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Research paper

Development of unique antibodies directed against each of the six different phosphotyrosine residues within the T cell receptor CD3ζ chain

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ABSTRACT

Signal transduction from the T cell antigen receptor (TCR)/CD3 complex involves six different immunoreceptor tyrosine-based activation motifs (ITAM) located within the cytoplasmic tails of the CD3 chains. Each ITAM possesses two conserved tyrosine residues that can undergo phosphorylation upon TCR/CD3 crosslinking and become a docking site for SH2-containing effector molecules. Specificity of the SH2 domains is determined by their ability to bind a phosphorylated tyrosine in the context of a longer peptide motif within the target protein. As a result, phosphorylation of different tyrosines within the CD3 cytoplasmic tails creates docking sites for distinct SH2-containing signaling proteins that differentially impact on the quality of the T cell response.

In the present study, we prepared antibodies specific for each of the six different phosphotyrosines of the mouse CD3ζ chain. The antibodies were characterized with respect to their cross-reactivity, ability to recognize the phosphorylated versus non-phosphorylated forms of tyrosine-containing motifs, and cross-reactivity with the homologous phospho-motifs on the human CD3ζ protein.

The antibodies were found to be specific and selective for phospho-CD3ζ. They can serve as useful tools for distinguishing between the six potential tyrosine phosphorylation sites on the CD3ζ chain, and for correlating the phosphorylation of specific CD3ζ tyrosine residues with activation of signaling pathways that dictate T cell differentiation into responding, anergic, or apoptotic cells.

1. Introduction

T cell activation is initiated upon the engagement of the T cell antigen receptor (TCR) with a peptide-bound major histocompatibility complex (MHC) molecule, plus costimulatory signals provided by the antigen-presenting cell (APC). These initial signals lead to a rapid and transient activation of protein tyrosine kinases (PTK) that phosphorylate the cytoplasmic tails of receptor subunits, which become docking sites for SH2-containing effector molecules. The association of SH2-containing proteins, including adaptor and effector molecules, with the phosphorylated receptor subunits, creates multimolecular complexes at the receptor site that rearrange into 'supra molecular activation complex' (SMAC) (Monks et al., 1998), or immunological synapse (Grakoui et al., 1999), at the T cell-APC interface. Further activation of effector molecules, such as phospholipase C (PLC), leads to membrane phospholipid hydrolysis and formation of second messengers that activate protein kinase C (PKC) and mobilize Ca^{2+} ions from intracellular stores (Isakov et al., 1987). Full activation of these cascades can culminate in T cell

Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif;TCR, T cell antigen receptor;CD3ζ, the zeta chain of the TCR/CD3 complex;pTyr-CD3ζ, tyrosine phosphorylated CD3ζ;IB, immunoblot;IP, immunoprecipitation

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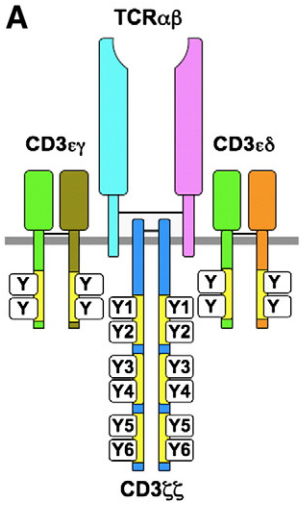
proliferation, differentiation, maturation and conversion into functionally distinct effector T cells.

The TCR includes a heterodimer of highly polymorphic α-β (or γ-δ) antigen recognition units, in association with the CD3 complex, comprising of the invariant γ-ε and δ-ε heterodimers and the ζ-ζ homodimer (see Fig. 1A). The intracellular tail of each of the CD3 heterodimeric proteins (γ-ε and δ-ε) contains an evolutionary conserved sequence, termed immunoreceptor tyrosine-based activation motif (ITAM), which serves as a substrate for PTKs and is implicated in the receptor activation response (Reth, 1989; Isakov, 1998). In addition, the ζ chain contains three copies of ITAMs arranged in tandem at the cytoplasmic portion of the receptor subunit. Each ITAM possesses two repeats of the conserved sequence Y-X-X-L/I (where Y equals tyrosine, L equals leucine, I equals isoleucine, and X represents any amino acid), spaced by six-to-eight residues. TCR triggering leads to phosphorylation of adjacent tyrosine residues within individual ITAMs, apparently by the Src-family PTKs, Lck or Fyn (Samelson et al., 1990; Straus and Weiss, 1992; van Oers et al., 1996). The phosphotyrosines serve as temporary binding sites for SH2-containing cytoplasmic effector molecules, such as the ζ chain-associated protein of 70 kDa (ZAP-70), which further transduces signals downstream of the activated receptor (Chan et al., 1991; Chan et al., 1992). The existing heterogeneity of amino acids surrounding the phosphotyrosines, in different CD3ζ ITAMs, suggests that individual phosphotyrosines interact with proteins which have distinct SH2 domains (Songyang et al., 1993).

CD3ζ is one of the earliest and most prominent tyrosine-phosphorylated proteins in TCR activated T cells (Baniyash et al., 1988). The requirement of multiple ITAMs possibly enables signal amplification or delivery of different types of signal information unique for each combination of phosphorylated tyrosines, which are sorted within the cell into distinct activation programs. Indeed, minute changes in the sequence of peptide antigens, such as one amino acid difference, could be interpreted by the TCR and translated into either a stimulatory or inhibitory response (Madrenas et al., 1995). Such peptides were found to induce the formation of distinct CD3ζ phosphorylation patterns, suggesting that quantitative or qualitative differences in CD3ζ tyrosine phosphorylation cascades may explain the different responses induced by TCR ligands that differ in just a single amino acid.

Indeed, reduced or altered patterns of tyrosine phosphorylation of the CD3ζ were found to correlate with a wide range of autoimmune diseases, including systemic lupus erythematosus (SLE) (Liossis et al., 1998; Pang et al., 2002), and rheumatoid arthritis (Maurice et al., 1997; Matsuda et al., 1998; Berg et al., 2000), as well as infectious (Stefanova et al., 1996; Trimble and Lieberman, 1998; Geertsma et al., 1999; Bronstein-Sitton et al., 2003) or malignant diseases (Mizoguchi et al., 1992; Finke et al., 1993; Matsuda et al., 1995; Chen et al., 2000).

Some of the important questions relevant to the CD3ζ include the characterization of the exact phosphorylation sites and the functional roles of the different forms of phosphorylated CD3ζ, and the rate and order of phosphorylation of the distinct tyrosine residues. Several attempts have been made to map the phosphorylation sites on the different pTyr-CD3ζ forms in T cells and thymocytes, using a combination of mutagenesis and transfection procedures, mass spectrometry, CD3ζ transgenic mice, and CD3ζ phosphotyrosine-specific antibodies (Frank et al., 1992; Koyasu et al., 1992; Kersh et al., 1998; van Oers et al., 2000). In one study, the two major phospho-CD3ζ forms in thymocytes, the 23- and



B

Mouse TCRζ-derived peptides used in this study

Peptide name	Targeted tyrosine	Peptide sequence	Peptide name	Peptide sequence	Amino acid sequence position	Homology to human TCRζ
Y1	Tyr72	c-PNQLYNELN	pY1	c-PNQLpYNELN	(68-76)	(8/9)
Y2	Tyr83	c-RREEYDVLE	pY2	c-RREEpYDVLE	(79-87)	(8/9)
Y3	Tyr111	c-QEGVYNALQ	pY3	c-QEGVpYNALQ	(107-115)	(7/9)
Y4	Tyr123	c-MAEAYSEIG	pY4	c-MAEApYSEIG	(119-127)	(9/9)
Y5	Tyr142	c-HDGLYQGLS	pY5	c-HDGLpYQGLS	(138-146)	(9/9)
Y6	Tyr153	c-TKDTYDALH	pY6	c-TKDTpYDALH	(149-157)	(9/9)

Fig. 1. A. Schematic model of the T cell antigen receptor (TCR)/CD3 complex on the surface of T cells. Each receptor includes a heterodimer of α-β highly polymorphic chains that form the clonotypic antigen recognition unit. It is associated with the CD3 complex consisting of the γ-ε and δ-ε heterodimers, and a homodimer of ζ-ζ invariant chains. Yellow regions indicate the position of the ITAMs in the CD3 chain cytoplasmic tails, and 'Y' indicates the positions of the tyrosine residues. B. Description of the six peptides and six phosphopeptides used in this study. Peptides are termed Y1-to-6 and phosphopeptides are termed pY1-to-6. The position of the tyrosine residue of each peptide, along the mouse CD3ζ chain is indicated in the second row. The third and fifth rows indicate the amino-acid sequences of each of the peptides, the sixth row indicates the position of the peptide sequence along the mouse CD3ζ chain, and the seventh row indicated the extent of homology between the human and mouse CD3ζ chains at the position of the relevant peptide. The human CD3ζ corresponding sequences are: Y1, c-QNQLYNELN; Y2, c-RREEYDVLD; Y3, c-QEGLYYNELQ (amino acids that differ between mouse and human are underlined). Full sequence identity exists between the mouse and human CD3ζ Y4, Y5 and Y6.