ZAP-70: A NEWLY IDENTIFIED JUNCTION BETWEEN T-CELL RECEPTOR AND GLUCOCORTICOID SIGNALLING PATHWAYS.

PHD THESIS

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1.1 The effects of the glucocorticoid hormone

1.1.1 Target organs of the glucocorticoid hormone

The name “glucocorticoid” derives from early observations that these hormones were involved in glucose metabolism [1]. Regarding the chemical structure of the glucocorticoid hormone (GC) it belongs to the family of the steroid hormones [2]. Glucocorticoids are mainly produced in mammalians in the cortex of the adrenal glands [2]. There are multiple slightly different steroid molecules produced here, which are characterised by the ability to bind the glucocorticoid receptor (GR) and trigger similar effects. The most important physiologically produced GC is cortisol, which has a great range of effects on the various target tissues. Among the GC target organs are the brain [3], bones [4], cardiovascular system [5], immune system [6], kidneys [7], skin [8], liver [1], adipose tissue [9] and skeletal muscles [10]. The GC exerts its effects primarily through the glucocorticoid receptor (GR) but recently new GR-independent mechanisms of actions have been described.

1.1.2 The structure of the glucocorticoid receptor (GR)

The GR belongs to the nuclear receptor superfamily [2]. This terminology refers to the fact that the active forms of these receptors reside in the nucleus. Ligand-free GR is inactive and located in the cytoplasm. Other hormones with steroid structure (e.g. Estrogen, progesterone, testosterone, aldosterone) and other, structurally non-steroid but lipophil hormones (eg. Tiroxin and retinoic acid) have receptors of similar structure. (Figure 1.) Nevertheless, there are data about proteins which are structurally similar to steroid receptors, but their ligands are unknown (orphan receptors) [2].

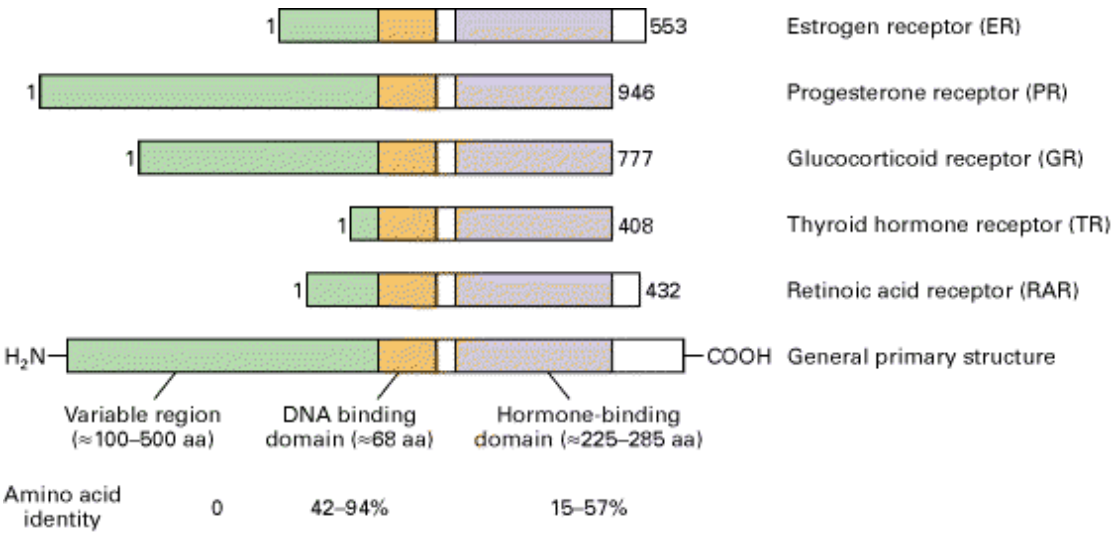

Figure 1. *Comparison of the primary structure of the nuclear receptors. Figure reproduced after Lodish et al. [11]*

The GR is an evolutionary conserved transcription factor. GR with high functional and structural homology can be found in fishes [12], amphibians [13], rodents, and men. The gene encoding the human GR (hGR) is located on the long arm of the 5th chromosome (locus 5q31p) [14]. The gene consists of 9 exons, of which the 2-9 exons are encoding the GR protein. 3 promoters have been identified before the hGR gene (1A, 1B and 1C). The exact regulatory function of these sequences is unknown yet, but presumably they play a role in the regulation of tissue-specific GR expression [15]. The alternative splicing of hGR mRNA results in the expression of two isoforms: hGRα and hGRβ. The α isoform is the “classical” 777 amino acid protein expressed almost ubiquitously among tissues. It is found in its inactive form in the cytoplasm in a macromolecular complex with other proteins [16].

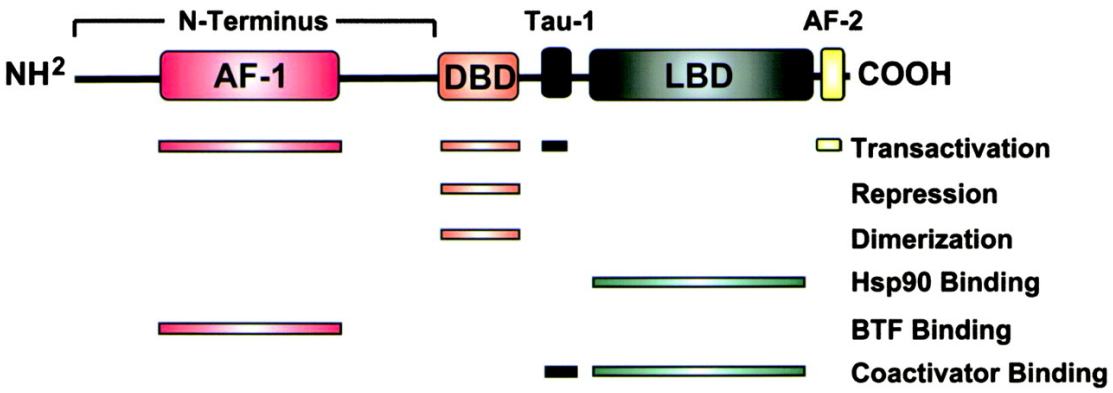

Figure 2. *The primary structure of the GR shown on a ribbon diagram. Figure reproduced after Necela and Cidlowsky [6].*

Functionally GR can be divided into 4 different domains. (Figure 2.) The N-terminal domain is the regulatory domain (amino acid residues 1-421). This region is responsible for the constitutive transcription activation function. The DNA binding domain (DBD, aa. residues 422-486) is the evolutionary most conserved part of the molecule possessing a zinc-finger structure. This part of the molecule binds to the specific palindrome DNA sequences called glucocorticoid response elements (GRE) thus regulating gene transcription. A short hinge region (aa. residues 487-526) connects the ligand binding domain (LBD, aa residues 527-777) which is also responsible for the formation of the homodimer.

The hGRβ isoform is formed via the alternative splicing of exon 9. It differs only in its C-terminal region from hGRα containing a 15 aa long unique sequence. The function of hGRβ is controversial: it does not bind ligand and localised exclusively in the nucleus. Some studies demonstrate the role of hGRβ in pathological GC resistance cases. It may take part in the fine tuning of GR-regulated gene expression [6].

1.1.3 Genomic signal transduction pathways of the nuclear receptors

The ligands of the nuclear receptors are lipophil molecules so they are able to penetrate freely through biological membranes. Most of them are chemically

steroids (glucocorticoids, mineralocorticoids, sex-steroides, Vitamin D) but there are also some non-steroid lipophilic molecules (retinoic acid, thyroxine and Triiodothyronine). [2] These hormones diffuse into the cytoplasm and bind to their intracellular receptors. The ligand bound receptors then actively translocate into the nucleus and bind specific regulatory DNA sequences. They act as transcription factors and regulate (either positively or negatively) the expression of certain genes. This way of transcription regulation is DNA-dependent. There are examples of DNA-independent transcription regulation e.g. ligand bound steroid receptor interaction with other transcription factors or signalling proteins thus modulating their activity. In case of GR there are examples of both DNA dependent and independent regulation mechanisms [6]. A common characteristic of the genomic signal transduction pathways are that the effects can be abolished by either antibiotics inhibiting transcription (eg. Actinomycin-D) [17] or drugs inhibiting protein synthesis (eg. Cycloheximide) [17]. The onset of genomic effects is always slow: it usually takes at least a few hours till the new protein is expressed and exerts its effect [18].

1.1.4 Molecular mechanisms of the genomic signal transduction pathway of the GR.

The inactive GR is associated with other proteins in the cytoplasm like the Hsp-90, Hsp-70, immunophillins like p59 and p23 [16]. Due to the comformation changes following ligand binding, the GR dissociates the Hsp-90 complex and translocates into the nucleus as a homodimer [19]. This process is actively supported by the elements of the cytoskeletal network, [20] e.g GR is known to be associated with tubulin [21]. The function of ligand-bound GR in the nucleus is mainly transcription regulation. It binds GRE sequences, and enhances the transcription of the genes containing this element. The GRE sequence is fairly well characterised: GGTACAnnnTGTTCT [22]. The GR may implement negative transcription regulation either as it may bind negative regulatory GRE (nGRE) as well. Such nGRE sequences were described in the promoters of HIV type 1 [23],

human osteocalcin [24] and human corticotrophin releasing hormone (CRH) genes [25]. However, these nGRE sequences are less well characterised yet.

The regulation of transcription by GR can be exerted via other ways: "communication" with other transcription factors. During this process, the GR establishes close physical contact with the other molecule and changes its activity. Many other transcription factors are known to interact with GR like the NFκB, AP-1, CREB, STAT-1, STAT-3 and STAT-5 (Figure 3, [6]).

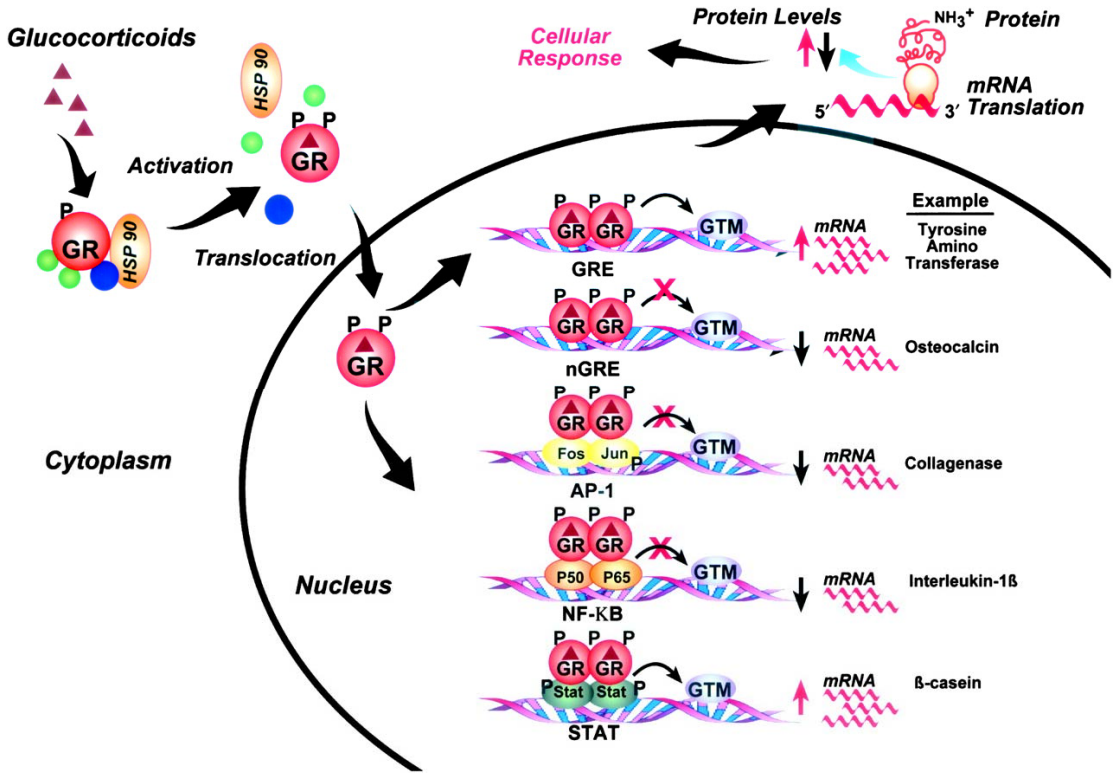

Figure 3.

Basic mechanisms of glucocorticoid receptor (GR) action. Left: The GR resides in the cytoplasm complexed with several chaperones including hsp90 and immunophilin p59. On binding to glucocorticoids, the activated receptor dissociates from the attached accessory proteins and translocates into the nucleus. Middle: The GR then regulates the expression of genes by several basic modes of action. From top to bottom: The GR binds as a dimer to glucocorticoid response elements (GREs) in target genes to activate gene transcription; the GR binds to negative GREs (nGREs) and inhibits target gene transcription; the GR physically interacts with the c-Jun subunit of the AP-1 complex to inhibit AP-1-mediated gene expression; the GR physically interacts with the p65 (RelA) subunit of NF-κB and represses NF-κB-regulated gene expression; the GR physically interacts with members of the STAT family (STAT1, STAT5, and STAT3) and synergistically enhances STAT-regulated gene transcription. GTM = general transcriptional machinery; P = phosphate. Right: Examples of genes regulated by the GR by the various mechanisms are shown. Figure reproduced after Necela and Cidlowsky [6]

1.1.5 Non-genomic effects of GC

Common feature of the non-genomic steroid hormone effects is that no *de novo* protein synthesis is necessary. Non-genomic effects are mainly characterised by post-translational modifications of already existing signal transduction molecules. These reactions may occur considerably fast, as they show within seconds or minutes. The non-genomic GC effects are divided into 3 groups according to Buttgereit and colleagues: (1) specific GC effects accomplished through the classical cytoplasmic GR (cGR). These cytoplasmic effects are mainly exerted via GR interactions with other signalling proteins. (2) Specific GC effects involving the putative membrane resident GR (mGR) and (3) non-specific GC effects via physicochemical membrane interactions. (Figure 4.) This latter one can be considered only by very high hormone concentration [26].

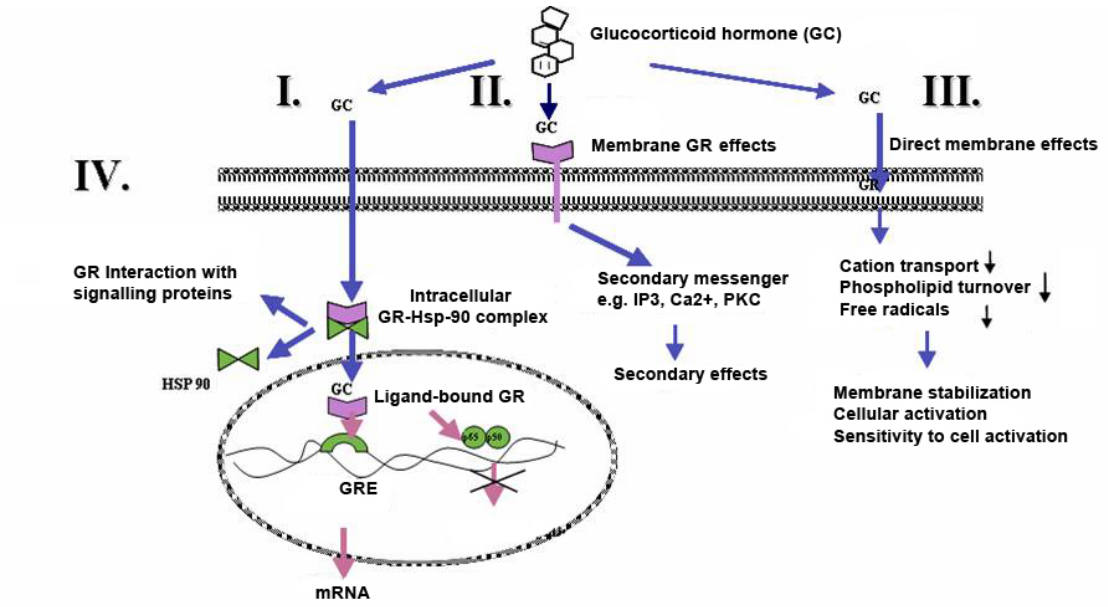

Figure 4.

Non-genomic effects of GCs. I.: non-genomic GC action involving cGR. II.: non-genomic GC effects accomplished via the putative mGR. III.: non-genomic GC effects exerted by direct physicochemical membrane actions.

1.1.6 Specific rapid GC effects directed by the cGR

Good examples for the rapid, non-genomic effects mediated by the cGR are the results described by Croxtall and co-workers: the cytosolic phospholipase

A_2 ($cPLA_2$) activation and arachidonic acid secretion following EGF stimulation can be rapidly inhibited by Dexamethasone (DX) treatment [27]. The process proved to be insensitive to actinomycin and sensitive to RU486 which clearly suggests non-genomic signalling pathway involving the cGR. The authors propose the participation of src-kinase in the signalling pathway. Since the src-family kinases are Hsp-90 associated and the inactive GR is localised in the same macromolecular compartment, the ligand-binding and the consequential dissociation of the ligand-bound GR may initialize mechanisms which result in the observed outcome [27].

1.1.7 Specific GC effects involving the putative mGR

Membrane resident receptors were described concerning every steroid hormone [28]. However, data about mGR are remarkably obscure. The presence of classic cGR-like antigen was shown in the membrane of murine T-cell leukaemia [29] and on the surface of human leukaemic cells [30]. Gametchu and co-workers proposed a relation between the presence of the mGR and the sensitivity of the cells to GC-induced cell death [31, 32]. They demonstrated that the mGR expression level is cell-cycle dependent [32]. Others detected mGR on the surface of human PBMC [33]. We were able to show membrane associated GR on the surface of Jurkat cells [34]. Evans *et al.* described the partial purification of a membrane-resident GC binding protein from the brain of the rough skin newt (*Taricha granulosa*), that exhibited different structure than that of the classical cGR. The attributes of this acidic glycoprotein of 63kDa resembled the 7TM G-protein linked receptors [35]. Other steroids also have been shown acting on membrane receptors of different structure. A caveola-associated estrogen receptor was described by Chambiss and colleagues on the surface of mammary tumor cells [36]. A progesterone membrane receptor of 7TM domain structure was isolated and cloned from spotted sea trout (*Cynoscion nebulosus*) eggs [37].

1.1.8 Non-specific GC effects due to physicochemical membrane interactions

Buttgereit and his colleagues take a clinical experience as a base point: namely, that daily 100-200 mg orally administered prednisolon equivalent GC is able to saturate the specific binding capacity of the GR. In contrast to this fact, concerning some particular cases better therapeutic effects were achieved by giving a high dose iv. bolus of 500-1000 mg methylprednisolone [26]. This observation raised the possibility that in the high-dose range there are different GC mechanisms of action. Studying the effects of high dose GC effects on mitogen-activated rat thymocytes they observed that methylprednisolone inhibited the oxidative phosphorylation (ATP-producing processes) and oxygen consumption [38]. GC proved to inhibit the ion-transport across the plasma membrane in mitogen-activated thymocytes [39]. They considered these effects as non-specific membrane effects, showing at extremely high GC concentrations. In these *in vitro* experimental systems the administered GC concentrations were 1-10mM.

1.1.9 The regulation of inflammatory response

Inflammation is the response of the body to tissue damage which can be the result of physical trauma, infection, or other pathological conditions. The main role of the inflammatory response is the isolation of the harmful agent so that it is prevented from spreading throughout the rest of the body, the neutralization/elimination of the potential infectious agent and the repair of the caused tissue damage. The local inflammation is often accompanied by systemic response, called acute phase reaction [40]. The local signs of inflammation are redness and heat (due to the blood flow of increased volume, but slower speed) and swelling (due to increased vascular permeability). In the site of the inflammation the vascular endothelium expresses many different adhesion molecules directing the migration and the extravasation of the cellular elements participating in the inflammatory reaction. [40] In the regulation of the inflammation chemokines, cytokines, and other soluble factors are participating. The inflammatory cytokines produced

by macrophages are of primary importance in this process. These are TNF-α, IL-1, IL-6, CXCL-8 (IL-8), and IL-12 [40].

Inflammatory cytokine	**IL-1**	**TNF-α**	**IL-6**	**CXCL-8**	**IL-12**
Local effect	Vascular endothelium activation Lymphocyte activation Tissue destruction	Vascular endothelium activation Increased vascular permeability: migration of inflammatory cells, IgG and complement	Lymphocyte activation Increase of antibody production	Chemotaxis: Enhancing the migration of Neutrophiles, Basophiles, T-Lymphocytes	Activation of NK cells Promoting Th1 differentiation
Systemic effect	Fever Increase of IL-6 production	Fever Mobilisation of metabolism	Fever Production of Acut phase proteins		
Signal transduction pathway	NFκB, AP-1	NFκB, AP-1	JAK - STAT	G-protein linked receptor	JAK - STAT

Table 1.

Physiological effects of inflammatory cytokines. Table modified after [40]

1.1.10 GR communication with AP-1 and NFκB signal transduction pathways

The transcription factor AP-1 is one of the key molecules of signal transduction pathways activated by the inflammatory cytokines (TNFα, IL-1β). The AP-1 complex consists of the heterodimer of c-fos and c-jun family member proteins. Both have leucin-zipper structure: c-fos family members have direct transcription-activating activity, while members of c-jun family have only regulatory role. The ligand-bound GR associates with the c-jun

member of the heterodimer and thereby inhibits the transcription of pro-inflammatory genes with AP-1 responsive element (eg. collagenase). (Figure 5. [6]).

Recent data suggest that the GR inhibits the mitogen-activated protein kinase (MAPK) signal transduction pathway as well. The members of the MAPK cascade are serine-threonine kinase enzymes, playing important role in the signalling originating from cytokine receptors. JNK is a member of the MAPK cascade, being responsible for the activation of the AP-1 complex, while the p38 subfamily have important function by maintaining the stability of the mRNA transcripts of the TNFα, IL-6, CXCL-8, VEGF genes. There are multiple data on GR interfering with kinases of the MAPK cascade. GR inhibits JNK activity, thus arresting c-jun phosphorylation and preventing AP-1 activation [41]. GR is known to increase the expression of MAPK-phosphatase-1, thereby inhibiting the activity of p38 MAPK. Because of the increased expression of phosphatases the mRNA transcripts of proinflammatory genes become instable [42].

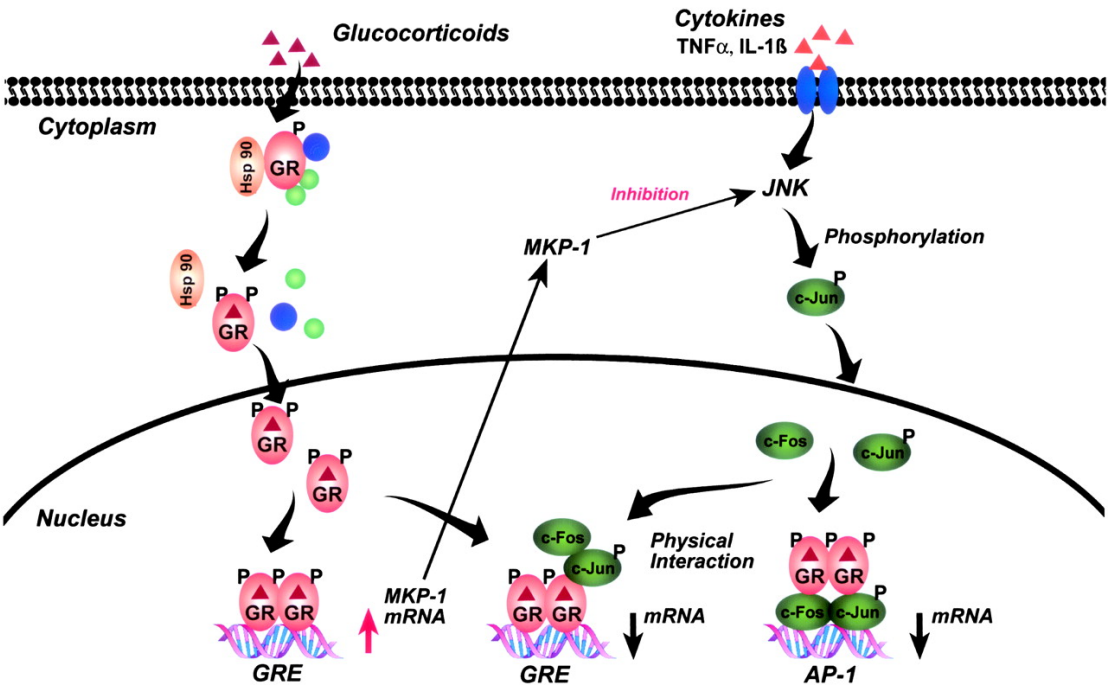

Figure 5.

Mechanisms of cross-talk between the glucocorticoid receptor and the AP-1 complex. Cytokines such as IL-1ß and TNF-α bind to and activate membrane-bound cytokine receptors. In turn, the cytokine receptor activates c-Jun N-terminal kinase (JNK), a member of the MAPK family. JNK phosphorylates the c-Jun subunit of AP-1, which is localized in the cytoplasm. Phosphorylation of c-Jun induces its activation and subsequent translocation into the nucleus. The c-Jun subunit then dimerizes with the transcriptionally active c-Fos subunit, and binds AP-1 response elements to regulate gene expression. The c-Jun subunit of AP-1 can also physically interact with the GR and repress GR-mediated gene transcription. Reciprocally, the GR can physically interact with c-Jun and repress AP-1-mediated gene expression. The mechanism by which physical interaction between c-Jun and the GR leads to mutual antagonism remains unclear. Alternatively, the GR can repress AP-1 signaling through induction of the MAPK phosphatase-1 (MKP-1) gene. MKP-1 prevents phosphorylation of JNK and its subsequent activation. Figure reproduced afterNecela and Cidlowsky [6].

NFκB is an ubiquitously expressed transcription factor playing key role in inflammatory reactions. NFκB is a dimeric molecule consisting of a p50 and a p65 subunit, the latter being transcriptionally active [43]. Inactive NFκB is associated with the inhibitory protein I-κB in the cytoplasm. Following the appropriate signal from the inflammatory cytokine, I-κB becomes phosphorylated (mediated by IKK) and dissociates the NFκB complex. The free NFκB complex then translocates to the nucleus, binding appropriate response elements. This initiates the transcription of pro-inflammatory genes, eg. cytokines (IL-1β, TNFα, GM-CSF) chemokines (CXCL-8), adhesion molecules (ICAM-1). GR may interact with the p65 subunit, preventing it from activating the transcription machinery. Interestingly both the p65 and c-jun are

mutually antagonistic with the GR: their association with it hinders the GR binding to GRE thereby preventing the GC induced genes from expression. (Figure 6. [6, 43]).

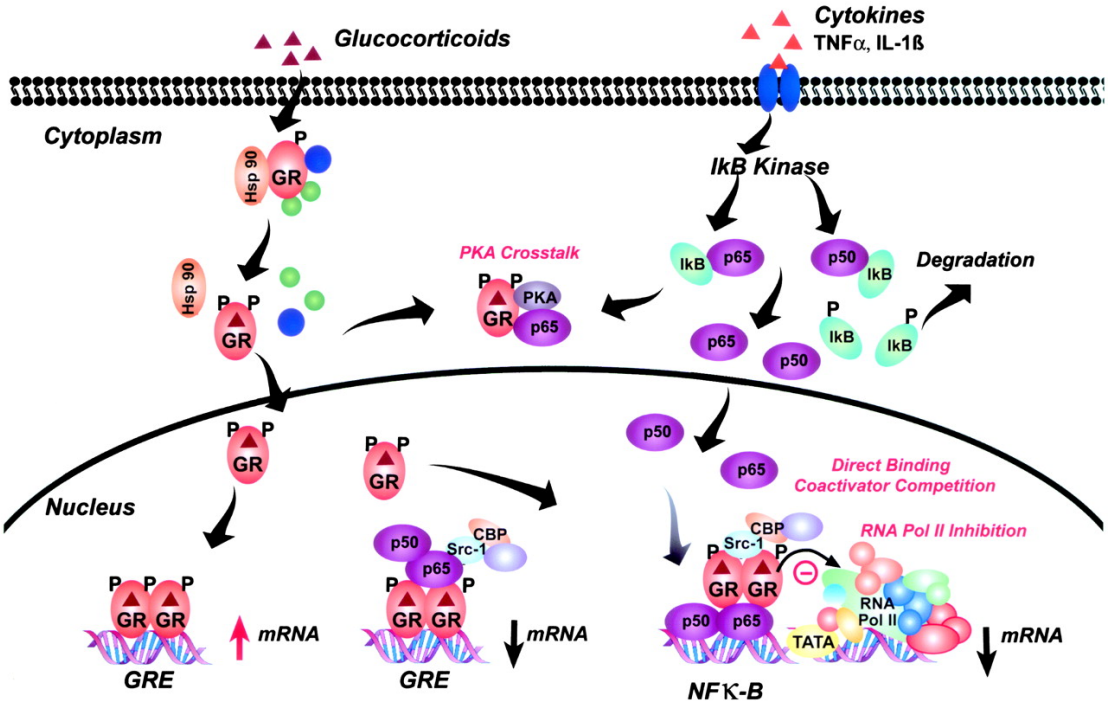

Figure 6.

Mechanisms of cross-talk between the glucocorticoid receptor and NF-κB. The NF-κB transcription factor is composed of two subunits, p50 and p65 (RelA). The subunits are sequestered in the cytoplasm through the interaction of the inhibitory protein I-κB. Binding of proinflammatory cytokines to their appropriate receptors induces the activation of a kinase cascade leading to the phosphorylation of I-κB by inhibitory κkinase (IKK). Phosphorylation of I-κB by IKK leads to the subsequent degradation of I-κB and release of the bound p65 and p50 subunits. The free NF-κB subunits translocate to the nucleus, dimerize, and bind NF-κB response elements to activate proinflammatory genes. The glucocorticoid receptor and the p65 subunit of NF-κB can physically interact and antago-nize each other's transcriptional activity. Several mechanisms have been proposed by which the physical interaction can lead to mutual antagonism: the glucocorticoid receptor and NF-κB may compete for coactivators SRC-1 (steroid receptor coactivator-1) and CBP (cAMP response element–binding protein (CREB)–binding protein) (direct binding coactivator competition); the cytoplasmic subunit of protein kinase A (PKA) may mediate the cross-repression of GR and NF-κB (PKA cross-talk); or the glucocorticoid receptor may directly or indirectly inhibit the phosphorylation and, thus, activity of RNA polymerase II (Pol II) on p65-occupied promoters (RNA Pol II inhibition). This GR-mediated inhibition prevents subsequent NF-κB–mediated gene transcription. Figure reproduced afte rNecela and Cidlowsky [6].

1.1.11 GR interaction with STAT family proteins

Proteins of the STAT-family participate in many signal transduction pathways initiated by cytokines of key importance such as IL-2 and IL-6. The activation of the STAT-proteins occur via the Janus kinase (JAK) [44]. Following phosphorylation by JAK, STAT-monomers dimerize and migrate into the nucleus. They regulate the transcription of a number of gene products controlling cell growth, survival, differentiation or apoptosis upon binding STAT responsive elements on DNA [44]. Numerous STAT-proteins are known to interact physically with GR, namely STAT-1, STAT-3 and STAT-5 (Figure 7, [6]). In general, the GR acts synergistically with the previously listed STATs. As a result of the interaction, GR and STATs enhance the expression of proteins regulated by STATs in a promoter-dependent way. Intriguingly, the STAT-family proteins regulate differently the transcription of genes controlled by the GR. For example, GR and STAT-3 synergistically increase the activity of the GR-responsive MMTV promoter and IL-6 responsive γ-fibrinogen and α_2-macroglobulin genes in rat after IL-6 stimulus. In contrast, STAT-5 being activated after IL-2 stimulus and GR inhibits the MMTV promoter activity (GR responsive) and increases synergistically the expression of β-casein (STAT-5 responsive) [45-48]. The interactions of the STAT-family proteins and GR highlight the existence of very diverse regulating mechanisms, which are recently not completely explored. It seems that GR-STAT interactions are not only to suppress but also to enhance the immune response in certain cases.

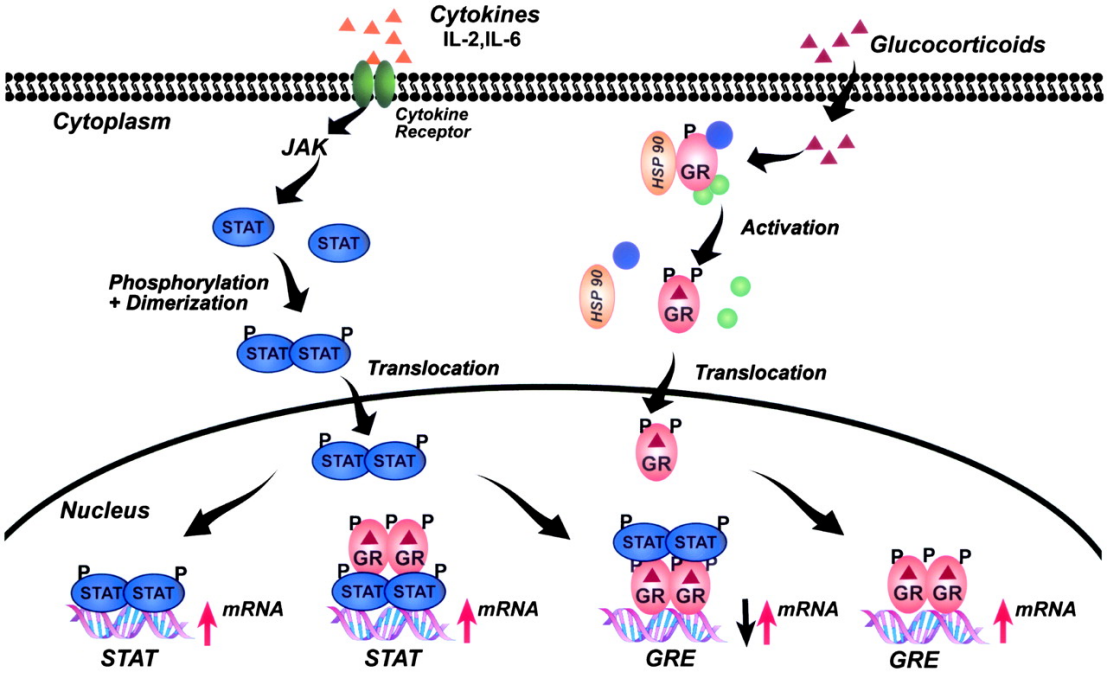

Figure 7. *Cross-talk between the glucocorticoid receptor and the STAT family. Cytokines such as IL-2 and IL-6 activate STAT proteins through induction of the Janus kinase (JAK) pathway. JAK phosphorylates STAT proteins that exist as monomers in the cytoplasm. JAK-mediated phosphorylation of STAT proteins induces their dimerization and translocation to the nucleus. The STAT proteins then bind response elements and regulate the expression of genes that influence growth, survival, apoptosis, host defense, stress, and differentiation functions. In some promoter contexts, the glucocorticoid receptor physically interacts with members of the STAT family such as STAT1, STAT3, and STAT5 (a and b), and synergistically enhances STAT-mediated gene expression. Reciprocally, STAT proteins can either enhance or repress GR-mediated gene activation depending on stimuli and the STAT protein involved. Figure reproduced after Necela and Cidlowsky [6]*

1.1.12 The regulatory role of GC mediated apoptosis in the immune system

The robust anti-inflammatory effects of GCs are exerted not only via the down-regulation of pro-inflammatory signal transduction processes but also causing the apoptosis of cells participating in the inflammatory reaction. This pro-apoptotic effect was extensively studied in thymocytes, monocytes and eosinophil granulocytes. However, GC may promote also anti-apoptotic effects in some cells, which are exerted through the activation of Bcl-2 and NF-κB pathways [49]. Recent experimental data suggest that for the GC-induced apoptosis an appropriate GR level is needed in the cell. If the GR expression is up-regulated after GC action, the result is apoptosis. This

phenomenon is characteristic for thymocytes and some T-cell lymphomas [50]. In contrast, when GC action is followed by GR down-regulation, (like in the majority of cells types) the cell is prevented from apoptosis [51].

1.1.13 The importance of the communication between GC and TcR signal transduction pathways

One of the most intensively studied GC induced apoptosis process is the selection of thymocytes in the thymus. The maturation and selection of thymocytes is recently the subject of intensive debate. Nowadays the most acknowledged model is the “mutual antagonism theory” [51]. According to the aforementioned theory, the GC modifies the signal originating from the TcR, thereby preventing the cell from apoptosis. Thymocyte clones receiving none or “subthreshold” signal from the TcR will be deleted, (“death by neglect”) presumably via GC induced apoptosis [52]. TcRs with very strong affinity towards the self MHC-peptide complex are potentially autoreactive. This “super-strong” TcR-signal can not be antagonized by GCs, so these clones are deleted via negative selection [53].

The importance of GR is controversial in thymocyte selection. Some authors deny the role of GR in the thymic selection process, because the maturation of the thymocites seems to be undisturbed in the thymi of GR-deficient mice [54, 55]. This is supported by the fact that in the thymus the CD4+CD8+ double positive (DP) cells are the most sensitive to GC-induced apoptosis although expressing the lowest level of GR [56, 57]. Administration of GR-antagonists do not influenced the GC effects on the thymic selection, suggesting a GR-independent process [56, 57].

Mature peripheral T-cells after antigen recognition also have to “decide” between activation or anergy/apoptosis [58]. Many experimental data supports the opinion that the TcR is not a simple molecular on/off switch. Peptide ligands with varying affinity towards the TcR induce various “phosphorylation programmes” in T-cells [59]. Phosphorylation after engaging high affinity TcR ligands cause saturated CD3 ζ-chain phosphorylation (pp23 isoform dominates), the docking of phosphorylated ZAP-70 and the phosphorylation of the LAT adapter is saturated, too. If the TcR was stimulated by a low-affinity ligand, the unsaturated pp21 ζ-isoform dominated and ZAP-70

phosphorylation did not occur [59]. According to Van Leathem and colleagues, TcR stimulation with full agonist in the presence of GC induced a phosphorylation pattern similar to that of stimulation with partial agonist. They report that Dexamethasone (DX) treatment inhibited the phosphorylation signal after TcR stimulation. Since the DX treatment did not cause alteration in the expression level or in the activity of src-family kinases, they suggest that DX changed the membrane compartmentalisation of key signal-transduction molecules [60]. The authors propose that the GC administration abrogated the palmitoylation of important signalling molecules thereby inhibiting their raft-association [61].

1.2 The signal transduction process originating from TcR

1.2.1 The antigen recognition of T-cells

The T-cell receptor (TcR) is the surface antigen receptor complex of the T-cells being able to specifically recognise antigen. The antigen recognition of T-cells is MHC-restricted, meaning that the TcR is only able to bind processed peptide antigens presented by the MHC complex. The antigen is not recognised by T-cells in its native form, but has to be taken up and processed by antigen presenting cells (APCs). The presented antigen can be either an endogenous protein degradation product of the APC itself or an exogenous antigen engulfed by phagocytosis. The endogenous peptides are presented via MHC Class I, the exogenous peptides are presented by MHC Class II. The MHC molecules of the APCs are recognised by CD8+ cytotoxic T-cells, and CD4+ helper T-cells, respectively [40].

1.2.2 Molecules participating in antigen recognition of T-cells

While the TcR recognises the unique parts of the antigen-primed MHC, the constant domain of the MHC is also bound by to co-receptors on the surface of T-cells (CD4, CD8). The variable (V) domains of TcR show extreme high diversity, and every T-cell clone bears a TcR specific for different antigen. This makes up the T-cell repertoire which has enormous variability of antigen-specific receptors. The TcR consists of a heterodimer of $\alpha\beta$ or $\gamma\delta$ chains the

CD3-complex, making up a functionally united molecular complex on the surface of the T-cells. Approximately 95% of the mature T-cells bear αβTcR, and 5% expresses γδTcR [40].

After antigen recognition by TcR, various signal transduction events occur involving the CD3-complex. Most of these proteins are members of the Immunoglobulin (Ig) superfamily, structurally consisting of globular Ig-domains. These are approximately of 110aa length and held together by disulphide (S-S) bonds. The molecular weight of TcR α chain is of 43-49 kDa while β chain is of 38-44 kDa. They both have 2 extracellular domains, one variable (V) and one constant (C). The V domains show high structural homology with the variable domains of immunoglobulin molecules, containing the hypervariable or complementarity determining regions, (CDRs) which directly participate in antigen recognition. The cytoplasmic parts of the chains are rather short, 5-12 aa residues altogether, so the TcRαβ dimer without the CD3 complex is incapable of signal transduction [40].

The αβ T-cells can be further divided concerning their function: helper T-cells and cytotoxic T-cells, bearing CD4 or CD8 co-receptors, respectively. CD4 is the member of the Ig superfamily and consists of one polypeptide chain of 55 kDa. It recognises Class II MHC molecules on the APCs. In humans CD4 is expressed by 2/3 of the peripheral T-cells, and monocytes, macrophages, dendritic cells are also CD4 positive. It is worth mentioning, that CD4 serves as the receptor of the Human Immunodeficiency Virus (HIV) gp120 envelope-protein.

The CD8 molecule is either a homodimer (CD8αα chains) or a heterodimer (CD8αβ). It associates with Class I MHC molecules thereby stabilizing the link between the T-cell and the APC. Both CD4 and CD8 molecules contain characteristic CXC motives in their cytoplasmic part allowing the association with the p56-lck kinase, a src-family kinase present in T-cells [40].

1.2.3 The structure and function of the CD3 complex

Because the short intracellular parts of the TcR chains are incapable of signal transduction, other molecules perform the transmission of the signals following antigen recognition. It became evident soon that the chains of the

CD3 complex play this role. This is confirmed by the fact that crosslinking the CD3 complex with antibodies leads to T-cell activation [62]. The CD3 complex is expressed on the surface of every T-cell. The complex consists of multiple chains, associated with each other and with the TcR with non-covalent bonds. The chains of the CD3 complex are termed γ, δ, and ε chains and there are either homodimeric (ζ–ζ) or heterodimeric (ζ–η) chains. The γ, δ, and ε chains have an extracellular Ig-like domain and a relatively long intracellular part with a so-called ITAM sequence (Immunoreceptor Tyrosine-based Activation Motif), while the ζ chains have 3 ITAM sequences. The ITAM tyrosines become phosphorylated by various kinases upon the signal transduction events and may serve as docking sites for other signalling molecules. The ITAMs are present not only in T-cells but also other cells and molecules, such as B-cell receptor, Fc-receptors and cytokine receptors [40].

1.2.4 Signal transduction events following antigen recognition in T-cells

The first signal transduction events following the antigen recognition by the TcR are the tyrosine phosphorylation of various molecules, which only needs a couple of seconds to occur. This activation signal triggers such biochemical events, which cause a significant change in the metabolism of T-cells. In two hours the activated T-cells express cytokine receptors and cytokines (the most characteristic is the IL-2 and its high-affinity receptor). Within 2-6 hours further cytokines are synthethised which is followed by DNA replication in 24 hours. Mitosis occurs within 48 hours [63]. The optimal T-cell activation requires the participation of a number of co-receptors and adhesion molecules. These are responsible for the stabilisation of the connection between T-cells and APCs, as well as providing co-stimulatory signals, which are indispensable for achieving physiological T-cell response to the antigen.

One of the earliest events following the antigen recognition by the TcR is the activation of the src-family kinases. The most studied src-kinases in T-cells are the p56-lck and p59-fyn associated with CD4/8 or with CD3 complex, respectively. Although they are structurally highly homologous, the p56-lck plays a role in the T-cell activation process, while the p59-fyn rather delivers

inhibitory/anergizing signals. Src-family kinases contain 3 domains, the SH3 domain on the N-terminus, which recognises proline rich regions on other proteins, the SH2 domain, which associates with phosphorylated tyrosine and the C-terminal kinase domain. Both the p56-lck and p59-fyn has a myristoylation signal in their N-terminus. The conjugation with myristyl acid containing long hydrophobic chain enables these molecules to permanently associate with lipid rafts in the membrane. The activity of src-family kinases is regulated by tyrosine phosphorylation. There are two main phosphorylation sites in src kinases: an N-terminal inhibitory site and a C-terminal activating site [64].

The chains of the CD3 complex, mainly the ζ chains become phosphorylated after antigen recognition. This phosphorylation is mediated by the p56-lck kinase. The phosphotyrosines serve as a docking site for ZAP-70 kinase (zeta-chain associated protein of 70 kDa). The chains of CD3 complex, together with src-family kinases and many other signalling molecules are the part of the so-called “immunological synapse”.

Membrane lipid rafts are cholesterol-rich parts of the cell membrane which have key role organising the immunological synapse formed between the T-cell and the APC. All of the signalling molecules participating in T-cell activation have a strict spatiotemporal organization schedule regardless they are excluded or included the lipid rafts. If this process is disturbed, the molecules can not reach their correct spatiotemporal position/conformation and the proper T-cell activation is prevented. [65-67]

1.2.5 The role of ZAP-70 kinase in T-cell activation

The ZAP-kinase is a T-and NK cell specific kinase of the Syk family. It is essential for proper T-cell development and function. In the absence of active ZAP-70 the thymocyte development is arrested at the DP stage in mice and there are no mature $\alpha\beta$T-cells in the periphery [68]. Both negative and positive selection is severely perturbed in ZAP-70$^{-/-}$ thymocytes. The reconstitution of these mice with human ZAP-70 restored the block in T-cell maturation and allowed the appearance of mature SP thymocytes and peripheral T-cells [69].

ZAP-70 deficiency in humans results in Severe Combined Immunodeficiency syndrome (SCID). SCID is a heterologous syndrome affecting both T and B-

cell function. SCID due to ZAP-70 deficiency is characterized by the lack of CD8+ T-cells either in the thymus and periphery. Peripheral CD4+ T-cells fail to mobilize calcium after anti-CD3 activation and do not proliferate in response to phytohemagglutinin or concanavalin-A (both lectins induce TcR-dependent proliferation) [70, 71]. Interestingly, phosphorylation of near-TcR molecules occur not only after TcR stimulation. It is known that ZAP-70 participates in CD27/CD70 [72], CD59 [73], CD44 [74], RANTES chemokine [75], CD26 [76], CD38 [77], CD5 [78], LFA-1 [79], CD28 [80], CXCR4 [81] and Fas/Fas-L [82], signal transduction pathways. We recently found that the glucocorticoid hormone may interfere to TcR signal transduction via the p56-lck/ZAP-70 kinase hierarchy [83].

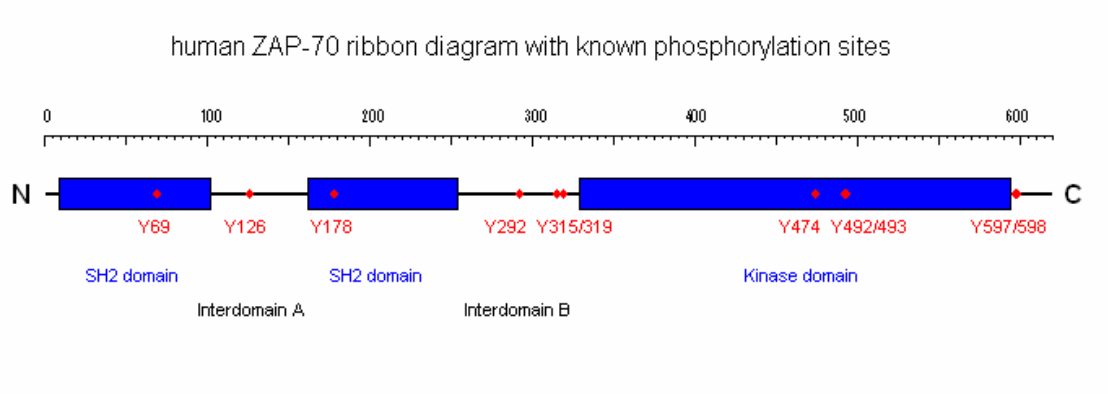

Figure 8. *The ribbon diagram of human ZAP-70 showing the known phosphorylated tyrosine residues*

Structurally the ZAP-70 kinase consists of two tandem SH2 domains and one C-terminal kinase domain (Figure 8). The ZAP-70 plays a crucial role transducing the signal derived from the TcR. After the TcR and the co-receptors (CD4 or CD8) engaged the MHC-antigen complex, the src-family kinases (primarily p56-lck) become activated and phosphorylate the tyrosine-containing ITAMs on the CD3 complex. These phosphorylated tyrosines provide docking sites for ZAP-70 kinase by its SH2 domains. Then the ZAP-70 itself is also phosphorylated by src-family kinases [84]. The ZAP-70 has two major substrates: LAT and SLP-76 adaptor proteins. LAT is a transmembrane adaptor protein, being phosphorylated on a number of tyrosine residues. Phosphorylated LAT molecules serve as docking platform

for other signalling proteins. There is a myristoilation motif on the N-terminus of LAT: the hydrophobic group ensures its association with the membrane lipid rafts [67]. SLP-76 is a cytoplasmic adaptor protein containing several phosphorylation sites. It also has a proline-rich region which allows the attachment of proteins with SH3 domain and a SH2 domain, which binds to phosphotyrosines on other proteins [85, 86]. The LAT and SLP-76 initiates a "molecular scaffold system" which provides platform in the cytoplasm for signalling molecules. These adaptor proteins and other signalling molecules provide a complex system which is highly organized to allow the fine tuning of the signalling processes which will result in T-cell activation.

1.2.6 The activity regulation of ZAP-70

The activity regulation of ZAP-70 and other proximal kinases is crucial: the pattern of near-TcR tyrosine phosphorylation depends on the "strength" of signal (antigen affinity towards the TcR) and thus determines the fate of the T-cell after antigen encounter: activation, anergy or apoptosis [59]. The activity regulation of the non-receptor PTKs occur mainly via phosphorylation. There are 30 tyrosine residues in ZAP-70 and 11 of them are known phosphorylation sites (Figure 8). Initial studies on TcR signalling revealed that the phosphotyrosine-content as well as the kinase activity of ZAP-70 increases rapidly after TcR stimulation [87]. The recently known tyrosine phosphorylation sites and literature data are summarized in Table 2. and briefly explained in the following sections.

Tyrosine on ZAP-70	Phosphorylation Induced by	Function	Literature
Y69	*In vitro*: p56-lck	unknown	[88]
Y126	*In vitro*: autophosphorylation p56-lck	unknown	[88]
Y178	*In vitro*: p56-lck	unknown	[88]
Y292	*In vitro*: autophosphorylation p56-lck OKT-3 activation	Docking site for Cbl (inhibition)	[88] [89] [90] [91] [92] [93]
Y315	Autophosphorylation Phosphorylated upon TcR triggering	Docking site for Vav Docking site for CrkII Allosteric regulation for SH2- ITAM binding	[94] [92] [95] [96] [97]
Y319	Autophosphorylation Phosphorylated upon TcR triggering	Docking site for p56-lck SH2 domain	[98] [99] [100] [96]
Y474	TcR triggering	Docking site for Shc	[101]
Y492	p56-lck *in vitro*	Inhibitory site	[102] [89]
Y493	p56-lck *in vitro*	Phosphorylation dependent activation	[102] [103] [89]
Y597	autophosphorylation	Negative regulatory site	[104]
Y598	autophosphorylation	Negative regulatory site	[104]

Table 2. *Summary of the ZAP-70 tyrosine phosphorylation sites*

1.2.7 N-terminal tyrosine phosphorylation sites of ZAP-70

Initially, Watts and colleagues identified some phosphorylation sites analyzing the tryptic fragments of ZAP-70 from Jurkat cells by electrospray ionization mass spectrometry [88]. Others detected different phosphorylation sites applying cyanogen-bromide cleavage. [98]

There is only one publication which identifies three N-terminal phosphorylation sites on ZAP-70: Y69, Y126 and Y178 [88]. There are no further information about the function of these phosphorylation sites. The Y69 site is located within the N-terminal SH-2 domain of ZAP-70. Although the sequence around this site in human ZAP-70 is highly homologous not only to that of in murine ZAP-70, but also to murine and human Syk, this tyrosine residue has not been shown being phosphorylated in murine Syk [105].

The Y126 site is located within the Interdomain A region of ZAP-70 kinase. The homologous tyrosine Y130 is phosphorylated in murine Syk kinase [105]. The phosphorylation of this tyrosine inhibits the association of Syk kinase with the ITAMs of the B cell antigen receptor complex. [106, 107] However, we have no information about the exact function of this tyrosine in ZAP-70.

The Y178 is located in the C-terminal SH-2 domain of ZAP-70. It's function is unknown, Watts and colleagues showed that it is phosphorylated in vitro in the presence of p56-lck [88]. Magistrelli et al. described that the ablation of the SH2 domains of ZAP-70 (thus abolishing all three aforementioned phosphorylation sites) increases the catalytic activity of ZAP-70 [108].

1.2.8 Phosphorylation sites located on Interdomain B of ZAP-70

The phosphorylation sites of interdomain B region were studied the most intensively. This region is important in the regulation of ZAP-70 intrinsic kinase activity as well as the intermolecular interactions with other signalling proteins. Watts et al. designated the Y292 as the principal autophosphorylation site of ZAP-70 [88]. It is known that this is the docking site of the Cbl protein which negatively regulates ZAP-70 function. [109, 110]. The pY292 phosphotyrosine

can be dephosphorylated by LMPT phosphatase, thus activating ZAP-70 [93]. Interestingly, when the interdomain B region is entirely removed containing the Y292, Y315 and Y319 phosphorylation sites, the mutated ZAP-70 retained full receptor binding affinity. Although the intrinsic kinase activity was markedly decreased, the cells transfected with the mutant ZAP-70 showed NF-AT induction, calcium signal and MAPK cascade activation [111].

The first *in vitro* functional studies of the of the Y315 phosphorylation site were performed by Wu and colleagues. They demonstrated that pY315 recruits the 95 kDa protooncogene product Vav. A Y>F mutation introduced at this site eliminated the ZAP-70-Vav interaction and caused deep impairment in T-cell activation. Moreover, this point mutation caused a marked reduction in the tyrosine phosphorylation of ZAP-70, Vav, SLP-76, and Shc [94]. Recent findings indicate, that the pY315 serves also for binding the CrkII adaptor protein [97]. The authors found that the CrkII and Vav binds to pY315 with affinity of similar magnitude. They propose that the two different signalling protein may link ZAP-70 to different signalling pathways. However, the exact role of the ZAP-70-CrkII linkage is not determined yet [97].

The Y319 functions as an important activating phosphorylation site on ZAP-70. The first in vitro study by DiBartolo et al. suggested that the phosphorylation of this site is induced both by autophosphorylation and by TcR stimulation. The Y319F mutation dramatically impaired anti-TcR-induced activation of the NF-AT and IL-2 production of T-cells. Additionally, substantial loss was observed in ZAP-70-(Y319F) activation-induced tyrosine phosphorylation and up-regulation of catalytic activity, as well as a decreased capacity to phosphorylate known ZAP-70 substrates, such as SLP-76 and LAT [98]. Later it was shown that this tyrosine is responsible for mediating the ZAP-70 co-clustering with p56-lck [99]. There are also data about CTLA-4 mediated suppression: the ligation of CTLA-4 selectively inhibited the phosphorylation of Y319 of ZAP-70 [100].

Hitherto there are three *in vivo* studies designed to elucidate the regulatory functions of the Interdomain B phosphorylation sites on ZAP-70. DiBartolo and colleagues demonstrated that the pY315 may function as an allosteric regulator for the SH2-ITAM association. Studying knock-in mice expressing the point mutant ZAP-70-(Y315F) they showed that the affinity of mutant ZAP-

70 is markedly decreased towards the ITAMs compared to wild type ZAP-70. Moreover, the authors found decreased phosphorylation of the TcR ζ chain, suggesting that the phosphotyrosines were more accessible to phosphatases. However, the defective ITAM-binding of the Y315F mutant ZAP-70 appeared to be independent of this residue being the binding site of Vav [95]. Magnan and co-workers generated mice with Y292F and Y315F mutations. The thymi of all transgenic mice showed normal cellularity and thymocytes appeared to have normal surface marker expression. Analyzing the signalling processes derived from the TcR the authors observed various phosphorylation changes both in the ZAP-70-(Y315F) and ZAP-70-(Y292F) mutant mice. Referring to the complex function of ZAP-70 during the T-cell maturation stages marked differences were described between the signalling processes in thymocytes and peripheral T-cells in both mutants. In general, the ZAP-70-(Y292F) mutant mice were “hypersensitive” to TcR stimulation showing increased phosphorylation and producing more IL-2 and IFNγ in response. In contrast, ZAP-70-(Y315F) mutant mice showed reduced rate of positive selection and delayed negative selection indicating the attenuated ZAP-70 function [92]. The other study by Gong Q et al. used transgenic mice expressing the Y315F and Y319F mutants of ZAP-70 under the control of the *lck* promoter. The authors described that CD4+ thymocytes were present in a significantly lower number in the thymus and both CD4+ and CD8+ cell number was lower in the peripheral lymphoid organs of the ZAP-70-(Y319F) mutant mice, than that of in the wild type controls. The thymocytes obtained from the ZAP-70-(Y319F) mutant mice showed attenuated calcium signal responding TcR stimulation. The results of this group showed on ZAP-70-(Y315F) mutant mice are consistent with those of the former group showed [96].

1.2.9 C terminal tyrosine phosphorylation sites of ZAP-70

The tyrosine residue 474 was identified as the binding site of Shc, which couples the activated TcR to Ras. Y>F point mutation of this site prevented ZAP-70 interaction with Shc and the subsequent binding of Shc to phospho-ζ. Neither ZAP-70 catalytic activity nor the pattern of protein phosphorylation induced by TcR triggering was affected by this mutation. However, expression

of the ZAP-70-(Y474F) mutant resulted in impaired TcR-dependent gene activation [101].

Tyrosine 492 and 493 as a phosphorylation site was first identified by Watts et al. [88]. First characterisation of the ZAP-70-(Y492F) point mutant showed 4 times higher basal kinase activity compared to wild type ZAP-70, [102] while the phosphorylation of the Y493 site was required for proper ZAP-70 activation mediated by p56-lck [103].

Virtually all PTKs are themselves substrates for tyrosine phosphorylation. In some PTKs, such as the Src family, there are tyrosine residues located outside of the kinase domain, which inhibit kinase activity when phosphorylated. Usually these tyrosines are the substrates of other regulatory PTKs. Other tyrosine residues present in the kinase domain of all PTKs are critical for full activation of the kinases [112]. Phosphorylation sites like this were first characterised in Src kinase. Here the tyrosine residue Y416 is the principal site of autophosphorylation with the sequence $EY^{416}TAR$. Highly homologuos phosphorylation site in p56-lck kinase is $EY^{394}TAR$ [113]. The amino acids around the corresponding tyrosine of the human and murine ZAP-70 sequence are homologous, too: $SY^{492}Y^{493}TAR$. Strikingly, the Syk $DENY^{525}Y^{526}KAG$ sequence around the corresponding phosphorylation site is different. This difference may cause the phenomenon that the ZAP-70 by itself is not active after being recruited to the TcR ζ ITAMs, but it needs to be phosphorylated by src family kinases (mainly by p56-lck) to achieve fully activated state. In contrast to ZAP-70, Syk kinase is able to autophosphorylate this residue once docked on the ITAMs, not requiring the presence of src family kinases [114].

Negative regulatory sites have been found in the C-terminal end of ZAP-70 by Zeitlmann and colleagues [104]. Consistently with the C-terminal tyrosines Y628, Y629 and Y630 of the Syk kinase, the adjacent tyrosines Y597 and Y598 of ZAP-70 exert a negative regulatory function when phosphorylated. Y>F mutations of these sites result in a prominent gain-of-function phenotype in Jurkat T cells, as increased cytokine promoter activation, elevated tyrosine phosphorylation of potential targets and augmented intracellular calcium mobilization [104].

2 Objectives

1. Studying the rapid non-genomic GC effects on T-cells using an *in vitro* cultured model cell line (Jurkat). Definition the alterations in the tyrosine phosphorylation pattern in Jurkat cells after high dose Dexamethasone (DX, GR agonist) treatment.
2. Characterisation of the rapid changes in the phosphotyrosine content of ZAP-70 kinase after high dose DX treatment. Determination of the GR dependency and the upstream kinase responsible for DX induced phosphorylation of ZAP-70. Studying the kinetics of the DX induced ZAP-70 phosphorylation.
3. Examination the possible physical linkage between GR and ZAP-70 using co-immunoprecipitation and confocal microscopy in the presence and absence of GR agonist in Jurkat cells. Clarification the relationship of the Hsp-90 chaperone toward the GR and ZAP-70.
4. Studying the relation of GR, ZAP-70 and Hsp-90 in a non-T cell environment applying HeLa cells stably transfected with ZAP-70.
5. Investigation of the crosstalk between the TcR-CD3 complex and GR-related signal transduction pathways.

3 Experimental procedures

3.1 Cell lines

We used Jurkat cells (human acute T-cell leukemia) and its p56-lck and ZAP-70 deficient subclone (JCaM1.6 and P116, respectively), a kind gift of E. Monostori (Biological Research Centre, Szeged, Hungary), and HeLa cells (human cervix adenocarcinoma). Cells were cultured in humidified atmosphere, containing 5 % CO2 at 37^{o}C, in RPMI medium supplemented with 5 % (Jurkat cells) and 10 % (JCaM1.6 and HeLa cells) fetal calf serum (Gibco).

3.2 Plasmid construct

Human full-length wild-type ZAP-70 cDNA has been cloned, sequenced and inserted in the pWPTS lentiviral transfer plasmid. In this construct ZAP-70 is under the control of an EF1 promoter. This late second generation lentiviral construct contains elements that increase transgene integration and expression, i.e. cPPT and WPRE [115, 116].

3.3 Lentivirus production and transduction

Briefly, an envelope construct (pMD.G), a packaging plasmid (R8.91) and the transfer plasmid (pWPTS with EF1-ZAP-70) were transiently co-transfected by calcium-phosphate method into 293T cells pre-treated with chloroquine (1mM final concentration). Following an overnight incubation and medium change, the supernatant of the virus producing cells was harvested after 24 hours, centrifuged (2000 rpm, 10 min, 4°C) and filtered (0.45mm pore-size PVDF-coated filters) to eliminate rough cellular debris [117-119]. P116 and HeLa cell lines cells were transduced by spinoculation. Briefly, viral supernatants were spun in 24-well plates (3600 rpm, 60 min, 4°C), then the supernatant was discarded and 20,000 cells were carefully layered into the wells in 2 ml fresh medium. The viral supernatant was previously titrated and biological viral titer was approx. 10^5 TU/ml. P116 and HeLa cell lines were transduced at MOI=10 (MOI: multiplicity of infection or virus/cell ratio) [120].

3.4 Chemicals and buffers

All fine chemicals were purchased from Sigma-Aldrich otherwise indicated. Dexamethasone as a glucocorticoid hormone receptor agonist and RU486 (Mifepristone) was used as glucocorticoid antagonist, both dissolved in DMSO at a concentration of 10 mM. For Western-blot experiments cells were lysed in lysis buffer (50 mM HEPES, 10 mM sodium pyrophosphate 10 mM EDTA, 100 mM sodium fluoride, 10 % glycerol, 1 % Triton X-100) or, in case of the co-immunoprecipitation experiments, we used TEM buffer containing 50 mM NaCl, 10 mM Tris-HCl pH7.6, 4 mM EDTA, 20mM sodium molybdate, 10 % glycerol. Aprotinin, leupeptin (Fluka) (10 μg/ml), PMSF (2 mM) were freshly added to the lysis buffers.

The samples were separated by SDS-PAGE after adding 2x sample buffer (125 mM Tris pH 6.8, 4 % SDS, 10 % glycerol, 0.006 % Bromo-phenol-blue 10 % mercaptoethanol). To saturate non-specific binding sites, the blots were soaked in blocking buffer (1 % bovine serum albumin, 10 mM Tris, 100 mM sodium chloride, 0.1 % Tween 20, pH 7.4) For washing the blots washing buffer was used (10 mM Tris, pH 7.4 100 mM sodium chloride, 0.1 % Tween-20). Western-blot visualisation was performed by enhanced chemiluminescence as described in the manufacturer's instructions (SuperSignal West Pico Chemiluminescent substrate, Pierce). The intracellular labelling for confocal microscopy was performed in saponine buffer (0.1% saponin, 0.1%BSA, 0,1% azide in PBS).

3.5 Antibodies

We used mouse monoclonal anti-human CD3 antibody (clone: OKT-3, 2,5 mg/ml stock solution in PBS) for cell activation. Immunoprecipitation was performed either with rabbit polyclonal anti-ZAP-70 antibody (kindly provided by E. Monostori, Biological Research Centre, Szeged, Hungary), or mouse monoclonal anti-GR antibody (clone 8E9) produced in our laboratory [121]. For western blotting mouse monoclonal anti-ZAP-70 (clone: 29, Transduction Laboratories) mouse monoclonal anti-GR antibody (clone 5E4, [121]), rabbit polyclonal anti-Hsp-90 antibody (Santa Cruz Biotechnology), HRPO-conjugated goat anti-mouse IgG (Hunnavix). For confocal microscopy

fluorescein conjugated anti-GR (5E4) and phycoerythrin-conjugated mouse monoclonal anti-ZAP-70 (eBioscience, clone 1E7.2) were used. FITC and PE-conjugated mouse IgG1 isotype control antibodies (DakoCytomation) were applied as negative controls. The tyrosine-phosphorylation pattern was examined by using biotinylated monoclonal anti-phosphotyrosine antibody (clone: PY20, Transduction Laboratories) and HRPO-conjugated streptavidin (Amersham) as a secondary reagent. To prove the equal protein loading we used monoclonal mouse anti-β-actin antibody (Sigma).

3.6 Geldanamycin treatment

Jurkat 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 once in serum-free RPMI and subjected to DX treatment as indicated below.

3.7 Glucocorticoid analogue/antagonist treatment

Jurkat or JCaM1.6 cells were harvested and resuspended in incomplete RPMI. The cells were plated and incubated for 4 hours at 37^{o}C in the presence or absence of RU486 (Mifepristone). After a washing step, final cell concentration was set to 10^{8}cells/ml and tubes were placed on an Eppendorf Thermomixer set at 37^{o}C. Dexamethasone (DX) or solvent (DMSO) were added at a concentration of 10μM for 5 minutes. The treatment was stopped by placing the tubes into liquid nitrogen. In case of confocal microscopy samples the final cell concentration was 10^{7}cells/ml and the DX treatment was stopped by addition of 9 volumes of ice-cold PBS supplemented with 0.1% sodium-azide.

3.8 Cell activation and lysis

50 μg OKT-3 anti - CD3 monoclonal antibody was added to the DX treated or untreated samples. Following incubation for the indicated time at 37^{o}C the activation was stopped by quickly freezing the cells in liquid nitrogen. Then the samples were lysed for 30 minutes in 500 μl ice-cold TX-100 or TEM lysis buffer. When TEM buffer was used for co-immunoprecipitation experiments, an additional sonication step was included for 5 times 5 seconds at 5W power.

After lysis the samples were centrifuged at 13000 rpm for 15 minutes at 4°C. Postnuclear supernatants were aspirated and boiled for 5 minutes with equal amounts of 2x sample buffer or subjected to immunoprecipitation.

3.9 Immunoprecipitation and Western blot

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 minutes. After the removal of the pre-clearing beads, 10 μl precipitating antibody was added to the lysates for 2 hours. Then Protein-G Sepharose beads were added for additional 2 hours. The beads were pelleted and five washes were performed with ice-cold washing buffer. After washing, beads were resuspended in 100 μl of SDS sample buffer and the immunoprecipitates were boiled for 10 minutes. The supernatants were collected and loaded on SDS-polyacrylamide gels. SDS-PAGE was performed using a BioRad MiniProtean elecrophoresis equipment. The gels were blotted overnight to nitrocellulose membranes (Bio-Rad) by wet transfer procedure. After blocking non-specific binding sites for 1 hour, blots were incubated with the primary antibody for two hours. Following 3x 10 minutes washing, the HRPO - conjugated secondary antibody or streptavidine was added for one hour. Western blots were visualised with SuperSignal West Pico Chemiluminescent substrate and the fluorescent signal was recorded using X-ray film. (Fujifilm).

3.10 Confocal microscopy

Dexamethasone treated or untreated Jurkat cells were fixed for 10 minutes in 4 % paraformaldehyde, washed once in ice-cold PBS-azide and once in saponine buffer. The cells were permeabilized in saponine buffer and the fluorochrome labelled monoclonal antibodies or the appropriate negative control antibodies were added at a concentration of 1 μg/ml. After one hour incubation on ice the cells were washed twice in saponine buffer, once in PBS-azide and layered onto slides. After the cells settled the remaining fluid was carefully aspirated and the slides were covered using 50 % glycerol-PBS. In case of HeLa cells the labeling procedure was carried out on monolayers grown on coverslips. An Olympus Fluoview 300 confocal microscope or later a

Olympus Fluoview FV1000S-IX81 system was used for the examination of the samples.

3.11 Calcium signal measurement

Intracellular free calcium was measured using Fluo-3 AM according to the protocol previously described [122], with some modifications [123]. Briefly: the fluorescence intensity of Fluo-3 AM dye was detected in the gated cell population at 526 nm (FL1 channel), which is proportional with the intracellular free calcium level [122]. After measuring basal fluorescence at 526 nm, anti-CD3 antibody was added to the tubes (each containing $5x10^5$ cells/500 µl RPMI, loaded by Fluo-3 AM for 30 min at RT prior to analysis) and the measurement was continued for a further 400 s, thus making possible to follow the alterations of intracellular free calcium level (FL1 fluorescence intensity) in time. Gates were created along the time axis of the activation dot plots at definite time points and the mean fluorescence intensity at 526 nm was statistically analyzed from every gate. These values were corrected with the basal fluorescence intensities measured in the same sample before the addition of the activating agent and represented as mean fluorescence intensity ratios (y-axis) [123].

4 Results

4.1 Dexamethasone alters the tyrosine phosphorylation pattern of Jurkat cells

First we examined how DX treatment influences the tyrosine-phosphorylation pattern of resting and anti - CD3 activated Jurkat cells. 2 minutes of 10μM DX treatment alone caused increased protein tyrosine - phosphorylation at the 34, 52, 59, 70 and 90 kDa molecular weight bands in whole Jurkat cell lysates, compared to the solvent-treated control (Figure 9.). Activation with monoclonal anti - CD3 antibody markedly increased the tyrosine-phosphorylation of several proteins in whole cell lysates at 25, 34, 38, 40, 52, 59, 70 and 90 kDa molecular weight ranges, respectively (Figure 9.). A 2 minute DX pre-treatment at 10 μM concentration inhibited the anti - CD3 induced tyrosine-phosphorylation the proteins of 34, 38, 40, 52, 59, and 90 kDa (Figure 9.). No significant signal was detected on negative control blots, incubated with biotinylated monoclonal anti-insulin antibody (data not shown).

4.2 Dexamethasone rapidly induces ZAP-70 phosphorylation

ZAP-70 plays a central role in the TcR signal transduction pathway, by phosphorylating a multiple substrates, and itself is also phosphorylated on several tyrosine residues. For this reason we investigated the tyrosine-phosphorylation of ZAP-70 after DX and / or anti - CD3 treatments. 5 minutes exposure to 10 μM DX caused an average of 4 fold (3.32 $\pm$ 1.72) increase in tyrosine-phosphorylation of the ZAP-70 kinase in the anti-ZAP-70 precipitated samples (Figure 10.). The anti - CD3 treatment also resulted in a 4 fold increase (3.59 $\pm$ 1.69) in the tyrosine-phosphorylation of ZAP-70 kinase (Figure 10.). The tyrosine-phosphorylation after combined DX + anti - CD3 treatment was higher (4.98 $\pm$ 2.83) than in case of anti - CD3 or DX treatment alone (5 fold versus 4 fold increase, respectively). Phosphorylation increase after single DX or anti - CD3 or combined treatments proved significant compared to the solvent treated control sample (Student's t-test, $P < 0.05$). We observed higher phosphorylation after combined DX + anti - CD3

treatment than single DX or anti - CD3 treatment in all of our independent experiments, but this further rise was statistically not significant by Student's t-test. (Figure 10.).

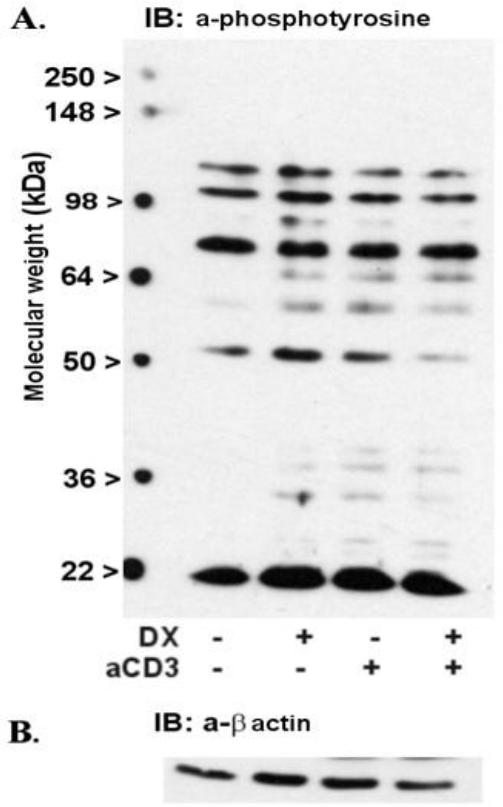

Figure 9.. *Dexamethasone and / or anti - CD3 treatment influenced the tyrosine-phosphorylation pattern in Jurkat cell lysates. Western blotting was performed with monoclonal anti-phosphotyrosine antibody. Then the blot was stripped and reprobed with monoclonal anti-beta actin antibody to check equal protein loading. Molecular weight markers are indicated on the left, treatment modalities on the bottom side of the blot. The experiment was repeated three times with similar results. Here a representative Western blot is depicted.*

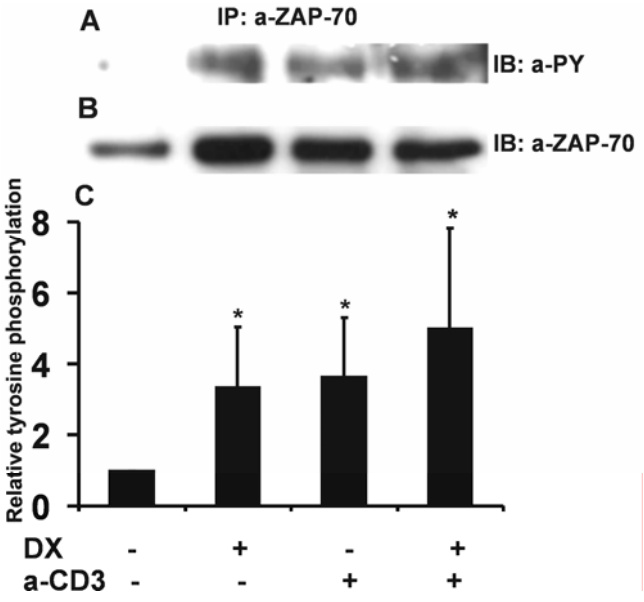

Figure 10.
Effect of Dexamethasone and / or anti-CD3 treatment on ZAP-70 tyrosine-phosphorylation.
Western blotting was performed on anti-ZAP-70 immuno-precipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: a-ZAP-70) (Panel B). The relative phosphotyrosine content of ZAP-70 is shown as relative density averages ± SD from the densitometry data of three separate experiments ($P < 0.05$) (Panel C). Treatment modalities are indicated below the diagram (Panel C).*

4.3 Time kinetics of Dexamethasone induced ZAP-70 phosphorylation

Since the kinetics of tyrosine-phosphorylation is a key feature to understand cellular signalling events, we further investigated the time course of DX induced ZAP-70 tyrosine-phosphorylation. The tyrosine-phosphorylation of ZAP-70 occurs rapidly after the addition of high dose DX. We measured a rise in tyrosine-phosphorylation after 1 and 2 minutes after DX administration, respectively (Figure 11.). Dephosphorylation occurred within 5 minutes (Figure 11.).

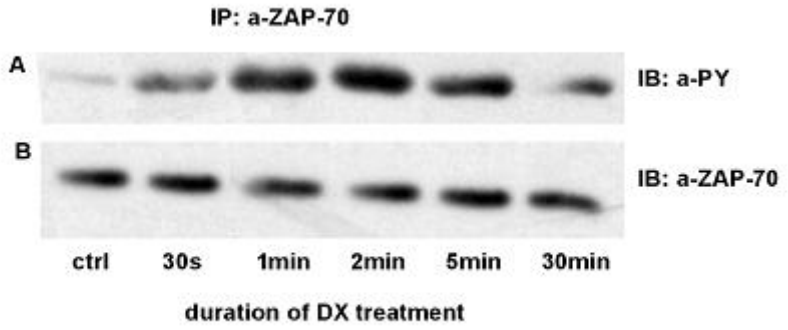

Figure 11. *Time dependent tyrosine-phosphorylation of ZAP-70 after high dose Dexamethasone treatment. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Duration of the Dexamethasone treatment is indicated below the figure. The experiment was repeated three times with similar results.*

4.4 Dexamethasone induced ZAP-70 phosphorylation is p56-lck dependent

In addition to acting as a tyrosine kinase, ZAP-70 is a substrate of p56-lck, a T-cell specific src-family tyrosine kinase. We therefore tested whether the DX-induced rapid tyrosine-phosphorylation of ZAP-70 was p56-lck dependent. When JCaM1.6, the p56-lck deficient subclone of Jurkat cells was subjected to high dose DX treatment, we observed no rise in the degree of tyrosine-phosphorylation of ZAP-70 kinase (Figure 12.). The anti - CD3 activation induced notable increase in tyrosine-phosphorylation of ZAP-70 in JCaM1.6 cells (Figure 12.). Additional DX stimulation did not alter the phosphotyrosine

increase caused by anti - CD3 activation in the p56-lck deficient cell line (Figure 12.).

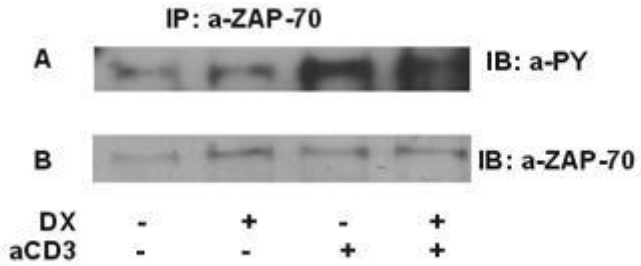

Figure 12. *ZAP-70 tyrosine-phosphorylation after Dexamethasone and / or anti - CD3 treatment in p56-lck kinase deficient Jurkat subclone JCaM1.6. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Treatment modalities are indicated below the blots. A representative Western blot is shown from 3 independent experiments with similar results.*

4.5 Dexamethasone triggered ZAP-70 phosphorylation is abolished by RU486 pre-treatment

Previous studies indicated the existence of various GR mediated or receptor independent non-genomic GC effects. Therefore we investigated whether DX induced tyrosine-phosphorylation alterations were GR dependent in Jurkat T-cells using RU486 (Mifepristone), a glucocorticoid receptor antagonist. DX alone caused markedly growth in the tyrosine-phosporylation of ZAP-70 (Figure 13.). Pre-treatment of Jurkat cells for 4 hours with equimolar RU486 prevented this rapid phosphorylation of ZAP-70 induced by subsequent high dose DX exposure. RU 486 treatment alone caused no remarkable tyrosine-phosphorylation change in ZAP-70 (Figure 13.).

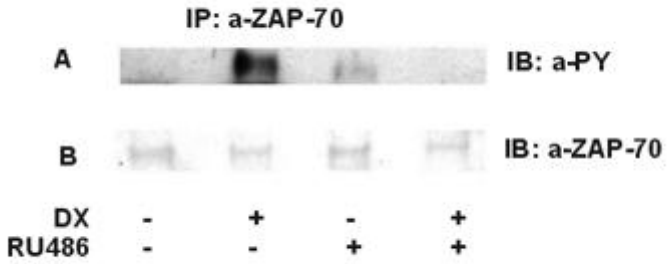

Figure 13. *Effect of RU486 (Mifepristone, glucocorticoid receptor antagonist) pre-treatment on the Dexamethasone induced tyrosine-phosphorylation of ZAP-70. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Treatment modalities are indicated below the blots. A representative Western blot is shown from 3 independent experiments with similar results.*

4.6 GR co-precipitates with ZAP-70 in the lysates of DX treated Jurkat cells

Since previously we have found that sort term high dose DX treatment increased the phosphotyrosine content of the ZAP-70 molecule, we sought a possible association between the ZAP-70 and the GR molecules in Jurkat cells. Therefore we performed immunoprecipitation on DX or vehicle treated Jurkat cell lysates, both with anti-ZAP-70 and anti-GR antibodies, to investigate whether there is a physical link between GR and ZAP-70 in the cytoplasm. In GR antibody precipitated lysates we found that ZAP-70 signal increased significantly upon DX treatment, compared to the vehicle treated samples, while the GR signal was unchanged (Figure 14.). In the ZAP-70 antibody precipitated lysates GR signal increased significantly upon DX treatment, when compared to vehicle treated cell lysates, while the ZAP-70 signal remained unchanged (Figure 14.). So in DX treated Jurkat cells the GR co-precipitated with ZAP-70 and *vica versa*, while the association of the two molecules was minimal in the vehicle-treated control lysates (Figure 14.).

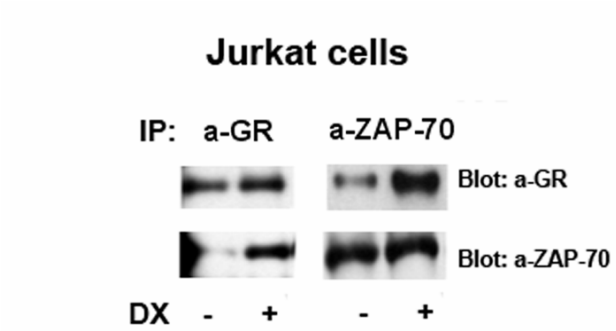

Figure 14. *Co-precipitation of liganded GR with ZAP-70 kinase occurs in Jurkat cells. When anti-GR antibody was used for immunoprecipitation (IP: a-GR), equal amount of GR precipitated (Blot:a-GR), but ZAP-70 co-precipitated only when DX was present. (Blot: a-ZAP-70) Similarly, when anti-ZAP-70 was the precipitating antibody (IP: a-ZAP-70) equal amount of ZAP-70 was present both in the control and DX treated samples but GR co-precipitated with ZAP-70 only in the DX-treated sample.*

4.7 The ligand-bound GR co-localises with ZAP-70 in the cytoplasm of Jurkat cells

To characterise the localization and relation of the ZAP-70 and GR molecules in the cytoplasm further, we visualised the two molecules parallel by confocal microscopy. In vehicle treated, resting Jurkat cells both ZAP-70 and GR showed even, mostly cytoplasmic distribution, with almost no co-localization. Upon 5 min high dose DX treatment we observed near-membrane staining of both the GR and ZAP-70 molecules with markedly increased co-localization of the two molecules (Figure 15.). Thus we confirmed our immunoprecipitation results with confocal microscopic pictures also.

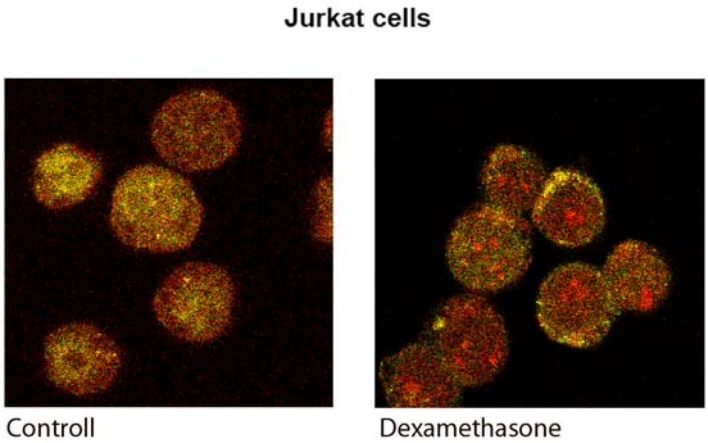

Figure 15. *Confocal microscopic examination of GR and ZAP-70 co-localization. Jurkat cells were fixed and labelled with a-GR-FITC (green channel) and a-ZAP-70-PE (red channel). The cells were sedimented on slides and examinated with a confocal microscope. In the DX-treated samples the GR and the ZAP-70 showed near-membrane co-localisation, while this phenomenon was absent in the untreated controls.*

4.8 The GR co-precipitates with ZAP-70 in the DX treated samples of HeLa-trZAP-70 cell lysates

As ZAP-70 is a T-and NK cell specific molecule and it is in close relation with other lymphocyte specific molecules e.g. the p56-lck, we wanted to investigate whether the co-precipitation of the GR and ZAP-70 molecules were dependent on lymphocyte specific factors. We used a novel 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 kinase. The ZAP-70 expression level in transfected P116 cells showed to be higher (MFI: 98) than that of Jurkat cells (MFI: 28) as tested by flow cytometry (Figure 16. A). The ZAP-70 construct showed to be fully functional in P116 cells as tested by anti-CD3 activation and consequent intracellular calcium signal measurement using flow cytometry (Figure 16. B). Despite the higher ZAP-70 expression in ZAP-70 transfected P116 cells, the kinetics and amplitude of the calcium signal was similar to that of wild type Jurkat cells (Figure 16. B).

The ZAP-70 expression level in transfected HeLa cells showed to be higher (MFI: 108) to that of Jurkat cells (MFI: 28) as tested by flow cytometry (Figure 17. A). Immunoprecipitations with anti-ZAP-70 and anti-GR antibodies were performed from HeLa cell lysates (Figure 15. B). The ZAP-70 kinase co-precipitated with the GR and *vica versa* in transgenic HeLa cells expressing ZAP-70 in the DX-treated samples but in the absence of its ligand the clustering of the two molecules was absent (Figure 17. B), similarly to what we have found in Jurkat cells. Co-localization of the two molecules was also verified with fluorescence confocal microscopy (Figure 18). We found the perinuclear co-localization of the transfected ZAP-70 molecule with the GR in ZAP-70 transfected Hela cells (Figure 18.). Upon 5 min high dose DX treatment we observed intensive cytoplasmic staining of both the GR and ZAP-70 molecules with markedly increased co-localization of the two molecules (Figure 18.).

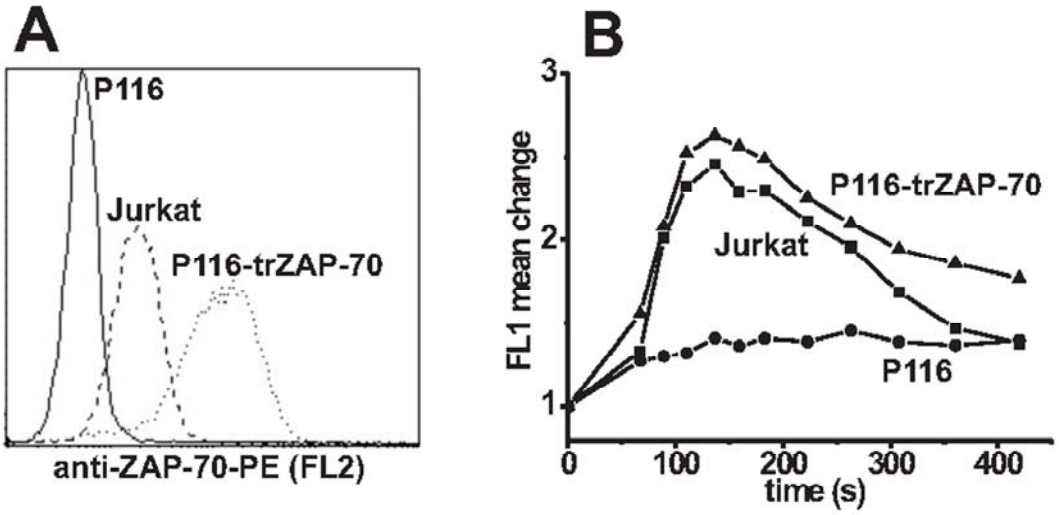

Figure 16. *Lentiviral transfection of ZAP-70 deficient P116 cells with ZAP-70.* ***Panel A:*** *ZAP-70 expression of ZAP-70 transfected P116 cells was compared to untransfected P116 and Jurkat cells by flow cytometry after intracellular phycoerythrine (PE) conjugated anti-ZAP-70 antibody labelling. Flow cytometric histogram shows PE conjugated anti-ZAP-70 fluorescence (FL2) of a representative measurement from three separate experiments.* ***Panel B:*** *Calcium signal of ZAP-70 transfected P116 cells (▲) was compared to untransfected P116 (●) and Jurkat cells (■) by flow cytometry after Fluo-3 loading of samples. Anti-CD3 antibody was added to the cells at zero time point, then calcium level changes were followed as FL1 fluorescence changes for 400 seconds. Curves show the result of a representative measurement from three separate experiments.*

4.9 ZAP-70 and Hsp-90 co-pecipitation in Jurkat and HeLa-trZAP-70 lysates could be inhibited by geldanamycin

Since the unliganded GR complexes Hsp-90 in the cytoplasm [124], and the upstream kinase p56-lck is also an Hsp-90 client protein [125], we aimed to check the relation of ZAP-70 to Hsp-90. We found that Hsp-90 co-precipitated with ZAP-70 from both Jurkat and HeLa-trZAP-70 cell lysates (Figure 19.). This co-precipitation was detectable both in DX and vehicle treated samples (Figure 19.). To further elucidate the relations of these three molecules: ZAP-70, GR and Hsp-90 in Jurkat cells we used geldanamycin (GA) to specifically inhibit Hsp-90 and we performed immunoprecipitation with anti-ZAP-70 antibody (Figure 20.). Hsp-90 failed to co-precipitate with ZAP-70 in the GA treated samples (Figure 20.). Although GA inhibited the co-precipitation of ZAP-70 with Hsp-90, it could not abrogate the DX induced GR-ZAP-70 association. (Figure 20.)

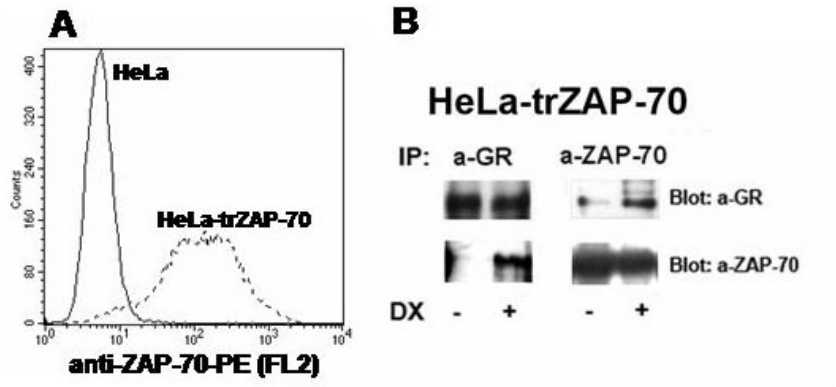

Figure 17. *Liganded GR clustering with ZAP-70 in ZAP-70 transfected HeLa cells.* ***Panel A:*** *Representative flow cytometric histograms show the ZAP-70 expression as FL2 fluorescence of control and ZAP-70 transfected HeLa cells after intracellular labelling with phycoerythrin (PE) conjugated anti-ZAP-70 antibody.* ***Panel B:*** *Anti-GR or anti-ZAP-70 Western blots are shown from anti-GR or anti-ZAP-70 precipitated ZAP-70 expressing HeLa cell lysates with or without DX pretreatment. Using anti-GR antibody for immunoprecipitation (IP: a-GR, left panels), equal amount of GR precipitated (Blot: a-GR, upper left panel), but ZAP-70 co-precipitated only when DX was present (Blot: a-ZAP-70, lower left), similary as we found in Jurkat cells. Vica versa, when the precipitating antibody was anti-ZAP-70 (IP: a-ZAP-70, right panels) equal amount of ZAP-70 was present both in the control and DX treated samples (Blot: a ZAP-70, lower right panel) but GR co-precipitated with ZAP-70 only in the DX-treated sample(Blot: a-GR, upper right panel). The figure shows a representative blot from three separate experiments.*

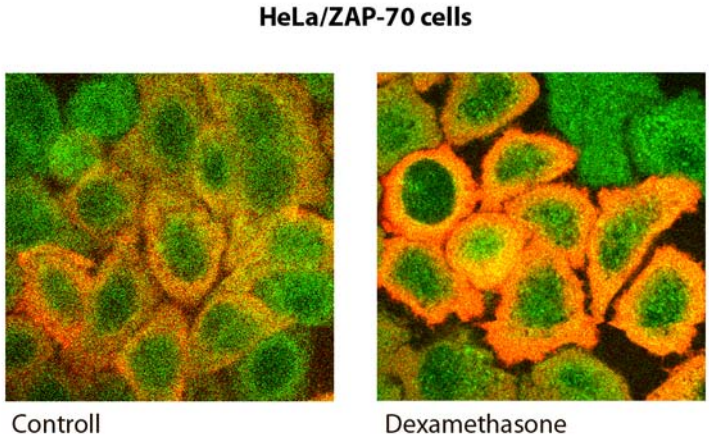

Figure 18. *Confocal microscopic examination of GR and ZAP-70 co-localisation in transgenic HeLa/ZAP-70 cells. DX treated or untreated monolayers of HeLa/ZAP-70 cells growing on a coverslip were fixed and subjected intracellular labelling with a-GR-FITC (green channel) and a-ZAP-70-PE (red channel). The coverslips were mounted on slides and examinated with a confocal microscope. In the DX-treated samples the GR and the ZAP-70 showed perinuclear co-localisation, while this phenomenon was absent in the untreated controls.*

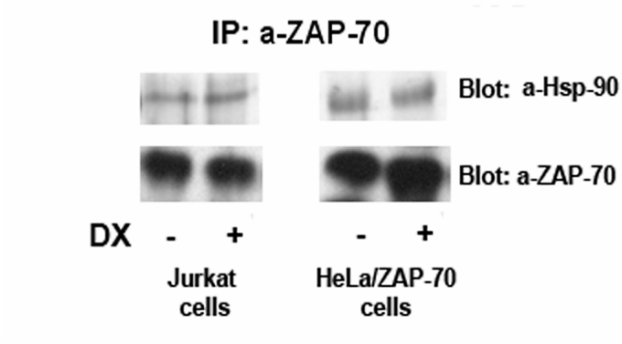

Figure 19. *ZAP-70 co-precipitates with Hsp-90 chaperone. We used anti-ZAP-70 as the precipitating antibody. (IP: a-ZAP-70) Equal amount of Hsp-90 co-precipitated both in the control and DX treated samples, suggesting that the ZAP-70 Hsp-90 co-clustering is independent of the presence or absence of GC hormone. The amount of precipitated ZAP-70 was also checked and found to be equal. (Blot: a-ZAP-70) The co-precipitation of Hsp-90 with ZAP-70 occurs both in Jurkat cells and ZAP-70 expressing HeLa cells.*

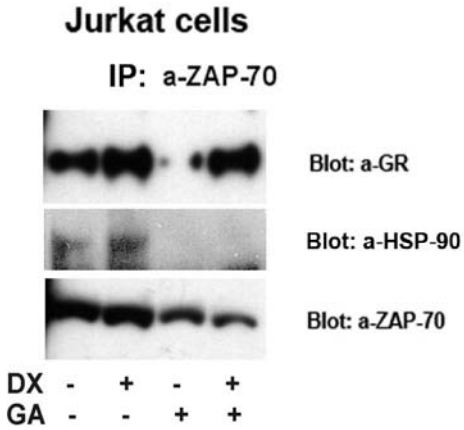

Figure 20. *Geldanamycin (GA) treatment does not inhibit the Dexamethasone induced ZAP-70 – GR co-precipitation. Anti-GR (upper panel), anti-Hsp-90 (middle panel) or anti-ZAP-70 (lower panel) Western blots are shown from anti-ZAP-70 precipitated (IP: a-ZAP-70) control or DX treated Jurkat cell lysates with or without GA pretreatment. More GR co-precipitated with ZAP-70 in the DX-treated sample compared to control (Blot: a-GR, upper panel), while equal amount of ZAP-70 was present in both samples (Blot: a-ZAP-70, lower panel). GR co-precipitation with the ZAP-70 was abrogated by GA in control samples, but was unaltered after DX treatment (Blot: a-GR, upper panel). Equal amount of Hsp-90 co-precipitated with ZAP-70 both in the control and DX treated samples, which was completely abrogated by GA pretreatment (Blot: Hsp-90, middle panel). The amount of precipitated ZAP-70 was found to be equal in control and DX treated samples with or without GA pretreatment (Blot: a-ZAP-70, lower panel). The panels of the figure show representative blots from three separate experiments.*

4.10 High dose DX treatment rapidly abrogates ZAP-70 association with the CD3 complex in Jurkat T-cells.

ZAP-70 plays a key role in the signal transduction pathways originating from the TcR-CD3 complex. T-cell activation caused by either TcR engaged by peptide primed MHC or cross-linking with anti-CD3 antibody triggers tyrosine phosphorylation events. After ZAP-70 molecules becomes phosphorylated (autophosphorylation and src-family kinase mediated phosphorylation) associate with the ITAM-tyrosines by their tandem SH2 domains. This step is crucial considering further activation events. Our results demonstrate that ZAP-70 association with the CD3 complex is abrogated in the presence of high dose DX, as it failed to co-precipitate in the DX treated samples. (Figure 21.)

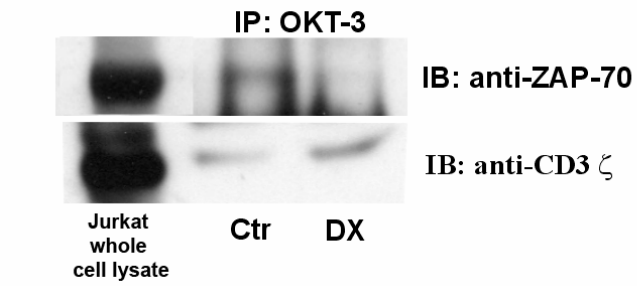

Figure 21. *DX treatment rapidly inhibits the association of ZAP-70 with the CD3 complex in Jurkat cells. CD3 complex and associated molecules were precipitated from lysates of anti-CD3 (OKT-3) activated Jurkat cells. (IP: OKT-3) The blots were probed (IB) with both anti-ZAP-70 and anti-CD3-ξ antibodies. More ZAP-70 is associated with the CD3 complex in the control sample (ctr) than that of in the DX treated sample(DX)*

5 Discussion

Our work was aimed at clarifying the possible linkage between the GR and TcR signal transduction pathways. Jurkat cells are a widely used *in vitro* model for T-cell signalling. The immunosuppressive effect of GC has been known for a long time. There are data about GC induced changes of tyrosine phosphorylation [60]. However, GC treatment applied by the authors in that experimental system was considerably longer (from a few hours to overnight time scale), although the effects were regarded to be non-genomic. The GC effects we studied occurred within minutes which definitely suggest a non-genomic way of action. We found that the rapid DX treatment induced phosphorylation events in Jurkat cells, while the DX pre-treatment abolished the phosphorylation of numerous proteins after anti-CD3 treatment. These results confirm the findings described by others [60], although on a completely different time scale.

ZAP-70 kinase plays a central role in the signalling processes originating from the TcR [84]. After the TcR engages the peptide primed MHC the ITAMs become rapidly phosphorylated. This is mediated by src-family kinases [63]. The ZAP-70 docks on the phosphorylated ITAM tyrosines and it is phosphorylated by src-family kinases. Then ZAP-70, being activated, phosphorylates other signalling proteins, e.g. the LAT, SLP-76 among others. We examined whether DX treatment changes the phosphorylation of ZAP-70. Our results demonstrated that DX caused marked increase in the phosphotyrosine content of ZAP-70. However, DX or anti-CD3 antibody induced similar level ZAP-70 phosphorylation, while combined DX+anti-CD3 treatment caused a further rise in ZAP-70 phosphotyrosine content. This observation suggests that anti-CD3 and DX treatment induces phosphorylation of different tyrosine residues on ZAP-70. Tyrosine phosphorylation is a common way of activity regulation in signal transduction. The human ZAP-70 consists of 619 amino acids and contains 30 tyrosine residues. Recently 11 tyrosines were identified as phosphorylation sites.

Some phosphorylated tyrosine residues serve as activatory or inhibitory sites, influencing directly the kinase activity. Others serve as docking sites for other regulatory proteins which exert various functions. Some phosphorylation sites' function is unknown yet. (Literature data summarized in Table 2).

Some authors describe that DX treatment inhibits the TcR induced proximal tyrosine phosphorylation events. Van Leathem et al. demonstrates that 6-16 hours of 1 μM DX treatment causes decreased phosphorylation of TcR ζ chain, ZAP-70 and LAT [60]. The authors suggested that this is because DX changes the compartmentalisation of important lipid-raft associated molecules by altering acylation [61]. Determining the concentration of GC used in various experimental systems is rather difficult. In our experiments 10μM (4μg/ml) Dexamethasone (DX) was applied. This is a pharmacological dose, much higher than the physiological GC level in the human serum. GCs are often applied in very high doses in clinical usage, especially when rapid effects are needed. (E.g.: spinal cord injury: 30mg/kg body weight methylprednisolone, osteoarthritis: 25-40mg triamcinolone acetonide or 2-4 mg dexamethasone intraarticulary (knee)). Very few publications are about the rapid, non-genomic effects of glucocorticoids. Buttgereit et al. [39] used methylprednisolone hemisuccinate-Na in a concentration range of 0.1-1.0 mg per 10^7 cells (1-10mM). This concentration is one hundred-one thousand times higher than we used. Although the physiologic GC concentration in the serum is much lower, T-cells may encounter high local GC concentrations in the thymus, where local GC production of the thymic epithel was observed [126].

The increased ZAP-70 phosphorylation after DX treatment opposes the results described by Van Leathem et al. To elucidate this controversy, we designed experiments to reveal the time kinetics of DX induced ZAP-70 phosphorylation. We observed that DX caused a rapid, transient increase in ZAP-70 phosphorylation which declined after 5 minutes. These rapidly occurring phosphorylation events indicate the participation of near-membrane molecules. The ZAP-70 is one of the main substrates of p56-lck, which is constantly directed to membrane lipid rafts by N-terminal palmitoylation. Our finding that the DX induced ZAP-70 phosphorylation was absent in p56-lck deficient JCaM1.6 cells clearly suggests the involvement of p56-lck in the

process. Intriguingly, the ZAP-70 phosphorylation after CD3 crosslinking seemed to be intact in JCaM1.6 cells. This might be due to the compensatory effects of other src-family kinases, presumably p59-fyn, which remains active in JCaM1.6 cells [127]. This confirms our theory that different tyrosine residues become phosphorylated after anti-CD3 and DX treatment.

Steroid hormones like GCs exert their effects via their cytoplasmic receptor, but presumably membrane receptors may also participate in mediating hormone action. Some authors propose the role of non-specific physicochemical effects in non-genomic steroid action. However, these effects were shown to occur only at extremely high steroid concentration [26]. We demonstrated that equimolar RU486 (Mifepristone, GR antagonist) treatment prevented the DX induced ZAP-70 phosphorylation. This refers to a classical cytoplasmic GR mediated process. The inactive GR associates with Hsp-90 in the cytoplasm. Others described the linkage of p56-lck and Hsp-90 [125]. Additionally, the Hsp-90 apparently plays an important role in maintaining the active conformation of p56-lck. The disruption of functional Hsp-90 with geldanamycin inhibits p56-lck kinase activity and consequently impairs TcR signalling [125]. So it raised the possibility that GR, ZAP-70 and Hsp-90 are closely related in the cytoplasm being parts of the macromolecular complex organised by Hsp-90. This theory is supported by our results that GR co-precipitates with the ZAP-70 and *vica versa* in Jurkat cells and in transgenic HeLa cells. This suggests the direct physical association of the two molecules, which is independent of p56-lck since this molecule is not expressed in HeLa cells. The fact that GR agonist promoted the clustering of ZAP-70 and GR might suggests that the transmission of the signal presumably occur via direct physical association of the two molecules. We also confirmed the close relation of these two molecules upon DX treatment in the near membrane compartment of the cytoplasm of Jurkat cells by confocal microscopy. We found minimal association between ZAP-70 and the GR in untreated cells. Both experimental approaches showed that the presence of GR ligand facilitated the GR association with the ZAP-70 kinase. It is interesting to observe that co-localization of GR and ZAP-70 occurred both in Jurkat and in HeLa-trZAP-70 cells, although showing different pattern. The co-clustering of ZAP-70 and GR in Jurkat cells occurred in the near-membrane compartment,

while that of in HeLa-trZAP-70 cells showed perinuclear co-localization pattern. Presumably in Jurkat cells, where the TcR-associated signaling 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 determining membrane orientation are absent resulting in different co-localization pattern.

Interestingly, our results show that ZAP-70 also co-precipitated with the Hsp-90 chaperone. Other authors also describe the co-clustering of ZAP-70 and Hsp-90 in B-CLL cells isolated from the blood of leukaemic patients [128]. 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, while the authors found no such phenomenon in healthy T-cells. Castro and colleagues proposed that the B-CLL cells, similarly to many other tumor cells, express the “activated” form of Hsp-90, with high ATPase activity [128], existing in multi-chaperone complexes [129], whereas normal cells express Hsp-90 in its non-activated form, which does not associate with ZAP-70 [128]. Our results 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 geldanamycin. As Jurkat cells themselves are of malignant origin, the ZAP-70-Hsp-90 association might represent a new aspect of the leukaemic phenotype. It is accepted that the GR dissociates the Hsp-90 after ligand binding [16]. Here we describe the ligand dependent association of the GR to ZAP-70, which cannot be blocked by geldanamycin. 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 hypothesize that the ZAP-70 might exist in two forms in the cytoplasm of Jurkat cells, whether or not it is associated to the chaperone Hsp-90. There seems to be 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. On the other hand, the non-Hsp-90 bound ZAP-70 fraction would associate with the ligand-bound GR and consequently might be involved in the cross talk between the TcR and GR signaling pathways. If we take into

consideration the findings of Castro and colleagues [128], we might speculate the “activated” Hsp-90 associated ZAP-70 fraction might represent an inactive molecular fraction in the cytoplasm of leukaemic Jurkat cells.

The fact that DX treatment inhibited the ZAP-70 kinase association with the CD3 complex in activated cells, raises the possibility that this non-genomic GC effect described here takes part in the well-known GC mechanism of suppressing T-cell activation and function. According to our recent knowledge in cellular signaling, the exact temporospatial organization of the participating molecules is of key importance in the proper signaling function. We found along with others [130], that DX treatment disrupts the CD3-associated signaling complex preventing ZAP-70 from the association with the TcR-CD3 complex. If the association of ZAP-70 with the CD3 chains is inhibited, the T-cell activation is at least defective or can lead to anergy.

In conclusion, our experiments described here suggest the existence of a previously unrevealed function of the ZAP-70: a junction between the GR and TcR signal transduction pathway that might also be affected by the chaperone Hsp-90. However, the exact role of ZAP-70, p56-lck and Hsp-90 at the molecular level needs further elucidation. We plan further examination of the GC mediated effects on signal transduction processes downstream of ZAP-70.

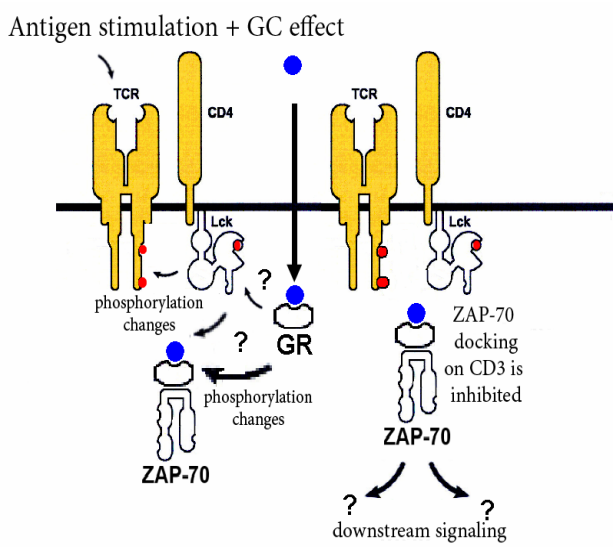

Figure 21.
Our concept on TcR-CD3- and GR signal transduction crosstalk mechanisms. We suggest that GC hormone causes multiple tyrosine phosphorylation changes as we demonstrated on Jurkat cell lysates and precipitated ZAP-70 samples. The GC hormone caused rapid phosphorylation changes of the ZAP-70 kinase. After ligand binding, the GR associates with the ZAP-70 kinase and ZAP-70 recruitment to the CD3 complex is inhibited. We assume that these processes might be partly responsible for the inhibitory effect of the GC hormone on T-cell activation.

6 References

1. Barf, T., *Intervention of Hepatic Glucose Production. Small Molecule Regulators of Potential Targets for Type 2 Diabetes Therapy.* Mini Reviews in Medical Chemistry 2004. **4**(8): p. 897-908.
2. Szollar, L.e., *Korelettan*. 1993, Budapest: Semmelweiss Kiado. 387.
3. Dallman, M.F., *Fast glucocorticoid actions on brain: Back to the future.* Frontiers in Neuroendocrinology, 2005. **26**(3-4): p. 103.
4. Cooper, M.S., *Sensitivity of bone to glucocorticoids.* Clin Sci (Lond), 2004. **107**(2): p. 111-23.
5. Seckl, J.R. and M.J. Meaney, *Glucocorticoid Programming.* Ann NY Acad Sci, 2004. **1032**(1): p. 63-84.
6. Necela, B.M. and J.A. Cidlowski, *Mechanisms of Glucocorticoid Receptor Action in Noninflammatory and Inflammatory Cells.* Proc Am Thorac Soc, 2004. **1**(3): p. 239-246.
7. Lang, F., et al., *Renal tubular transport and the genetic basis of hypertensive disease.* Clinical and Experimental Nephrology, 2005. **9**(2): p. 91-99.
8. Schafer-Korting, M., et al., *Glucocorticoids for human skin: new aspects of the mechanism of action.* Skin Pharmacology and Physiology, 2005. **18**(3): p. 103-14.
9. Seckl, J.R., et al., *Glucocorticoids and 11beta-Hydroxysteroid Dehydrogenase in Adipose Tissue.* Recent Prog Horm Res, 2004. **59**(1): p. 359-393.
10. Mitch, W.E. and S.R. Price, *Mechanisms activating proteolysis to cause muscle atrophy in catabolic conditions.* The Journal of Renal Nutrition, 2003. **13**(2): p. 149-52.
11. Lodish, H., et al., *Molecular Cell Biology*. 2000, W.H. Freeman.
12. Baker, M.E., *Evolution of Glucocorticoid and Mineralocorticoid Responses: Go Fish.* Endocrinology, 2003. **144**(10): p. 4223-4225.
13. May, F.E. and B.R. Westley, *Glucocorticoid receptor of X. laevis: possible effect of phosphorylation on hormone binding.* Molecular and Cellular Endocrinology, 1982. **26**(1-2): p. 103-17.
14. Encio, I.J. and S.D. Detera-Wadleigh, *The genomic structure of the human glucocorticoid receptor.* J. Biol. Chem., 1991. **266**(11): p. 7182-7188.
15. Breslin, M.B., C.-D. Geng, and W.V. Vedeckis, *Multiple Promoters Exist in the Human GR Gene, One of Which Is Activated by Glucocorticoids.* Mol Endocrinol, 2001. **15**(8): p. 1381-1395.
16. Pratt, W.B. and D.O. Toft, *Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones.* Endocr Rev, 1997. **18**(3): p. 306-360.
17. Guetierrez, M., et al., *Genomic and non-genomic effects of steroidal drugs on smooth muscle contraction in vitro.* Life Sciences, 1994. **55**(6): p. 437-43.
18. Falkenstein, E. and M. Wehling, *Nongenomicalli initiated steroid actions.* European Journal of Clinical Investigation, 2000. **Suppl. 3**: p. 51-4.
19. Yang, J. and D.B. DeFranco, *Assessment of glucocorticoid receptor-heat shock protein 90 interactions in vivo during nucleocytoplasmic trafficking.* Molecular Endocrinology, 1996. **10**(1): p. 3-13.
20. Harrell, J.M., et al., *Evidence for Glucocorticoid Receptor Transport on Microtubules by Dynein.* J. Biol. Chem., 2004. **279**(52): p. 54647-54654.

21. Sanchez, E.R., et al., *Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells.* Molecular Endocrinology, 1988. **2**(8): p. 756-60.
22. Chandler, V.L., B.A. Maler, and K.R. Yamamoto, *DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo.* Cell, 1983. **33**(2): p. 489.
23. Soudeyns, H., et al., *Identification of a Novel Glucocorticoid Response Element within the Genome of the Human Immunodeficiency Virus Type 1.* Virology, 1993. **194**(2): p. 758.
24. Morrison, N. and J. Eisman, *Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter.* Journal of Bone and Mineral Research, 1993. **8**(8): p. 969-75.
25. Malkoski, S.P., C.M. Handanos, and R.I. Dorin, *Localization of a negative glucocorticoid response element of the human corticotropin releasing hormone gene.* Molecular and Cellular Endocrinology, 1997. **127**(2): p. 189.
26. Buttgereit, F. and A. Scheffold, *Rapid glucocorticoid effects on immune cells.* Steroids, 2002. **67**(6): p. 529.
27. Croxtall, J.D., Q. Choudhury, and R.J. Flower, *Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism.* 2000. **130**(2): p. 289.
28. Norman, A.W., M.T. Mizwicki, and D.P.G. Norman, *Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model.* Nature Reviews Drug Discovery 2004. **3**(1): p. 27-41.
29. Gametchu, B., et al., *Studies on the arrangement of glucocorticoid receptors in the plasma membrane of S-49 lymphoma cells.* Steroids, 1991. **56**(8): p. 411.
30. Gametchu, B., C.S. Watson, and S. Wu, *Use of receptor antibodies to demonstrate membrane glucocorticoid receptor in cells from human leukemic patients.* FASEB J., 1993. **7**(13): p. 1283-1292.
31. Gametchu, B., *Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis.* Science, 1987. **236**(4800): p. 456-61.
32. Sackey, F.N., B. Gametchu, and C.S. Watson, *Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis.* The American Journal of Physiology, 1997. **273**(3 Pt 1): p. E571-83.
33. Bartholome, B., et al., *Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis.* FASEB J., 2004. **18**(1): p. 70-80.
34. Bartis, D., et al., *Sejtfelszini glukokortikoid receptor kimutatasa JN2B4D sejteken.*, in *XXXIV. Membrántranszport Konferencia*. 2004: Sumeg, Hungary.
35. Evans, S.J., T.F. Murray, and F.L. Moore, *Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain.* The Journal of Steroid Biochemistry and Molecular Biology, 2000. **72**(5): p. 209.
36. Chambliss, K.L., et al., *ER{beta} Has Nongenomic Action in Caveolae.* Mol Endocrinol, 2002. **16**(5): p. 938-946.

37. Zhu, Y., et al., *Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes.* PNAS, 2003. **100**(5): p. 2231-2236.
38. Buttgereit, F., et al., *The effects of methylprednisolone on oxidative phosphorylation in Concanavalin-A-stimulated thymocytes. Top-down elasticity analysis and control analysis.* European Journal of Biochemistry, 1994. **223**(2): p. 513-9.
39. Buttgereit, F., S. Krauss, and M.D. Brand, *Methylprednisolone inhibits uptake of Ca2+ and Na+ ions into concanavalin A-stimulated thymocytes* Biochemical Journal, 1997. **326**: p. 329-332.
40. Gergely, J. and A. Erdei, *Immunbiologia*. 1998, Budapest: Medicina.
41. Johnson, G.L. and R. Lapadat, *Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases.* Science, 2002. **298**(5600): p. 1911-1912.
42. Stellato, C., *Post-transcriptional and Nongenomic Effects of Glucocorticoids.* Proc Am Thorac Soc, 2004. **1**(3): p. 255-263.
43. Ghosh, S., M.J. May, and E.B. Kopp, *NF-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses.* Annual Review of Immunology, 1998. **16**(1): p. 225-260.
44. Levy, D.E. and J.E. Darnell, *STATS: TRANSCRIPTIONAL CONTROL AND BIOLOGICAL IMPACT.* Nature Reviews Molecular Cell Biology, 2002. **3**(9): p. 651.
45. Lechner, J., et al., *Promoter-dependent Synergy between Glucocorticoid Receptor and Stat5 in the Activation of beta -Casein Gene Transcription.* J. Biol. Chem., 1997. **272**(33): p. 20954-20960.
46. Zhang, Z., et al., *STAT3 Acts as a Co-activator of Glucocorticoid Receptor Signaling.* J. Biol. Chem., 1997. **272**(49): p. 30607-30610.
47. Fuller, G.M. and Z. Zhang, *Transcriptional Control Mechanism of Fibrinogen Gene Expression.* Ann NY Acad Sci, 2001. **936**(1): p. 469-479.
48. Takeda, T., et al., *Crosstalk between the interleukin-6 (IL-6)-JAK-STAT and the glucocorticoid-nuclear receptor pathway: synergistic activation of IL-6 response element by IL-6 and glucocorticoid.* J Endocrinol, 1998. **159**(2): p. 323-330.
49. Amsterdam, A., K. Tajima, and R. Sasson, *Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action.* Biochemical Pharmacology, 2002. **64**(5-6): p. 843.
50. Denton, R.R., et al., *Differential autoregulation of glucocorticoid receptor expression in human T- and B-cell lines.* Endocrinology, 1993. **133**(1): p. 248-256.
51. Zacharchuk, C.M., et al., *Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic.* J Immunol, 1990. **145**(12): p. 4037-4045.
52. Vacchio, M.S., J.Y.M. Lee, and J.D. Ashwell, *Thymus-Derived Glucocorticoids Set the Thresholds for Thymocyte Selection by Inhibiting TCR-Mediated Thymocyte Activation.* J Immunol, 1999. **163**(3): p. 1327-1333.
53. Stephens, G.L., J.D. Ashwell, and L. Ignatowicz, *Mutually antagonistic signals regulate selection of the T cell repertoire.* Int. Immunol., 2003. **15**(5): p. 623-632.

54. Cole, T.J., et al., *Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation.* Genes & Development, 1995. **9**(13): p. 1608-21.
55. Purton, J.F., et al., *Intrathymic T Cell Development and Selection Proceeds Normally in the Absence of Glucocorticoid Receptor Signaling.* Immunity, 2000. **13**(2): p. 179.
56. Berki, T., et al., *Glucocorticoid (GC) sensitivity and GC receptor expression differ in thymocyte subpopulations.* Int. Immunol., 2002. **14**(5): p. 463-469.
57. Boldizsar, F., et al., *Antigen and glucocorticoid hormone (GC) induce positive selection of DP thymocytes in a TcR transgenic mouse model.* Immunology Letters, 2003. **90**(2-3): p. 97.
58. Germain, R.N. and I. Stefanova, *THE DYNAMICS OF T CELL RECEPTOR SIGNALING: Complex Orchestration and the Key Roles of Tempo and Cooperation.* Annual Review of Immunology, 1999. **17**(1): p. 467-522.
59. Sloan-Lancaster, J. and P.M. Allen, *ALTERED PEPTIDE LIGANDS INDUCED PARTIAL T CELL ACTIVATION: Molecular Mechanisms and Role in T Cell Biology.* Annual Review of Immunology, 1996. **14**(1): p. 1-27.
60. Van Laethem, F., et al., *Glucocorticoids Attenuate T Cell Receptor Signaling.* J. Exp. Med., 2001. **193**(7): p. 803-814.
61. Van Laethem, F., et al., *Glucocorticoids Alter the Lipid and Protein Composition of Membrane Rafts of a Murine T Cell Hybridoma.* J Immunol, 2003. **170**(6): p. 2932-2939.
62. Weber, W.E., et al., *Activation through CD3 molecule leads to clonal expansion of all human peripheral blood T lymphocytes: functional analysis of clonally expanded cells.* J Immunol, 1985. **135**(4): p. 2337-2342.
63. Cantrell, D., *T cell antigen receptor signal transduction pathways.* Annual Review of Immunology, 1996. **14**(1): p. 259-274.
64. Roskoski, J.R., *Src protein-tyrosine kinase structure and regulation.* Biochemical and Biophysical Research Communications, 2004. **324**(4): p. 1155.
65. Bunnell, S.C., et al., *T cell receptor ligation induces the formation of dynamically regulated signaling assemblies.* J. Cell Biol., 2002. **158**(7): p. 1263-1275.
66. Bromley, S.K., et al., *The immunological synapse.* Annual Review of Immunology, 2001. **19**: p. 375-96.
67. Horejsi, V., W. Zhang, and B. Schraven, *TRANSMEMBRANE ADAPTOR PROTEINS: ORGANIZERS OF IMMUNORECEPTOR SIGNALLING.* Nature Reviews Immunology, 2004. **4**(8): p. 603.
68. Elder, M.E., et al., *Distinct T Cell Developmental Consequences in Humans and Mice Expressing Identical Mutations in the DLAARN Motif of ZAP-70.* J Immunol, 2001. **166**(1): p. 656-661.
69. Negishi, I., et al., *Essential role for ZAP-70 in both positive and negative selection of thymocytes.* 1995. **376**(6539): p. 435-438.
70. Chan, A.C., et al., *ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency.* Science, 1994. **264**(5165): p. 1599-601.
71. Elder, M.E., et al., *Severe Combined Immunodeficiency with Absence of Peripheral Blood CD8+ T Cells Due to ZAP-70 Deficiency.* Cellular Immunology, 1995. **165**(1): p. 110.

72. Kobata, T., et al., *CD27 is a signal-transducing molecule involved in CD45RA+ naive T cell costimulation.* J Immunol, 1994. **153**(12): p. 5422-5432.
73. Deckert, M., et al., *The glycosylphosphatidylinositol-anchored CD59 protein stimulates both T cell receptor zeta/ZAP-70-dependent and -independent signaling pathways in T cells.* European Journal of Immunology, 1995. **25**(7): p. 1815-22.
74. Taher, T.E.I., et al., *Signaling through CD44 Is Mediated by Tyrosine Kinases.* J. Biol. Chem., 1996. **271**(5): p. 2863-2867.
75. Bacon, K.B., et al., *RANTES induces tyrosine kinase activity of stably complexed p125FAK and ZAP-70 in human T cells.* J. Exp. Med., 1996. **184**(3): p. 873-882.
76. Hegen, M., et al., *Cross-linking of CD26 by antibody induces tyrosine phosphorylation and activation of mitogen-activated protein kinase.* Immunology 1997. **90**(2): p. 257-64.
77. Zubiaur, M., et al., *CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-zeta/zeta-associated protein-70 signaling pathways in Jurkat T lymphocytes.* J Immunol, 1997. **159**(1): p. 193-205.
78. Gary-Gouy, H., et al., *In vivo association of CD5 with tyrosine-phosphorylated ZAP-70 and p21 phospho-zeta molecules in human CD3+ thymocytes.* J Immunol, 1997. **159**(8): p. 3739-3747.
79. Soede, R.D.M., et al., *ZAP-70 Tyrosine Kinase Is Required for LFA-1-dependent T Cell Migration.* J. Cell Biol., 1998. **142**(5): p. 1371-1379.
80. Salojin, K.V., J. Zhang, and T.L. Delovitch, *TCR and CD28 Are Coupled Via ZAP-70 to the Activation of the Vav/Rac-1-/PAK-1/p38 MAPK Signaling Pathway.* J Immunol, 1999. **163**(2): p. 844-853.
81. Ottoson, N.C., et al., *Cutting Edge: T Cell Migration Regulated by CXCR4 Chemokine Receptor Signaling to ZAP-70 Tyrosine Kinase.* J Immunol, 2001. **167**(4): p. 1857-1861.
82. Zhong, L., et al., *{zeta}-Associated Protein of 70 kDa (ZAP-70), but Not Syk, Tyrosine Kinase Can Mediate Apoptosis of T Cells through the Fas/Fas Ligand, Caspase-8 and Caspase-3 Pathways.* J Immunol, 2004. **172**(3): p. 1472-1482.
83. Bartis, D., et al., *Dexamethasone induces rapid tyrosine-phosphorylation of ZAP-70 in Jurkat cells.* The Journal of Steroid Biochemistry and Molecular Biology, 2006. **98**(2-3): p. 147.
84. Mustelin, T. and K. Tasken, *Positive and negative regulation of T-cell activation through kinases and phosphatases.* Biochem J, 2003. **371**(Pt 1): p. 15-27.
85. Wardenburg, J.B., et al., *Phosphorylation of SLP-76 by the ZAP-70 Protein-tyrosine Kinase Is Required for T-cell Receptor Function.* J. Biol. Chem., 1996. **271**(33): p. 19641-19644.
86. Wu, J.N. and G.A. Koretzky, *The SLP-76 family of adapter proteins.* Seminars in Immunology, 2004. **16**(6): p. 379.
87. Chan, A., et al., *The {zeta} Chain is Associated with a Tyrosine Kinase and Upon T-Cell Antigen Receptor Stimulation Associates with ZAP-70, a 70-kDa Tyrosine Phosphoprotein.* PNAS, 1991. **88**(20): p. 9166-9170.
88. Watts, J., et al., *Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the*

protein tyrosine kinase ZAP-70. J. Biol. Chem., 1994. **269**(47): p. 29520-29529.
89. Kong, G., et al., *Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function.* Mol. Cell. Biol., 1996. **16**(9): p. 5026-5035.
90. Zhao, Q. and A. Weiss, *Enhancement of lymphocyte responsiveness by a gain-of-function mutation of ZAP-70.* Mol. Cell. Biol., 1996. **16**(12): p. 6765-6774.
91. Lupher Jr, M.L., et al., *The Cbl Phosphotyrosine-binding Domain Selects a D(N/D)XpY Motif and Binds to the Tyr292 Negative Regulatory Phosphorylation Site of ZAP-70.* J. Biol. Chem., 1997. **272**(52): p. 33140-33144.
92. Magnan, A., et al., *T Cell Development and T Cell Responses in Mice with Mutations Affecting Tyrosines 292 or 315 of the ZAP-70 Protein Tyrosine Kinase.* J. Exp. Med., 2001. **194**(4): p. 491-506.
93. Bottini, N., et al., *Activation of ZAP-70 through Specific Dephosphorylation at the Inhibitory Tyr-292 by the Low Molecular Weight Phosphotyrosine Phosphatase (LMPTP).* J. Biol. Chem., 2002. **277**(27): p. 24220-24224.
94. Wu, J., et al., *The Vav Binding Site (Y315) in ZAP-70 Is Critical for Antigen Receptor-mediated Signal Transduction.* J. Exp. Med., 1997. **185**(10): p. 1877-1882.
95. Di Bartolo, V., et al., *Tyrosine 315 determines optimal recruitment of ZAP-70 to the T cell antigen receptor.* European Journal of Immunology, 2002. **32**(2): p. 568-575.
96. Gong, Q., et al., *Requirement for Tyrosine Residues 315 and 319 within {zeta} Chain-associated Protein 70 for T Cell Development.* J. Exp. Med., 2001. **194**(4): p. 507-518.
97. Gelkop, S., et al., *T Cell Activation-Induced CrkII Binding to the Zap70 Protein Tyrosine Kinase Is Mediated by Lck-Dependent Phosphorylation of Zap70 Tyrosine 315.* J Immunol, 2005. **175**(12): p. 8123-8132.
98. Di Bartolo, V., et al., *Tyrosine 319, a Newly Identified Phosphorylation Site of ZAP-70, Plays a Critical Role in T Cell Antigen Receptor Signaling.* J. Biol. Chem., 1999. **274**(10): p. 6285-6294.
99. Pelosi, M., et al., *Tyrosine 319 in the Interdomain B of ZAP-70 Is a Binding Site for the Src Homology 2 Domain of Lck.* J. Biol. Chem., 1999. **274**(20): p. 14229-14237.
100. Guntermann, C. and D.R. Alexander, *CTLA-4 Suppresses Proximal TCR Signaling in Resting Human CD4+ T Cells by Inhibiting ZAP-70 Tyr319 Phosphorylation: A Potential Role for Tyrosine Phosphatases.* J Immunol, 2002. **168**(9): p. 4420-4429.
101. Pacini, S., et al., *Tyrosine 474 of ZAP-70 Is Required for Association with the Shc Adaptor and for T-cell Antigen Receptor-dependent Gene Activation.* J. Biol. Chem., 1998. **273**(32): p. 20487-20493.
102. Wange, R.L., et al., *Activating and Inhibitory Mutations in Adjacent Tyrosines in the Kinase Domain of ZAP-70.* J. Biol. Chem., 1995. **270**(32): p. 18730-18733.
103. Chan, A.C., et al., *Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function.* The EMBO Journal, 1995. **14**(11): p. 2499-508.
104. Zeitlmann, L., et al., *T Cell Activation Induced by Novel Gain-of-function Mutants of Syk and ZAP-70.* J. Biol. Chem., 1998. **273**(25): p. 15445-15452.

105. Furlong, M., et al., *Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk.* Biochimica et Biophysica Acta, 1997. **1355**: p. 177-190.
106. Keshvara, L., et al., *Syk activation and dissociation from the B-cell antigen receptor is mediated by phosphorylation of Tyrosine 130.* The Journal of Biological Chemistry, 1997. **272**(16): p. 10377-10381.
107. Keshvara, L., et al., *Syk-and Lyn-dependent Phosphorylation of Syk on multiple tyrosines following B cell activation includes a site that negatively regulates signaling.* The Journal of Immunology, 1998. **161**: p. 5276-5283.
108. Magistrelli, G., et al., *Role of the Src homology 2 domains and interdomain regions in ZAP-70 phosphorylation and enzymatic activity.* European Journal of Biochemistry, 1999. **266**(3): p. 1166-1173.
109. Fournel, M., et al., *Association of tyrosine protein kinase Zap-70 with the protooncogene product p120c-cbl in T lymphocytes 10.1084/jem.183.1.301.* J. Exp. Med., 1996. **183**(1): p. 301-306.
110. Lupher Jr., M.L., et al., *The Cbl Phosphotyrosine-binding Domain Selects a D(N/D)XpY Motif and Binds to the Tyr292 Negative Regulatory Phosphorylation Site of ZAP-70.* J. Biol. Chem., 1997. **272**(52): p. 33140-33144.
111. Zhao, Q., et al., *Interdomain B in ZAP-70 Regulates but Is Not Required for ZAP-70 Signaling Function in Lymphocytes.* Mol. Cell. Biol., 1999. **19**(1): p. 948-956.
112. Courtneidge, S.A., *Protein tyrosine kinases, with emphasis on the Src family.* Seminars in Cancer Biology, 1994. **5**(4): p. 239-46.
113. Smart, J.E., et al., *Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src).* PNAS, 1981. **78**(10): p. 6013-7.
114. Couture, C., et al., *Identification of the Site in the Syk Protein Tyrosine Kinase That Binds the SH2 Domain of Lck.* J. Biol. Chem., 1996. **271**(39): p. 24294-24299.
115. Zennou, V., et al., *HIV-1 Genome Nuclear Import Is Mediated by a Central DNA Flap.* Cell, 2000. **101**(2): p. 173.
116. Zufferey, R., et al., *Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors.* J. Virol., 1999. **73**(4): p. 2886-2892.
117. Bovia, F., et al., *Efficient transduction of primary human B lymphocytes and nondividing myeloma B cells with HIV-1-derived lentiviral vectors.* Blood, 2003. **101**(5): p. 1727-1733.
118. Klages, N., R. Zufferey, and D. Trono, *A Stable System for the High-Titer Production of Multiply Attenuated Lentiviral Vectors.* Molecular Therapy, 2000. **2**(2): p. 170.
119. Kvell, K., et al., *Transduction of CpG DNA-stimulated primary human B cells with bicistronic lentivectors.* Molecular Therapy, 2005. **12**(5): p. 892.
120. O'Doherty, U., W.J. Swiggard, and M.H. Malim, *Human Immunodeficiency Virus Type 1 Spinoculation Enhances Infection through Virus Binding.* J. Virol., 2000. **74**(21): p. 10074-10080.
121. Berki, T., et al., *Production and flow cytometric application of a monoclonal anti-glucocorticoid receptor antibody.* Journal of Immunological Methods, 1998. **214**(1-2): p. 19.

122. Minta, A., J.P. Kao, and R.Y. Tsien, *Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores.* J. Biol. Chem., 1989. **264**(14): p. 8171-8178.
123. Boldizsar, F., et al., *Effect of hyperglycemia on the basal cytosolic free calcium level, calcium signal and tyrosine-phosphorylation in human T-cells.* Immunology Letters, 2002. **82**(1-2): p. 159.
124. Ziemiecki, A., et al., *Association of the heat shock protein HSP90 with steroid hormone receptors and tyrosine kinase oncogene products.* Biochemical and Biophysical Research Communications, 1986. **138**(3): p. 1298.
125. Schnaider, T., et al., *The Hsp90-specific inhibitor geldanamycin selectively disrupts kinase-mediated signaling events of T-lymphocyte activation.* Cell Stress & Chaperones, 2000. **5**(1): p. 52-61.
126. Vacchio, M.S., V. Papadopoulos, and J.D. Ashwell, *Steroid production in the thymus: implication for thymocyte selection.* J. Exp. Med., 1994. **179**(6): p. 1835-46.
127. Fusaki, N., et al., *Physical and functional interactions of protein tyrosine kinases, p59fyn and ZAP-70, in T cell signaling.* J Immunol, 1996. **156**(4): p. 1369-1377.
128. Castro, J.E., et al., *ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia.* Blood, 2005. **106**(7): p. 2506-2512.
129. Kamal, A., et al., *A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors.* Nature, 2003. **425**(6956): p. 407.
130. Lowenberg, M., et al., *Glucocorticoids cause rapid dissociation of a T-cell-receptor-associated protein complex containing LCK and FYN.* EMBO rep. , 2006. **7**(10): p. 1023-9.

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Appendix A: List of figures and tables.

Figures:

Tables:

Appendix B: List of Abbreviations

7TM	7 transmembrane domain containing receptors
aa	amino acid
AP-1	activator protein-1
ATP	adenosine triphosphate
B-CLL	B-cell chronic lymphoid leukaemia
BSA	Bovine Serum Albumin
Cbl	protein product of *c-cbl* oncogene
CD	cluster of differentiation
C-domain	Constant domain
cGR	cytoplasmic GR
CREB	cAMP-response element binding protein
CRH	corticotropin releasing hormone
Crk	protein product of CT10 sarcoma oncogene cellular homolog
DBD	DNA-binding domain
DMSO	dimethyl sulphoxide
DNA	dezoxyribonucleic acid
DP	double positive thymocytes (CD4+CD8+)
DX	Dexamethasone
ECL	enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
F	Phenilalanine
Fc	Fragment crystallibile
FCS	Fetal Calf Serum
FITC	fluorescein-isothyocyanate
GA	geldanamycin
GC	Glucocorticoid hormone
GEF	guanozin exchange factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hGRα	human glucocorticoid receptor isoform

hGRβ	human glucocorticoid receptor isoform
HIV-1	Type-1 Human Immunodeficiency Virus
HRPO	Horseradish Peroxidase
Hsp-90	Heat shock protein of 90 kilodaltons
IB	immunoblotting
ICAM-1	Intercellular Adhesion molecule-1
IFNγ	Interferon gamma
Ig	Immunoglobulin
I-kB	Inhibitor-kappa-B
IL	interleukin
IL-2R	interleukin-2 receptor
IP	Immunoprecipitation
ITAM	Immunoreceptor Tyrosine based activation motif
JAK	Janus kinase
JCaM-1	p56-lck kinase deficient Jurkat subclone
JNK	c-jun N-terminal kinase
kDa	Kilodalton
LAT	Linker of Activation in T-cells
LBD	ligand-binding domain
LMPTP	Low Molecular Weight Phosphotyrosine Phosphatase
MAPK	Mitogen Activated Protein Kinase
mGR	membrane GR
MHC	main histocompatibility complex
MKP-1	MAPK-phosphatase-1
MMTV	mouse mammary tumor virus
mRNA	messenger RNA
NFAT	nuclear factor of activated T-cells
NFkB	nuclear factor kappa-B
nGRE	negative regulatory GRE
NK	natural killer cells
P116	ZAP-70 deficient Jurkat subclone
p56-lck	lymphocite-cell specific protein tyrosine kinase of 56 kDa
p59-fyn	protein product of the *c-fyn* oncogene
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PE	phycoerythrine

PKA	Protein Kinase A
PMSF	Phenyl-Methyl-Sulphonyl-Fluoride
PTK	Protein Tyrosine kinase
PY	Phosphotyrosine
RNA	ribonucleic acic
RNA pol II	RNA polymerase II
RPMI	cell culture medium developed at Rapid Prototyping and Manufacturing Institute
RU486	Glucocorticoid receptor antagonist (Mifepristone)
SDS	Sodium Dodecyl Sulphate, ionic detergent
SH	src-homology
Shc	SH2 containing collagen-related protein
SLP-76	SH2 domain containing leukocyte protein of 76kDa
Src	protein product of the *c-src* cellular oncogene
SRC-1	steroid receptor coactivator-1
STAT	signal transduction and activator of transcription
Syk	spleen tyrosine kinase
TcR	T-cell receptor
Th1	T-helper 1
Th2	T-helper 2
TNF	Tumor Necrosis Factor
Tris	Tris(hydroxymethyl)aminomethane
TX-100	Triton-X-100 non-ionic detergent
V-domain	variable domain
VEGF	Vascular Endothelial Growth Factor
Y	Tyrosine
ZAP-70	zeta-chain associated protein of 70 kilodalton

Appendix C: List of publications

Articles:

1. **Domokos Bartis**, Ferenc Boldizsár, Krisztián Kvell, Mariann Szabó, László Pálinkás, Péter Németh, Éva Monostori and Tímea Berki: *Intermolecular relations between the glucocorticoid receptor, ZAP-70 and Hsp-90,* Biochem Biophys Res Commun 354 (2007) 253-258

2. Ferenc Boldizsár, László Pálinkás, Tamás Czömpöly, **Domokos Bartis**, Peter Németh, Tímea Berki: Low glucocorticoid receptor (GR), high Dig2 and low Bcl-2 expression in double positive thymocytes of Balb/c mice indicates their endogenous glucocorticoid hormone exposure, Immunobiology, 211 (2006), 785-796

3. **Domokos Bartis**, Ferenc Boldizsár, Mariann Szabó, László Pálinkás, Péter Németh and Tímea Berki: *Dexamethasone induces rapid tyrosine-phosphorylation of ZAP-70 in Jurkat cells,* J Steroid Biochem Mol Biol 98. (2006) 147-154

4. Ferenc Boldizsár, László Pálinkás, **Domokos Bartis**, Péter Németh, Tímea Berki: *Antigen and glucocorticoid hormone (GC) induce positive selection of DP thymocytes in a TcR transgenic mouse,* Immunol Lett. (2003) 90. (2-3):97-102.

Citable abstracts:

1. Boldizsár, F, Berki, T., Pálinkás, L., **Bartis, D.**, Németh, P: *Antigen and glucocorticoid hormone (GCs) induce positive selection of DP thymocytes in a TCR transgenic mouse model.* Immunol Lett. Special Issue: Abstracts of the 15th European Immunology Congress, EFIS 2003, June 8-12, 2003, Vol. 87.(1-3) W11.09

2. Boldizsár, F, Czömpöly, T., Berki, T., Pálinkás, L., **Bartis, D.**, Németh, P: *Real time PCR analysis of murine thymocyte glucocorticoid receptor (GCR) mRNA expression.* Immunol Lett. Special Issue: Abstracts of the 15th European Immunology Congress, EFIS 2003, June 8-12, 2003, Vol. 87.(1-3) W01.10

3. **D. Bartis**, F. Boldizsár, M. Szabó, L. Pálinkás, P. Németh and T. Berki: *Glucocorticoid hormone elicited tyrosine-phosphorylation events involving T-cell specific kinases* . The FEBS Journal, July 2005. 272(Suppl.1): 482 Abstracts of the 30th FEBS-Congress 9th IUBMB Conference 2-7.

Appendix D: The thesis is based on the following publications:

Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Steroid Biochemistry & Molecular Biology 98 (2006) 147–154
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Dexamethasone induces rapid tyrosine-phosphorylation of ZAP-70 in Jurkat cells

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Abstract

Steroid hormones are known to mediate rapid non-genomic effects occurring within minutes, besides the classical genomic actions mediated by the nuclear translocation of the cytoplasmic glucocorticoid receptor (GR). The glucocorticoid hormone (GC) has significant role in the regulation of T-cell activation; however, the cross-talk between the GC and T-cell receptor (TcR) signal transducing pathways are still to be elucidated. We examined the rapid effects of GC exposure on in vitro cultured human T-cells. Our results showed that Dexamethasone (DX), a GC analogue, when applied at high dose (10 μM), induced rapid (within 5 min) tyrosine-phosphorylation events in Jurkat cells. Short DX pre-treatment strongly inhibited the tyrosine-phosphorylation stimulated by CD3 cross-linking. Furthermore, we also investigated the phosphorylation status of ZAP-70, an important member of tyrosine kinase mediated signalling pathway of TcR-elicited T-cell activation. Here, we demonstrate that high dose DX induced a rapid ZAP-70 tyrosine-phosphorylation in Jurkat T-cells. DX-induced ZAP-70 phosphorylation could be inhibited by RU486 (GR antagonist), suggesting that this process was GR mediated. DX-induced ZAP-70 phosphorylation did not occur in the absence of active p56-lck as examined in the p56-lck kinase-deficient Jurkat cell line JCaM1.6. Our results show that DX, at a high dose, can rapidly influence the initial tyrosine-phosphorylation events of the CD3 signalling pathway in Jurkat cells, thereby modifying TcR-derived signals. Lck and ZAP-70 represent an important molecular link between the TcR and GC signalling pathways.

Keywords: Glucocorticoid hormone; Glucocorticoid hormone receptor; Non-genomic effects; ZAP-70; Tyrosine-phosphorylation; Jurkat cells

1. Introduction

Glucocorticoid hormones (GCs) participate in the regulation of differentiation, activation, survival and apoptosis of T lymphocytes [1]. GCs modulate the immune response by inhibiting the cellular (Th1) branch of the immune response while promoting humoral (Th2) response [2]. For these reasons GCs have been used as immunosuppressive therapeutic agents for 50 years in autoimmune and other inflammatory diseases, organ transplantations and haematological malignancies [2,3].

The major physiological source of GCs is the adrenal glands [4], but extraadrenal GC production was also found in the thymus [5], the chicken bursa [6] and the intestinal epithelial cells [7]. Depending on other simultaneous signalling processes, extraadrenally produced GCs can posses local immunomodulatory effects, eliciting both stimulatory and inhibitory responses [7,8].

In addition to a number of other GC-sensitive tissues like lung, spleen, brain, liver, kidney, heart, adrenal, testis [11], muscle, different cell lines and tumors [12], mature peripheral T-cells [9] and thymocytes [10] also express glucocorticoid receptor (GR). The inactive form of GR is associated with Hsp-90 in the cytoplasm [13]. Upon ligand binding, GR dissociates from Hsp-90 and translocates to the nucleus, where it forms dimers and functions as transcription factor, binding to specific conserved palindromic DNA sequences (GGTACAnnnTGTTCT), called glucocorticoid response elements (GRE) [14]. The receptor translocation to the nucleus occurs within 30 min in HepG2 cell line [15], and subsequent gene transcription effects develop within hours.

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Accumulating evidence show that besides the classical genomic GC action, other, non-genomic mechanisms also exist. The recent opinion is that rapid GC effects might be mediated through three different mechanisms: (1) specific cytoplasmic protein–protein interactions involving the cytoplasmic GR (cGR), (2) specific signalling mechanisms through the membrane-bound GR and (3) non-specific physico-chemical GC interactions with the cell membrane [16]. Croxtall et al. observed that epidermal growth factor (EGF)-stimulated cPLA2 (cytosolic PLA2) activation with subsequent arachidonic acid release could be inhibited by Dexamethasone [17]. This effect was considered to be a glucocorticoid receptor-dependent (RU486-sensitive), but transcription-independent (actinomycin-insensitive) mechanism [17]. Hitherto all steroid hormone classes (glucocorticoids, progesterone, estrogens, androgens, neurosteroids, mineralocorticoids, Vitamins D3, T3 and T4) have been shown to mediate rapid, non-genomic effects [18]; however, the exact signalling mechanisms are still to be identified.

It has been shown that GCs inhibit the early steps of TcR signalling events such as the calcium flux, inositol phosphate production and phospholipase-Cγ1 tyrosine-phosphorylation following TcR ligation in murine T-cell hybrids [19]. GCs also inhibit the phosphorylation of some early T-cell signalling molecules such as the T-cell receptor (TcR) zeta chain and the ZAP-70 kinase in T-cell hybridomas and murine thymocytes [20]. The authors proposed a non-genomic GC signalling mechanism through the cytoplasmic GR which affected the membrane compartmentalisation of src-family kinases [20]. They also found that GC hormone treatment altered the lipid composition of lipid rafts of a murine T-cell hybridoma, thereby altering the raft association and palmitoylation of key signalling molecules [21]. However, the GC effects described in the above works [19–21] needed hours to manifest. Only few data are available about the signalling mechanisms induced by rapid steroid effects which occur within minutes. Particularly, the modulatory effects of steroid hormones on the phosphorylation status of key signalling elements are sparse, therefore we set out to investigate this process in more depth.

It has been shown that the Vitamin D receptor mediates rapid src tyrosine-phosphorylation and consequent MAP-kinase activation in muscle cells following ligand binding [22,23]. Boonyaratanakornkit et al. found that the progesterone receptor interacts with the src-family tyrosine kinases via their SH3 domains and stimulates the activation of the Ras/Raf-1/MAP-kinase pathway [24].

In this paper, we report that Dexamethasone (DX), a glucocorticoid hormone analogue, rapidly diminishes the tyrosine-phosphorylation events caused by CD3 cross-linking. We assayed the phosphorylation status of ZAP-70, a key T-cell signalling protein and found that high dose DX rapidly induced its phosphorylation on tyrosine residues. The DX-induced phosphorylation process proved to be sensitive to the GR antagonist RU486 and was absent in p56-lck-deficient Jurkat cells.

2. Materials and methods

2.1. Cell lines

We used Jurkat cells (human acute T-cell leukemia) and its p56-lck-deficient subclone (JCaM1.6), both a generous gift from E. Monostori (Biological Research Centre, Szeged, Hungary). Cells were cultured in humidified atmosphere, containing 5% CO_2 at 37 °C, in RPMI medium supplemented with 5 and 10% fetal calf serum (Gibco, Gaithersburg, MD, USA), respectively.

2.2. Chemicals and buffers

All fine chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) otherwise indicated. Dexamethasone was used as glucocorticoid hormone agonist and RU486 (Mifepristone) was used as glucocorticoid antagonist, both dissolved in DMSO at a concentration of 10 mM. For the Western blot experiments Jurkat cells were lysed in ice-cold lysis buffer (50 mM HEPES, 10 mM sodium pyrophosphate 10 mM EDTA, 100 mM sodium fluoride, 10% glycerol and 1% Triton X-100). Aprotinin, leupeptin (Fluka, Buchs, Switzerland) (10 μg/ml), PMSF (2 mM) and sodium orthovanadate (2 mM) were freshly added to the lysis buffer. The cell lysates were separated by SDS-PAGE after adding equal amount of 2× sample buffer (125 mM Tris, 4% SDS, 10% glycerol, 0.006% Bromo-phenol-blue and 10% mercaptoethanol) or subjected to immunoprecipitation. The gels were blotted overnight to nitrocellulose membranes using a Trans-Blot cell blotting equipment (both from Bio-Rad, Hercules, CA, USA). To saturate non-specific binding sites, the blots were soaked in blocking buffer (1% bovine serum albumin, 10 mM Tris, 100 mM sodium chloride and 0.1% Tween 20, pH 7.4) For washing the blots washing buffer was used (10 mM Tris, 100 mM sodium chloride and 0.1% Tween-20, pH 7.4). Western blot visualisation was performed by enhanced chemiluminescence as described in the manufacturer's instructions (SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) or, in case of anti-ZAP-70 immunoblots with bromo-chloro-indolyl-phosphate/nitro-blue tetrazolium (BCIP/NBT), in a 100 mM sodium chloride, 50 mM Tris, 5 mM $MgCl_2$, pH 9.5, buffer. For stripping the blots we used Restore Stripping Buffer (Pierce).

2.3. Antibodies

We used mouse monoclonal anti-human CD3 antibody (clone: UCHT-1, 1 mg/ml stock solution in PBS) for cell activation. Immunoprecipitation was performed with purified rabbit polyclonal anti-ZAP-70 antibody (kindly provided by E. Monostori, Biological Research Centre, Szeged, Hungary). The tyrosine-phosphorylation pattern was examined by using biotinylated monoclonal anti-phosphotyrosine antibody (clone: PY20, Transduction Laboratories, Lexington,

KY, USA) and HRPO-conjugated streptavidin (Amersham, Little Chalfont, UK) as a secondary reagent. To prove the equal protein loading we used monoclonal mouse anti-β-actin antibody (Sigma). ZAP-70 immunoblots were probed with monoclonal anti-ZAP-70 antibody (clone: 29, Transduction Laboratories) and alkaline-phosphatase labelled rabbit polyclonal anti-mouse (DakoCytomation, Glostrup, Denmark) secondary antibody. Biotinylated monoclonal anti-insulin antibody (clone: 2D11) was used as negative control.

2.4. Glucocorticoid analogue and glucocorticoid antagonist treatment

Jurkat cells were cultured for 4 h in RPMI supplemented with 10^{-5} M RU486 or solvent. After the exposure, cells were harvested, and 5×10^6 to 10^7 cells per sample were resuspended in 500 μl RPMI. 10^{-5} M Dexamethasone was added and cells were incubated at 37 °C for various periods as indicated.

2.5. Cell activation and lysis

Fifty micrograms UCHT-1 anti-CD3 monoclonal antibody was added to the DX-treated or untreated samples. Following 1 or 2 min of incubation at 37 °C the activation was stopped by quickly freezing the cells in liquid nitrogen. Then, the samples were lysed for 30 min in 500 μl ice-cold lysis buffer. Following lysis the samples were centrifuged at 13,000 rpm for 15 min at 4 °C. Postnuclear supernatants were aspirated and boiled for 5 min with equal amounts of 2× sample buffer or subjected to immunoprecipitation.

2.6. Immunoprecipitation and Western blot

Equal amounts of cell lysates were incubated on a rotator platform at 4 °C for 2 h with 10 μl of the precipitating antiserum. Then, 30 μl slurry of Protein G coupled Sepharose beads were added for additional 2 h. The beads were pelleted and five washes were performed with ice-cold washing buffer. After washing the beads were resuspended in 100 μl of SDS sample buffer and the immunoprecipitates were boiled for 5 min. After a quick spin the supernatants were collected and loaded on 7.5 or 10% SDS-polyacrylamide gels. SDS-PAGE was performed using a BioRad MiniProtean elecrophoresis equipment. The gels were blotted overnight to nitrocellulose membranes by wet transfer procedure. After blocking non-specific binding sites for 1 h, the blots were incubated for 2 h with 0.2 μg biotinylated monoclonal anti-PY antibody. Western blot negative controls were incubated with biotinylated monoclonal anti-insulin antibody for the same time period at the same concentration. Following extensive washing, the HRPO-conjugated streptavidin (1:5000 dilution) was added for another hour. Western blots were visualised with SuperSignal West Pico Chemiluminescent substrate. Then stripping of the membranes was performed and blots were reprobed with 0.5 μg of monoclonal anti-ZAP-70 antibody. Following extensive washing the alkaline-phosphatase labelled rabbit anti-mouse antibody (1:2000 dilution) was added for another 1 h. Reprobed Western blots were visualised with BCIP/NBT substrate.

2.7. Analysis of blots

Densitometry of the blots was carried out with the ScionImage software (Scion Corporation, Frederick, MD, USA). Relative densities of phosphotyrosine blots were corrected with relative densities of ZAP-70 blots of the same sample, to obtain the relative phosphorylation levels of ZAP-70 in each sample.

2.8. Statistical analysis

Each experiment was repeated three times and representative blots are shown in the figures. The effect of treatments was tested for statistical significance using Student's *t*-test. $P < 0.05$ denoted statistical significance.

3. Results

3.1. Dexamethasone alters the tyrosine-phosphorylation pattern of Jurkat cells

First we examined how DX treatment influences the tyrosine-phosphorylation pattern of resting and anti-CD3 activated Jurkat cells. Two minutes of 10 μM DX treatment alone caused increased protein tyrosine-phosphorylation at the 34, 52, 59, 70 and 90 kDa molecular weight bands in whole Jurkat cell lysates, compared to the solvent-treated control (Fig. 1). Activation with monoclonal anti-CD3 antibody markedly increased the tyrosine-phosphorylation of several proteins in whole cell lysates at 25, 34, 38, 40, 52, 59, 70 and 90 kDa molecular weight ranges, respectively (Fig. 1). A 2 min DX pre-treatment at 10 μM concentration inhibited the anti-CD3-induced tyrosine-phosphorylation the proteins of 34, 38, 40, 52, 59 and 90 kDa (Fig. 1). We got no significant signal on negative control blots, incubated with biotinylated monoclonal anti-insulin antibody (data not shown).

3.2. High dose Dexamethasone rapidly induces ZAP-70 phosphorylation

ZAP-70 plays a central role in the TcR signal transduction pathway, by phosphorylating a broad range of substrates, and itself is also phosphorylated on several tyrosine residues [25]. For this reason, we investigated the tyrosine-phosphorylation of ZAP-70 after DX and/or anti-CD3 treatments. Five-minute exposure to 10 μM DX caused an average of four-fold (3.32 ± 1.72) increase in tyrosine-phosphorylation of the ZAP-70 kinase in the anti-ZAP-70 precipitated samples (Fig. 2). The anti-CD3 treatment also resulted in a four-fold increase (3.59 ± 1.69) in the tyrosine-phosphorylation of

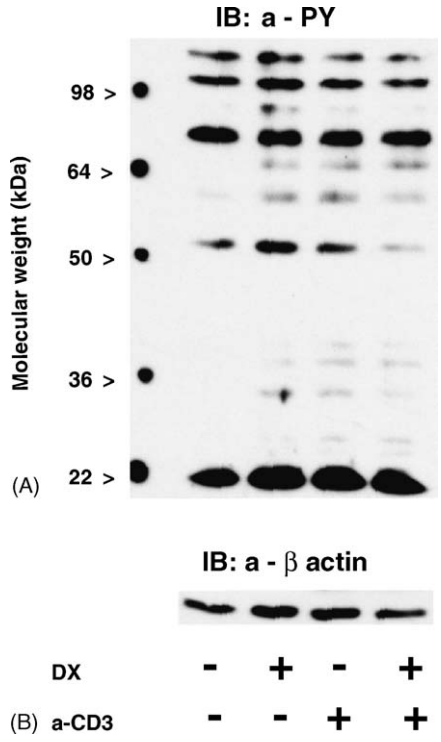

Fig. 1. Dexamethasone and/or anti-CD3 treatment influenced the tyrosine-phosphorylation pattern in Jurkat cell lysates. Western blotting was performed with monoclonal anti-phosphotyrosine antibody (Panel A). Then, the blot was stripped and reprobed with monoclonal anti-beta actin antibody to check equal protein loading (Panel B). Molecular weight markers are indicated on the left, treatment modalities on the bottom side of the blot. The experiment was repeated three times with similar results. Here, a representative Western blot is depicted.

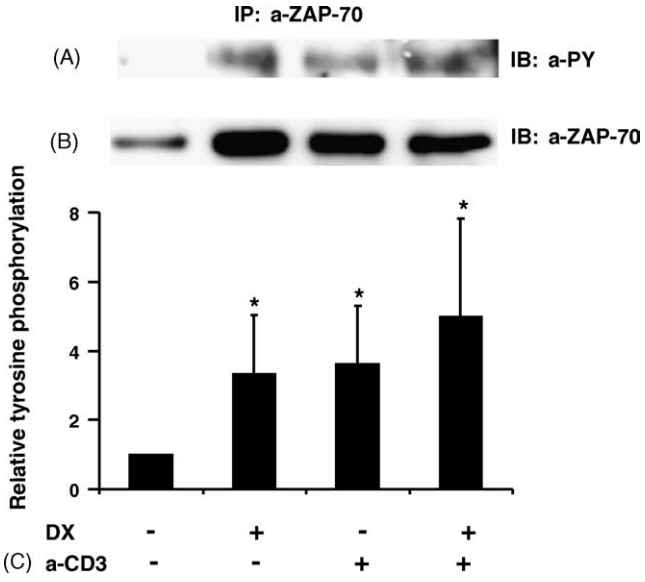

Fig. 2. Effect of Dexamethasone and/or anti-CD3 treatment on ZAP-70 tyrosine-phosphorylation. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: a-ZAP-70) (Panel B). The relative phosphotyrosine content of ZAP-70 is shown as relative density averages ± S.D. from the densitometry data of three separate experiments ($^{*}P < 0.05$) (Panel C). Treatment modalities are indicated below the diagram (Panel C).

ZAP-70 kinase (Fig. 2). The tyrosine-phosphorylation after combined DX + anti-CD3 treatment was higher (4.98 ± 2.83) than in case of anti-CD3 or DX treatment alone (five-fold versus four-fold increase, respectively). Phosphorylation increase after single DX or anti-CD3 or combined treatments proved significant compared to the solvent-treated control sample (Student's *t*-test, $P < 0.05$). We observed higher phosphorylation after combined DX + anti-CD3 treatment than single DX or anti-CD3 treatment in all of our independent experiments, but this further rise was statistically not significant by Student's *t*-test (Fig. 2).

3.3. Temporal kinetics of ZAP-70 tyrosine-phosphorylation following single high dose Dexamethasone treatment

Since the kinetics of tyrosine-phosphorylation is a key feature to understand cellular signalling events [26], we further investigated the time course of DX-induced ZAP-70 tyrosine-phosphorylation. The tyrosine-phosphorylation of ZAP-70 occurs rapidly after the addition of high dose DX. We measured a rise in tyrosine-phosphorylation after 1 and 2 min after DX administration, respectively (Fig. 3). Dephosphorylation occurred within 5 min (Fig. 3).

3.4. ZAP-70 phosphorylation induced by Dexamethasone is p56-lck dependent

In addition to acting as a tyrosine kinase, ZAP-70 is a substrate of p56-lck, a T-cell specific src-family tyrosine kinase [27]. We therefore tested whether the DX-induced rapid tyrosine-phosphorylation of ZAP-70 was p56-lck dependent. When JCaM1.6, the p56-lck-deficient subclone of Jurkat cells was subjected to high dose DX treatment, we observed no rise in the degree of tyrosine-phosphorylation of ZAP-70 kinase (Fig. 4). The anti-CD3 activation induced notable increase in tyrosine-phosphorylation of ZAP-70 in JCaM1.6 cells (Fig. 4). Additional DX stimulation did not alter the

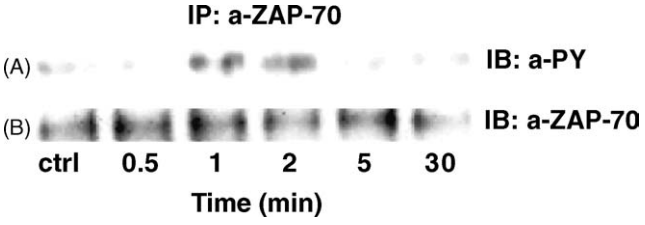

Fig. 3. Time-dependent tyrosine-phosphorylation of ZAP-70 after high dose Dexamethasone treatment. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Duration of the Dexamethasone treatment is indicated below the figure. The experiment was repeated three times with similar results.

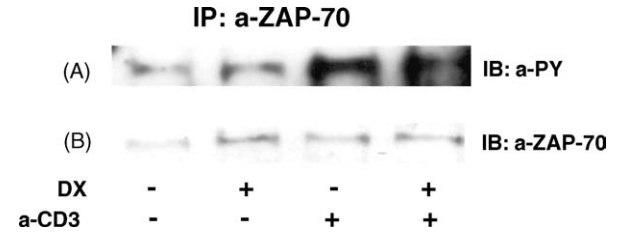

Fig. 4. ZAP-70 tyrosine-phosphorylation after Dexamethasone and/or anti-CD3 treatment in p56-lck kinase-deficient Jurkat subclone JCaM1.6. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Treatment modalities are indicated below the blots. A representative Western blot is shown from three independent experiments with similar results.

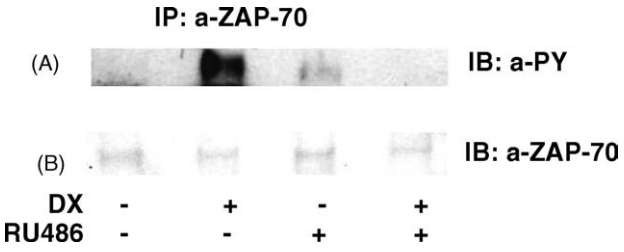

Fig. 5. Effect of RU486 (Mifepristone, glucocorticoid receptor antagonist) pre-treatment on the Dexamethasone-induced tyrosine-phosphorylation of ZAP-70. Western blotting was performed on anti-ZAP-70 immunoprecipitated (IP: a-ZAP-70) samples with anti-phosphotyrosine antibody (IB: a-PY) (Panel A). The blots were stripped and reprobed with monoclonal anti-ZAP-70 antibody (IB: ZAP-70) (Panel B). Treatment modalities are indicated below the blots. A representative Western blot is shown from three independent experiments with similar results.

phosphotyrosine increase caused by anti-CD3 activation in the p56-lck-deficient cell line (Fig. 4).

3.5. ZAP-70 phosphorylation can be elicited by the glucocorticoid receptor

Previous studies indicated the existence of various non-genomic GC effects, either GR mediated or receptor independent. Therefore, we investigated whether tyrosine-phosphorylation alterations in Jurkat T-cells induced by DX were GR-dependent using RU 486 (Mifepristone), a glucocorticoid receptor antagonist [28]. DX alone caused markedly growth in the tyrosine-phosphorylation of ZAP-70 (Fig. 5). Pre-treatment of Jurkat cells for 4 h with equimolar RU486 prevented this rapid phosphorylation of ZAP-70 induced by subsequent high dose DX exposure (Fig. 5). RU 486 treatment alone caused no remarkable tyrosine-phosphorylation change in ZAP-70 (Fig. 5).

4. Discussion

Our work was aimed at clarifying the GC-TcR signal cross-talk in Jurkat cells in vitro. It has been known for a long time that GCs exhibit immunosuppressive effects [2]. As a model for TcR engagement, the cross-linking of the CD3 signalling complex with anti-CD3 antibody increases the phosphotyrosine content in T-cells [29]. Others have shown that GC alters the tyrosine-phosphorylation after TcR activation in T-cells [20]. However, those DX effects needed hours to manifest, although they proved to be non-genomic. The GC effects we demonstrate here occurred within 5 min, which strongly suggest that these responses were non-genomic in nature. The phosphotyrosine content of DX-treated Jurkat cells increased, while the addition of DX reduced the degree of tyrosine-phosphorylation in anti-CD3 activated Jurkat cells. This demonstrated the rapid suppressive effects of GCs on T-cell activation, which is supported by previous findings [20], however on a considerably different time scale.

The ZAP-70 kinase has a central role in TcR signalling [25]. After the MHC–peptide complex engaged the TcR, the ITAMs on the CD3 complex are rapidly phosphorylated [25]. These phosphorylation events are mediated by the src-family kinases [27]. Following CD3 phosphorylation, ZAP-70 kinase is recruited to the phosphotyrosines by its tandem SH2 domains and additional phosphorylation events occur [26]. The ZAP-70 kinase phosphorylates LAT and other adaptor proteins, which leads to the augmentation of the cell activation signal [30]. We investigated whether the GC hormone was able to influence ZAP-70 phosphorylation. Our experiments showed that DX caused a marked rise in ZAP-70 tyrosine-phosphorylation within 5 min. ZAP-70 kinase activity is regulated by site-specific tyrosine-phosphorylation [31,32]. Increase in the phosphotyrosine content of ZAP-70 might lead to either the increase or inhibition of kinase activity [32]. ZAP-70 is a member of the Syk-family tyrosine kinases, which contains 30 tyrosine residues. Several of these are potential phosphorylation sites. The known activation sites are Y315, Y319 [33], Y493 [34], some of them have inhibitory function Y292 [35], Y492 [34], others have unknown function (Y69, Y126 and Y178), respectively [31].

Some authors previously showed that the application of DX abrogated the early events of TcR-CD3 signalling. Van Laethem et al. demonstrated that 1 μM DX treatment for 6–16 h inhibited the phosphorylation of the TcR-zeta chain, ZAP-70, and the transmembrane adaptor LAT [20]. The authors suggested that these effects were due to altered acylation and lipid raft compartmentalisation of key signalling molecules [21]. In our experiments, we used high dose DX (10 μM) for 0.5–30 min, which was considerably shorter than reported in other experimental systems. The increased ZAP-70 tyrosine-phosphorylation after DX treatment seemed in contrast to what Van Laethem et al. have found on DX attenuated ZAP-70 tyrosine-phosphorylation [20]. To elucidate this controversy, we analysed the kinetics of ZAP-70 phosphorylation response induced by DX. We found that 10 μM DX induced rapid, transient ZAP-70 phosphorylation, within 1 min and dephosphorylation occurred already after 5 min. Such rapid GC actions suggested the involvement of near-membrane structures. Structural data indicate that ZAP-70 is

localised adjacent to the cytoplasmic membrane both in its active and inactive form [36,37]. ZAP-70 is a key substrate of src-family kinases, which also localise in lipid rafts of the lymphocyte membranes [38], which supports this interpretation.

Next, we determined whether the DX-induced tyrosine-phosphorylation of ZAP-70 occurred in the absence of active p56-lck, a key kinase in T-cell activation with ZAP-70 as one of its important substrates. Our results revealed that DX was unable to induce ZAP-70 phosphorylation in the absence of p56-lck, suggesting that phosphorylation events initiated by DX in T-cells involve the p56-lck kinase. Interestingly, the anti-CD3-induced phosphorylation of ZAP-70 in JCaM1.6 cells was not defective. This might be due to the compensatory effect of other src-family kinases for example p59-fyn which might remain active in JCaM1.6 cells and participate in ZAP-70 phosphorylation [39–41]. DX treatment could not increase anti-CD3-induced tyrosine-phosphorylation of ZAP-70 in the p56-lck-deficient cell line, in contrast to wild-type Jurkat cells. This suggests that anti-CD3 activation and DX treatment may have caused the phosphorylation of different tyrosine residues of ZAP-70.

Steroid hormones mediate their rapid non-genomic effects either through the classical cytoplasmic receptor by specific protein–protein interactions or through their putative membrane receptor. Moreover, direct physico-chemical interactions may also participate in hormone action [16]. Some studies showed the rapid suppressive effect of GCs on mitogen activated human peripheral blood mononuclear cells (PBMC) [42] and rat thymocytes [43]. They showed that the oxygen consumption of the ConA-activated PBMC [42] or rat thymocytes [43] rapidly decreased in response to high dose methylprednisolone. The administration of high dose GC diminished the sodium and calcium ion transport across the plasma membrane and also inhibited the calcium signal following the ConA-activation in rat thymocytes [43]. These effects were thought to be due to direct physicochemical interactions with the plasma membrane and occurred at very high GC concentrations (1–10 mM) [43].

Our results showed that GC action was inhibited by the application of equimolar Mifepristone, a GR antagonist, suggesting that the rapid phosphorylation events induced by DX was mediated by the GR. The participation of GR in the rapid steroid signalling process of Jurkat cells raises the question whether the receptor is membrane-bound (mGR) or localises in the cytoplasm (cGR). Hsp-90 provides a binding site for the inactive form of cGR, which complex is dissociated by the ligand binding to GR [13]. The src-family member p56-lck also binds Hsp-90, which might have an important function in arranging the active conformation of the src-family kinases [44]. Moreover, the disruption of functional Hsp-90 with geldanamycin inhibits T-cell activation and decreases the phosphorylation of lck and Raf-1 kinases [44]. These data imply that the cGR and the p56-lck might localise in the same multimolecular compartment organised by the Hsp-90, thereby influencing each other's function.

Gametchu demonstrated the presence of GR-like antigens in the plasma membrane of a mouse T-lymphoma cell line [45] and in human leukaemia cells [46], suggesting that the GR may reside in the plasma membrane, too. The presence of membrane GR has been shown in the membrane of normal mononuclear blood cells [47]. Others have partially purified and characterised a novel membrane resident GC binding protein from amphibian brain [48]. This novel mGR protein is different from the classical cGR, since it resembles the properties of receptors containing seven transmembrane domains [48]. Similarly, Wong et al. recently identified and cloned a novel protein termed Modulator of Nongenomic Activity of ER (MNAR), a previously uncharacterised scaffold protein that modulates ER interaction with src-family protein tyrosine kinases [49]. They showed that this interaction leads to src activation and the stimulation of MAPK pathway. These data, together with our findings, raise the possibility of the mGR participating in the mediation of rapid glucocorticoid actions.

In conclusion, we established that the hierarchy of p56-lck and ZAP-70 represents an important link between the GC and TcR signalling pathways in Jurkat cells. Our data may provide a better insight to the molecular events underlying the rapid effects of high dose GC treatment.

Acknowledgements

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References

[1] H.M. Reichardt, Immunomodulatory activities of glucocorticoids: insights from transgenesis and gene targeting, Curr. Pharm. Des. 10 (23) (2004) 2797–2805.
[2] D. Franchimont, Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies, Ann. N. Y. Acad. Sci. 1024 (2004) 124–137.
[3] O. Frankfurt, S.T. Rosen, Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates, Curr. Opin. Oncol. 16 (6) (2004) 553–563.
[4] S.S. Nussey, S.A. Whitehead, Endocrinology: An Integrated Approach, BIOS Scientific Publishers Ltd., Oxford, UK, 2001.
[5] M.S. Vacchio, V. Papadopoulos, J.D. Ashwell, Steroid production in the thymus: implication for thymocyte selection, J. Exp. Med. 179 (1994) 1835–1846.

[6] O. Lechner, H. Dietrich, G.J. Wiegers, M. Vacchio, G. Wick, Glucocorticoid production in the chicken bursa and thymus, Int. Immunol. 13 (6) (2001) 769–776.
[7] I. Cima, N. Corazza, B. Dick, A. Fuhrer, S. Herren, S. Jakob, E. Ayuni, C. Mueller, T. Brunner, Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation, J. Exp. Med. 200 (12) (2004) 1635–1646.
[8] J.D. Ashwell, F.W. Lu, M.S. Vacchio, Glucocorticoids in T cell development and function, Annu. Rev. Immunol. 18 (2000) 309–345.
[9] J.F. Purton, J.A. Monk, D.R. Liddicoat, K. Kyparissoudis, S. Sakkal, S.J. Richardson, D.I. Godfrey, T.J. Cole, Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death, J. Immunol. 173 (6) (2004) 3816–3824.
[10] T. Berki, L. Palinkas, F. Boldizsar, P. Nemeth, Glucocorticoid (GC) sensitivity and GC receptor expression differ in thymocyte subpopulations, Int. Immunol. 14 (5) (2002) 463–469.
[11] J.E. Kalinyak, R.I. Dorin, A.R. Hoffman, A.J. Perlman, Tissue-specific regulation of glucocorticoid receptor mRNA by Dexamethasone, J. Biol. Chem. 262 (22) (1987) 10441–10444.
[12] P. Norgaard, H.S. Poulsen, Glucocorticoid receptors in human malignancies: a review, Ann. Oncol. 2 (8) (1991) 541–557.
[13] D.F. Smith, D.O. Toft, Steroid receptors and their associated proteins, Mol. Endocrinol. 7 (1993) 4–11.
[14] J.M. Berg, DNA binding specificity of steroid receptors, Cell 57 (1989) 1065–1068.
[15] T. Berki, L. Grama, P. Németh, Detection of steroid induced glucocorticoid receptor (GR) translocation and expression with a FITC labeled monoclonal antibody, Cytometry Suppl. 10 (2000) 99.
[16] F. Buttgereit, A. Scheffold, Rapid glucocorticoid effects on immune cells, Steroids 67 (2002) 529–534.
[17] J.D. Croxtall, Q. Choudhury, R.J. Flower, Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism, Br. J. Pharmacol. 130 (2000) 289–298.
[18] R.M. Lösel, E. Falkenstein, M. Feuring, A. Schultz, H.C. Tillmann, K. Rossol-Haseroth, M. Wehling, Nongenomic steroid action: controversies, questions, and answers, Physiol. Rev. 83 (2003) 965–1016.
[19] E. Baus, F. Andris, P.M. Dubois, J. Urbain, O. Leo, Dexamethasone inhibits the early steps of antigen receptor signaling in activated T lymphocytes, J. Immunol. 156 (1996) 4555–4561.
[20] F. Van Laethem, E. Baus, L.A. Smyth, F. Andris, F. Bex, J. Urbain, D. Kioussis, O. Leo, Glucocorticoids attenuate T cell receptor signaling, J. Exp. Med. 193 (7) (2001) 803–814.
[21] F. Van Laethem, X. Liang, F. Andris, J. Urbain, M. Vandenbranden, J.M. Ruysschaert, M.D. Resh, T.M. Stulnig, O. Leo, Glucocorticoids alter the lipid and protein composition of membrane rafts of a murine T cell hybridoma, J. Immunol. 170 (2003) 2932–2939.
[22] C. Buitrago, R. Boland, A.R. de Boland, The tyrosine kinase c-src is required for 1,25(OH)2-Vitamin D3 signalling to the nucleus in muscle cells, BBA-Mol. Cell. Res. 1541 (3) (2001) 179–187.
[23] C.G. Buitrago, V.G. Pardo, A.R. de Boland, R. Boland, Activation of RAF-1 through Ras and protein kinase Calpha mediates 1alpha,25(OH)2-Vitamin D3 regulation of the mitogen-activated protein kinase pathway in muscle cells, J. Biol. Chem. 278 (4) (2003) 2199–2205.
[24] V. Boonyaratanakornkit, M.P. Scott, V. Ribon, L. Sherman, S.M. Anderson, J.L. Maller, W.T. Miller, D.P. Edwards, Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-src-family tyrosine kinases, Mol. Cell. 8 (2) (2001) 1269–1280.
[25] J.E. van Leeuwen, L.E. Samelson, T cell antigen-receptor signal transduction, Curr. Opin. Immunol. 11 (3) (1999) 242–248.
[26] A.C. Chan, B.A. Irving, J.D. Fraser, A. Weiss, The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein, Proc. Natl. Acad. Sci. U.S.A. 88 (20) (1991) 9166–9170.
[27] T. Mustelin, K. Tasken, Positive and negative regulation of T-cell activation through kinases and phosphatases, Biochem. J. 371 (2003) 15–27.
[28] I. Jung-Testas, E.E. Baulieu, Inhibition of glucocorticosteroid action in cultured L-929 mouse fibroblasts by RU 486, a new anti-glucocorticosteroid of high affinity for the glucocorticosteroid receptor, Exp. Cell. Res. 147 (1) (1983) 177–182.
[29] A. Weiss, D.R. Littman, Signal transduction by lymphocyte antigen receptors, Cell 76 (2) (1994) 263–274.
[30] W. Zhang, J. Sloan-Lancaster, J. Kitchen, R.P. Trible, L.E. Samelson, LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation, Cell 92 (1) (1998) 83–92.
[31] J.D. Watts, M. Affolter, D.L. Krebs, R.L. Wange, L.E. Samelson, R. Aebersold, Identification by electrospray ionization mass spectrometry of the sites of tyrosine-phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70, J. Biol. Chem. 269 (47) (1994) 29520–29529.
[32] G. Kong, M. Dalton, J.B. Wardenburg, D. Straus, T. Kurosaki, A.C. Chan, Distinct tyrosine-phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function, Mol. Cell. Biol. 16 (9) (1996) 5026–5035.
[33] V. Di Bartolo, D. Mege, V. Germain, M. Pelosi, E. Dufour, F. Michel, G. Magistrelli, A. Isacchi, O. Acuto, Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signalling, J. Biol. Chem. 274 (10) (1999) 6285–6294.
[34] D. Mege, V. Di Bartolo, V. Germain, L. Tuosto, F. Michel, O. Acuto, Mutation of tyrosines 492/493 in the kinase domain of ZAP-70 affects multiple T-cell receptor signaling pathways, J. Biol. Chem. 271 (51) (1996) 32644–32652.
[35] A. Magnan, V. Di Bartolo, A.M. Mura, C. Boyer, M. Richelme, Y.L. Lin, A. Roure, A. Gillet, C. Arrieumerlou, O. Acuto, B. Malissen, M. Malissen, T cell development and T cell responses in mice with mutations affecting tyrosines 292 or 315 of the ZAP-70 protein tyrosine kinase, J. Exp. Med. 194 (4) (2001) 491–505.
[36] J. Sloan-Lancaster, W. Zhang, J. Presley, B.L. Williams, R.T. Abraham, J. Lippincott-Schwartz, L.E. Samelson, Regulation of ZAP-70 intracellular localization: visualization with the green fluorescent protein, J. Exp. Med. 186 (10) (1997) 1713–1724.
[37] R.D. Huby, M. Iwashima, A. Weiss, S.C. Ley, ZAP-70 protein tyrosine kinase is constitutively targeted to the T cell cortex independently of its SH2 domains, J. Cell. Biol. 137 (7) (1997) 1639–1649.
[38] T. Magee, N. Pirinen, J. Adler, S.N. Pagakis, I. Parmryd, Lipid rafts: cell surface platforms for T cell signalling, Biol. Res. 35 (2) (2002) 127–131.
[39] N. Fusaki, K. Semba, T. Katagiri, G. Suzuki, S. Matsuda, T. Yamamoto, Characterization of p59fyn-mediated signal transduction on T cell activation, Int. Immunol. 6 (8) (1994) 1245–1255.
[40] N. Fusaki, S. Matsuda, H. Nishizumi, H. Umemori, T. Yamamoto, Physical and functional interactions of protein tyrosine kinases, p59fyn and ZAP-70 in T cell signalling, J. Immunol. 156 (4) (1996) 1369–1377.
[41] D. Filipp, M. Julius, Lipid rafts: resolution of the “fyn problem”? Mol. Immunol. 41 (6–7) (2004) 645–656.
[42] D. Schmid, G.R. Burmester, R. Tripmacher, G. Fici, P. von Voigtlander, F. Buttgereit, Short-term effects of the 21-aminosteroid lazaroid tirilazad mesylate (PNU-74006F) and the pyrrolopyrimidine lazaroid PNU-101033E on energy metabolism of human peripheral blood mononuclear cells, Biosci. Rep. 21 (1) (2001) 101–110.
[43] F. Buttgereit, S. Krauss, M.D. Brand, Methylprednisolone inhibits uptake of Ca^{2+} and Na^{+} ions into concanavalin A-stimulated thymocytes, Biochem. J. 326 (1997) 329–332.
[44] T. Schnaider, J. Somogyi, P. Csermely, M. Szamel, The Hsp90-specific inhibitor geldanamycin selectively disrupts kinase-mediated signaling events of T-lymphocyte activation, Cell Stress Chaperones 5 (1) (2000) 52–61.

[45] B. Gametchu, Glucocorticoid receptor-like antigen in lymphoma cell membranes: correlation to cell lysis, Science 236 (4800) (1987) 456–461.
[46] B. Gametchu, C.S. Watson, S. Wu, Use of receptor antibodies to demonstrate membrane glucocorticoid receptor in cells from human leukemic patients, FASEB J. 7 (13) (1993) 1283–1292.
[47] B. Bartholome, C.M. Spies, T. Gaber, S. Schuchmann, T. Berki, D. Kunkel, M. Bienert, A. Radbruch, G.R. Burmester, R. Lauster, A. Scheffold, F. Buttgereit, Membrane glucocorticoid receptors (mGR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis, FASEB J. 18 (1) (2004) 70–80.
[48] S.J. Evans, T.F. Murray, F.L. Moore, Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain, J. Steroid Biochem. Mol. Biol. 72 (5) (2000) 209–221.
[49] C.W. Wong, C. McNally, E. Nickbarg, B.S. Komm, B.J. Cheskis, Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with src/Erk phosphorylation cascade, Proc. Natl. Acad. Sci. U.S.A. 99 (23) (2002) 14783–14788.

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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-

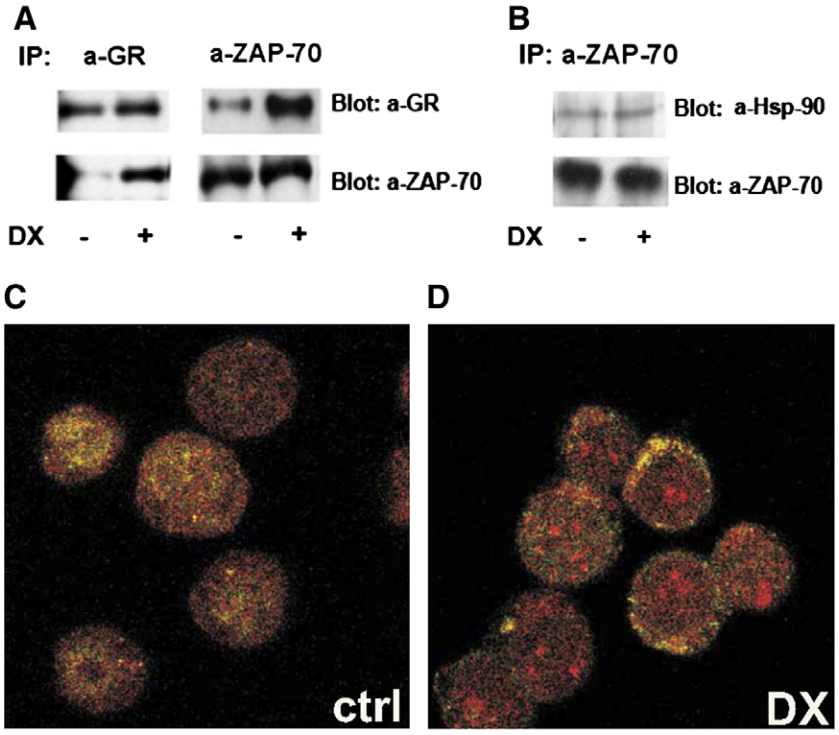

Fig. 1. Association of ligand-bound GR with ZAP-70 in Jurkat cells. (A) Anti-GR and anti-ZAP-70 Western blots are shown from anti-GR or anti-ZAP-70 precipitated Jurkat cell lysates with or without DX treatment. Equal amount of GR and ZAP-70 precipitated when anti-GR and anti-ZAP-70 antibodies were used 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

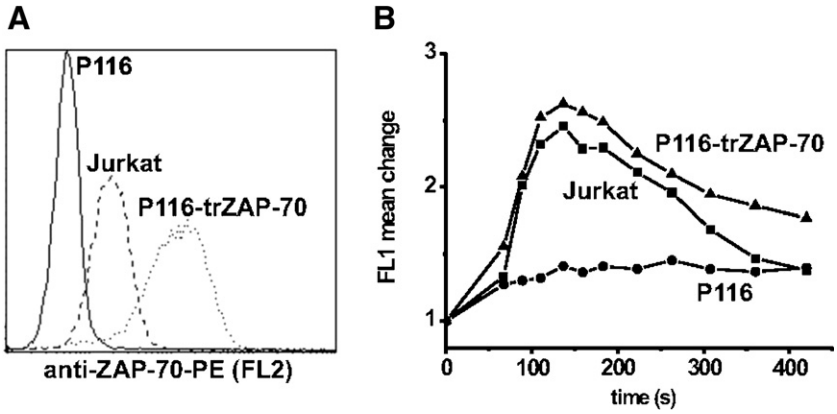

Fig. 2. Lentiviral transfection of P116 (ZAP-70 deficient) cells with ZAP-70. (A) ZAP-70 expression of ZAP-70 transfected P116 cells was compared to untransfected P116 and Jurkat cells by flow cytometry after intercellular phycoerythrin (PE)-conjugated anti-ZAP-70 antibody labelling. Flow cytometric 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.

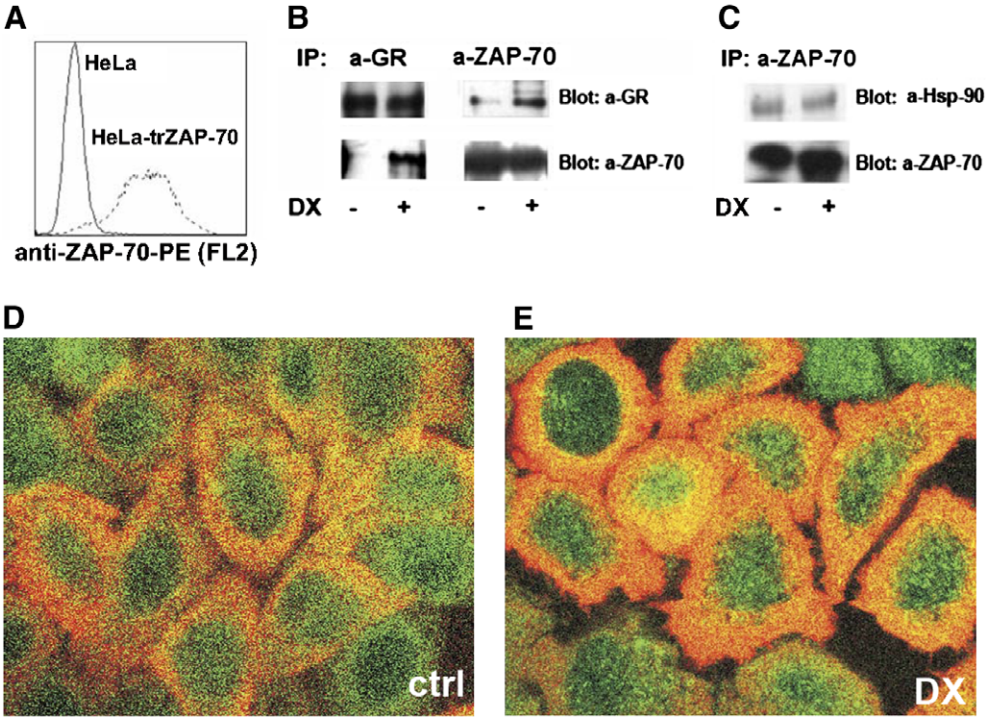

Fig. 3. Liganded GR clustering with ZAP-70 in HeLa–trZAP-70 cells. (A) Representative flow cytometric histograms show the ZAP-70 expression of control and HeLa–trZAP-70 cells after labelling with phycoerythrin (PE)-conjugated anti-ZAP-70 antibody. (B) Anti-GR and anti-ZAP-70 blots are shown from anti-GR 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).

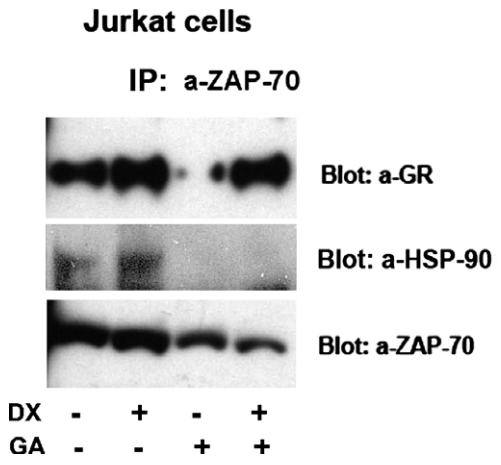

Fig. 4. Geldanamycin treatment does not inhibit the Dexamethasone-induced ZAP-70–GR co-precipitation. Anti-GR, anti-Hsp-90, or anti-ZAP-70 blots are shown from anti-ZAP-70 precipitated control or DX-treated Jurkat cell lysates with or without Geldanamycin pretreatment. Geldanamycin treatment did not 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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References

[1] R.M. Losel, E. Falkenstein, M. Feuring, A. Schultz, H.-C. Tillmann, K. Rossol-Haseroth, M. Wehling, Nongenomic steroid action: controversies, questions, and answers, Physiol. Rev. 83 (2003) 965–1016.

[2] P. Csermely, T. Schnaider, C. Soti, Z. Prohaszka, G. Nardai, The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review, Pharmacol. Ther. 79 (1998) 129–168.

[3] W.B. Pratt, E.R. Sanchez, E.H. Bresnick, S. Meshinchi, L.C. Scherrer, F.C. Dalman, M.J. Welsh, Interaction of the glucocorticoid receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation, Cancer Res. 49 (1989) 2222s–2229s.

[4] J.M. Berg, DNA binding specificity of steroid receptors, Cell 57 (1989) 1065.

[5] B.M. Necela, J.A. Cidlowski, Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells, Proc. Am. Thorac. Soc. 1 (2004) 239–246.

[6] E.D. Hall, J.E. Springer, Neuroprotection and acute spinal cord injury: a reappraisal, NeuroRx 1 (2004) 80–100.

[7] A.F.T. Brown, D. McKinnon, K. Chu, Emergency department anaphylaxis: a review of 142 patients in a single year, J. Allergy Clin. Immunol. 108 (2001) 861.

[8] K.L. Drain, G.W. Volcheck, Preventing and managing drug-induced anaphylaxis, Drug Saf. 24 (2001) 843–853.

[9] E. Baus, F. Andris, P.M. Dubois, J. Urbain, O. Leo, Dexamethasone inhibits the early steps of antigen receptor signaling in activated T lymphocytes, J. Immunol. 156 (1996) 4555–4561.

[10] F. Van Laethem, E. Baus, L.A. Smyth, F. Andris, F. Bex, J. Urbain, D. Kioussis, O. Leo, Glucocorticoids attenuate T cell receptor signaling, J. Exp. Med. 193 (2001) 803–814.

[11] F. Van Laethem, X. Liang, F. Andris, J. Urbain, M. Vandenbranden, J.-M. Ruysschaert, M.D. Resh, T.M. Stulnig, O. Leo, Glucocorticoids alter the lipid and protein composition of membrane rafts of a murine T cell hybridoma, J. Immunol. 170 (2003) 2932–2939.

[12] F. Buttgereit, A. Scheffold, Rapid glucocorticoid effects on immune cells, Steroids 67 (2002) 529–534.

[13] G. Akner, K. Mossberg, A.C. Wikstorm, K.G. Sundgvist, J.A. Gustafsson, Evidence for colocalization of glucocorticoid receptor with cytoplasmic microtubules in human gingival fibroblasts, using two different monoclonal anti-GR antibodies, confocal laser scanning microscopy and image analysis, J. Steroid Biochem. Mol. Biol. 39 (1991) 419–432.

[14] T. Schnaider, J. Somogyi, P. Csermely, M. Szamel, The Hsp90-specific inhibitor geldanamycin selectively disrupts kinase-mediated signaling events of T-lymphocyte activation, Cell Stress Chaperon. 5 (2000) 52–61.

[15] A.C. Chan, D.M. Desai, A. Weiss, The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction, Ann. Rev. Immunol. 12 (1994) 555–592.

[16] M. Lowenberg, J. Tuynman, J. Bilderbeek, T. Gaber, F. Buttgereit, S. van Deventer, M. Peppelenbosch, D. Hommes, Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn, Blood 106 (2005) 1703–1710.

[17] D. Bartis, F. Boldizsar, M. Szabo, L. Palinkas, P. Nemeth, T. Berki, Dexamethasone induces rapid tyrosine-phosphorylation of ZAP-70 in Jurkat cells, J. Steroid Biochem. Mol. Biol. 98 (2006) 147–154.

[18] F. Bovia, P. Salmon, T. Matthes, K. Kvell, T.H. Nguyen, C. Werner-Favre, M. Barnet, M. Nagy, F. Leuba, J.-F. Arrighi, V. Piguet, D. Trono, R.H. Zubler, Efficient transduction of primary human B lymphocytes and nondividing myeloma B cells with HIV-1-derived lentiviral vectors, Blood 101 (2003) 1727–1733.

[19] K. Kvell, T.H. Nguyen, P. Salmon, F. Glauser, C. Werner-Favre, M. Barnet, P. Schneider, D. Trono, R.H. Zubler, Transduction of CpG DNA-stimulated primary human B cells with bicistronic lentivectors, Mol. Ther. 12 (2005) 892–899.

[20] U. O'Doherty, W.J. Swiggard, M.H. Malim, Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding, J. Virol. 74 (2000) 10074–10080.

[21] T. Berki, G. Kumanovics, A. Kumanovics, A. Falus, E. Ujhelyi, P. Nemeth, Production and flow cytometric application of a monoclonal anti-glucocorticoid receptor antibody, J. Immunol. Methods 214 (1998) 19–27.

[22] F. Boldizsar, T. Berki, A. Miseta, P. Nemeth, Effect of hyperglycemia on the basal cytosolic free calcium level, calcium signal and tyrosine-phosphorylation in human T-cells, Immunol. Lett. 82 (2002) 159–164.

[23] J.E. Castro, C.E. Prada, O. Loria, A. Kamal, L. Chen, F.J. Burrows, T.J. Kipps, ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia, Blood 106 (2005) 2506–2512.

[24] A. Kamal, L. Thao, J. Sensintaffar, L. Zhang, M.F. Boehm, L.C. Fritz, F.J. Burrows, A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors, Nature 425 (2003) 407–410.