Immunology Letters 130 (2010) 43–50

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Analysis of novel phospho-ITAM specific antibodies in a S2 reconstitution system for TCR–CD3 signalling

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ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form 1 December 2009
Accepted 2 December 2009
Available online 6 January 2010

Keywords:
Phosphorylation
TCR signalling
Lck
Fyn
S2 cells
EM-26

ABSTRACT

The T cell antigen receptor (TCR–CD3) complex contains 12 different cytoplasmic tyrosines, each of which is part of an immunoreceptor tyrosine-based activation motif and thus occurs in similar sequence context. Since phosphorylation of individual tyrosines can be correlated with the quality of the T cell response, monitoring their phosphorylation is important. We thus generated novel antibodies against phospho-tyrosines of the TCR–CD3 complex and tested the specificity in a synthetic biology approach. We utilized the Drosophila S2 reconstitution system testing several kinases and stimulation conditions that lead to optimal phosphorylation of the TCR–CD3 subunit ζ. Expressing TCR–CD3 subunits and tyrosine mutants thereof we tested the specificity of the novel antibodies in Western blot and immunopurification experiments. In particular, we generated and characterized the monoclonal antibody EM-26 that specifically recognizes phosphorylation of the membrane proximal tyrosine of ζ (phospho-ζY1) and antisera raised against the first and the second phospho-tyrosine of CD3ε (phospho-εY1 and phospho-εY2).

1. Introduction

Many cellular processes are regulated by post-translational modifications of proteins. The most prominent one is phosphorylation on histidine, serine, threonine and tyrosine residues that controls proliferation, differentiation, metabolism, motility or activation. Protein phosphorylations can be detected using phospho-specific antibodies. In fact, phospho-reactive antibodies that recognize one certain phosphorylation site in proteins, but not related sites, are important tools in cell biology. The antibodies can be of monoclonal or polyclonal origin. Some recognize phospho-tyrosine in many different sequence contexts, as e.g. the 4G10 antibody. Others are very specific for one certain epitope (or sequence) when phosphorylated. Phospho-specific antibodies are used in many experimental procedures. These include Western blotting (WB) after SDS-PAGE, immunopurification (IP), intracellular FACS staining, ELISA and fluorescent microscopy.

In order to obtain phospho-specific antibodies mice or rabbits can be immunized with synthetic peptides (serving as the antigens) carrying a phosphorylated amino acid. Coupling of the peptide to a carrier protein is essential to mount an immune response. In case of rabbits, the serum is collected. In case of mice, monoclonal antibodies are generated by fusing the splenocytes with a myeloma cell line [1]. Hundreds of the resulting hybridomas have then to be tested for the secretion of antibodies of the desired specificity.

The first test is to check whether the new antisera or monoclonal antibodies recognize the phosphorylated antigen, but not the non-phosphorylated counterpart. In addition, similar sequences as the one used for immunization might occur in the same or a related protein from which the antigen was derived. Thus, an assay is needed to validate and test the exact specificity of the antisera or antibodies, including thorough checks for the cross-reactivity to similar phospho-sequences. For this we used a synthetic biology approach to test the specificity of novel phospho-specific antibodies raised against peptides derived from the T cell antigen receptor (TCR–CD3) complex.

The TCR–CD3 consists of the TCRαβ, CD3εγ, CD3δε and ζζ dimers in a αβεγδεζζ stoichiometry [2–4]. Its ζ, CD3ε, CD3δ and CD3γ subunits contain immunoreceptor tyrosine activation motifs (ITAMs) of the consensus sequence YxxL/IxxxxxxxYxxL/I in which both tyrosines can be phosphorylated [5]. The ζ chain contains three ITAMs (Fig. 2A) and CD3ε, CD3δ and CD3γ each one ITAM. Thus, the

Abbreviations: BCR, B cell antigen receptor; ITAM, immunoreceptor tyrosine activation motif; TCR–CD3, T cell antigen receptor; WB, Western blotting.

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doi:10.1016/j.imlet.2009.12.011

TCR–CD3 complex has 12 ITAM tyrosines in a similar sequence context [6–8]. These tyrosines are phosphorylated by Src-kinase family members as Lck and Fyn [9]. Human, but not mouse ζ contains an additional tyrosine (ζY0).

Phosphorylation of the ITAMs is critical for TCR signalling, since they serve as docking sites for SH2 domain containing proteins, as e.g. Syk and ZAP70 [9,10] which contain tandem SH2 domains capable of binding to the two phospho-tyrosines of an ITAM with high affinity. Together with a structural change in CD3 [11,12], ITAM phosphorylation is the main activation event of the TCR–CD3 complex [13,14].

Although very similar in sequence, not all phospho-tyrosines of the TCR–CD3 complex might fulfill the same function. Recognition of an agonistic ligand by TCRαβ results in the phosphorylation of all six ζ tyrosines (Y1–Y6) [15,16] and in the activation of the T cell. In contrast, recognition of antagonistic ligands only leads to the phosphorylation of the four membrane distal ζ tyrosines (Y3–Y6) without T cell activation. Phosphorylated ζ can be recognized by general anti-phospho-tyrosine antibodies [15,16]. However, the distinction of the individual tyrosines is not possible with these antibodies. Since ζ shows reduced mobility in SDS-PAGE upon phosphorylation, a correlation between the amount of phospho-tyrosines and the migration distance can be made. In addition, antisera against all six phospho-tyrosine-containing sequences in ζ have been described [17].

Phosphorylation of the other subunits (CD3ε, CD3δ and CD3γ) cannot easily be detected by anti-phospho-tyrosine WB, since commercially anti-phospho-tyrosine antibodies do not recognize these chains and since these proteins do not show a gelshift upon phosphorylation. However, phosphorylation was recently shown by mass spectroscopy [18]. Thus, new phospho-specific antibodies reactive against TCR–CD3 tyrosines are desirable.

Here, we generated two phospho-specific antisera and one monoclonal antibody that are specific for certain phospho-tyrosines of the TCR–CD3 complex. To test the specificity of these novel reagents, we used a Drosophila S2 cell reconstitution system developed in our lab [19–22]. S2 cells can be transiently co-transfected with several different plasmids and thus are an ideal tool for the reconstitution of small signalling modules. Indeed, co-expression of the B cell antigen receptor (BCR) with B cell kinases led to the phosphorylation of the BCR and new insights into the interaction of the BCR with the kinases was obtained [19].

2. Materials and methods

2.1. Cells and reagents and antibodies

Schneider S2 cells (gift from Klaus Karjalainen) were grown in Schneider's Drosophila medium revised (SERVA) supplemented with 5% FCS and glutamine as described [19].

Besides the new antibodies described here, the following ones were used: anti-CD3ε (M20, Southern Biotech.), anti-phosphotyrosine (4G10, UBI) and anti-GFP (full length a.v. polyclonal antibody, Living Colors). The rabbit anti-ζ antiserum 448 has been described [23]. HRPO-conjugated secondary antibodies were purchased from Pierce.

2.2. Immunization of mice

In order to obtain monoclonal antibodies, Balb/c mice were biweekly immunized subcutaneously with 30 μg and intrasplenicly with 10 μg of KLH conjugated synthetic peptide surrounding the membrane proximal phospho-tyrosine of ζ (tyrosine number 1, ζY1). The sequence of the peptide was CLQDPNQLY(p)NELNLGR corresponding to the murine sequence. The first subcutaneous immunization was performed with complete Freud's adjuvant and followed by two injections in incomplete Freud's adjuvant. Intrasplenic immunization was done in incomplete Freud's adjuvant. Ten days after the second and third immunization, the animals were tail-bled and the immune response to antigen measured by ELISA.

2.3. Cell fusion

The mouse selected for generation of monoclonal antibodies was boosted i.v. with 5 μg of antigen in saline. Four days later the spleen was harvested and used for the hybridoma production. The 350×10^6 spleen cells were fused to 60×10^6 Sp2/0 myeloma cells using polyethyleneglycol 1500 (STEMCELL Technologies, Canada) according to the manufacturer's manual. The fused cells were initially seeded in tissue culture plates containing semi-solid ClonaCell®-HY Selection and Cloning Medium D (STEMCELL Technologies, Canada). 672 hybridoma clones were picked from semi-solid medium after 14 day of growth. Primary screening to test positive clones for the production of anti-phospho-tyrosine 1 antibodies was performed using an ELISA assay. All animal experiments were performed according to Czech Central Commission for Animal Welfare guidelines.

2.4. ELISA

ELISA plates were coated for 5 h with 1 μg/ml of phospho-peptide-coupled BSA, dephosphorylated-peptide-coupled BSA (the peptide was identical to the one used for the immunizations) or 2.5 μg/ml of an irrelevant phospho-peptide-coupled BSA in carbonate buffer at 37 °C. Blocking was performed with 1% BSA at 4 °C overnight. Culture tissue supernatants samples of individual hybridoma clones were added (50 μl per well) and the plates incubated for 1 h at 37 °C. The presence of antigen-specific antibodies was detected with horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG (Jackson Immunoresearch, USA) diluted 1:4000. 50 μl of HRPO substrate *o*-phenylenediamine (OPD) (Sigma–Aldrich, USA) was added to each well and the reaction was stopped 10 min later with 50 μl 2 M H_2SO_4. The absorbance of the ELISA plates was read at 492 and 630 nm.

2.5. Generation of expression vectors

The cDNAs of the proteins of interest were inserted into the Drosophila expression vector pRmHa-3 containing an inducible metallotionein promotor [24]. pRmHa-3 is abbreviated as pD.

pDhζ encodes for human ζ. The cDNA was amplified from pSRαhζ (gift of Balbino Alarcon) with AGCTCGAGCAAGATGAAGTGGAAGGC and GCGATATCTGTTAGCGAGGGGGCAGGGCC and subcloned into pBluescript. Subsequently, it was cut with EcoR1 and Kpn1 and ligated into the same sites of pRmHa-3. pDhζYxyF mutants in which the tyrosines with the number *x* and *y* were changed to phenylalanine (Fig. 2A) were generated from pDhζ by site directed mutagenesis using the Quickchange-Kit from Stratagene. In pDhζY0F a tyrosine that is between the transmembrane part and the membrane proximal tyrosine of the membrane proximal ITAM (Y1) was mutated. Y0 does not exist in mouse.

To obtain pDhε the plasmid pSRαhε (gift of Balbino Alarcon) was cut with XhoI and the cDNA-encoding fragment cloned into the SalI site of pRmHa-3. pDhεY1F/D/E and pDhεY2F/D/E mutants in which the ITAM tyrosines 1 (membrane proximal) or 2 (membrane distal) were changed to phenylalanine, aspartic acid or glutamic acid were generated from pDhε by site directed mutagenesis.

The plasmid pUC19Fyn was cut with BamHI and the sequence coding for Fyn was ligated into pRmHa-3 cut with the same enzyme to yield pDFyn. The plasmid pSMLck (gift of Arthur Weiss) was