Molecular Immunology 46 (2009) 2167–2177

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

CD45 recruits adapter protein DOK-1 and negatively regulates JAK–STAT signaling in hematopoietic cells

Liangtang Wu[a], Krikor Bijian[b], Shi-Shiang Shen[a,b,*]

[a] Department of Medicine, McGill University, Montreal, Quebec, Canada H3G 1A4
[b] Mammalian Cell Genetics, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2

ARTICLE INFO

Article history:
Received 8 March 2009
Received in revised form 29 April 2009
Accepted 29 April 2009

Keywords:
CD45
DOK-1
JAKs
STATs
T-cells
Negative regulation

ABSTRACT

It has been extensively documented that CD45 positively regulates T cell receptor-mediated signaling through the activation of Src-family kinases. The mechanism whereby CD45 negatively regulates the JAK/STAT pathway, however, has not been fully elucidated. Here we describe the mechanism by which CD45 negatively regulates the JAK/STAT pathway through the recruitment of the inhibitory molecule Downstream of Kinase 1 (DOK-1) in hematopoietic cells. We present evidences that CD45 recruits DOK-1 to associate with tyrosine-phosphorylated DOK-1, and that the DOK-1-Y296F mutant completely abrogates its interaction with CD45. Moreover, CD45 expression is required for DOK-1 targeting to the plasma membrane in response to anti-CD3 stimulation. Functional studies further showed that stable expression of DOK-1 in K562 cells markedly decreased both JAK-2 and STAT-3/5 phosphorylation following IL-3 and IFN-α stimulation. Likewise, stable expression of DOK-1 in Jurkat cells significantly decreased JAK-2 phosphorylation. Similarly, both IL-3 and IFN-α-induced JAK-2 phosphorylations were significantly increased in CD45 deficient Jurkat cells. Consistently, silencing of the DOK-1 gene resulted in rescue of MAP kinases and JAKs activities in CD45 positive Jurkat cells. Accordingly, CD45 recruits adaptor DOK-1 to the proximal plasma membrane to serve as a downstream effector, resulting in negative regulation of the JAK/STAT signaling pathway.

1. Introduction

Adequate immune cell expansion and differentiation require T cell receptor (TCR) activation, which mediates intracellular signaling events necessary for a proper immune response. One of most striking events during such a response is the inducible phosphorylation of tyrosine residues within the TCR cytoplasmic domain, thereby creating binding sites for SH2 domain containing proteins such as Syk family kinases (Syk kinase for B cells and Zap-70 kinase for T cells). This procedure is though to be mediated by Src-family kinases, p56 Lck and p59 Fyn (Kane et al., 2000; Koretzky, 2003). CD45 plays a key role in the activation and regulation of TCR-mediated signaling pathways. It has been suggested that CD45 predominantly functions as a positive regulator of TCR signaling through its ability to dephosphorylate negative regulatory sites of Src-family kinases (Alexander, 2000; Frearson and Alexander, 1997; Huntington and Tarlinton, 2004; Mustelin et al., 2004), and the activity of p56 Lck is differentially regulated by CD45 during T cell development (Falahati and Leitenberg, 2007). Previously we have observed that the immune-restricted adaptor Src kinase associated protein, SKAP55, mediates the interaction between CD45 and Fyn kinase (Wu et al., 2002a,b), and we hypothesized that the CD45-mediated regulation of Src-family kinases might require other novel protein–protein interactions possibly through intermediate players, such as the adaptor SKAP55 (Wu et al., 2002a,b).

While CD45 plays a role as a positive effector in TCR-mediated signaling, recent studies have shown that CD45 can negatively regulate T cell and macrophage function by dephosphorylating Janus-kinases (JAK)s, leading to the inactivation of the JAK/STAT pathway (Fujii et al., 2003; Irie-Sasaki et al., 2003; Penninger et al., 2001; Wu et al., 2002a,b). JAK/STAT signaling has been shown to be activated in CD45 deficient mice (Irie-Sasaki et al., 2001). Moreover, JAKs, such as JAK-1 and JAK-2, and downstream substrates, such as STAT3 and STAT5, are hyperphosphorylated upon stimulation with IL-3 and INF-α in CD45−/− mice when compared to CD45+/+ mice. However, the underlying mechanism of CD45 regulation of the JAK/STAT signaling pathway is largely unknown.

Intracellular adaptor proteins play a pivotal role in linking one or more signaling components upon cell activation, capable of integrating multiple signaling pathways used to coordinate signals within and between cells (Jordan et al., 2003; Koretzky and Myung, 2001; Rudd, 1998; Wilkinson et al., 2004). For instance, a

* Corresponding author at: Mammalian Cell Genetics, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2. Tel.: +1 514 496 6318; fax: +1 514 496 6319.
E-mail address: shi.shen@cnrc-nrc.gc.ca (S.-S. Shen).

0161-5890/$ – see front matter.
doi:10.1016/j.molimm.2009.04.032

hyper-phosphorylated protein Downstream of Kinase 1 (DOK-1), with a molecular weight of 62 kDa, interacts with Abelson tyrosine kinase (ABL) in hematopoietic cells (Carpino et al., 1997; Yamanashi and Baltimore, 1997). Despite DOK-1 being ubiquitously expressed, the highest amount of DOK-1 expression is found in T cell and macrophages (Songyang et al., 2001; Tamir et al., 2000; Yamakawa et al., 2002; Yamanashi et al., 2000). DOK-1 contains several features consistent with its function in signal transduction. It has been well characterized that the DOK-1 gene contains a PH domain at the N-terminus for lipid membrane targeting, a PTB domain in the central region for binding of tyrosine-phosphorylated proteins, and a sequence at the C-terminus that is readily tyrosine phosphorylated. Functional data arising from DOK-1 overexpression and DOK-1 gene knockout mice show that DOK-1 is a negative regulator of the mitogen-activated protein kinase (MAPK) pathway in B cells and other hematopoietic cells (Ott et al., 2002; Tamir et al., 2000; Yamanashi et al., 2000; Zhao et al., 2006), although it has a positive effect in insulin signaling and tumor cell migration (Noguchi et al., 1999). Nonetheless, DOK-1 has consistently been shown to be negatively involved in TCR/BCR signaling (Tamir et al., 2000; Yamanashi et al., 2000; Yasuda et al., 2007). Furthermore, DOK-1 is tyrosine phosphorylated by Lck kinase (Nemorin and Duplay, 2000; Nemorin et al., 2001) in response to anti-CD2 and CD3 stimulation. It is thought that once DOK-1 is phosphorylated, it binds to a Ras GTPase-activating protein (Ras-GAP), thereby increasing intrinsic Ras hydrolysis activity and hence converting Ras-GTP to Ras-GDP.

In order to further investigate the negative regulatory function of CD45 in the JAK/STAT pathway, we employed a CD45 trapping mutant as bait in a yeast two-hybrid system to identify novel interacting partners of CD45. We report here that DOK-1 associates with CD45 in a tyrosine phosphorylation dependent manner. DOK-1 is dephosphorylated by CD45 in the presence of wild-type CD45. Moreover, site-directed mutagenesis led to the identification of tyrosine residue (Y296) in Dok-1 as a pivotal site for CD45/Dok-1 interaction. Upon anti-CD3/TCR stimulation, DOK-1 translocates from the cytoplasm to the plasma membrane, an event that requires CD45 expression. Stable expression of DOK-1 in K562 and Jurkat cells dramatically decreases phosphorylation of JAK and STAT3/5 upon IL-3 and IFN-α stimulation.

2. Materials and methods

2.1. Antibody reagents and cell lines

Anti-CD3 and anti-CD45 were purchased from BD Pharmingen and rat anti-hemagglutinin (HA) monoclonal antibody (clone 3F10) was bought from Boehringer Mannheim (Mannheim, Germany). Anti-Myc (9E10) monoclonal and anti-DOK-1 as well as SLP-76 and ERK1/2 monoclonal antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (New York, USA). Phospho-JAK-1 and JAK-2 were purchased from QCB. Anti-phospho-MAPK antibody was from New England Biolabs. Both Jurkat cell line and Jurkat CD45 deficient cells (J45.01) were obtained from American Type Culture Collection (ATCC, USA) and cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine (GIBCO, Maryland, USA), 50 mM Hepes.

2.2. Yeast two-hybrid screen system

Briefly, the intracellular domain of CD45 cDNA (residues 597–1304) containing the entire two tandem catalytic domains where the aspartic acid residue 819 was mutated to valine (D819V) in its catalytic domain I by site-directed PCR mutagenesis (Stratagene, La Jolla, CA). The CD45-D819V trapping mutant was fused with the LexA DNA binding domain in-frame in pBTM116 vector with independently coexpressing c-Src kinase for further phosphorylating its potential binding targets in the yeast (Keegan and Cooper, 1996). The Jurkat cDNA library with the activation domain of GAL4 (pACT2) was amplified according to the manufacturer's instructions (BD, Clonetech, CA, USA). For two-hybrid screening, L40 strain yeast cells were sequentially transformed with the bait (pBTM116/Src-CD45-D819V) and the prey Jurkat cDNA library was selected for growth following standard's protocols. Growing yeast clones were screened by β-galactosidase assay and targeted genes were further confirmed by sequencing analysis.

2.3. Plasmid construction and gene mutagenesis

Truncated CD45-D819V was expressed in N-terminal HA tag into pAcTAG2 vector (Invitrogen, CA) while full-length DOK-1 was constructed in pcDNA3.1 vector (Invitrogen, CA). Fyn (p59) in pRK5 vector was a kind gift from Dr. S. Stamm (University Erlangen, Erlangen, Germany). For the cellular distribution experiment, DOK-1 and its mutants were cloned and fused in-frame with the C-terminus of the green fluorescence protein (GFP) gene in pEGFP-C1 vector (BD Clontech, CA). For site-directed mutagenesis, the tyrosine residues of DOK-1 at 296, 337, 362, 398 and 449 sites were selected and mutated into phenylalanine (F) by PCR. These mutants DOK-1-Y296F, DOK-1-Y337F, DOK-1-Y362F DOK-1-Y398F and DOK-1-Y449F were expressed using pcDNA3.1 vector. All individual DOK-1 mutants were confirmed by DNA sequencing.

2.4. Immunoprecipitation and Western blot analysis

Approximately 1×10^8 Jurkat wild type and CD45 deficient Jurkat (CD45-) cells were transfected with 50 μg plasmid DNA by electroporation with 300 V, 10 ms in 300 μl volume using a BTX820 electroporator (BTX Corp., CA). For stable cells resistant to G418 were pooled together from each independent transfection experiment. A similar, but harsher electroporation condition was used for transient transfection in Jurkat cells over 70% of which was killed to obtain effective transfections. For anti-CD3 stimulation, Jurkat cells were treated with anti-CD3 antibody (10 μg/ml) (BD Pharmingen, CA) in cell culture and the secondary antibody (rabbit anti-mouse antibody, 20 μg/ml) for 5 min followed by adding ice-cold PBS quick wash once buffer (see below).

For immunoprecipitation, cells were washed with cold phosphate-buffered saline (PBS) once and lysed with lysis buffer containing 1% Triton, 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 1 mM PMSF, 2 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin on ice for 30 min. The lysed cells were pelleted in a microcentrifuge for 15 min at 13,000 rpm. The supernatants were immunoprecipitated and subjected to immunoblotting with appropriate antibodies as described before (Su et al., 1996). At least three Western blots were performed for each experiment to obtain consistent results, and representative results were presented in the figures.

2.5. Protein subcellular localization and confocal microscopy

Normal Jurkat and CD45 deficient Jurkat cell lines were cultured in RPMI 1640 medium as described above. Cells were transfected with 10 μg DNA of GFP-DOK-1 (or its mutants) plus 20 μl of Superfects (Qiagen Inc., Germany) in 60 mm plates overnight. The transfection efficiency was approximately 50%. For confocal microscopic examination, glass slides were pretreated and coated with 3-aminopropyltriethoxy-saline (Sigma). The transfected cells were seeded on glass slides in RPMI 1640 medium, grown overnight, and then treated with anti-CD3 antibody for 10 min, or left untreated. Cell images were taken using a Multi-