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Research paper

Measuring the specific activity of the protein tyrosine phosphatase Lyp

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ABSTRACT

Altered function of the protein tyrosine phosphatase (PTP) Lyp (PTPN22) has been implicated in the pathogenesis of a number of human diseases, and so accurate assessment of its functional activity is needed to further our understanding of its biology. We have developed an *in vitro* method to measure the specific catalytic activity of the Lyp phosphatase. Lyp is captured from cell lysates using an anti-Lyp monoclonal antibody coated 96-well plate, and activity measured by dephosphorylation of a fluorescent substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). The amount of protein is measured using an anti-Lyp HRP conjugate, with reference to a standard curve generated with purified Lyp. These two measurements are then used to calculate the specific phosphatase activity. We used this assay to show that the specific activity of the Lyp phosphatase is decreased by H_2O_2 in Jurkat T cells and primary CD4+ T cells. We also modified this assay to measure the specific activity of CD45, the other main PTP regulating T cell receptor (TCR) signalling, in order to compare the relative susceptibility of CD45 and Lyp to oxidation by H_2O_2. By measuring specific activity in Jurkat T cells and primary CD4+ T cells, we demonstrated that CD45 is more susceptible to oxidation by H_2O_2 when compared to Lyp. Reduced function of CD45 and Lyp has been associated with human immune mediated inflammatory diseases, and a differential susceptibility to oxidation could be an important regulatory mechanism associated with both physiological and pathological changes in signalling through the TCR.

1. Introduction

Protein tyrosine phosphatases (PTP's) play an important role in regulating the nature and duration of physiological immune responses, through their influence on immune cell signalling. The cytoplasmic PTP Lyp, encoded by the PTPN22 gene, is involved in the negative regulation of signal transduction through the T cell antigen receptor, via its ability to down-regulate activity of the Src family kinase Lck (Hill et al., 2002). Mice lacking PEP, the mouse homologue of Lyp, exhibit increased function and expansion of the T effector cell population, elevated serum antibody levels, and spontaneous formation of germinal centres (Hasegawa et al., 2004) suggesting that the normal role for Lyp is to suppress immune responses. This phenotype is likely to be a result of increased signalling and responses mediated through the T cell receptor (TCR), as the negative regulatory effect of Lyp on Lck is absent in these mice. The other main PTP regulating TCR signalling is the membrane PTP CD45. Using cell lines lacking CD45, a positive regulatory role for this PTP has been identified (Desai et al., 1994), which is confirmed by observations in human subjects deficient in CD45, who develop a severe-combined immunodeficiency (SCID) (Kung et al., 2000). Similarly to Lyp, CD45 is able to regulate the activity of Lck, but with an opposing effect of increasing the activity of the kinase.

Alterations in the function of CD45 and Lyp have been associated with human disease. Impaired CD45 function has been observed in patients with HIV and SLE (Takeuchi et al., 1997; Giovannetti et al., 2000), and even in healthy ageing individuals (Rider et al., 2003), implicating alteration in PTP

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activity as a contributor to the decline in T lymphocyte function associated with ageing. In comparison to CD45, the function and regulation of Lyp are less well known. Lyp activity is known to be altered by a common single nucleotide polymorphism (SNP) in the PTPN22 gene, known as PTPN22 R620W, the consequences of which are reviewed in Burn et al. (2011). This functional polymorphism is perhaps the leading example of a gene in which a polymorphic variant confers risk of developing a diverse range of human immune mediated inflammatory diseases. However, the effect of the SNP on Lyp activity and the mechanism by which this occurs remain unclear. In addition, reduced Lyp function through oxidation by nitric oxide donors has been observed *in vitro* (Karver et al., 2011), but it is not known if oxidation by this mechanism or others occurs *in vivo* in human cells. It is thought that oxidative regulation of PTP's is an important physiological and pathological mechanism (Ostman et al., 2011). Based on these observations, accurate measurement of the activity of CD45 and Lyp is likely to be important in understanding their role in the context of human disease.

At present there is no suitable assay which allows measurement of the specific activity of the Lyp phosphatase in primary cells. Techniques commonly used include immunoprecipitation (Orrú et al., 2009; Fiorillo et al., 2010) of purified protein, following transfection (Cohen et al., 1999) or over-expression (Hill et al., 2002). However, in these approaches there is no accurate correlation between the amount and activity of Lyp.

We have developed a sensitive and specific method to assess Lyp activity which does not require the use of potentially confounding methodological techniques, such as over-expression. The increased sensitivity provided by our new assay means that low cell numbers can be used to compare the specific activity of CD45 and Lyp from cells cultured in different conditions, or cells taken directly from diseased individuals. This provides novel insights into how the opposing functions of CD45 and Lyp are affected by specific treatments, and in different human disease states. In addition, this method provides a platform for further investigation of how these changes in CD45 and Lyp function alter overall signalling through immune cell receptors.

2. Materials and methods

All reagents were purchased from Sigma unless otherwise stated.

2.1. Experimental procedures

2.1.1. Cell culture

Jurkat T cells were grown in RPMI 1640 supplemented with 10% HIFCS and 1% GPS in a 5% CO_2 humidified incubator. All experiments were carried out with cells that had received fresh medium 24 h previously, and cultures were maintained at a density between 1.0×10^6 and 2.0×10^6 cells/ml. Peripheral blood mononuclear cells (PBMC's) were obtained from healthy volunteers following informed consent, and isolated by Ficoll-Paque Plus (GE Healthcare, UK) density gradient centrifugation. Human CD4+ T cells were isolated from the PBMC population by negative selection using a CD4+ T cell isolation kit (Miltenyi Biotech, Germany). T cell purity was >90% as assessed by flow cytometry using antibodies to CD3 and CD4 (Immunotools, Germany). Human synovial fibroblasts were isolated as described previously (Salmon et al., 1997), and cultured in RPMI 1640 supplemented with 10% FCS, 1% GPS, MEM-non-essential amino acids and 1 mM sodium pyruvate in a 5% CO_2 humidified incubator.

2.1.2. Procedure for measuring Lyp phosphatase activity and amount captured

The activity of the Lyp phosphatase was assessed using a fluorimetric method modified from Rider and Young (2003) by measuring dephosphorylation of an alternative fluorescent substrate 6, 8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Invitrogen, USA). Protein A (10 μg/ml) in carbonate–bicarbonate buffer (50 mM, pH 9.6) was added to wells of a 96-well Maxisorp™ plate (Nunc, Denmark) overnight at 4 °C. Wells were washed three times using PBS/0.05% Tween 20, and 5 μg/ml anti-human Lyp mAb, (Clone 340113, R and D Systems, USA) in 1% BSA/PBS/0.05% Tween 20 was added for 2 h at 37 °C to allow antibody binding to protein A. Wells were washed as before and blocked with 2% BSA/PBS/0.05% Tween 20 for 1 h at 37 °C. Jurkat T cells or purified CD4+ T cells were washed twice in Hank's buffered salt solution (HBSS), once in ice-cold PBS/0.05% Tween 20 and then lysed in TNE buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) supplemented with protease inhibitors (Roche Diagnostics, USA). Cell debris was removed by centrifugation at 13,000 $\times g_{av}$ for 10 min at 4 °C. After aspiration of the blocking solution, cell lysate was added to the wells and incubated for 3 h at 37 °C. Wells were washed three times and the activity of Lyp was detected by the addition of 0.2 mM DiFMUP in phosphate reaction mixture (0.1 M sodium acetate, 1 mM EDTA, 0.2% Triton X-100, pH 6.0) with 50 mM DTT. The accumulation of fluorescent product was measured at 37 °C over a 2 hour time period using a Fluoroskan Ascent plate reader (Labsystems, UK) with the 355/460 nm filter pair. Parallel wells bound with Lyp protein from identical lysates were probed with chicken anti-human Lyp-HRP polyclonal antibody for 1 h at 37 °C. The antibody was made by setting up a conjugation reaction using anti-Lyp pAb and HRP (Innova Biosciences, UK). The standard ELISA substrate tetramethylbenzidine (TMB) was added, and the amount of Lyp was determined by comparison to a standard curve using purified Lyp protein (Abnova, Taiwan).

2.1.3. Comparing the susceptibility of CD45 and Lyp to oxidation

For comparative purposes, CD45 phosphatase activity was detected by modifying the method above described for measurement of Lyp phosphatase activity. CD45 protein was captured using anti-CD45 mAb (MCAP87 AbD Serotec, UK) and amount bound detected using sheep anti-human CD45-HRP polyclonal antibody (Binding Site, UK).

2.1.4. Measurement of maximum fluorescence intensity

Serial dilutions of alkaline phosphatase (AP) were mixed with various concentrations of DiFMUP and readings taken every 30 s at 355/460 nm using a Fluoroskan Ascent plate reader. The increase in fluorescence intensity (FI) following cleavage of 1 nmol of substrate was calculated by dividing the maximum FI obtained for each concentration of AP by the number of moles of DiFMUP present in the wells.