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Echinococcus multilocularis: Molecular characterization of EmSmadE, a novel BR-Smad involved in TGF-β and BMP signaling ☆

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ABSTRACT

Smad transcription factors are central components of transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signaling pathways in metazoans, and regulate key developmental processes such as body axis formation or regeneration. In the present study, we have identified and characterized a novel member of this protein family, EmSmadE, in the human parasitic cestode *Echinococcus multilocularis*, the causative agent of alveolar echinococcosis. The cDNA of the corresponding gene, *emsmadE*, was fully sequenced and shown to encode a protein with considerable homologies to known members of the receptor regulated Smad (R-Smad) family of a wide variety of organisms. EmSmadE contains highly conserved MH1- and MH2-domains and, on the basis of sequence features around the L3 loop region, could be assigned to the BR-Smad subfamily that typically transmits BMP signals. RT-PCR analyses indicated expression of *emsmadE* in all larval stages that are involved in the infection of the intermediate host. Yeast two-hybrid interaction studies demonstrated that EmSmadE can form homodimers, and is capable of heterodimer formation with the previously identified common Smad (Co-Smad) EmSmadD and the R-Smads, EmSmadA, and EmSmadB. In a heterologous expression system, EmSmadE was specifically phosphorylated at a conserved C-terminal SSVS motif by the human BMP type I receptor and, despite being structurally a BR-Smad, also by the human TGF-β type I receptor. Taken together, these data indicate that EmSmadE is a functionally active R-Smad that is involved in larval *Echinococcus* development. The data presented herein will be important for further analyses on the role of TGF-β/BMP signaling pathways in *Echinococcus* pattern formation and differentiation.

1. Introduction

Alveolar echinococcosis (AE), one of the most dangerous parasitic infections of the Northern Hemisphere, is caused by the metacestode larval stage of the fox-tapeworm *Echinococcus multilocularis* (McManus, 2010). Upon initial infection of the intermediate host (rodents, humans) by oral uptake of infectious eggs, the embryonic larval stage (oncosphere) is activated and undergoes a metamorphic transition towards the metacestode within the host's liver. The metacestode subsequently grows into the surrounding host tissue like a malignant tumor and, at a later stage of the infection, usually forms protoscoleces that are passed on to the definitive host when it takes the prey (Brehm et al., 2006; Brehm, 2010a). During recent years, several sophisticated *in vitro* cultivation systems have been developed by which the *E. multilocularis* oncosphere–metacestode transition, initiated by totipotent parasite stems cells, the proliferation of metacestode vesicles, and their differentiation towards protoscoleces can be mimicked under laboratory conditions (Brehm and Spiliotis, 2008; Brehm, 2010a; Spiliotis et al., 2008; Spiliotis and Brehm, 2009). Using these cultivation systems, it has been shown that parasite development is governed by soluble factors that are secreted by co-cultivated host cells and/or are present in host serum (Brehm, 2010a; Spiliotis and Brehm, 2009). In particular, evidence has been obtained that host-derived hormones and cytokines such as insulin, epidermal growth factor (EGF), BMP2, and an as yet uncharacterized serum component can interact with evolutionarily conserved parasite receptors of the insulin (EmIR; Konrad et al., 2003), the EGF (EmER; Spiliotis et al., 2006), the TGF-β (EmTR1; Zavala-Gongora et al., 2006), and the nuclear hormone receptor (EmNHR1; Förster et al., 2011) families, respectively.

The cytokines of the TGF-β superfamily (e.g., TGF-β *sensu stricto*; activins; inhibins; BMPs) are evolutionarily conserved and regulate a wide variety of developmental processes such as proliferation, differentiation, apoptosis, pattern formation or stem cell renewal in metazoans (Bragdon et al., 2011; Brehm, 2010b; Loverde et al., 2007; Moustakas and Heldin, 2009; Sieber et al., 2009). They interact with membrane-associated complexes of so-called TGF-β/BMP types I and II receptors, which subsequently leads to the

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phosphorylation, and thus activation, of intracellular transcription factors of the R-Smad family (Funaba and Mathews, 2000). Upon phosphorylation, which occurs at a highly conserved C-terminal SSXS-motif, R-Smads can form homodimers and heterodimers with other R-Smads but also, and most importantly, with members of the Co-Smad family. Once formed, the R/Co-Smad complexes are translocated into the nucleus where they regulate the expression of target genes through interaction with Smad binding elements (SBEs) in the promoter regions of TGF-β/BMP responsive genes (Bragdon et al., 2011; Loverde et al., 2007; Moustakas and Heldin, 2009). On the basis of the sequence context of the so-called L3 loop, R-Smads can be subdivided into two groups. The AR-Smads are typically activated in response to cytokines of the TGF-β/activin/inhibin branch whereas the BR-Smads are usually involved in BMP signaling (Bragdon et al., 2011; Loverde et al., 2007; Moustakas and Heldin, 2009; Sieber et al., 2009). Characteristic for all members of the R- and Co-Smad family is a domain structure of well conserved MH1 (N-terminal) and MH2 (C-terminal) domains that are separated by a variable linker region (Bragdon et al., 2011; Moustakas and Heldin, 2009). The major function of the MH1 domain lies in DNA binding through interaction with SBEs, thus ensuring transcription specificity, whereas the MH2 domain is mostly involved in homo- and heteromeric interactions among Smads and other transcription factors as well as R-Smad-receptor interactions (Bragdon et al., 2011; Moustakas and Heldin, 2009).

In flatworms, TGF-β/BMP signaling has already been studied to a certain extent, particularly in free-living planarians, in the trematode *Schistosoma mansoni*, and in *E. multilocularis*. Although no members of the TGF-β/BMP receptor families or Smads have been biochemically characterized in planarians so far, an important role of BMP signaling in defining dorso-ventral polarity in these animals is well established (Gavino and Reddien, 2011). In schistosomes, one member each of the TGF-β types I and II receptor families (Beall and Pearce, 2001; Forrester et al., 2004; Osman et al., 2006), one Co-Smad (Osman et al., 2004), several R-Smads (Beall et al., 2000; Carlo et al., 2007; Osman et al., 2001), and two TGF-β/BMP-like ligands (Freitas et al., 2007, 2009) have been described and biochemically characterized (LoVerde et al., 2007). In particular, it has been shown that the schistosome TGF-β receptors are able to interact with host-derived TGF-β (Beall and Pearce, 2001; Osman et al., 2006), that TGF-β signaling is crucially involved in the control of embryonic development of *S. mansoni* (Freitas et al., 2007), and that it is most probably also involved in the production of vitellocytes in female schistosomes (Knobloch et al., 2007). Our studies on *E. multilocularis* so far identified one member of the TGF-β type I receptor family, EmTR1, which was able to functionally interact with host derived BMP2 (Zavala-Gongora et al., 2006), thus indicating that host BMPs might have an influence on *Echinococcus* development. Furthermore, we could characterize two biochemically unusual members (lacking the MH1 domain) of the AR-Smad family, EmSmadA, and EmSmadC, one member of the BR-Smad family, EmSmadB, and one Co-Smad, EmSmadD (Zavala-Gongora et al., 2003, 2008). We also established cross-interactions of *Echinococcus* TGF-β/BMP signaling with mitogen-activated protein kinase (MAPK; Spiliotis et al., 2006) and nuclear hormone receptor (Förster et al., 2011) signaling, and identified a member of the SNW/SKIP family of transcriptional regulators that interacts with all three R-Smads (Gelmedin et al., 2005).

The recently initiated whole genome sequencing project for *E. multilocularis* is presently in a very advanced stage (Brehm, 2010a,b) and will greatly facilitate further studies into evolutionarily conserved signaling pathways in this parasite. As an initial step to further characterize TGF-β/BMP signaling pathways, we utilized the currently available assembly version in a genomic approach to screen for further Smad components. This led us to the identification of an as yet unknown R-Smad, EmSmadE, in *E. multilocularis*, the biochemical properties of which we will present in this study.

2. Materials and methods

2.1. Organisms and culture methods

All experiments were performed with the natural *E. multilocularis* isolate GH09 that has been obtained from the liver of an infected Rhesus monkey (*Macaca mulatta*) from a breeding enclosure in Göttingen, Germany (Tappe et al., 2007). Parasite material was continuously passaged in mongilian jirds (*Meriones unguiculatus*) as previously described (Spiliotis and Brehm, 2009). The *in vitro* cultivation of metacestode vesicles was performed essentially as described (Spiliotis and Brehm, 2009) and primary cell cultures were established and maintained according to the protocol of Spiliotis et al. (2008). Protoscoleces were isolated as described (Brehm et al., 2003) and were activated by pepsin/low pH treatment according to Gelmedin et al. (2010).

2.2. Genomic analyses and in silico procedures

The latest assembly version (AUG-12-2011) of the *E. multilocularis* genome project was used as accessible via http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html. In the case of *Echinococcus granulosus*, the assembly version of SEPT-24-2010 was used which is accessible under the same address. For the identification of TGF-β signaling components, tBLASTN searches were carried out using the WinBlast software (v.0.2.0) with an *E*-value cut-off at 1.0. Amino acid comparisons were performed using the basic local alignment research tool (BLAST) on the nr-aa database collection available under http://blast.genome.ad.jp. Pileups and CLUSTAL W alignments were constructed employing the software BioEdit Sequence Alignment Editor (version 7.0.0) using the BLOSUM62 matrix. Prediction of protein domains was performed using the simple modular architecture research tool (SMART) available under http://smart.embl-heidelberg.de. For phylogenetic tree construction, CLUSTAL W2 software was utilized as accessible under http://www.ebi.ac.uk/Tools/msa/clustalw2/.

2.3. Cloning of the emsmadE cDNA

Total RNA was isolated from *in vitro* cultivated *E. multilocularis* metacestode vesicles using TRIzol reagent (Invitrogen) and reverse transcribed as previously described (Brehm et al., 2003). The resulting cDNA served as a template to PCR amplify the *emsmadE* open reading frame using oligonucleotides SE-ORF-5′ (5′-GTG TAC AGG AGA CGA TC-3′) and SE-ORF-3′ (5′-GCT TAT CAC AGA AAT TTT ATG-3′), which was cloned using the PCR cloning kit (Qiagen). The remaining parts of the cDNA were subsequently amplified and cloned using primers SE-up3 (5′-GGT AGA ACT GGG TAA TCA ACG-3′) and SE-dw5 (5′-GAG AAT ACG CGG AAG CAG-3′). Sequences were verified by sequencing on an ABI 3130 Genetic Analyzer (Applied Biosystems). The *emsmadE* cDNA sequence has been submitted to the GenBank database and assigned Accession No. FR856863.

2.4. RT-PCR analyses

Total RNA was isolated from *in vitro* cultivated metacestode vesicles (Spiliotis and Brehm, 2009), from developing primary cell cultures (Spiliotis et al., 2008), as well as from non-activated and activated protoscoleces (Gelmedin et al., 2010) as described in Section 2.3., and was reverse transcribed (Brehm et al., 2003). For