of *E. multilocularis*, we were in this study interested in signaling molecules that could act downstream of EmER and EmIR.

The best characterized signaling pathway that transduces signals from EGF- and insulin-receptors to the nucleus is the receptor tyrosine kinase (RTK)/Ras/MAP kinase cascade [8,9]. This pathway controls key mechanisms such as growth and proliferation in mammalian cells, eye development in *Drosophila* and vulva development in *Caenorhabditis elegans* [8–11]. Essential components of this pathway are the members of the Ras subfamily of GTP binding proteins which, in mammals, comprise the highly related proteins H-Ras, K-Ras and N-Ras and of which homologues have already been described in a variety of eukaryotes, including yeast [12]. All Ras proteins characterized so far are small (21 kDa) monomeric GTPases which function as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. This process is tightly regulated by the activities of guanine nucleotide exchange factors (GEFs) which accelerate GTP loading and GTPase activating proteins (GAPs) which increase the rate of GTP hydrolysis [12]. Activation of the Ras subfamily members leads to their interaction with a variety of downstream effector proteins of which the best characterized are the intracellular serine/threonine kinases of the Raf family (e.g. the three kinases Raf-1, A-Raf and B-Raf of mammals). Upon interaction with GTP-bound, farnesylated Ras, Raf-kinases are recruited to the cell membrane and are further activated. Downstream targets for activated Raf are then the dual-specific kinases of the MAP kinase kinase family (or MEKs) which, in turn, phosphorylate and activate MAP kinases (or ERKs). Activated MAP kinases eventually translocate to the nucleus whereby they modulate gene expression [8,9,13,14].

The interaction mechanisms and functions of the Ras/Raf/MAP kinase cascade have already been intensely studied in vertebrates and, to some extent, in animal model systems such as *Drosophila* or *C. elegans* [8–11]. Very little information is available, however, concerning such systems in parasitic helminths. Up to now, biochemical evidence has been provided for the presence of Ras-, MAP kinase- and GAP-homologues in the trematode *Schistosoma mansoni* [15] and the respective Ras-factor has been characterized to some extent [16,17]. However, no Raf-homologue has so far been described in platyhelminths. The sequence of such a factor has, on the other hand, been reported for the parasitic nematode *Brugia malayi* [18] although data on a Ras-homologue of this species is still missing. In the present study, we have characterized both the Ras- and Raf-homologues from the cestode *E. multilocularis*. We provide detailed information on the corresponding genes and proteins and we show that the parasite-derived factors can physically interact like their mammalian orthologues.

2. Materials and methods

2.1. Organisms and culture methods

The properties of the two natural *E. multilocularis* isolates H95 and K188 have been described by Konrad et al. [7]. Isolation and maintenance of the clinical isolate MP1 was described by Brehm et al. [5]. The isolates were propagated and continuously kept in mongolian jirds (*Meriones unguiculatus*) essentially as described [19]. In vitro cultivation of metacestode vesicles under axenic conditions was performed according to a previously established protocol [3] and protoscoleces were isolated from in vivo cultivated parasite material as described [5].

2.2. Nucleic acid isolation, cloning procedures and sequencing

Chromosomal DNA and total RNA were isolated from parasite material after cultivation in laboratory animals as described [19]. For RNA isolation from in vitro cultivated metacestode vesicles and protoscoleces, the RNEasy kit (Qiagen) was used according to the manufacturer's instructions. PCR products were cloned employing the TOPO-TA-cloning kit (Invitrogen) and sequenced using vector-specific primers on an ABI-prism 377 DNA sequencer (Perkin Elmer).

2.3. GTP overlay and in vitro prenylation assays

For Western blot detection and GTPase assays, the *emras* reading frame was PCR amplified using primers Ras-5PR-mod (5′-GTG CCT GAG TAC AAA CTC G-3′) and Ras-3PR-mod (5′-GAG CAG ATG GCA TTT CCT CTT G-3′) and was directly cloned into the expression vector pBAD/Thio-TOPO (Invitrogen) giving rise to plasmid pRas-TEX1. In this expression system, EmRas was translationally fused with thioredoxin at the N-terminus and with an