Molecular Immunology 53 (2013) 111–117

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

ZAP-70 tyrosines 315 and 492 transmit non-genomic glucocorticoid (GC) effects in T cells

F. Boldizsar*,[1], M. Szabo[1], K. Kvell, T. Czompoly, G. Talaber, J. Bjorkan, D. Bartis, P. Nemeth, T. Berki

University of Pecs, Department of Immunology and Biotechnology, H-7624, Pecs, Szigeti ut 12, Hungary

ARTICLE INFO

Article history:
Received 3 May 2012
Received in revised form 20 July 2012
Accepted 21 July 2012
Available online 13 August 2012

Keywords:
Non-genomic GC action
ZAP-70
TcR signaling
SLP-76
Cbl

ABSTRACT

ZAP-70 kinase is a key regulator of early T-cell signaling; moreover, it also participates in non-genomic glucocorticoid (GC) signaling. Short-term high-dose GC-analogue treatment induces the phosphorylation of the kinase, and its association with the GC receptor (GR).

In the present work, first, we identified those tyrosine (Y) residues of the ZAP-70 kinase which were involved in non-genomic GC signaling using an array of P116 cells (ZAP-70-deficient Jurkat subclone) lentivirally-transfected with wild type or point-mutated ZAP-70 constructs where Y-residues were replaced with phenylalanine (F) at positions 069, 126, 178, 238, 292, 315, 492 or 493. Then, we characterized the GC-analogue-induced Y-phosphorylation of 3 key substrates of the ZAP-70 kinase: SLP-76, LAT and Cbl. Finally, we studied the cross talk between the non-genomic GC- and TcR/CD3 signaling pathways.

Y-F mutations at positions 315 or 492 abolished the short high-dose Dexamethasone (DX) treatment-induced ZAP-70 phosphorylation suggesting that these Y-residues were involved in ZAP-70-mediated non-genomic GC actions. DX treatment alone induced Y-phosphorylation of LAT, SLP-76 and Cbl; moreover, in F315- and F492-ZAP-70 mutated cells decreased DX-induced Y-phosphorylation of SLP-76 and Cbl was observed indicating that these molecules might transmit downstream non-genomic GC signals in a ZAP-70 dependent manner. Short, high dose DX treatment influenced significantly the anti-CD3-induced signaling events: we observed alterations in LAT, SLP-76 and Cbl Y-phosphorylation and a decreased Ca^{2+}-signal.

These results confirm that ZAP-70 represents an important link between the non-genomic GC and TcR/CD3 signaling pathways. Importantly, the DX-induced effects on resting and activated T-cells are differentially mediated. These fine molecular details help to better understand the complex mechanism of non-genomic GC effects in T-cells.

1. Introduction

Physiologically, glucocorticoid (GC) hormones are produced in the adrenal cortex, and their analogues are widely used as therapeutic agents (Singer, 1972; Swartz and Dluhy, 1978) in the treatment of autoimmune diseases, allergy and asthma, and in the prevention of graft rejection in transplantation (Flammer and Rogatsky, 2011; Krishnan et al., 2009; Hricik et al., 1994) based on their immunosuppressive and anti-inflammatory effects. GC-analogue treatment results in the apoptosis of many cell types in the immune system, especially mature (Th1 or Th2) and immature ($CD4^+CD8^+$ double positive thymocytes) T cells, and, as a consequence, the number of circulating T cells decreases. Moreover, GCs inhibit T cell signaling and cytokine – especially IL-2 – production (Stahn et al., 2007; Zen et al., 2011).

GCs exert most of their effects via the GC receptor (GR) through distinct intracellular signaling pathways (Buttgereit and Scheffold, 2002; Boldizsar et al., 2010). In the classical genomic pathway, ligand molecules induce the dissociation of the GR from chaperone molecules in the cytoplasm and this receptor–ligand complex translocates to the nucleus where it associates to GC responsive elements (GRE) in the promoter region of several genes, leading to changes in the transcription of cells (Nicolaides et al., 2010).

Abbreviations: ATPase, adenosine-triphosphatase; Cbl, Casitas B-lineage Lymphoma; CD, cluster of differentiation; DX, dexamethasone; DMSO, dimethyl sulfoxide; F, phenylalanine; DP, double positive; Fluo-3AM, Fluo-3-acetoxymethyl ester; GC, glucocorticoid; GR, GC receptor; Hsp-90, heat shock protein-90; IP, immunoprecipitation; ITIM, immunoreceptor Y-based inhibitory motif; Itk, IL2-inducible T cell kinase; IL2, interleukin 2; LAT, linker of activated T cells; Lck, lymphocyte-specific protein Y kinase; MAPK, mitogen activated protein kinase; PKB, protein kinase B; PKC, protein kinase C; PLCγ, phospholipase Cγ; PVDF, RPMI, Roswell Park Memorial Institute tissue culture medium; RT, room temperature; SH2, Src homology 2; SLP-76, SH2 domain containing leukocyte protein of 76 kDa; Syk, spleen Y kinase; TcR, T cell receptor; Th1, T-helper1; Th2, T-helper2; WB, Western blot; WT, wild type; Y, Y; ZAP-70, zeta-chain-associated protein kinase of 70 kDa.

* Corresponding author. Tel.: +36 72 536 288; fax: +36 72 536 289.
E-mail address: ferenc.boldizsar@aok.pte.hu (F. Boldizsar).

[1] These authors contributed equally to the work.

0161-5890/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2012.07.007

However, not all effects of the GCs can be explained by this genomic mechanism. Recently, accumulating evidence is available about non-genomic GC effects usually precipitated by high-dose GC treatment within a short period of time (Falkenstein et al., 2000). Non-genomic GC effects include: (i) direct effects in the plasma membrane; (ii) interactions between the activated GR and other cytoplasmic signaling molecules; (iii) signals mediated through the membrane GR; or (iv) mitochondrial effects (Buttgereit and Scheffold, 2002; Boldizsar et al., 2010). T lymphocytes are key regulators of the immune response; thus, inhibiting their activation is in the focus of immunosuppressive therapies. Importantly, some of the non-genomic GC effects were originally described in T-cells, which raised the possibility that the T cell suppressive effects of GCs might, at least partially, be mediated by non-genomic effects (Reichardt et al., 2006; Schweingruber et al., 2012).

For example, in activated murine thymocytes GCs inhibited the activation of Na^+–K^+-ATPase and Ca^{2+}-ATPase (Buttgereit and Scheffold, 2002), and the Na^+/H^+ exchange could also be inhibited with GCs (Chang et al., 2010). Membrane GR expression has been observed first in the S-49 mouse T lymphoma cells and the CCRF-CEM human T cell lymphoblast like cell line, and later on human peripheral lymphocytes and monocytes; however, its exact structure and signaling mechanisms are still unclear (Gametchu et al., 1993). According to recent results, the membrane and the cytoplasmic forms of the human GR are both encoded in the same gene, moreover, the mGR induces p38 MAPK phosphorylation upon ligand binding (Strehl et al., 2011).

Mitochondrial translocation of the GR was observed in GC sensitive cells including DP thymocytes upon short time GC treatment (Sionov et al., 2006b,a; Talaber et al., 2009). In activated human $CD4^+$ T-cells short high dose GC treatment inhibited the phosphorylation of several signaling molecules of the TcR/CD3 pathway, including early participants like Lck and Fyn as well as downstream targets including PKB, PKC and members of the MAPK cascade (Lowenberg et al., 2005). Moreover, GCs disrupted the activation induced TcR–Lck–Fyn complex containing the Hsp-90 and the GR (Lowenberg et al., 2006).

Our earlier observations in Jurkat cells (human T-cell leukemia) showed that short-term high-dose Dexamethasone (henceforth DX) – a widely used synthetic GC-analogue – treatment led to Y-phosphorylation of the ZAP-70 kinase, a key molecule of T-cell activation, and enhanced the association between the ZAP-70 and GR most likely as a consequence of non-genomic GC effect. Moreover DX further increased the anti-CD3 induced Y-phosphorylation of the kinase (Bartis et al., 2006, 2007). This was a rather unexpected result, since Y-phosphorylation of the ZAP-70 is usually associated with T cell activation, whereas GC analogues are used as inhibitors of T cell activation. These observations led us to hypothesize that the non-genomic GC effects or the TcR/CD3 pathways could lead to the phosphorylation of different (possibly inhibitory) Y-residues in the ZAP-70 kinase. These fine Y-phosphorylation pattern differences could, in turn, regulate T cell activation.

In our present work, we aimed to elucidate which Y residues of the ZAP-70 kinase were involved in non-genomic GC actions. For this purpose, we used transgenic P116 cell lines expressing point-mutated ZAP-70, where Y at residues 069,126, 178, 238, 292, 315, 492 and 493 were replaced with phenylalanine (F). Here, we show that Y315 and Y492 of ZAP-70 kinase are two candidate residues participating in non-genomic GC signaling. Short high dose DX treatment led to the Y-phosphorylation of important downstream ZAP-70 target molecules LAT, SLP-76 and Cbl, which was disrupted by Y-F point mutations at residues 315 and 492. Finally, GC-analogue treatment influenced the CD3 activation-induced phosphorylation of SLP-76, LAT and Cbl and the intracellular Ca^{2+} signal. These results contribute to the better understanding of the fine molecular details of non-genomic GC action in T cells.

2. Materials and methods

2.1. Chemicals and buffers

All fine chemicals were obtained from Sigma unless otherwise stated.

2.2. Cell lines

We used Jurkat (ATCC TIB-152) or transgenic P116 (ZAP-70 deficient Jurkat subclone, ATCC CRL-2676) cells lentivirally transfected with the WT or point mutated ZAP-70. In the point-mutant cells Ys were replaced with F at residues 069, 126, 178, 238, 292, 315, 492 and 493 as described (Szabo et al., 2012). Cells were cultured in RPMI supplemented with 10% fetal calf serum (Gibco), sodium-pyruvate (1 mM) and glucose (4.5 g/l), penicillin and streptomycin under conventional conditions (37 °C, humidified atmosphere, containing 5% CO_2).

2.3. Antibodies

For immunoprecipitation the following antibodies were used: rabbit polyclonal anti-ZAP-70 (a kind gift from Prof. E. Monostori, Szeged, Hungary), mouse monoclonal anti-SLP-76 (clone F-7; 2 μg/sample), mouse monoclonal anti-LAT (clone 11B.12; 2 μg/sample) and mouse monoclonal anti-Cbl (clone A-9, 2 μg/sample) antibodies from Santa Cruz Biotechnology.

The following antibodies were used for Western blotting: mouse monoclonal anti-phospho-Y (clone PY20, 1:5000) and anti-ZAP-70 (clone 29/ZAP-70 kinase, 1:5000) antibodies from BD Pharmingen (San Jose, CA, USA); mouse monoclonal anti-β-actin (clone AC-74, 1:50,000) was from Sigma; rabbit polyclonal anti-SLP-76 (1:1000), anti-LAT (1:500) and anti-Cbl (1:500) antibodies were from Santa Cruz Biotechnology (Santa Cruz CA, USA). Mouse monoclonal anti-GR (clone 5E4, 1:2000) was produced in our laboratory (Berki et al., 1998). HRPO conjugated goat anti-mouse IgG (Hunnavix, Hungary, 1:1000) or anti-rabbit IgG (Pierce 1:1000) were used as secondary antibodies.

2.4. DX and anti-CD3 treatment of the cells

Before activation, cells were resuspended in serum free RPMI. DX was added to 10^7 cells in 100 μl RPMI at a final concentration of 10^{-5} M (corresponding to high-dose treatment in human therapy (Buttgereit et al., 2004; Buttgereit and Scheffold, 2002) for 2 min. For the activation of the TcR/CD3 pathway, anti-human-CD3 (clone OKT-3; ATCC CRL-8001) 5 μg/10^6 cells was used for 2 min. For combined treatment cells were pre-incubated with DX for 2 min followed by anti-CD3 activation for additional 2 min. Treatments were performed at 37 °C under continuous shaking (Thermo Mixer, Eppendorf, Germany). The reactions were stopped in liquid nitrogen. For the intracellular Ca^{2+} measurement, cells were pre-treated with 10^{-5} M DX for 10 min followed by activation with anti-CD3.

2.5. Western blot, immunoprecipitation

Resting (untreated control), DX-, and/or anti-CD3-treated cells were lysed in Triton-X lysis buffer (50 mM HEPES, 10 mM sodium pyrophosphate, 10 mM EDTA, 100 mM sodium fluoride, 10% glycerol and 1% Triton-X 100, pH 7.3) complemented freshly with protease inhibitor and Na-orthovanadate. Lysis was performed for 30 min on ice then samples centrifuged for 10 min at 13,000 rpm. The supernatant was either used for immunoprecipitation or boiled immediately in SDS sample buffer (125 mM Tris, 4% SDS, 10% glycerol, 0.006% Bromo-phenol-blue and 10% mercaptoethanol) for 10 min.