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HELMINTHOLOGY - ORIGINAL PAPER

Gene structure and expression patterns of *Acdaf-1*, a TGF-β type I receptor in *Ancylostoma caninum*

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Abstract

The components of the transforming growth factor β (TGF-β) signaling pathway in parasitic nematodes remain unknown. In this research, a type I receptor for TGF-β was isolated from the hookworm *Ancylostoma caninum*. The new gene was designated as *Acdaf-1*, a *Caenorhabditis elegans daf-1* homolog. The full-length cDNA of *Acdaf-1* encodes a 595-amino-acid protein with an NH_2-terminal signal peptide. This protein has a cytoplasm tail (209-595aa region) which corresponds to the type 1a membrane topology. Between amino acid position 295–500, the protein contains the ATP binding site, substrate binding sites, and PKC-kinase-like domain. Real-time RT-PCR showed that the transcript was expressed in three main stages of *A. caninum.* It reached the maximal level in the female adult worm stage with lower transcript level in the first and second larvae (L1/L2) and intermediate level in L3 stages as well as in the male worms. After serum activation, the activity of *Acdaf-1* was decreased in L3 larvae. These data implied that *Acdaf-1* might relate to the infection ability of the larvae. Immunolocalization revealed that AcDAF-1 was present in eggs, intestine, and epidermis cells of larvae (L1, L2, and L3 stages) with strong signal in primordium of the gonads in L3 and was abundant in epidermis, intestine, and ovary of adult worm. These results suggested that Acdaf-1 might be involved in the interaction of the parasite and host relationship and provide a potential target for parasite control.

Keywords *Ancylostoma caninum, Acdaf-1* · Phylogenetic analysis · Expression

Abbreviations

TGF-β	Transforming growth factor-β
ORF	Open reading frame
PKC	Protein kinase C
aa	Amino acid
RT-PCR	Reverse transcription-PCR
PCR	Polymerase chain reaction
CLM	Cutaneous larvae migrans
IPTG	Isopropyl β-D-1-thiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ES	Excretory/secretory

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Introduction

The transforming growth factor-β (TGF-β) signaling pathway comprises a family of genes which regulate a variety of

biological activities including cell growth, proliferation and differentiation, morphogenesis, and apoptosis (Gumienny and Savage-Dunn 2013; Padgett et al. 1998; Padgett et al. 1997; Savage-Dunn 2005). These genes are highly conserved from nematodes to flies, mice, and humans (Luo et al. 2010; Padgett 1999). The TGF-β pathway is initiated by a ligand bind to cell surface type II receptor and then combines with type I receptor to form a heteromeric complex. The type II receptor phosphorylates the type I receptor, a serine/threonine kinase, and activates its kinase activity, then the type I receptor can phosphorylate specific Smad proteins allowing them to translocate to the nucleus, where Smad proteins activate or repress transcription to regulate gene expression (Savage-Dunn 2005; Viney et al. 2005).

The members of TGF-β signal transduction pathway are involved in the development and homeostasis of both vertebrate and invertebrate organisms (Crook et al. 2005; Viney 2009). In *Caenorhabditis elegans*, the TGF-β signaling pathway has been demonstrated to regulate reproductive development by controlling dauer formation (Inoue and Thomas 2000a, b) and lifespan extension under unfavorable condition (Shaw et al. 2007). The TGF-β signaling pathway contains genes which encode a ligand (*daf-7*) (Crook and Grant 2013), type I (*daf-1*) and type II (*daf-4*) (Estevez et al. 1993) receptors, and three Smads (*daf-8*, *daf-14*, *daf-3*) (McGehee et al. 2015). The biochemical relationships of these gene products have not been determined; however, based on the vertebrate TGF-β signaling pathway system, it is thought that DAF-7 could bind to the type II receptor in order to activate the receptor complex DAF-1/DAF-4 and thus resulting in phosphorylation of the Smads. Although the *C. elegans* Smad proteins DAF-8 and DAF-14 contain the conserved phosphorylation motif (SSXS) at the COOH-terminus, they represent divergent Smads since they are unable to interact directly with DNA following entry into the nucleus (Inoue and Thomas 2000a). DAF-8 and DAF-14 appear to antagonize the activity of the third Smad, DAF-3 which functions to induce dauer formation in the absence of signaling (Savage-Dunn 2005).

Hookworms are important parasitic nematodes which are of medical and veterinary importance. *Ancylostoma caninum* parasitize in the intestine of dogs, but their larvae can cause zoonoses in humans. Infections of human with *A. caninum* can provoke eosinophilic enteritis and creeping eruptions caused by migrating larvae in skin, a hypersensitivity reaction resulted from cutaneous larvae migrans (CLM).

As soil-transmitted helminth (STH), the non-feeding infective larvae can survive in moist soil for a relatively long time until they meet the host. This phenomenon corresponds to the *C. elegans* dauer stage; the mechanism of how the developmental arrest and egress from the arrest are regulated in the infective larvae of hookworm still remains unclear.

In previous reports, two TGF-β ligands, *Ac-dbl-1* and *Ac-daf-7*, were characterized from *A. caninum* (Freitas and Arasu 2005). The TGF-β isoforms 1 and 2 had significant stimulatory effects on feeding and reactivation response of *A. caninum* arrested, hypobiotic larvae, and these effects could be blocked by pre-incubation with anti-TGF-β antibodies (Arasu 2001). Also they found that *Ac-daf-7* with a length of 355 amino acids showed high homology to Ce-DAF-7 and other TGF-β molecules. *Ac-daf-7* mRNA was highly expressed in infective third stage larvae (L3) and serum stimulated L3 and weakly expressed in L1 and adult worms as described by Brand et al. (Brand et al. 2005). These authors also found a parasite-encoded receptor that can sense the host-derived TGF-β signal to provoke the reactivation of tissue-arrested larvae (Arasu 2001).

There are a lot of similarities between *C. elegans* dauer arrested larvae and infective larvae of *A. caninum*, which led to the hypothesis (Rajan 1998) that they are alike stages. In *C. elegans*, the dauer larvae formation is well studied and several signaling pathways are involved in these processes, including the TGF-β signaling pathway (Ludewig and Schroeder 2013). Whether the parasitic nematodes use same routes and receptors especially in *A. caninum* still remains unknown.

In this study, we reported the identification of *Ac-daf-1*, a TGF-β type 1 receptor from *A. caninum*, the protein expression level, and the localization of Ac-daf-1 in different stages of worm.

Materials and methods

Preparation of the parasites and RNA extraction

Fecal cultures from infected dogs were incubated at room temperature to recover the first-stage larvae, second-stage larvae (L2), and infective third-stage larvae (L3) as previously described (Yang et al. 2011). L3 larvae were used to infect the dogs. *A. caninum* adult worms were obtained from dog intestine at necropsy after 30 days of post-infection. Dogs were housed in the animal room according to the guidelines of the Laboratory animal care in China. This study was conducted following the guidelines of Xiamen University for animal care and approved by Ximen University (XMU) (approval no. 20101206). Total RNA was extracted from L1/L2, L3, infective stage larvae, and male and female adult worm using TRIzol reagents (MRC), respectively. The precipitated RNA pellet was then resuspended in 30 μL of RNase-free DEPC-treated water. Subsequently, the ethidium bromide (0.5 μg/ml)-stained agarose gel was run using 5 μL of RNA to determine the integrity of RNA confirmed by the presence of two discrete 18S and 28S ribosome bands.

Preparation of first strand cDNA and RT-PCR

First-strand cDNA was synthesized from 8 μL of adult worm RNA in a total reaction volume of 20 μL using Fermentas First-strand cDNA Synthesis Kit (Fermentas, USA) with oligo (dT) 18 primer according to the manufacturer's instructions. Full length TGF-β type I receptor cDNA from adult worm was then amplified using RT-PCR with Acdaf1F (5′-TTAT GAATCGGGTCACATAAGTCT-3′) as a forward primer and Acdaf1R (5′-CTCTCAGGAGAACCGCAAGT-3′) as a reverse primer. PCR reactions were performed in a final volume of 100 μL using 10 μL of 10× buffer containing 20 mM $MgCl_2$ (Takara Inc., Dalian), 5 μL dNTP mixture, 0.5 μL ExTaq polymerase, 4 μL of each primer (20 pmol/L Acdaf1F and Acdaf1R), 0.5 μL of adult worm cDNA, and 76 μL of deionized H_2O. The reaction was performed in a Bio-Rad Thermocycler under following conditions: predenaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 61.2 °C for 45 s, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. The resulting PCR products were run in ethidium bromide-stained agarose gels and photographed under UV transillumination. The amplified cDNA product was purified and cloned into the pMD18-TA cloning vector (Takara Inc., Dalian, China) and transformed into *Escherichia coli* DH5α, which were then incubated on amp+ plates. For screening for the presence of PCR inserts, plasmid DNA was extracted, digested using restriction enzymes, and sequenced to confirm its identity. The sequence was then deposited in GenBank (accession no. JF826239).

Sequence analysis of Ac-daf-1 and phylogenetic analysis

A homology search was performed using Basic Local Alignment Sequence Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the identity of the plasmid DNA. The cDNA was translated into the theoretical amino acid sequence using the ORF Finder program from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the translated protein sequence was determined to confirm as a TGF-beta type I receptor. The signal peptide sequence was predicted using SignalP v2.0 (Nielsen et al., 1997) that is available at the Center for Biological Sequence Analysis online (www.cbs.dtu.dk/services/SignalP). Amino acid sequences of full-length cDNA of TGF-β type I receptor from *A. caninum* (accession no. JF826239), *Homo sapiens* (NP_001124388.1), *Bos taurus* (NP_777046.1), *Mus musculus* (BAA05023.1), *Ixodes scapularis* (XP_002412676.1), *Rattus norvegicus* (NP_036907.2), *Sus scrofa* (BAD91020.1), *Equus caballus* (XP_001494957.1), *Canis familiaris* (XP_538750.2), *Taeniopygia guttata* (XP_002190425.1), *Danio rerio* (NP_001032772.2), *Aedes aegypti* (XP_001653836.1), *Gallus gallus* (NP_989577.1), *Xenopus (Silurana) tropicalis* (NP_001015961.1), *Brugia malayi* (XP_001900442.1), *Ornithorhynchus anatinus* (XP_001512345.1), *Pan troglodytes* (XP_001159204.1), and *Macaca mulatta* (XP_001112590.1) were aligned using ClustalW (Thompson et al. 1994) with a maximum parsimony (MP) and neighbor-joining (NJ) methods by PAUP v 4.0b10 (Swofford 2002). A bootstrap analysis (using 1000 replicates) was conducted using TBR-Branch swapping in order to determine the relative support for clades in the consensus tree.

Transcript level of Ac-daf-1 in different stages of *A. caninum* by real-time RT-PCR

To determine the expression level of *Acdaf-1* mRNA in each stage, total RNA was separately extracted from L1/L2, infective L3 larvae, serum-treated L3 infective larvae, and adult *A. caninum*, respectively. Infective L3 larvae were first treated with dog serum for 30 min and then collected for RNA extraction. The first-strand cDNA was synthesized using total RNA treated with DNase I as template according to the protocol obtained from PrimeScript RT Master Mix Perfect Real Time kit (Takara Inc., Japan). Single-strand cDNA was synthesized from 500 ng of total RNA in a final volume of 10 μL containing 2 μL of 5× PrimeScript RT Master Mix (for Real Time). The reactions were first incubated at 37 °C for 15 min and then terminated by heating the mixture at 85 °C for 5 s. The terminated reactions were finally stored at − 20 °C. The cDNA mixture was diluted 1:10 for subsequent experiments. There were three replicates for each stage of hookworm. Two daf-1 gene-specific primers, daf1rF (5′-AAAG TCTGGAGGAGAAGGTGGAGTC-3′) and daf1rR (5′-TGGCAGTA AGCCGAGAAGATGGATT-3′), were used to amplify a 321-bp PCR product. A set of actin primers, actinF (5′-CACGAGACCACCTACCAATCCATC-3′) and actinR (5′-GGAAGGTTG AGAGGGAAGCAAGAA T-3′), was used to amplify a 235-bp PCR product as an internal control. The real-time PCR reaction was performed using SYBR^{R}Premix Ex Taq™ (Perfect Real Time) (Takara Inc., Dalian) according to the manufacturer's guidelines. The result was analyzed using the software Rotor-Gene 6. All data were given in terms of relative mRNA expression as means ± S.E.

Protein expression and purification of AcDAF-1 in *E. coli* and antibody production

The full ORF encoding AcDAF-1 was sub-cloned into pET-32m that had been digested with the same two enzymes, EcoR1 and Hind III, resulting in expression vector pETAcdaf-1. The construct was then confirmed by restriction analysis and nucleotide sequencing. Recombinant protein was expressed in *E. coli* BL21 and induced with 0.3 μm IPTG for 4 h at 37 °C. The crude protein preparations were separated on

a 10% SDS-PAGE preparative gel, and the bands were isolated from Coomassie blue-stained gels by elution and re-analyzed on stained SDS-PAGE gels to verify the purity of this protein. Mouse polyclonal anti-AcDAF-1 antiserum was raised against purified rAcDAF-1 protein. Three BALB/c mice (6–8 weeks of age) were immunized intraperitoneally with the eluted protein suspended in the adjuvant as described in our previous paper. Mice were boosted three times. The anti-antiserum was collected 7 days after the final boost.

Immunostaining

The eggs were collected from infected dog feces using flotation with saturated saline water. The L1, L2, and L3 were collected from cultured infected dog feces. The eggs and larvae were immune-stained following a protocol adapted from *C. elegans* (Yang et al. 2009). Larvae and eggs were frozen in liquid nitrogen and immersed in fixative solution (4% formaldehyde 10 min, cold 100% MeOH 5 min). After washed in PBS for 10 min, the slides were incubated in block solution (PBS containing 1% BSA) for 1 h and incubated in primary antibody (1:500 diluted in block solution) for 2 h, followed by three times wash in PBST (5/10/15 min each). The slides were stained with cy3-conjugated goat anti-mouse-IgG 1:500 in block solution for 2 h, followed by four times washes in PBST, and mounted for microscopy. Adult worms were fixed with 10% formaldehyde. The samples were embedded in paraffin. Sections were de-paraffinized and processed for staining with pre-immuno mouse serum (normal mouse immunoglobulins) or with mouse anti-AcDAF-1 antibodies (1:500 diluted in block solution) for 2 h. Antibody binding was detected with cy3 -conjugated goat anti-mouse-IgG 1:500 in block solution for 2 h, followed by four times washes in PBST, and mounted with glycerol for microscopy.

Results

Identification of a TGF-β type I receptor from *A. caninum and* cloning

The PCR product of *Acdaf-1* was about 1927 bps and cloned into pMD-18 T vector. The sequence analysis revealed that the open reading frame of *Acdaf-1* was about 1788 bp and encodes 595 amino acids. In addition, 5′UTR is about 95 bps, the ATG start codon is at the 96–98 bp, and 3′UTR is about 44 bps; the stop codon is at 1881–1883 bp (Fig. 1a). The N terminal end contained a signal peptide, the most likely cleavage site is between 28 A and 29 H, and a transmembrane region is from 192 to 208 amino acid (aa) and from 209 to 595 amino acids is the cytoplasm tail. The predicted *Acdaf-1* encodes a TGF-β type 1 receptor with a catalytic domain of the serine/threonine kinases, transforming growth factor beta family type I receptors (STKc_TGFβR_I), PKc-like superfamily domain, and Pkinase superfamily domain in 300 aa to 500 aa area. This region also has ATP binding site and substrate binding site as well as TGF-β GS motif site, which has a highly conserved GSGSGLP signature amino acid site in the cytoplasmic juxtamembrane region immediately preceding the protein's kinase domain (Fig. 1b). The cDNA sequence of *Acdaf-1* was deposited in GenBank (accession no. JF826239).

Fig. 1 The deduced amino acid sequence of transforming growth factor beta receptor I *Acdaf-1* from *A. caninum* (Fig. 1a). The first underlined region was a transmembrane region of AcDAF-1, and the second underlined was the signature amino acids of TGF-β GS motif. The conserved domains of STKc_TGFbR_I, PKc_like superfamily domain, and Pkinase superfamily domain have been detected (Fig. 1b), and the ATP binding sites and TGF-β GS motif were shown in Fig. 1b

Sequence analysis and phylogenetic analysis

The translated sequence of *Acdaf-1* was used in a BLASTp search against GenBank and identified as a TGF-β type 1 receptor homolog due to the high similarity with this protein from *C. elegans*. The amino acid sequences from a variety of species, including zebra fish, *Xenopus*, mouse, human, and *B. taurus*, were aligned using ClustalW. The phylogenetic analysis revealed that *Acdaf-1* is homologous to the TGF-β type 1 receptor family of proteins, with a conserved region between the amino acid position of 190 and 490 (Fig. 2), which has several active sites, including ATP binding sites and substrate binding sites and conserved domain of STKc-TGFbR-1 domain, and PKc-like superfamily domain as well as Pkinase superfamily domain. *Acdaf-1* is 42.4% identical to *B. malayi* homolog (50.1% similarity). *Acdaf-1* also has 31.9% identity and 42.4% similarity to mouse and human (29.9% in identity and 39.6% in similarity) TGF-β type 1 receptor. *Acdaf-1* has 31.7% identity and 42.7% similarity to *B. taurus* TGF-β type 1 receptor. Besides that, phylogenetic analysis also revealed that *Acdaf-1* is highly similar to *B. malayi* bone morphogenetic protein type 1 receptor. The TGF-β type 1 receptor homologs form two clades, an invertebrate and vertebrate clade (Fig. 3).

Transcript levels of *Acdaf-1* in different stages of *A. caninum* by real-time RT-PCR

To evaluate the expression of *Acdaf-1* in different stages of *A. caninum*, real-time RT-PCR was performed using first-strand cDNA from L1/L2, L3 larval stages, and adult worm of *A. caninum* to determine the transcript levels of *Acdaf-1* during the hookworm life cycle. In the adult worm of *A. caninum*, for both female and male, the expression of the *Acdaf-1* mRNA is remarkably higher in L1/L2 and L3 larval stage (Fig. 4). In the larval stages, *Acdaf-1* shows a higher

a

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   1 tgccaagcttgcatgcctgcaggtcgacgattggtttaattacccaagtttgagagagttctactaaattgtttta
   1                     M  K  P  S  Y  L  Y  L  T  N  S  Y  G  T  I  V  L  L  F
  76 ttagacttattggacatgacATGAAGCCGTCCTATCTTTACCTTACTAACTCTTATGGAACAATAGTTCTTCTTT
  20   E  V  L  L  S  I  S  F  A  H  E  L  T  V  H  N  S  S  T  F  D  V  D  T  T
 151 TTGAAGTATTATTATCTATATCATTTGCTCATGAGCTGACCGTACATAACTCGAGCACGTTTGACGTTGATACAA
  45   T  I  S  T  T  L  I  E  N  G  S  N  P  H  S  S  L  V  A  E  R  I  A  R  G
 226 CGACCATTTCTACAACGCTAATCGAAAATGGATCAAATCCTCATTCATCGTTAGTAGCTGAGAGAATTGCGCGTG
  70   N  A  I  L  E  E  M  K  S  H  I  F  G  T  E  E  Q  V  H  E  S  D  L  I  C
 301 GAAATGCTATACTAGAAGAAATGAAGTCACACATCTTTGGCACGGAAGAACAAGTGCATGAATCGGATCTGATTT
  95   N  C  N  G  A  L  C  D  N  E  L  V  H  L  L  G  D  S  Y  R  G  K  C  R  A
 376 GCAATTGTAATGGTGCACTGTGCGATAACGAATTAGTCCATTTACTGGGAGATTCTTATAGGGGCAAGTGTAGGG
 120   K  R  G  R  C  F  K  V  Y  D  Q  Q  H  N  Y  H  M  S  V  G  C  V  D  H  K
 451 CAAAGCGCGGTCGTTGCTTTAAAGTCTATGACCAGCAGCATAACTACCACATGTCAGTAGGGTGCGTTGACCACA
 145   D  L  F  P  V  P  I  F  C  L  G  H  P  N  Y  H  M  T  C  C  N  Y  S  F  C
 526 AAGATCTTTTCCCAGTGCCCATTTTCTGTCTTGGACATCCAAACTATCACATGACGTGCTGCAACTATTCATTCT
 170   D  E  E  I  S  L  N  V  V  E  G  I  T  L  E  D  A  I  P  W  H  W  V  L  I
 601 GCGATGAAGAAATCTCGCTAAATGTGGTGGAAGGAATCACGCTAGAGGATGCTATTCCATGGCACTGGGTTCTCA
 195   G  A  L  I  F  L  G  F  V  A  V  I  I  M  A  L  L  R  H  P  R  V  A  S  V
 676 TAGGAGCGCTCATCTTTCTTGGATTTGTGGCTGTTATCATCATGGCTCTTCTTCGACACCCCAGAGTTGCATCAG
 220   T  R  K  Y  L  R  L  G  P  T  M  S  A  A  L  N  H  I  S  P  R  E  D  E  V
 751 TTACGCGGAAGTATCTCCGTTTAGGCCCGACGATGTCTGCTGCATTGAATCACATATCCCCTAGAGAAGATGAAG
 245   P  M  I  E  T  M  S  Q  T  T  T  T  T  A  P  A  I  Q  L  L  L  L  K  G  L
 826 TGCCAATGATTGAAACAATGTCGCAGACGACCACTACTACTGCTCCAGCAATTCAACTCTTACTGCTGAAGGGAT
 270   E  S  T  D  A  G  S  G  S  G  S  G  L  P  L  L  T  Q  R  N  I  A  R  Q  I
 901 TGGAGTCCACCGATGCTGGATCGGGTTCTGGTTCTGGCTTGCCTTTGCTTACGCAACGAAACATCGCGAGACAGA
 295   E  L  V  R  E  I  G  Q  G  R  F  G  D  V  W  L  G  A  W  K  G  D  M  V  A
 976 TTGAACTTGTTCGAGAGATCGGCCAAGGACGTTTCGGTGATGTATGGTTGGGAGCGTGGAAGGGTGATATGGTTG
 320   V  K  I  F  S  S  R  D  E  G  S  W  S  H  E  V  E  T  F  Q  T  H  M  L  R
1051 CTGTAAAGATTTTCTCGTCACGTGATGAAGGATCATGGTCACATGAAGTGGAGACCTTCCAGACACATATGCTTC
 345   H  P  N  I  L  Q  F  Y  A  S  D  S  K  D  T  G  T  T  M  Q  L  W  L  I  T
1126 GTCATCCTAATATTCTACAGTTCTACGCTAGTGATAGCAAAGATACCGGTACTACTATGCAACTATGGTTGATTA
 370   E  Y  H  S  H  G  S  L  F  D  Y  L  S  S  S  T  V  N  V  V  T  L  V  Q  M
1201 CGGAGTATCATAGCCATGGATCTTTATTCGATTACTTATCAAGCTCTACTGTCAATGTTGTGACACTCGTGCAGA
 395   V  R  G  I  S  N  G  L  S  F  L  H  T  E  L  M  G  I  Q  K  K  P  A  I  A
1276 TGGTACGAGGTATCTCTAATGGACTCTCATTTTTGCACACAGAATTAATGGGGATTCAAAAAAAACCAGCGATTG
 420   H  R  D  I  K  S  K  N  I  L  V  K  A  D  L  T  C  A  I  A  D  L  G  L  A
1351 CACATCGAGACATCAAAAGCAAAAACATTCTTGTTAAAGCTGATTTGACTTGTGCTATAGCAGATTTGGGACTTG
 445   V  R  Y  E  S  G  H  I  S  L  P  N  S  N  K  C  G  T  V  R  Y  L  P  P  E
1426 CTGTACGTTATGAATCGGGTCACATAAGTCTACCGAATAGTAATAAATGTGGAACAGTGCGTTATCTTCCACCGG
 470   I  L  E  E  K  V  E  S  T  R  F  E  F  Y  R  M  G  D  M  Y  A  I  G  L  V
1501 AAATTCTGGAGGAGAAGGTGGAGTCCACAAGGTTCGAGTTCTATCGCATGGGTGATATGTACGCTATTGGTTTAG
 495   I  W  E  I  A  R  R  T  T  C  S  A  G  P  A  S  P  F  A  E  S  L  P  Y  Y
1576 TGATTTGGGAAATAGCACGACGTACGACGTGTTCCGCCGGTCCCGCCTCTCCGTTCGCAGAGTCTTTACCGTATT
 520   D  L  V  S  R  D  P  T  I  K  E  M  R  E  V  V  V  T  Q  G  V  R  P  Y  E
1651 ATGATCTGGTTTCGCGAGATCCAACTATAAAAGAGATGCGAGAAGTGGTTGTGACACAAGGAGTTCGACCTTACG
 545   S  Y  H  W  R  E  N  N  V  L  R  D  V  S  R  V  M  R  E  C  W  S  A  N  P
1726 AGTCTTACCATTGGCGAGAAAATAACGTACTACGCGATGTGTCTCGCGTAATGCGCGAATGCTGGTCAGCAAATC
 570   S  S  R  L  T  A  M  N  V  R  L  S  M  D  R  L  A  Q  T  E  L  N  L  R  F
1801 CATCTTCTCGGCTTACTGCCATGAATGTTCGTCTCTCTATGGATCGTTTGGCTCAAACTGAGCTCAACTTGCGGT
 595   S  *
1876 TCTCCTGAgagaatctctagaggatccccgggtaccgagctcgaattcgaat
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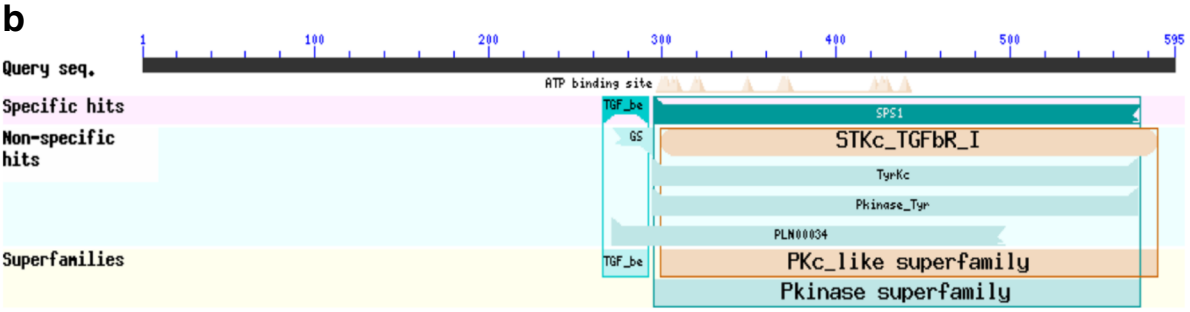
b



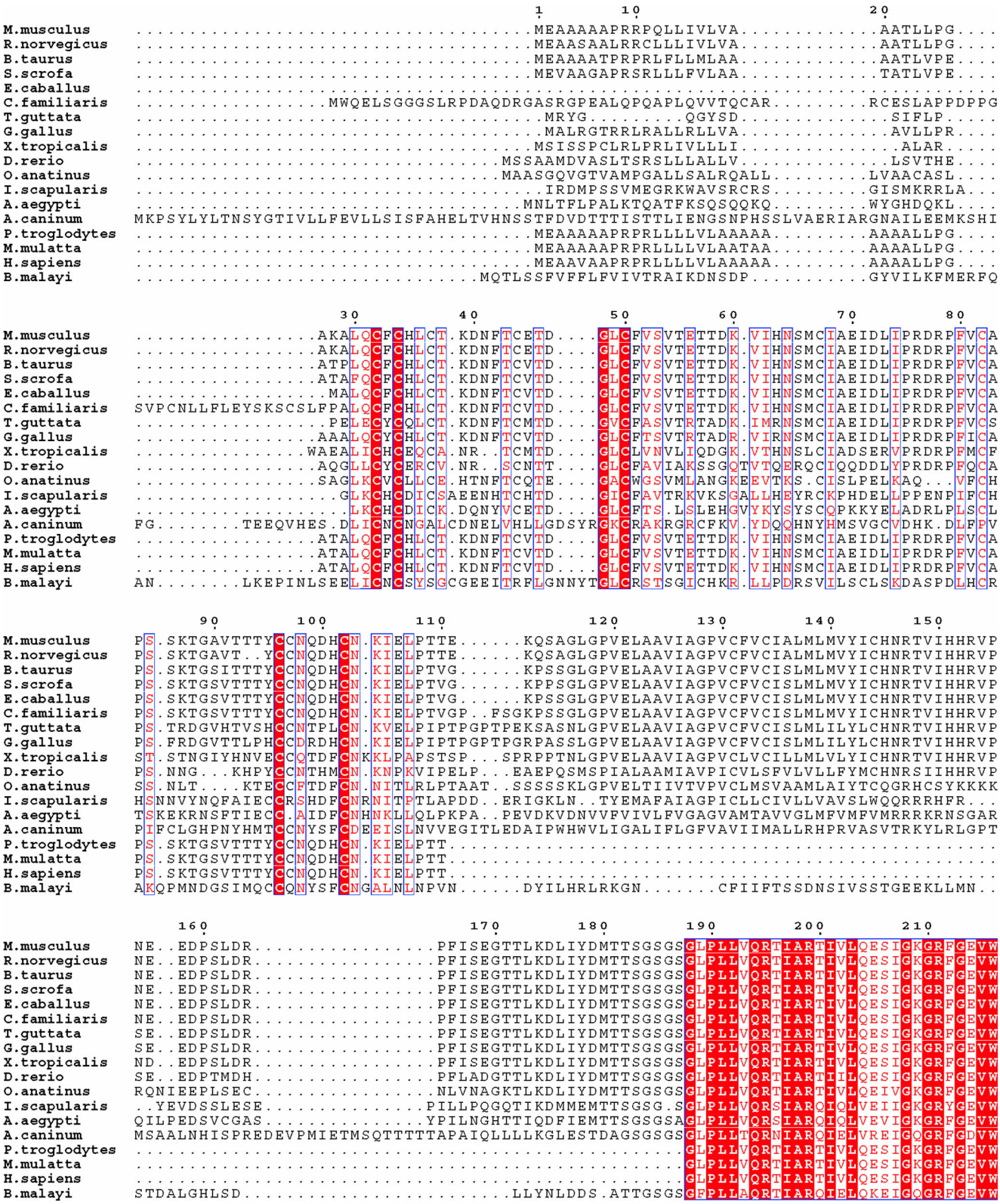


Fig. 2 Sequence alignment of TGF-b receptor 1. The AcDAF-1 protein has been aligned with transforming growth factor beta receptor I sequences from other organisms including accession number, *Homo sapiens* (NP_001124388.1), *Bos taurus* (NP_777046.1), *Mus musculus* (BAA05023.1), *Ixodes scapularis* (XP_002412676.1), *Rattus norvegicus* (NP_036907.2), *Sus scrofa* (BAD91020.1), *Equus caballus* (XP_001494957.1), *Canis familiaris* (XP_538750.2), *Taeniopygia guttata* (XP_002190425.1), *Danio rerio* (NP_001032772.2), *Aedes aegypti* (XP_001653836.1), *Gallus gallus* (NP_989577.1), *Xenopus (Silurana) tropicalis* (NP_001015961.1), *Brugia malayi* (XP_001900442.1), *Ornithorhynchus anatinus* (XP_001512345.1), *Pan troglodytes* (XP_001159204.1), and *Macaca mulatta* (XP_001112590.1) using the ClustalW program. Residues that are identical in half or more sequences are shaded in red. Those that are similar are shaded in light red

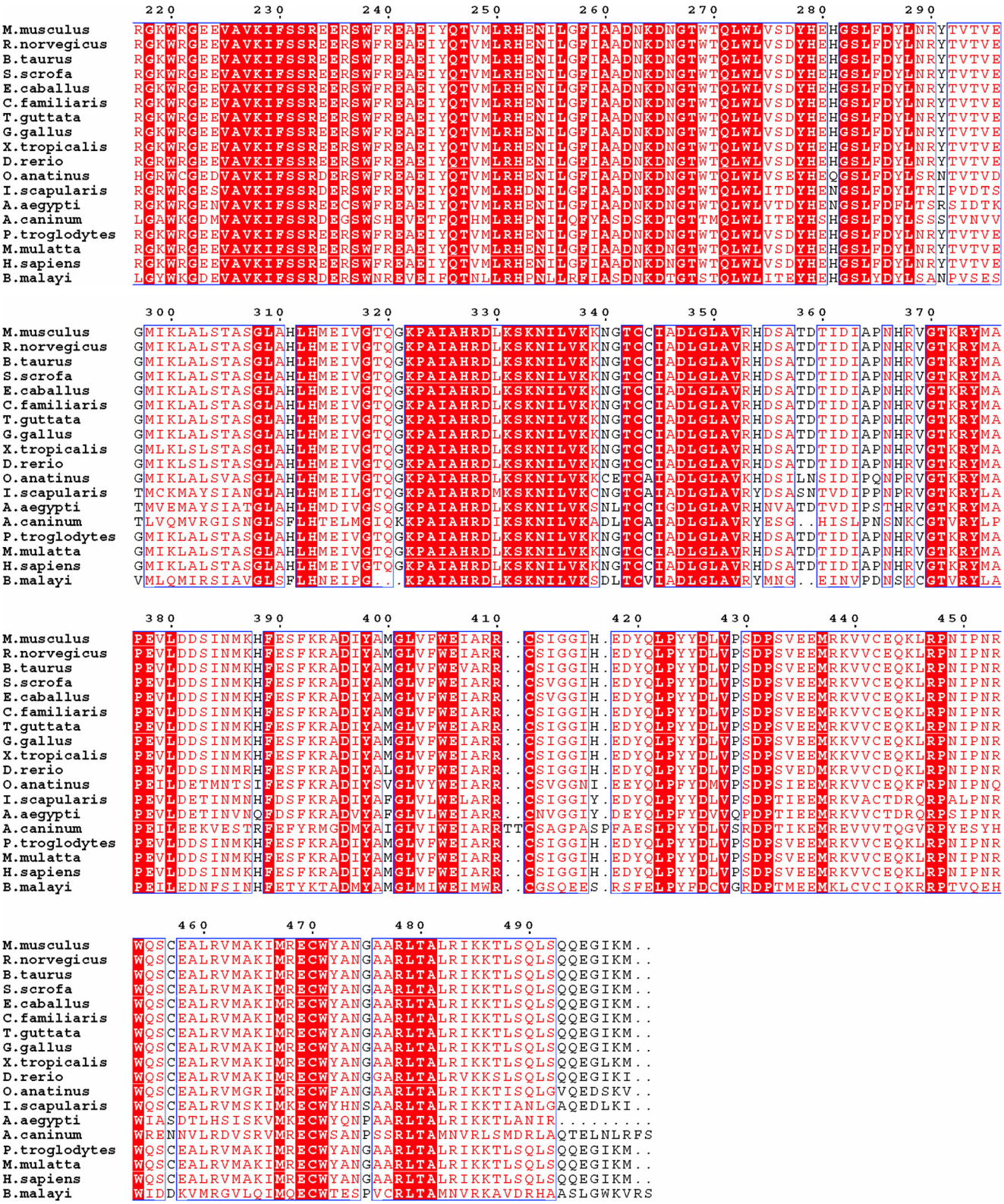


Fig. 2 continued.

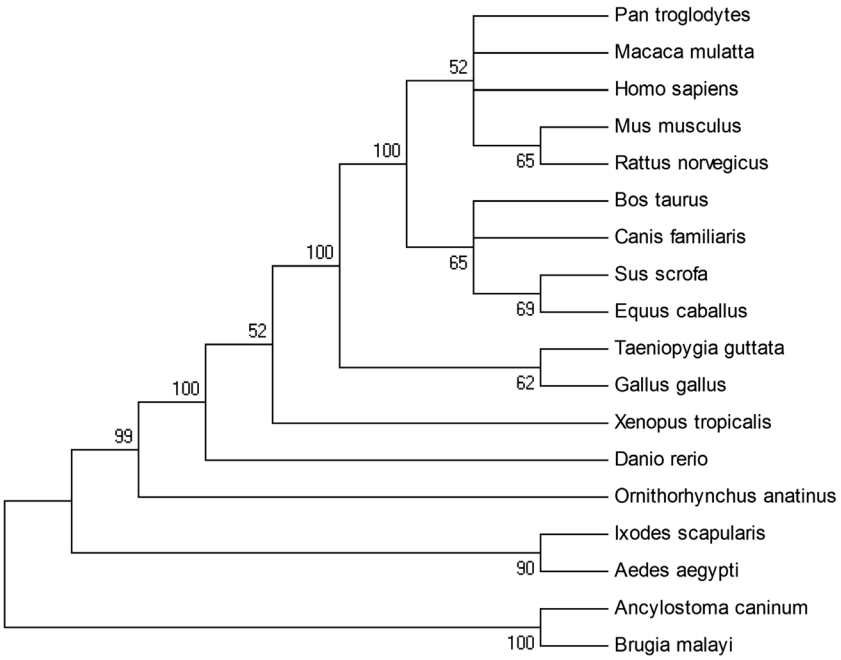


Fig. 3 Phylogenetic tree of AcDAF-1 protein with other proteins from 17 species. The phylogenetic analysis conducted using a maximum parsimony (MP) and neighbor-joining (NJ) methods employing PAUP v 4.0b10. A heuristic search with tree bisection–reconnection (TBR) branch swapping was used to infer the shortest trees. The length and retention index (C.I.), excluding uninformative characters, and retention index (R.I.) of the most parsimonious trees were recorded. A bootstrap analysis (using 1000 replicates) was conducted using heuristic searches and TBR-Branch swapping with the MulTrees option to determine the relative support for clades in the consensus tree. Numbers indicate the percentage association from bootstrap replication

mRNA expression in L3 stage compared to the L1/L2 stage of *A. caninum*. After serum activation, the expression of *Acdaf-1* was decreased in L3 infective larvae.

Expression and purification of *Acdaf-1* in *E. coli*

To obtain a recombinant AcDAF-1 protein for immunological and functional studies, the full ORF encoding AcDAF-1 was cloned into vector pET-32m resulting in the expression vector pETAcdaf-1. The construct was confirmed by restriction analysis and nucleotide sequencing. The cell extracts from *E. coli* BL21 cultures which induced with IPTG showed a major protein band of about 58 kDa in the SDS-PAGE analysis (Fig. 5). The recombinant AcDAF-1 was purified and used to raise specific antibodies in mouse.

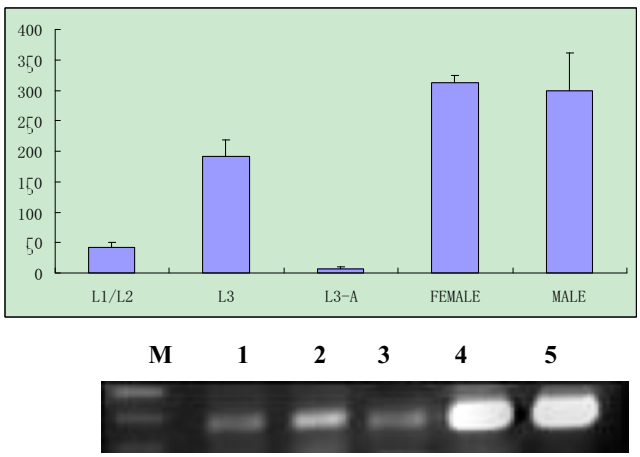


Fig. 4 The transcript levels of *Acdaf-1* in different stages of *A. caninum* detected by real-time RT-PCR. Lane 1: transcript of *Acdaf-1* from L1/L2 larvae. Lane 2: *Acdaf-1* transcript from L3. Lane 3: *Acdaf-1* transcript from female adult worms. Lane 4: transcript of *Acdaf-1* in male adult worms

Immunolocalization of AcDAF-1 in different stages of *A. caninum*

Eggs, L1, L2, and L3 of *A. caninum* were incubated with anti-rAcDAF-1 antibodies and AcDAF-1 was found to be present in the embryos and larval cuticle. When the eggs of *A. caninum* were incubated with anti-rAcDAF-1 antibodies, red fluorescence was observed and present in the surface of the blastomeres of embryo (Fig. 6, A3). On the slides of L1 larvae stage, red fluorescence was observed in the worm cuticle, epidermis, and intestine of the larvae (Fig. 6, B3). However, the red fluorescence was absent in esophagus (Fig. 6, B3), especially in muscle esophagus bulb (Fig. 6, C3). In L3 larvae, red fluorescence signal was present in the cuticle, epidermis, intestine, and pore of excretory organ (Fig. 6, D3) and primordial germ cells (Fig. 6, E3), as well as anus (Fig. 6, F3) of the worm.

Adult worm sections incubated with anti-rAcDAF-1 polyclonal antibody showed intense staining in the basal layer of

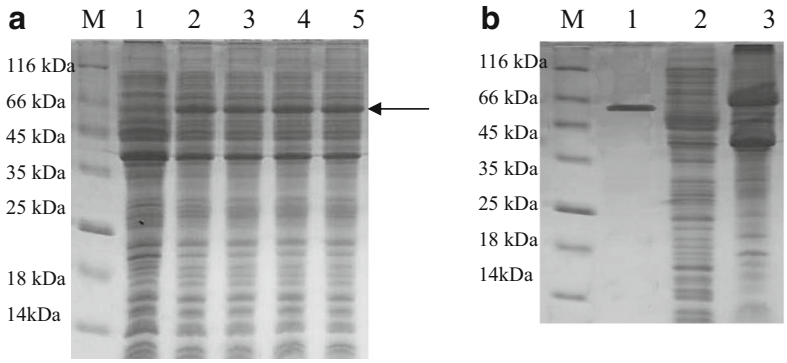


Fig. 5 Expression and purification of recombinant AcDAF-1 in *E. coli.* **a** Analysis of recombinant protein in an SDS-PAGE. Lane 1: uninduced cell lysate of pETAcDAF-1. Lanes 2–4: induced cell lysate of pETAcDAF-1 with 0.05 mM (lane 2), 0.1 mM (lane 3), 0.2 mM (lane 4), and 0.3 mM (lane 5) IPTG for 4 h. **b** Purification of recombinant AcDAF-1. M: protein marker. Lane 1: the purification of recombinant AcDAF-1. Lane 2: supernatant of cell culture. Lane 3: precipitate lysate of pETAcDAF-1 induced with 0.3 mM IPTG for 4 h

the intestine and ovary (Fig. 6, G3). Besides that, a clear staining was also present in the epidermis and eggs in the ovary (Fig. 6, H3) of the adult worm in sections. No fluorescence was detected using pre-immune mouse serum.

Discussion

In this study, we structurally and functionally characterized the *daf-1* orthologue from *A. caninum.* Sequence analysis revealed that the open reading frame of *Acdaf-1* was about 1788 bp, encodes 595 amino acids. The N terminal end contains a signal peptide with the most likely cleavage site between 28 A and 29 H, and a transmembrane region is from 192 to 208 amino acids and from 209 to 595 amino acids is the cytoplasm tail. The predicted *Acdaf-1* encodes a TGF-β type-1 receptor with a catalytic domain of the STKc_TGFβR_I and PKc-like superfamily domain as well as Pkinase domain in 300aa to 500aa region. There are several ATP binding sites and substrate binding sites in this region. In addition, the GS motif site of the TGF-β type I receptor is found immediately preceding the protein's kinase domain. The GS motif is mainly from its highly conserved GSGSGLP signature in the cytoplasmic juxtamembrane region. Point mutation in the GS motif could modify the signaling ability of type I receptor (Huse et al. 1999). Whether the GS motif in parasitic nematodes has the same function still remains unclear.

The protein with catalytic domain of STKc_TGFβR_I catalyzes the transfer of the gamma-phosphoryl group from ATP to serine/threonine residues on protein substrates. This subfamily is composed of type I receptors for the TGF-β family of secreted signaling molecules including TGF-β, bone morphogenetic proteins, activins, growth and differentiation factors, and anti-Mullerian hormone (Gupta et al. 2004). These receptors contain an extracellular domain that binds ligands, a single transmembrane (TM) region, and a cytoplasmic catalytic kinase domain (Allendorph et al. 2006). AcDAF-1 also has a transmembrane region from 192 to 208 amino acids and catalytic kinase domain. Normally type I receptors are low-affinity receptors that bind ligands only after they are recruited by the ligand/ type II high-affinity receptor complex. Following activation through transphosphorylation by type II receptors, they start intracellular signaling to the nucleus by phosphorylating SMAD proteins (Allendorph et al. 2006; Huse et al. 1999). Whether AcDAF-1 has this feature need to be clarified in the future.

Phylogenetic analysis revealed that *Acdaf-1* is homologous to the TGF-β type 1 receptor family proteins, with a conserved region localized in 190aa to 490aa (Fig. 2), which has several active sites, including ATP binding sites and substrate binding sites and conserved protein kinase domain. *Acdaf-1* is 42.4% identical to *B. malayi* homolog with a similarity of 50.1%. *Acdaf-1* also has 31.9% identity and 42.4% similarity to mouse and human (29.9% in identity and 39.6% in similarity) TGF-β type 1 receptor. *Acdaf-1* has 31.7% identity and 42.7% similarity to *B. taurus* TGF-β type 1 receptor. Moreover, the phylogenetic analysis also revealed that *Acdaf-1* is highly similar to *B. malayi* bone morphogenetic protein type 1 receptor, and the TGF-β type 1 receptor homologs form two clades, an invertebrate and vertebrate clade (Fig. 3). Most of the TGF-β type 1 receptors in sequence identity show significant disparity and low similarity. This implies that TGF-β type 1 receptor represents high diversity, and parasitic nematodes use different type I receptor, which might provide a new strategy to control parasites.

In *C. elegans*, *daf-1* regulated dauer larva formation and *daf-1* mutations result in dauer larvae even in abundant food. *C. elegans* DAF-1 has a transmembrane domain and a C-terminal protein kinase domain. The *daf-1* product is a 669 amino acid cell-surface receptor and has an ability to transduct environmental signals into cell (Georgi et al. 1990). We also found that AcDAF-1 localized in the cuticle and surface of intestine of *A. caninum*. The full length of *Acdaf-1* encodes 595 amino acids, which looks shorter than *C. elegans* DAF-1

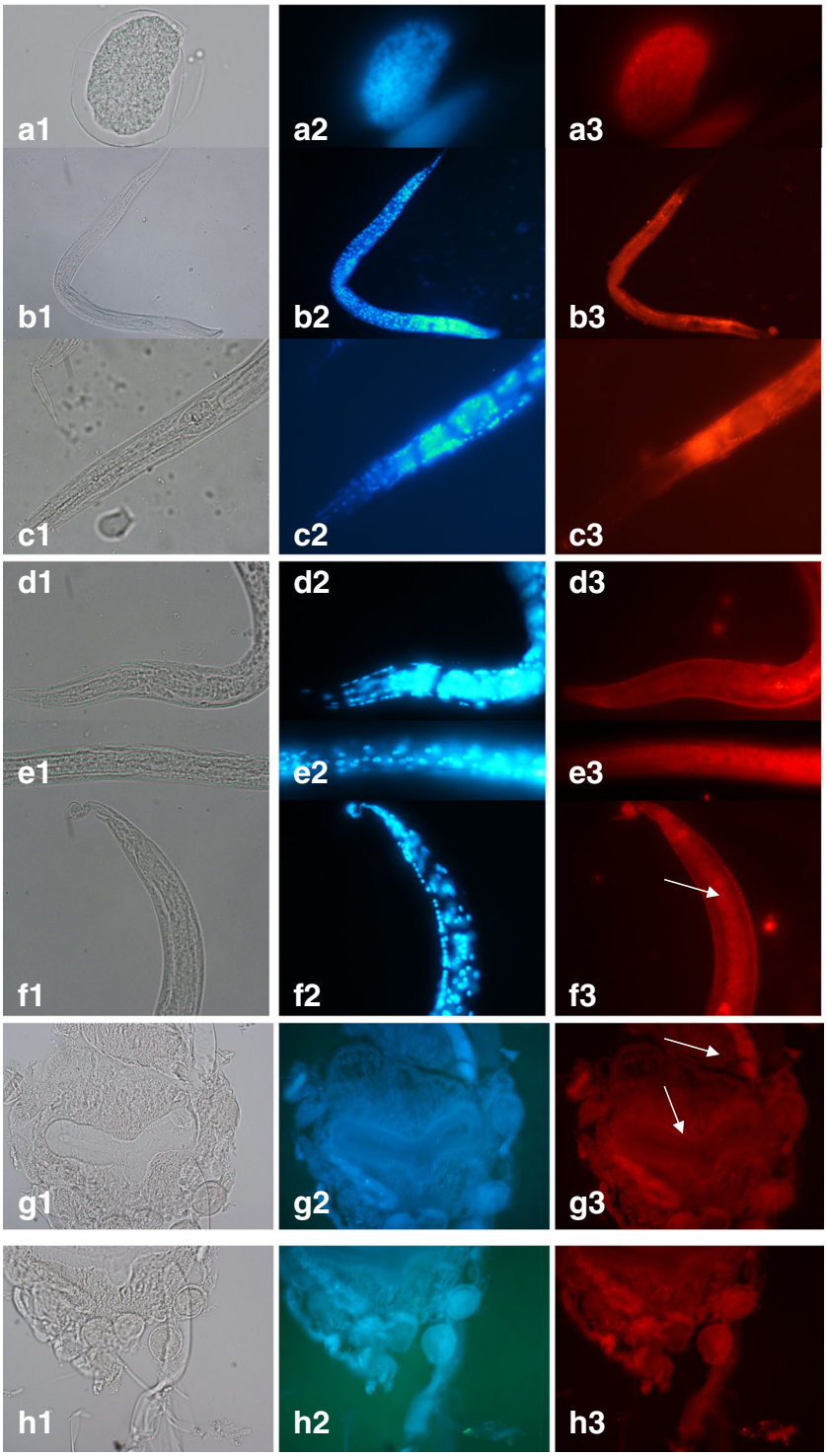


Fig. 6 The immunofluorescence of AcDAF-1 in egg, L1/L2, L3, and adult worm section of *A. caninum*. Egg (**a1**–**a3**), L1 larvae (**b1**–**b3**), and the head of L1 larva (**c1**–**c3**). Head of L3 larva (**d1**–**d3**), middle part (**e1**–**e3**), and tail (**f1**–**f3**) of L3 larva of *A. caninum*. The sections of *A. caninum* adult worm (**g1**–**g3**, **h1**–**h3**). Embryos and larvae were fixed with 4% formaldehyde for 10 min and cold with 100% MeOH for 5 min, respectively, and incubated with polyclonal anti-rAcDAF-1 mouse serum. Antibody binding was detected using cy3-conjugated anti-mouse immunoglobulin and DAPI. Panels **a1**, **b1**, **c1**, **d1**, **e1**, **f1**, **g1**, and **h1** were phase micrographs and panels **a2**, **b2**, **c2**, **d2**, **e2**, **f2**, **g2**, and **h2** were DAPI-stained fluorescence micrographs. Panels **a3**, **b3**, **c3**, **d3**, **e3**, **f3**, **g3**, and **h3** were stained with anti-AcDAF-1 antibodies. Intestine (arrow shows)

protein. In addition, *Acdaf-1* possesses the identified kinase domain, suggesting that it might bind to the same regions of their target genes.

In *C. elegans*, the dauer is an alternative developmentally arrested third larval stage (Patterson and Padgett 2000), which has been supposed to be similar to infective larvae stage of parasitic nematodes (Crook 2014; Crook et al. 2005; Gilabert et al. 2016; Viney et al. 2005). Entry into dauer state is modulated by environmental cues, including food deprivation, temperature, and the concentration of dauer pheromone (Fielenbach and Antebi 2008; Gallo and Riddle 2009). Three signaling pathways are involved in controlling this process in *C. elegans*. TGF-β (Gumienny and Savage-Dunn 2013; Patterson and Padgett 2000), insulin signaling

pathways(Gerisch et al. 2001), and pheromone-dependent cGMP signaling pathway (Barna et al. 2012) are involved in regulating dauer formation and recovery in *C. elegans* (Fielenbach and Antebi 2008; Gerisch et al. 2001; Ren et al. 1996). An insulin-like signaling pathway also mediates L3 activation in hookworms (Kiss et al. 2009).

In *C. elegans*, the TGF-β pathway consists of DAF-1, DAF-3, DAF-4, DAF-5, DAF-7, DAF-8, and DAF-14 proteins, which are involved in the transduction of the environmental signals to the nucleus of the Amphid Single I (ASI) chemosensory neuron (Gumienny and Savage-Dunn 2013; Inoue and Thomas 2000a). The DAF-4 protein is a type II receptor in *C. elegans* regulating body size, male tail patterning, and dauer larva formation (Estevez et al. 1993; Gunther et al. 2000). The partner of DAF-4 is DAF-1 receptor (Georgi et al. 1990). DAF-1 could sense the signal without DAF-4 activity, and overexpression of *daf-1* could partially rescue a *daf-4* mutant (Gunther et al. 2000). However, DAF-1 could not fully substitute DAF-4 activity, implying that non-dauer development needs both receptors activities (Gunther et al. 2000). It was also found that the GFP reporters driven by *daf-1* and *daf-4* promoter were also expressed in the nervous system, suggesting that the receptor complex DAF-1/DAF-4 may play a role in a neuronal pathway (Gunther et al. 2000), which is vital for nematode to sense the environment changes.

In *C. elegans*, TGF-β pathway is initiated by the ligand DAF-7/TGF-β binding with the DAF-1/DAF-4 receptor complex to activate the DAF-8/DAF-14 Smads (Monsivais et al. 2017). At present, only two TGF-beta ligands have been identified in hookworm (Brand et al. 2005; Freitas and Arasu 2005). *Ac-daf-7* was highly expressed in L3 and serum activated L3 but low in L1 and adult worm. Ac-DAF-7 mature protein was detected present in L3 and adult worms but absent in ES products of serum-activated L3 or adult worms. It was also found that the *Ac-daf-7* increased expression in arrested L3, implying that *Ac-daf-7* might be required for arrest larvae development (Brand et al. 2005).

Another ligand, *Ac-dbl-1*, displayed 60% in sequence identity to the *C. elegans dbl-1*, which controls growth, while *Ac-daf-7* revealed 46% sequence identity to *Ce-daf-7*, which modulated development arrest (Freitas and Arasu 2005). It was also found that the arrangement of exon/intron in *Ac-dbl-1* and *Ac-daf-7* was distinctive from that of *C. elegans* genes with nine and ten exons separately and intron size ranging from 56 to 2556 bp. The transcripts of *Ac-dbl-1* and *Ac-daf-7* were present in all stages, especially in egg, L1/L2, infective L3, and serum-activated L3, as well as adult worms. *Ac-dbl-1* displayed the highest expression in the male adult worm, implying that *Ac-dbl-1* and *Ac-daf-7* might play the same role as *Ce-dbl-1* in male tail formation. The highest expression of *Ac-daf-7* was in the arrest iL3 and reactivated ssL3, which was different from the expression of *Ce-daf-7*; this might be distinctive for parasitic nematodes which need to live through arrested development (Freitas and Arasu 2005).

It has been reported that antibodies of *A. caninum* TGF-β ligands could recognize proteins in iL3, ssL3, and adult worms. Antibody staining revealed that *Ac-daf-7* was presented in the anterior part of the iL3 similar to *Ce-daf-7*, which is concentrated in the ASI chemosensory neurons (Freitas and Arasu 2005). In hookworm, infective L3 receives a signal from host during invasion and the signal would re-activate pendent developmental pathways. Reply to this signal is crucial for the parasites to establish the infection in the host successfully and to complete the life cycle. In *C. elegans*, the response of dauer larvae recovery from the arrested stage to environmental signals is similar to the recovery of development of hookworm L3 invading in the host. In adult worm of *A. caninum*, both female and male, the expression of the Acdaf-1 mRNA is remarkable higher than L1/L2 and L3 stage. In the larvae stage, Acdaf-1 shows a higher mRNA expression in L3 stage of *A. caninum* than L1/L2 stage. After serum activation, the expression of Acdaf-1decreased in L3 infective larvae, which implied that the expression of Acdaf-1 was related to dauer state, and after serum activation, the expression of Acdaf-1 was downregulated. These results imply that the expression of Acdaf-1 might be important for the dauer stage survival.

At present, the other components of TGF-β signaling pathway still remain unclear in hookworm. Further analyses are required as how the *Acdaf-1* interact with type 2 receptor and initiate signaling through TGF-β pathway. In the meanwhile, how ligands bind receptors to start the pathway, and how the worm senses the changes of the environment to response need to be further identified in the future. The expression pattern of Acdaf-1 reveals that it might be involved in the interaction of the parasite and host relationship and provides a potential target for parasite control.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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