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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-

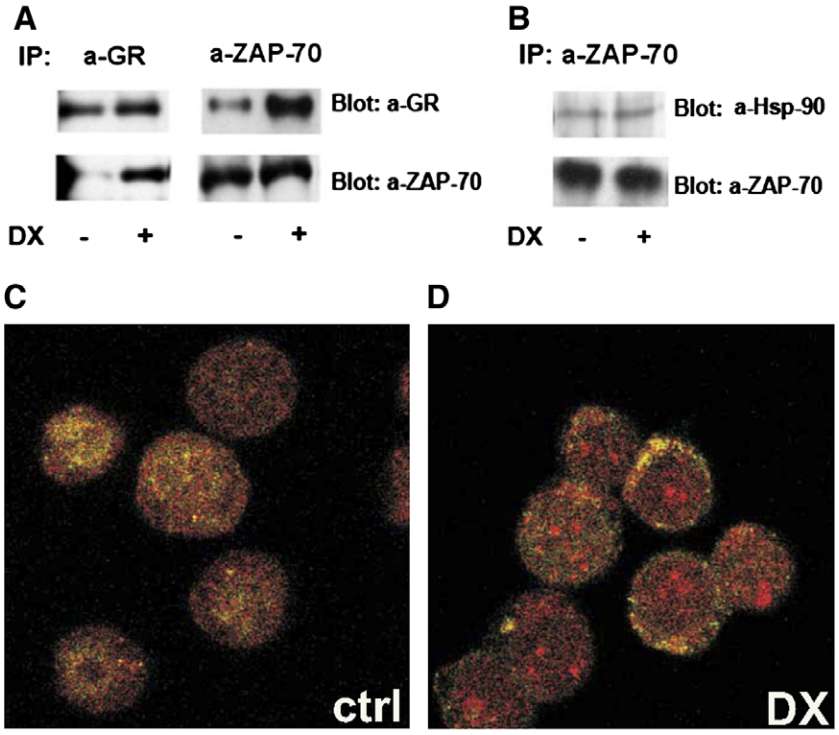

Fig. 1. Association of ligand-bound GR with ZAP-70 in Jurkat cells. (A) Anti-GR and anti-ZAP-70 Western blots are shown from anti-GR or anti-ZAP-70 precipitated Jurkat cell lysates with or without DX treatment. Equal amount of GR and ZAP-70 precipitated when anti-GR and anti-ZAP-70 antibodies were used for precipitation, respectively, while GR and ZAP-70 co-precipitation markedly increased in the DX-treated samples. (B) Anti-Hsp-90 and anti-ZAP-70 Western blots are shown from anti-ZAP-70 precipitated Jurkat cell lysates with or without DX pretreatment. Anti-ZAP-70 was used as precipitating antibody in all samples. Equal amount of Hsp-90 co-precipitated both in the control and DX treated samples. The amount of precipitated ZAP-70 was found to be equal in all samples. The Western blots shown on the figure are representatives from three separate experiments. (C,D) Jurkat cell samples were prepared after fixation and intracellular a-GR-FITC (green channel) and a-ZAP-70-PE (red channel) labelling, followed by sedimentation onto slides and examination with a confocal microscope. In the DX-treated samples, the GR and the ZAP-70 showed near-membrane co-localisation (DX, D), while this phenomenon was absent in the untreated controls (Ctrl, C). The figure shows representative images from three separate experiments. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

itated with ZAP-70 in Jurkat cells, but DX treatment did not alter the association of the two molecules (Fig. 1B).

The GR associates with ZAP-70 expressed in transgenic HeLa cells

ZAP-70 is a T-and NK-cell specific molecule and it is also in close relation with other lymphocyte specific molecules e.g. p56-lck. We aimed to investigate whether the co-precipitation of the GR and ZAP-70 molecules were dependent on these lymphocyte specific factors. We used a lentiviral vector construct for transfecting ZAP-70 deficient Jurkat cells (P-116) and HeLa human epithelial carcinoma cells to stably express the full-length ZAP-70. The ZAP-70 expression level in transfected P116 cells was higher (MFI:98) than in Jurkat cells (MFI:28) as tested by flow cytometry. (Fig. 2A) Moreover, the ZAP-70 construct was fully functional in P-116 cells as shown by anti-CD3 activation and calcium signal measurement. (Fig. 2B) Despite the higher ZAP-70 expression in ZAP-70 transfected P116 cells, the calcium signal was similar to that of wild-type Jurkat cells (Fig. 2B).

The ZAP-70 expression level in transfected HeLa cells was higher (MFI:108) than that of Jurkat cells (MFI:28) as tested by flow cytometry. (Fig. 3A) Reciprocal immunoprecipitations and immunoblotting with anti-ZAP-70 and anti-GR antibodies were performed from HeLa–trZAP-70 cell lysates (Fig. 3B). ZAP-70 co-precipitated with GR in HeLa–trZAP-70 cells. The clustering required the presence of GR ligand (Fig. 3B), similarly to what we have found in Jurkat cells (Fig. 1A). Co-localisation of the two molecules was also verified with confocal microscopy. We observed intensive cytoplasmic staining of both the GR and ZAP-70 molecules in both DX untreated and treated HeLa–trZAP-70 cells (Fig. 3D and E, respectively). We found a perinuclear co-localisation of ZAP-70 with GR in Hela–trZAP-70 cells upon 5 min high dose DX treatment, (Fig. 3E) while in the untreated samples both molecules showed even distribution throughout the cytoplasm (Fig. 3D). Examining Hsp-90 relation to ZAP-70 by immunoprecipitation, we found that Hsp-90 co-precipitated with ZAP-70 in HeLa–trZAP-70 cells, similarly to Jurkat cells. DX treatment did not alter the association of the two molecules (Fig. 3B).

ZAP-70 and Hsp-90 co-pecipitation in Jurkat lysates are inhibited by GA

To better clarify the role of Hsp-90 in this signalling process, we aimed to investigate whether GA, a specific

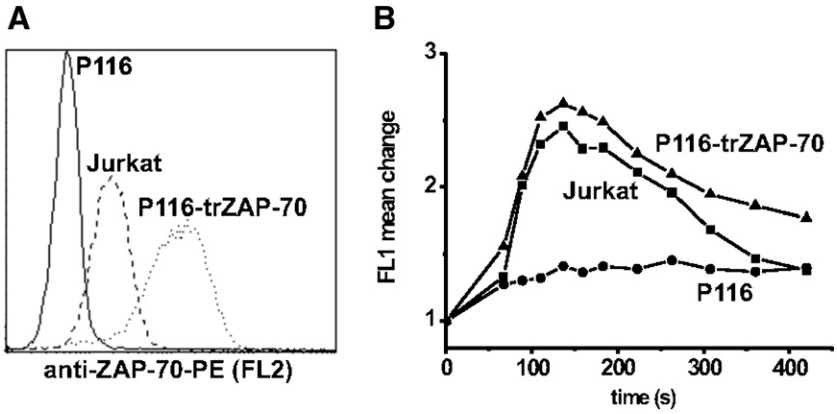

Fig. 2. Lentiviral transfection of P116 (ZAP-70 deficient) cells with ZAP-70. (A) ZAP-70 expression of ZAP-70 transfected P116 cells was compared to untransfected P116 and Jurkat cells by flow cytometry after intercellular phycoerythrin (PE)-conjugated anti-ZAP-70 antibody labelling. Flow cytometric histogram shows PE conjugated anti-ZAP-70 fluorescence (FL2) of a representative measurement from three separate experiments. (B) Calcium signal of ZAP-70 transfected P116 cells (▲) was compared to untransfected P116 (●) and Jurkat cells (■). Diagrams show the result of a representative measurement from three separate experiments.

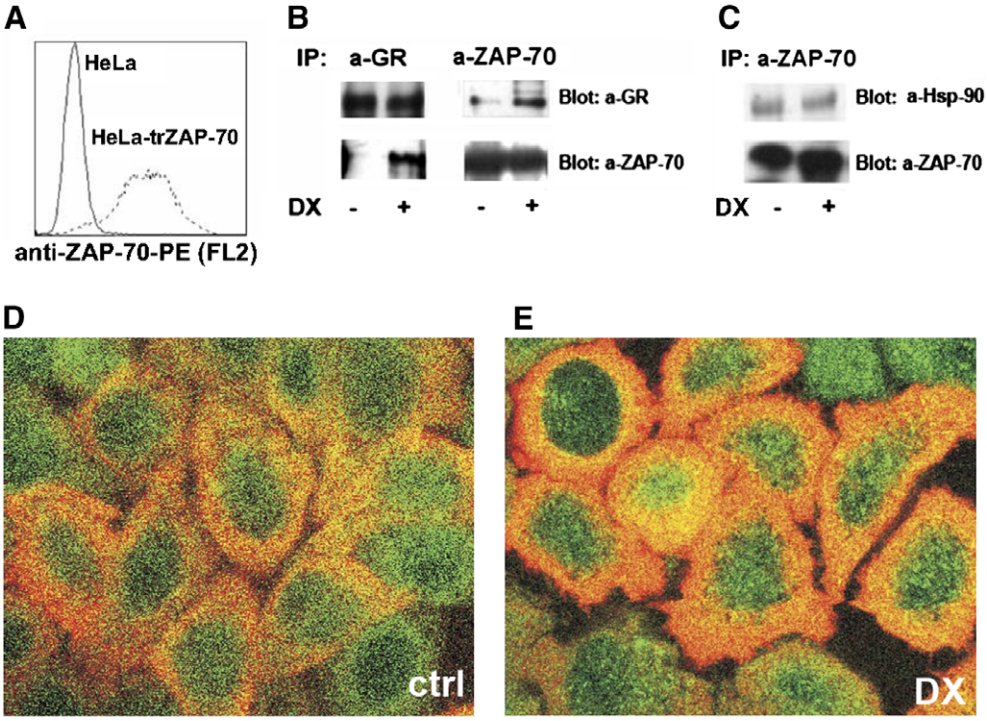

Fig. 3. Liganded GR clustering with ZAP-70 in HeLa–trZAP-70 cells. (A) Representative flow cytometric histograms show the ZAP-70 expression of control and HeLa–trZAP-70 cells after labelling with phycoerythrin (PE)-conjugated anti-ZAP-70 antibody. (B) Anti-GR and anti-ZAP-70 blots are shown from anti-GR or anti-ZAP-70 precipitated HeLa–trZAP-70 cell lysates with or without DX treatment. Equal amount of GR and ZAP-70 precipitated when anti-GR and anti-ZAP-70 antibodies were used for precipitation, respectively, while GR and ZAP-70 co-precipitated only in the DX-treated samples. (C) Anti-Hsp-90 and anti-ZAP-70 blots are shown from anti-ZAP-70 precipitated HeLa–trZAP-70 cell lysates with or without DX pretreatment. Anti-ZAP-70 was used as precipitating antibody in all samples. Equal amount of Hsp-90 co-precipitated both in the control and DX treated samples. The amount of precipitated ZAP-70 was found to be equal in all samples. The Western blots shown in the figure are representatives from three separate experiments. (D,E) Representative confocal microscopic pictures show HeLa–trZAP-70 cells grown in a monolayer after fixation and intracellular labelling with phycoerythrin conjugated anti-ZAP-70 antibody (red channel) and FITC-conjugated anti-GR antibody (green channel). Control cells are depicted left (D) and Dexamethasone-treated cells are depicted right. (E) In the DX-treated samples the GR and the ZAP-70 showed perinuclear co-localisation (E), while this phenomenon was absent in the untreated controls (D). The figure shows representative images from three separate experiments. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Hsp-90 inhibitor, interferes with GR–ZAP-70 association. We performed immunoprecipitation with anti-ZAP-70 antibody. Although GA inhibited the co-precipitation of ZAP-70 with Hsp-90, it did not impair the DX-induced GR–ZAP-70 association (Fig. 4).

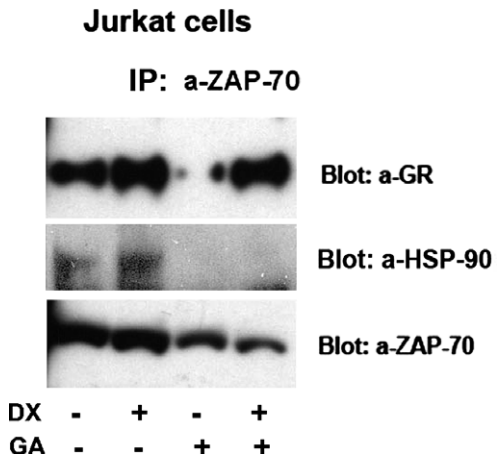

Fig. 4. Geldanamycin treatment does not inhibit the Dexamethasone-induced ZAP-70–GR co-precipitation. Anti-GR, anti-Hsp-90, or anti-ZAP-70 blots are shown from anti-ZAP-70 precipitated control or DX-treated Jurkat cell lysates with or without Geldanamycin pretreatment. Geldanamycin treatment did not influence the increased association of ZAP-70 with GR upon DX treatment. Equal amount of Hsp-90 co-precipitated with ZAP-70 both in the control and DX treated samples, which was completely abrogated by Geldanamycin pre-treatment. The amount of precipitated ZAP-70 was found to be equal in control and DX treated samples with or without Geldanamycin pretreatment. The blots shown are representatives from three separate experiments.

Discussion

Steroid hormones can exert rapid, non-genomic and genomic hormone effects [1]. Our recent finding was that 5 min, high dose GR agonist (Dexamethasone) treatment causes rapid p56-lck-dependent tyrosine phosphorylation of ZAP-70 in Jurkat cells [17]. In this study, we set out to investigate the possible molecular link between the GR and ZAP-70. We demonstrate here, that after short term, high dose DX treatment GR co-precipitates with ZAP-70. We also confirmed the close relation of these two molecules upon DX treatment in the submembrane compartment of the cytoplasm by confocal microscopy in Jurkat cells. Minimal association was found between ZAP-70 and the GR in DX untreated cells. This association offers a plausible explanation for the rapid GC-induced ZAP-70 tyrosine-phosphorylation as described earlier [17]. GR associating with ZAP-70 upon ligand binding, may modify latter's conformation or molecular environment, which in turn may lead to the tyrosine-phosporylation of ZAP-70.

ZAP-70 and p56-lck are lymphocyte specific molecules, while the Hsp-90 and GR are ubiquitous. To clarify whether the association of the GR and ZAP-70 was dependent upon lymphocyte specific factors, we performed the same investigations on HeLa–trZAP-70 cells. The ligand-bound GR showed similar co-clustering with ZAP-70 in the HeLa–trZAP-70 cells than in Jurkat cells. Interestingly, we found different co-localisation patterns in Jurkat and HeLa–trZAP-70 cells. While the co-clustering of the two molecules in Jurkat cells occurred in the submembrane compartment, in HeLa–trZAP-70 cells perinuclear co-localisation was observed. Presumably in Jurkat cells, where the TcR-signalling machinery is complete, ZAP-70 is directed towards the membrane during the signal transduction processes. In HeLa cells, where the transgenic expressed ZAP-70 is somewhat incompatible, the partner molecules sorting for membrane orientation are absent. Based on this we conclude that the molecular association between the liganded GR and the ZAP-70 is independent of lymphocyte specific factors, but takes place in distinct cellular compartments.

Hsp-90 is a molecular chaperone which associates with several other proteins [2]. We found, that ZAP-70 is associated with Hsp-90 in both Jurkat and transgenic HeLa cells. Similarly to this, others have recently shown that ZAP-70 is physically associated with Hsp-90 in B-CLL cells [23]. Although ZAP-70 is a T- and NK-cell specific molecule, it is pathologically expressed by malignant B-CLL cells, where it is associated to the activated form of Hsp-90 [23]. Castro and colleagues proposed that B-CLL cells, similarly to many other tumor cells, express the "activated" form of Hsp-90, with high ATPase activity [23], existing in multi-chaperone complexes [24], whereas normal cells express Hsp-90 in its non-activated form, which does not associate with ZAP-70 [23]. Our results here show that the association of ZAP-70 with the Hsp-90 was independent from the presence or absence of the GR agonist, but it could be abrogated with the Hsp-90 antagonist GA. As Jurkat cells are of malignant origin, the ZAP-70–Hsp-90 association might represent a new aspect of the leukemic phenotype. It is accepted that the GR dissociates from the Hsp-90 after ligand binding [3]. Here we describe the ligand-dependent association of GR to ZAP-70, which cannot be blocked by GA. Based on these data, we conclude that Hsp-90 complexes a lot of client proteins in the cytoplasm, both ligand-free GR and ZAP-70 among others. When GR ligand is present, the ZAP-70–GR association increases and becomes Hsp-90 independent. We hypothesise that ZAP-70 might exist in two forms in the cytoplasm of Jurkat cells, depending on its association with Hsp-90. It appears that there is a relatively constant amount of ZAP-70 bound to Hsp-90, which is not affected by GR agonist treatment and therefore possibly is not involved in the rapid GR-induced phosphorylation events. The non-Hsp-90 bound ZAP-70 fraction, on the other hand, would associate with the ligand-bound GR and, consequently, may be involved in the cross talk between the TcR and GR signalling pathways. If we take into consideration the findings of Castro and colleagues [23], we can also speculate that the fraction of ZAP-70 that associates to the "activated" Hsp-90 molecules present in the cytoplasm may represent an inactive molecular fraction.

In conclusion, the results of our past [17] and present work suggest the existence of a hitherto unknown function of ZAP-70: it is posed at the junction between the GR and TcR signal transduction pathways which may also be affected by the chaperone Hsp-90. However, the exact roles of ZAP-70, p56-lck, GR, and Hsp-90 in the balance between these events need further elucidation.

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