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Intermolecular relations between the glucocorticoid receptor, ZAP-70 kinase, and Hsp-90

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Abstract

The glucocorticoid receptor (GR) participates in both genomic and non-genomic glucocorticoid hormone (GC) actions by interacting with other cytoplasmic signalling proteins. Previously, we have shown that high dose Dexamethasone (DX) treatment of Jurkat cells causes tyrosine phosphorylation of ZAP-70 within 5 min in a GR-dependent manner. By using co-immunoprecipitation and confocal microscopy, here we demonstrate that the liganded GR physically associates with ZAP-70, in addition to its phosphorylation changes. The association of the ligand-bound GR and ZAP-70 was also observed in HeLa cells transfected with ZAP-70, suggesting that this co-clustering is independent of lymphocyte specific factors. Furthermore, the ZAP-70 was found to also co-precipitate with Hsp-90 chaperone both in Jurkat and transgenic HeLa cells, independent of the presence of DX. These findings raise the possibility that ZAP-70 may serve as an important link between GC and TcR-induced signaling, thereby transmitting non-genomic GC action in T-cells.

Keywords: ZAP-70; Glucocorticoid receptor (GR); Hsp-90; Non-genomic glucocorticoid effects; Jurkat cells

Glucocorticoid hormones (GCs) are lipophilic steroid molecules that can freely diffuse through biological membranes [1]. The ligand-free glucocorticoid receptor (GR) is a component of a multimeric protein complex in the cytoplasm. This multi-protein complex consists of heat-shock proteins, several immunophillins, and the inactive GR [2]. Upon ligand binding, the GR dissociates from this multi-protein complex, dimerises and translocates into the nucleus, where it serves as transcription factor [3]. In the nucleus the GR binds to specific DNA sequences called glucocorticoid response elements (GRE) [4]. This signalling mechanism of the GCs is called the classical, genomic pathway. The activated GR also interacts with a number of other transcription factors e.g., STATs, AP-1, NFκB, and octamer transcription factors [5].

GC effects exerted through the genomic pathway result in changes in the gene expression pattern and they need *de novo* protein synthesis to occur [5]. Nevertheless, there are GC effects that cannot be explained on the basis of the above, genomic pathway. In the clinical practice, GCs are used in the treatment of neurotraumatic cases, such as acute spinal cord injury [6] and acute allergic diseases e.g., anaphylactic reactions [7,8], where rapid actions are required. Due to the lack of time for gene expression changes, these prompt GC effects are considered to be non-genomic effects.

It has been shown that the early steps of TcR-signalling events are inhibited by GC exposure [9]. GCs also inhibit the phosphorylation of proximal T-cell signalling molecules in T-cell hybridomas and murine thymocytes [10]. GC hormone treatment altered the lipid composition of membrane lipid rafts of a murine T-cell hybridoma, thereby altering the raft association and palmitoylation of key signalling molecules [11]. However, the GC effects needed

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hours to develop in the above works and only few data were available until recently about the signalling mechanisms induced by rapid steroid effects which occur within minutes.

Protein–protein interactions are a possible way to exert non-genomic GC functions as proposed in the model by Buttgereit and colleagues [12]. Some protein–protein interactions are already known concerning the GR. The unliganded GR is complexed with Hsp-90 chaperone in the cytoplasm [2]. The translocation of the liganded GR dimers into the nucleus is supported by cytoskeletal elements [13]. The Hsp-90 also participates maintaining the signalling function of the p56-lck kinase [14]. Since ZAP-70 is one of the main substrates of p56-lck [15], and the ligand-free GR is complexed by Hsp-90, we assume that these molecules are in the same macromolecular compartment in the cytoplasm. A recent study described that the glucocorticoid hormone modulates the activity of lck and fyn kinases in peripheral blood Th cells [16].

In a previous work, we have shown that Dexamethasone (DX), a GR agonist causes rapid p56-lck dependent tyrosine phosphorylation of ZAP-70 in Jurkat cells, which could be inhibited by GR antagonist pre-treatment [17]. Here, we report that in the presence of its agonist, the GR co-precipitates with ZAP-70 in Jurkat cells and in HeLa cells transfected with ZAP-70. We confirmed this GC-induced molecular association with confocal microscopy. The co-localization of the GR, ZAP-70, and Hsp-90 may explain our previous findings [17] suggesting possible functional cross-link between the signaling pathways of TcR–CD3 complex and glucocorticoid hormone, which may participate in the fine tuning of T-cell response, thymic selection, and apoptosis processes.

Materials and methods

Cell lines. Jurkat cells, P116 (ZAP-70 deficient Jurkat subclone), and HeLa cells were cultured at 37 °C in humidified atmosphere, containing 5% CO_2, in RPMI medium supplemented with 5% (Jurkat cells) and 10% (HeLa and P116 cells) fetal calf serum (Gibco).

Lentivirus production and transduction. Human full-length wild-type ZAP-70 cDNA has been inserted in the pWPTS lentiviral transfer plasmid under the control of an EF1 promoter as published elsewhere [18]. Lentivirus production and transduction of P116 and HeLa cell lines were performed as described elsewhere [19,20].

Chemicals and buffers. All fine chemicals were purchased from Sigma–Aldrich, otherwise indicated. Dexamethasone (DX) and Geldanamycin (GA) were dissolved in DMSO at a concentration of 4 and 1 mg/ml, respectively. For intracellular free calcium measurement Fluo-3 AM (Molecular Probes), 1 mg/ml stock solution was dissolved in Pluronic-F-127 + DMSO. For the Western blot experiments, cells were lysed in an ice-cold TEM buffer containing 50 mM NaCl, 10 mM Tris–HCl, pH 7.6, 4 mM EDTA, 20 mM sodium molybdate, and 10% glycerol). Aprotinin, leupeptin (10 μg/ml), and PMSF (2 mM) were freshly added to the buffer. For washing Western blots washing buffer (10 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Tween-20) was used. The intracellular labelling for confocal microscopy was performed in saponine buffer (0.1% saponine, 0.1% BSA, and 0.1% azide in PBS).

Antibodies. Immunoprecipitation was performed either with polyclonal anti-ZAP-70 antibody generated by immunizing rabbits with a peptide corresponding to the amino acids 485–499 of ZAP-70 sequence, or mouse monoclonal anti-GR antibody (clone 8E9) produced in our laboratory [21]. For Western blotting, mouse monoclonal anti-ZAP-70 (clone: 29, Transduction Laboratories) mouse monoclonal anti-GR antibody (clone 5E4, [21]), and rabbit polyclonal anti-HSP-90 (Santa Cruz Biotechnology) were used. HRPO-conjugated goat anti-mouse IgG (Hunnavix) and anti-rabbit IgG (Pierce) were applied as secondary antibodies. For confocal microscopy, anti-GR-FITC (5E4), and phycoerythrin-conjugated mouse monoclonal anti-ZAP-70 antibodies (eBioscience, 1E7.2) were used. FITC and PE-conjugated mouse IgG1 isotype control antibodies (DakoCytomation) were applied as negative controls.

Geldanamycin and Dexamethasone treatment of cells. Cells were incubated overnight in complete RPMI medium in the presence of 1.78 μM Geldanamycin or solvent (DMSO). After the incubation, cells were washed in serum-free RPMI and subjected to DX treatment as described previously [17]. Briefly, cells were resuspended in RPMI at a concentration of 10^8/ml and incubated at 37 °C with 10 μM DX or solvent for 5 min. After incubation, the reaction was stopped by placing the tubes in liquid nitrogen (for Western blots) or with ice-cold PBS-azide (for microscopy).

Lysis, immunoprecipitation and Western blot. Ten million cells were lysed in 500 μl TEM buffer by sonication on ice. Postnuclear supernatants were aspirated and subjected to immunoprecipitation. Equal amounts of cell lysates were incubated on a rotator platform at 4 °C with 30 μl slurry of protein G coupled Sepharose beads (Amersham) for 30 min. After the removal of the pre-clearing beads, 10 μl antibody was added for 2 h. Then protein G–Sepharose beads were added for additional 2 h. Beads were washed five times with ice-cold washing buffer. The electrophoresis and Western blotting of the samples were performed as described previously [17].

Confocal microscopy. Cells were fixed in 4% paraformaldehyde and washed in saponine buffer. The labelling of the cells was performed in saponine buffer with an antibody concentration of 1 μg/ml. After 1 h incubation on ice, the cells were washed twice in saponine buffer and layered onto slides. The excess fluid was carefully aspirated and the slides were covered using 50% glycerol–PBS. In case of HeLa–trZAP-70 cells, the above process was performed on cell monolayers. The examination of the samples was carried out using an Olympus Fluoview 300 confocal microscope or later a Olympus Fluoview FV1000S-IX81 system.

Ca-signal measurement. Intracellular free calcium was measured according to the protocol previously described by Boldizsar et al. [22].

Results

The GR and Hsp-90 co-precipitates with ZAP-70 in the lysates of DX treated Jurkat cells

As our previous results [17] indicated, GC induced rapid phosphorylation of ZAP-70, therefore we investigated the possible association of ZAP-70 and GR in Jurkat cells. Immunoprecipitation was performed on lysates of DX or vehicle-treated Jurkat cells, reciprocally with anti-ZAP-70 and anti-GR antibodies. Upon DX treatment, the co-precipitation of the two molecules increased, indicating that agonist promoted the GR association with ZAP-70. (Fig. 1A) To further characterise the subcellular localisation and relation of ZAP-70 and GR in the cytoplasm, we visualised the two molecules simultaneously by confocal microscopy. In vehicle-treated, resting Jurkat cells both ZAP-70 and GR showed even, mostly cytoplasmic distribution, (Fig. 1C) with almost no co-localisation. Upon 5 min high dose DX treatment, we observed co-localisation of the two molecules clustered underneath the cell membrane. (Fig. 1D) Investigating Hsp-90 relation to ZAP-70 by immunoprecipitation we found, that Hsp-90 co-precip-