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The Src tyrosine kinase Lck binds to CD2, CD4-1, and CD4-2 T cell co-receptors in channel catfish, *Ictalurus punctatus*

Erin B. Taylor, Melanie Wilson, Eva Bengten*

Department of Microbiology, University of Mississippi Medical Center, Jackson, MS 39216, United States

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ABSTRACT

The binding of the lymphocyte specific protein tyrosine kinase (Lck) to T cell co-receptors is required for T cell development and activation. In mammals, Lck initiates signal transduction by binding to CD4 and CD8 co-receptors and phosphorylating ITAMs in the cytoplasmic tail of the CD3 molecules and the ζ chains. In addition, Lck can also bind to the adhesion molecule CD2 and trigger T cell activation. In this study, Lck and CD2 homologs were identified and characterized in channel catfish, *Ictalurus punctatus*. Lck and CD2 mRNAs were specifically expressed by clonal T cell lines, including both $CD4^+$ and $CD4^-CD8^-$ CTL lines, and in mixed lymphocyte cultures (MLC). Western blot analyses using anti-trout Lck and anti-human p-Lck antibodies demonstrated that Lck protein is expressed in catfish clonal CTL and is phosphorylated at a conserved tyrosine residue. Because of the lack of $CD8^+$ CTL lines as well as the absence of CD8 message in MLC, we performed magnetic bead binding assays to correlate CD2, CD4, and CD8 co-receptor expression with Lck binding ability. Recombinant Lck reproducibly bound to CD2, CD4-1, and CD4-2, but not to CD8α or CD8β. These data provide one possible explanation for the apparent low numbers of $CD8^+$ CTL and the presence of $CD4^+$ and $CD4^-CD8^-CD2^+$ CTL in catfish.

1. Introduction

The lymphocyte-specific protein tyrosine kinase (Lck) is a member of the Src protein kinase family, and it is expressed in all stages of thymocyte development. Lck plays a critical role in αβ T cell maturation as well as in T cell differentiation, survival, and activation (Rudd et al., 1993; Williams et al., 1998; Palacios and Weiss, 2004; Salmond et al., 2009). Structurally, Lck is an approximately 56 kDa protein that consists of an N-terminal unique (SH4) domain, an SH2 domain, an SH3 domain, and a protein tyrosine kinase (SH1) domain. The N-terminal region of Lck is myristoylated and palmitoylated, which allows the protein to tether to the inner leaflet of the plasma membrane (Rudd et al., 1993; Williams et al., 1998; Palacios and Weiss, 2004; Resh, 1994). Lck associates with several cell surface molecules, including the T cell co-receptors CD4 and CD8, as well as the adhesion molecule CD2, the tyrosine phosphatase CD45, and the interleukin-2 receptor (IL-2R); however, the majority of Lck protein will coimmunoprecipitate with CD4 or CD8 after antibody-mediated crosslinking of either of these molecules (Zamoyska et al., 2003). Lck interacts with the T cell co-receptors CD4 and CD8α using a CXXC motif in its SH4 domain, which forms a complex with a CXC motif within the cytoplasmic tails of CD4 or CD8 in a Zn^{2+}-dependent interaction (Lin et al., 1998; Campbell et al., 1995; Turner et al., 1990). When the TCR and a co-receptor molecule are brought together in close proximity by binding to the peptide–MHC complex on an antigen presenting cell (APC), co-receptor-associated Lck phosphorylates the immunoreceptor tyrosine activation motifs (ITAMs) in the cytoplasmic tail of the CD3 molecules and the ζ chains. The tyrosine kinase zeta chain-associated protein of 70 kD (ZAP-70) then binds to the phosphorylated ITAMs on the ζ chains and it is also phosphorylated by Lck. The phosphorylated ZAP-70 acts as a docking site for other signaling molecules such as linker for activation of T cells (LAT). Subsequent signaling events result in the activation of transcription factors NFκB, AP-1, and NFAT, all of which act to induce the proliferation and differentiation of T cells. Lck activity is controlled by the differential phosphorylation of two tyrosine residues. First, Y^{505}, located in the short C-terminal tail, binds to the SH2 domain when it is in its phosphorylated state. This maintains Lck in its inactive form. The C-terminal Src kinase (Csk) phosphorylates Lck at Y^{505}, while CD45 phosphatase opposes the action of Csk and dephosphorylates Y^{505}. This action results in the dissociation of the SH2

* Corresponding author at: Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, United States. Tel.: +1 601 984 1719.
E-mail address: ebengten@umc.edu (E. Bengten).

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domain and the activation of Lck. Y^{394} is subsequently autophosphorylated, allowing for a conformational change and activation of Lck. Activated Lck then initiates the T cell signaling cascade (Bergmann et al., 1992; Perlmutter et al., 1993; Hermiston et al., 2002). An additional tyrosine in the SH2 domain, Y^{192}, is also transiently phosphorylated upon TCR stimulation (Couture et al., 1996; Ziemba et al., 2009).

In contrast, CD2–Lck interactions are mediated by the SH3 domain of Lck, which binds to the proline-rich motifs in the CYT of CD2 (Bell et al., 1996). CD2 is a type I integral membrane protein consisting of a membrane distal non-disulfide bonded V-type immunoglobulin (Ig) domain that is responsible for binding to the APC ligand and a membrane proximal C2 Ig domain that contains two interchain disulfide bonds (Tangye et al., 2000; Seed and Aruffo, 1987). CD2 is expressed on virtually all T and NK cells and its ligand is CD58 (LFA-3) in humans and CD48 (SLAMF2) in rodents. Their interaction not only facilitates adhesion between the T cells and APC (Barclay et al., 1997; Selvaraj et al., 1987), but it is also thought to provide the optimal spacing for TCR antigen recognition (Davis and van der Merwe, 1996; Evans et al., 2006; Kearney et al., 2007). CD2 has been shown to localize to lipid rafts upon stimulation with anti-CD2 mAb or CD58 and its recruitment is dependent on Lck binding (Nunes et al., 2008; Yang and Reinherz, 2001). Similarly, CD2 on NK cells mediates effector:target conjugate formation and in turn the downstream signaling through the ζ chains results in exocytosis of cytotoxic granules (Schmidt et al., 1988; Vivier et al., 1991). In addition to functioning as an adhesion molecule, CD2 also participates in T cell activation, *e.g.* when T cells are incubated with either anti-CD2 mAb or the cognate ligand, they become activated, as assessed by increased proliferation and cytokine secretion as well as induction of non-specific cytolytic activity (Clark et al., 1988; Meuer et al., 1984; Siliciano et al., 1985). More recently, stimulation through the CD2 co-receptor has been shown to induce phosphorylation of the TCR-proximal signaling complex, as measured by phosphoflow cytometry and western blot analyses (Skanland et al., 2014). Furthermore, the development and function of inducible Treg cells is dependent on CD2 signaling, and polymorphisms in the CD2 ligand CD58 correlates with increased risk for multiple sclerosis and rheumatoid arthritis (Marson et al., 2007; De Jager et al., 2009).

Lck has been identified in several teleost genomes including the pufferfish, *Takifugu rubripes* (Brenner et al., 2002), zebrafish, *Danio rerio* (Langenau et al., 2004), and rainbow trout, *Oncorhynchus mykiss* (Laing et al., 2007). The pufferfish *lck* gene was first identified and found to contain a short 4.2 kb promoter region. This promoter was shown to direct specific expression of EGFP in lymphoid tissues in a transgenic mouse model as well as in human clonal T but not B cell lines (Brenner et al., 2002). In trout, two distinct Lck sequences, designated Lck1 and Lck2, were identified that are homologous to mammalian Lck. Both Lck1 and Lck2 were found to be expressed in thymus and in IgM^- lymphocytes. A monoclonal (mAb) reactive to the N-terminal region of trout Lck was developed, and used to immunoprecipitate Lck from trout thymocytes and stain permeabilized lymphocytes in flow cytometry (Laing et al., 2007). While gene equivalents of CD4 and CD8 have been identified in many species of bony fish, very little is known about the interaction of Lck with these molecules and CD2 in teleosts (Laing and Hansen, 2011). CD4-like molecules were first described in the pufferfish and trout (Laing et al., 2006), and later in the channel catfish, *Ictalurus punctatus* (Edholm et al., 2007), common carp, *Cyprinus carpio* (Sun et al., 2007), sea bass, *Dicentrarchus labrax* (Buonocore et al., 2008), Atlantic salmon, *Salmo salar* (Moore et al., 2009), and Atlantic halibut, *Hippoglossus hippoglossus* (Patel et al., 2009). All of these fish expressed a conserved ortholog of mammalian CD4 (CD4-1) that contains four extracellular Ig domains (Laing et al., 2006; Edholm et al., 2007; Sun et al., 2007; Buonocore et al., 2008; Moore et al., 2009; Patel et al., 2009). However, an additional CD4-like molecule (CD4-2) has been identified in trout and catfish (Laing et al., 2006; Edholm et al., 2007). Trout CD4-2 contains two Ig domains and catfish CD4-2 has three Ig domains. Both CD4-1 and CD4-2 molecules contain a CXC motif in their cytoplasmic tails and are expressed in lymphoid tissues, including the thymus, as well as IgM^- lymphocytes. Because of their similar expression patterns, it is unclear which of the two CD4 molecules would serve as a T cell co-receptor in trout and catfish. CD8α and CD8β homologs have also been described in many fish species, including trout (Moore et al., 2005a), Atlantic salmon (Moore et al., 2005a), pufferfish (Suetake et al., 2007), common carp (Sun et al., 2007), sea bass (Buonocore et al., 2006), sea bream, *Sparus aurata* (Costantini et al., 2008), Atlantic halibut (Patel et al., 2008), and catfish (Quiniou et al., 2011). Like mammalian CD8 molecules, teleost CD8α and CD8β consist of a single extracellular Ig domain, a TM, and a cytoplasmic tail. However, teleost CD8 molecules lack the consensus CXC Lck binding motif that is present in mammalian CD8α. Instead both CD8 co-receptors contain a CXH motif and it may be that in teleosts both CD8α and CD8β can bind Lck and unlike mammalian CD8β, teleost CD8β can function in the absence of CD8α (Sun et al., 2007; Moore et al., 2005a; Suetake et al., 2007; Buonocore et al., 2006; Costantini et al., 2008; Patel et al., 2008; Quiniou et al., 2011). Additionally, Hayashi et al. (2010) used surface plasmon resonance to show that the trout CD8α CXH motif can bind Lck in a Zn^{2+}-dependent manner (Hayashi et al., 2010). Thus, even though the identification of both Lck and T cell co-receptors CD4 and CD8 suggests that teleost T cells function in a manner analogous to mammalian T cells, we contend that additional binding studies, as well as the identification and analysis of other potential signaling receptors, such as CD2, are needed to advance our understanding of teleost T cell subsets and their function.

Catfish are an established model for the study of immune responses in ectothermic vertebrates, primarily because it is one of the few species for which an *in vitro* cell culture system and clonal leukocyte cell lines has been developed, including alloantigen-specific cytotoxic T lymphocyte (CTL) lines (Stuge et al., 2000). Herein, we identify a catfish homolog of Lck, IpLck, and examine its expression in lymphoid tissues and cell lines. In addition, we use a rat anti-trout Lck mAb (Laing et al., 2007) and anti-human p-Lck to show that Lck is expressed in catfish clonal CTL and is phosphorylated at a conserved tyrosine residue. We also identify a CD2 homolog, IpCD2, that is specifically expressed in catfish CTL lines. Notably, neither CTL lines nor mixed lymphocyte cultures (MLC) from immunized fish express CD8, although they do express both CD4 molecules and CD2. Because of this lack of CD8 expression and the absence of the consensus Lck CXC binding motif in the cytoplasmic tail of catfish CD8, we examine IpLck's ability to bind to the cytoplasmic tails of co-receptor molecules CD2, CD4, and CD8 in immunoprecipitation studies.

2. Materials and methods

2.1. Animals, cell lines, and immunizations

Outbred and homozygous gynogenetic (Hogan et al., 1999) catfish (1–2 kg) were obtained from the Warmwater Aquaculture Research Unit, USDA-ARS (Stoneville, MS) and maintained in individual tanks as previously described (van Ginkel et al., 1992). Experiments involving live catfish were performed in accordance with relevant institutional and national guidelines and regulations, and approved by the UMMC IACUC. Briefly, catfish were anesthetized with tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO) and bled from the caudal vein or were euthanized with an overdose of MS-222 and mesonephros (trunk kidney, TK) were collected. The tissue was immediately snap frozen in liquid nitrogen