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Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from *Echinococcus multilocularis* ☆

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Abstract

Members of the transforming growth factor-β/bone morphogenetic protein (TGF-β/BMP) family of cytokines play crucial roles in animal development and are candidate molecules for host–parasite cross-communication in helminth diseases. TGF-β/BMP-signalling involves binding of the cytokines to receptor kinases which subsequently activate intracellular transcription factors of the Smad family. We have previously characterized two members of the receptor-regulated Smad (R-Smad) family, EmSmadA and EmSmadB, from the human parasitic cestode *Echinococcus multilocularis* and now present evidence for two additional Smads that are expressed by the larval stages of the parasite. The full-length cDNAs coding for a third R-Smad, EmSmadC, and a common mediator Smad (Co-Smad), EmSmadD, were characterized. While EmSmadD displayed a typical Co-Smad structure, EmSmadC lacked the N-terminal MH1 domain which is typically found in Smads. In yeast two-hybrid analyses, EmSmadC and EmSmadD were capable of homo- and heterodimer formation with other *Echinococcus* Smads. Furthermore, EmSmadC displayed autonomous transcription activation activity and interacted with EmSkip, a member of the SNW/SKIP family of transcriptional regulators. In a heterologous expression system, EmSmadC was specifically phosphorylated by mammalian TGF-β receptors, indicating that it is a member of the AR-Smad sub-family. Finally, in activity assays, the parasite's Erk-like kinase EmMPK1 phosphorylated EmSmadD, indicating cross-regulation between mitogen-activated protein kinase cascade- and TGF-β/BMP-signalling in *Echinococcus*. The data presented herein significantly broaden our knowledge of Smad-signalling factors in *E. multilocularis* and will facilitate studies on TGF-β/BMP-regulated genes in the parasite as well as TGF-β/BMP mediated host–parasite cross-interaction during alveolar echinococcosis.

Keywords: *Echinococcus*; Cestode; Parasite; TGF-β; BMP; Smad; Erk; SKIP

1. Introduction

Echinococcus multilocularis is a human parasitic cestode whose larval stage causes alveolar echinococcosis (AE), one of the most dangerous zoonoses of the northern hemisphere (Eckert and Deplazes, 2004). In addition to the worm's adult stage, which resides in the intestine of definitive hosts (e.g. foxes, dogs), the *E. multilocularis* life-cycle comprises the three developmental stages – oncosphere, metacestode and protoscolex – of which the latter two are formed within the organs of natural intermediate hosts (small rodents) during an infection (Craig, 2003; McManus et al., 2003; Brehm et al., 2006). Humans become infected by the occasional ingestion of infective eggs, resulting in an almost unrestricted growth of the metacestode within the liver (Brehm et al., 2006).

☆ *Note.* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL, and DDJB databases under the Accession Nos. AM269924 and AM231608.

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At least part of the *E. multilocularis* life-cycle can be mimicked under laboratory conditions using in vitro cultivation systems which we (Spiliotis et al., 2004; Brehm et al., 2006) and others (Deplazes and Gottstein, 1991; Hemphill et al., 2002) have developed. Using these cultivation systems, evidence has been obtained that parasite development is governed by soluble factors that are secreted by co-cultivated host-cells and/or are present in host serum (Hemphill et al., 2002; Spiliotis et al., 2004; Brehm et al., 2006). Although the molecular nature of these host factors has not yet been worked out in great detail, recent studies of our group demonstrated that at least two host hormones/cytokines, insulin and bone morphogenetic protein 2 (BMP2), can interact with *Echinococcus* surface receptors (Konrad et al., 2003; Zavala-Góngora et al., 2006) and that host-derived epidermal growth factor (EGF) induces the parasite's mitogen-activated protein (MAP) kinase cascade when added to metacestode vesicles (Spiliotis et al., 2006). Molecular interactions between host-derived transforming growth factor-β (TGF-β) and surface receptor systems are also known for the related parasite *Schistosoma mansoni* (Beall and Pearce, 2001; Osman et al., 2006), which underscores the particular role that members of the TGF-β/bone morphogenetic protein (BMP) family of cytokines could play in flatworm–host interactions.

The TGF-β cytokines (e.g. TGF-β, BMP, activin) regulate cell fate by controlling proliferation, differentiation and apoptosis in a wide variety of metazoans (for recent reviews see Shi and Massague, 2003; Nohe et al., 2004; ten Dijke and Hill, 2004; Feng and Derynck, 2005; Massague et al., 2006). Signalling is initiated by binding of the cytokines to TGF-β/BMP receptors which consist of two transmembrane serine/threonine kinases called the type I and type II receptors. Upon activation, the type I/type II receptor complex recruits, phosphorylates and activates intracellular signal transducers of the R-Smad family which then associate with Co-Smads to form a gene regulatory complex (Shi and Massague, 2003; Feng and Derynck, 2005; Massague et al., 2006). The R-Smads can be grouped into two different sub-families. The AR-Smads are activated in response to receptors of the TGF-β/activin pathway while the BR-Smads are phosphorylated through BMP receptors (Massague et al., 2006). In both sub-families, phosphorylation through the type I receptor occurs at a conserved SxS motif located at the R-Smad C-terminus. Although Co-Smads, like the R-Smads, are composed of conserved N-terminal MH1 (Mad homology 1) and C-terminal MH2 domains, separated by a variable linker, they do not contain the C-terminal SxS motif and are not phosphorylated by type I receptors. In general, the MH1 domain of all Smads functions in DNA binding through interaction with Smad binding elements (SBEs) in the promoters of TGF-β/BMP-regulated genes. The MH2 domain, on the other hand, is responsible for protein–protein interactions such as receptor recognition, homo- and heterodimer formation, and the formation of transcription complexes with co-repressors and co-activators. The Smad MH1 and MH2 domains also interact with each other, and this interaction is thought to be involved in negative regulation of the MH2 domain. A third group of Smads, the inhibitory Smads, act as antagonists which counteract the functions of the R-Smads (Shi and Massague, 2003; Nohe et al., 2004; ten Dijke and Hill, 2004; Feng and Derynck, 2005; Massague et al., 2006).

In platyhelminths, TGF-β-signalling has best been studied in the human parasite *S. mansoni* in which two R-Smads (Beall et al., 2000; Osman et al., 2001), one Co-Smad (Osman et al., 2004), one type I receptor (SmTβRI; Beall and Pearce, 2001) and one corresponding type II receptor (SmTβRII; Forrester et al., 2004; Osman et al., 2006) have been characterized. Interestingly, the SmTβRII/SmTβRI receptor complex was shown to be expressed on the surface of adult schistosomes and to interact with host TGF-β which resulted in elevated expression of *SmGCP*, a gynaecophoral canal protein encoding gene of male schistosomes (Beall and Pearce, 2001; Osman et al., 2006). This suggests a role for the schistosomal TGF-β receptors in host–helminth cross-communication. Further data on TGF-β/BMP-signalling in flatworms have been provided by us for the fox-tapeworm *E. multilocularis*. We have previously characterized two members of the R-Smad family of this parasite, EmSmadA and EmSmadB, and showed that both Smads are phosphorylated through mammalian TGF-β/BMP receptors upon expression in human cells (Zavala-Góngora et al., 2003). Structurally, EmSmadB is a typical BR-Smad with both MH1 and MH2 domains, whereas EmSmadA is an untypical AR-Smad which lacks an N-terminal MH1 domain and is activated by both TGF-β and BMP receptors (Zavala-Góngora et al., 2003). In addition, we recently characterized a type I receptor, EmTR1, in *Echinococcus* larvae which is activated by human BMP2 and transmits the signal to EmSmadB (Zavala-Góngora et al., 2006), suggesting a role for BMP-signalling in host–parasite interactions during AE. Although EmTR1 and EmSmadB clearly form part of a BMP-signalling mechanism in *Echinococcus*, our studies did not yield clear-cut evidence for the presence of TGF-β-signalling in this parasite. Furthermore, evidence for a central component of TGF-β/BMP-signalling, a Co-Smad orthologue which would be necessary to study promoter regions of TGF-β/BMP regulated genes, was still missing. These gaps are filled with the present study in which we characterized another unusual AR-Smad without an MH1 domain and a Co-Smad orthologue from *E. multilocularis*.

2. Materials and methods

2.1. Organisms and culture methods

All experiments were performed with the *E. multilocularis* isolate H95 which was kept continuously in mongolian jirds (*Meriones unguiculatus*) as previously described (Jura et al., 1996). In vitro cultivation of metacestode ves-