Short communication

Cloning and radiation hybrid mapping of bovine toll-like receptor-4 (TLR-4) signaling molecules

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Abstract

Toll-like receptor (TLR)-4 is a transmembrane receptor for lipopolysaccharide, a highly pro-inflammatory component of the outer membrane of Gram-negative bacteria. To date, molecules of the TLR-4 signaling pathway have not been well characterized in cattle. The goal of this study was to clone and sequence the full-length coding regions of bovine genes involved in TLR-4 signaling including CASP8, IRAK1, LY96 (MD-2), TICAM2, TIRAP, TOLLIP and TRAF 6 and to position these genes, as well as MyD88 and TICAM1, on the bovine genome using radiation hybrid mapping. Results of this work indicate differences with a previously published bovine sequence for LY96 and a predicted sequence in the GenBank database for TIRAP based on the most recent assembly of the bovine genome. In addition, discrepancies between actual and predicted chromosomal map positions based on the Btau_2.0 genome assembly release were identified, although map positions were consistent with predicted locations based on the current bovine-human comparative map. Alignment of the bovine amino acid sequences with human and murine sequences showed a broad range in conservation, from 52 to 93%. Overall, this work should assist in the assembly and annotation of the bovine genome sequence, the identification of variations in genes critically involved in host innate immunity, and facilitate the study of TLR-4 signaling pathways in cattle.

Keywords: Apoptosis; Mastitis; Endotoxin; Inflammation
1. Introduction

Gram-negative bacteria are responsible for several economically important diseases in cattle, including enteric colibacillosis, coliform septicemia, brucellosis, metritis, pneumonia, salmonellosis, campylobacteriosis, and mastitis (Cullor, 1992). It is estimated that almost 40% of the clinical cases of mastitis are caused by Gram-negative bacteria (Erskine et al., 1991; Ziv, 1992). Many of the cows with these infections develop septic shock, an exaggerated inflammatory response elicited largely by a highly pro-inflammatory component of the Gram-negative bacterial envelope known as endotoxin or bacterial lipopolysaccharide (LPS).

LPS induces inflammation, in part, through its binding and activation of toll-like receptor (TLR)-4 (Chow et al., 1999), which leads to the downstream activation of the transcription factor NF-κB and corresponding pro-inflammatory cytokine production (Baldwin, 1996). Following LPS recognition by TLR-4 and its associated proteins MD-2 and CD-14, the adapter protein myeloid differentiation factor 88 (MyD88) is recruited to the cytoplasmic domain of TLR-4 through homotypic binding of respective toll receptor-interleukin-1 receptor (TIR) domains contained within each protein (Medzhitov et al., 1998). Human MyD88 contains a highly conserved death domain (DD) that facilitates its interaction with another DD-containing signaling molecule, IL-1 receptor-associated kinase (IRAK). In the absence of MyD88, TIR domain-containing adapter protein (TIRAP) (also known as MAL) has been shown to function in a redundant role to MyD88 (Horng et al., 2001). Following recruitment to MyD88, IRAK-1 undergoes rapid autophosphorylation and dissociation from the signaling complex. Phosphorylated IRAK subsequently interacts with TNF receptor-associated factor-6 (TRAF-6), initiating the activation of a kinase cascade involving IkB kinase (IKK) (Swantek et al., 2000). Activation of this cascade culminates in the phosphorylation and degradation of the NF-κB inhibitor, IkB, enabling NF-κB to translocate to the nucleus and promote pro-inflammatory gene expression. Adding to the level of complexity is the finding that another molecule, TOLLIP, is involved in negatively regulating TLR-4-induced NF-κB activation (Zhang and Ghosh, 2002).

In addition to their involvement in NF-κB activation, MyD88, TIRAP, IRAK-1, and TRAF-6 have been implicated in mediating TLR-4-induced apoptosis (Bannerman and Goldblum, 2003; Dauphinee and Karsan, 2006). TICAM-2, which in concert with TICAM-1 promotes TLR-4-elicited activation of the IRF-3 transcription factor that regulates IFN-β production (Vogel et al., 2003; Takeda and Akira, 2005), has recently been implicated in mediating TLR-4-induced pro-apoptotic signaling (Kaiser and Offermann, 2005). There is evidence to suggest that the ability of these various TLR-4 signaling molecules to induce apoptosis is through FADD-mediated activation of caspase-8 (Bannerman and Goldblum, 2006).
2003; Kaiser and Offermann, 2005; Dauphinee and Karsan, 2006). A schematic of select TLR-4 intracellular signaling molecules involved in NF-κB and IRF-3 activation (Vogel et al., 2003; Takeda and Akira, 2005), as well as apoptosis, is shown (Fig. 1).

Molecules involved in intracellular TLR-4 signaling have not been well characterized in cattle and information is lacking regarding their roles in mastitis and other diseases of cattle caused by Gram-negative bacteria. Therefore, the goal of this study was to clone and sequence the full-length coding regions of bovine genes involved in TLR-4 signaling, including interleukin-1 receptor-associated kinase 1 (IRAK1), MD-2 protein (LY96), toll-like receptor adaptor molecule 2 (TICAM2), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), toll interacting protein (TOLLIP), TNF receptor-associated factor 6 (TRAF6), and caspase 8 (CASP8), to facilitate study of these molecules in cattle. In addition, the chromosomal locations of these seven genes, as well as myeloid differentiation primary response gene 88 (MyD88), and toll-like receptor adaptor molecule 1 (TICAM1) were determined by radiation hybrid mapping.

2. Materials and methods

2.1. Cloning and sequencing

For the cloning and sequencing of each gene target, total RNA was obtained using the RNeasy kit (Qiagen, Valencia, CA) from primary cultures of aortic and mammary endothelial cells isolated from separate Holstein cows. To clone TRAF6, TIRAP, CASP8 and TOLLIP, reverse transcription was performed using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) in a 20-μL reaction volume. Polymerase chain reactions using cDNAs from both endothelial cell sources as template were performed in a 25-μL reaction volume using 2 μL of first strand cDNA and 1× iQ Supermix (BioRad): 3 mM MgCl₂; 0.8 mM dNTPs; 0.625 U iTaq DNA polymerase and 0.4 μM each primer described in Table 1. Thermocycling conditions consisted of 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. For the amplification of LY96, IRAK-1 and TICAM2, single step RT-PCR was conducted on each RNA source using the StrataScript One-Tube RT-PCR kit (Stratagene) according to kit instructions. To obtain the complete 5’ end of the IRAK-1 transcript, 5’ rapid amplification of cDNA ends (RACE) was performed using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA), 1 μg of total RNA isolated from bovine pulmonary artery endothelial cells, and gene-specific primer 5’-CGGTTGATCCACGGCCA-CAG-3’, according to manufacturer’s instructions. Reaction products for each gene were separated by agarose gel electrophoresis and the products of the expected size were extracted using the QIAquick Gel Extraction kit (Qiagen). Purified DNA was cloned into the pCR2.1 vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Multiple plasmid clones were isolated for each gene and endothelial cell line using the Fastplasmid Mini kit (Eppendorf, Westbury, NY) and sequenced in both directions using the CEQ8000 automated DNA sequencer and DTCS Quickstart chemistry (Beckman Coulter, Fullerton, CA). Sequences were submitted to the GenBank database and assigned accession numbers presented in Table 2.

2.2. Radiation hybrid mapping

For physical mapping of each gene target, PCR screening of DNA from 94 clone lines of a 3000-rad
bovine × hamster radiation hybrid (RH) panel (ResGen Invitrogen) consisted of 25 ng DNA; 0.4 mM of each gene-specific primer described in Table 3; 200 mM dNTPs; 1 × reaction buffer with MgCl₂ (Promega, Madison, WI, USA) and 0.5 U Taq polymerase (Promega). The thermocycling profile was 95 °C for 5 min; 10 cycles of 94 °C for 30 s, 65 or 68 °C (−1 °C per cycle) for 30 s, 72 °C for 30 s–1 min; followed by 20–25 cycles of 94 °C for 30 s, 55 or 58 °C for 30 s, 72 °C for 30 s–1 min and a final extension of 72 °C for 5 min. Amplification products from each cell line of the RH panel were evaluated by agarose gel electrophoresis and concordancy data were submitted to the Roslin RH database (http://databases.roslin.ac.uk.radhyb) for two-point analysis as previously described (Connor et al., 2004). Primers used for amplification of each gene target and amplicon sizes are presented in Table 3.
3. Results and discussion

For each gene locus, the GenBank accession number for the bovine nucleotide sequence and percentage nucleotide and amino acid sequence identities with human and mouse are presented in Table 2. A search of PubMed, the NCBI Gene database (http://www.ncbi.nlm.nih.gov/), the ARK database (http://texas.thearkdb.org/), and the INRA BOVmap database (http://locus.jouy.inra.fr/) did not indicate that any of the genes in the present work had previously been positioned within the bovine genome by radiation hybrid mapping. Recently, 10 bovine TLR’s have been physically mapped (McGuire et al., 2006). The same 3000-rad bovine × hamster RH panel was used in both that and the present study, but the genes mapped were different. Whereas that study mapped the genes encoding the receptors, we have mapped the genes encoding the intracellular proteins that enable TLR-4 signaling. The chromosomal assignments of these various bovine TLR-4 signaling molecules are shown in Table 3. Bovine chromosomal assignments of CASP8, LY96, MyD88, TICAM1, TIRAP, and TRAF6 were consistent with predicted locations based on the bovine-human comparative map (Hayes et al., 2003) and the March 2005 Bos taurus draft genome assembly (http://genome.ucsc.edu/). In contrast, discrepancies were found in the bovine chromosomal locations of TICAM2, TOLLIP, and IRAK1 based on RH mapping conducted in the present study and those predicted from the bovine draft genome assembly and/or the comparative map of Hayes et al. Specifically, the predicted location for TICAM2 based on the bovine draft genome assembly was tentatively BTA5, whereas the comparative map by Hayes et al. indicated a location of either BTA10 or BTA7. Mapping performed in the current study suggests that TICAM2 is located on BTA10. TOLLIP’s predicted position was on either BTA15 or BTA29 according to the comparative map and on BTA29 using the draft genome assembly. RH mapping in the present report suggests that TOLLIP is indeed on BTA29. Finally, the draft bovine genome assembly assigned IRAK1 to BTA7. However, human IRAK1 is known to be positioned on chromosome X. The current study provides RH mapping evidence that bovine IRAK1 is located on chromosome X. Together, the RH mapping assignments of these genes should contribute to the extension of regions of conserved synteny on the bovine-human comparative maps, the integration of the bovine physical and sequence maps, and the identification of causative genes underlying immune-related quantitative trait loci.

The current study provides the first full-length sequences of bovine CASP8, IRAK1, TICAM2 and TIRAP and confirms recently released sequences for TOLLIP (BT021785) and TRAF6 (NM_001034661). A predicted sequence for bovine TIRAP based on the current assembly of the bovine genome was available (XM_609564), however, it lacked 16 amino acids in the amino terminus. In addition, cloning and sequencing of LY96 from two different endothelial cell types, each of which was isolated from different Holstein cows, revealed a consensus sequence that shares only 82% nucleotide identity and 71% amino acid identity with a provisional reference sequence in the GenBank database for bovine LY96 (NM_174111). The provisional reference sequence originated from a single clone from a normalized pooled tissue library and shares 77% similarity with human LY96. In contrast to the provisional reference sequence, our reported sequence shares 99.9% identity with a predicted bovine LY96 sequence (XM_864835) on BTA14.

Predicted functional domains within the bovine translated sequences of each gene were compared with the published human protein sequences using Prosite (http://ca.expasy.org/prosite/) and found to be highly conserved, suggesting functional similarity of each of the proteins across the two species. For several of the bovine genes, including CASP8, IRAK1, MyD88, and TIRAP, single amino acids in the translated proteins of the respective human orthologs identified as critical for enabling signaling activity and/or functioning were found to be conserved in cattle. For instance, the predicted amino acid sequence of bovine CASP8 revealed conservation of His323 and Cys366 residues, which are required for proteolytic activity within the caspase domain and its corresponding ability to induce apoptosis (Stennicke and Salvesen, 1999). Alignment of the human and bovine predicted protein sequences of IRAK-1, MyD88, and TIRAP, identified the conservation of the amino acids Thr66, F56, and Pro136, respectively, each of which has been shown to be essential for proper functioning of the respective
proteins (Burns et al., 1998; Horng et al., 2001; Ross et al., 2002). Whether amino acid sequence differences that exist in other regions affect functioning of bovine TLR-4 signaling molecules remains unknown.

TLR-4 has been demonstrated to play a critical role in host innate immunity. TLR-4 knockout mice or those with naturally occurring genetic mutations in TLR-4 are hyporesponsive to LPS and highly susceptible to infection (Poltorak et al., 1998; Hoshino et al., 1999). Amino acid polymorphisms have also been identified in humans and have been associated with diminished pulmonary responsiveness to inhaled LPS and increased susceptibility to Gram-negative bacteremia and septic shock (Vogel et al., 2005). In addition, mutations have been identified in human TLR-4 signaling molecules, including IRAK-4 and other downstream effector proteins that promote NF-kB activation. Similarly, these mutations are associated with an impaired ability to respond to LPS and enhanced susceptibility to infection.

The numerous diseases of cattle in which Gram-negative bacteria are the etiological cause confers a critical importance to understanding the role of TLR-4 and its intracellular signaling molecules in mediating bovine innate immunity. The recent finding that TLR-4 expression is upregulated in cows with mastitis supports a link between TLR-4 and the pathogenesis of an infectious disease of cattle (Goldammer et al., 2003). The results of the present study should facilitate the development of reagents needed for more comprehensive functional studies of TLR-4 signaling in cattle.

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