Evidence for the Involvement of Tyrosine Kinase ZAP 70 in Nuclear Retinoid Receptor-dependent Transactivation in T Lymphocytes*

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Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are transcription factors that control diverse cellular functions during development and homeostasis. The biochemical role of these proteins in T lymphocytes is not well known. Here we have studied the role of protein-tyrosine kinase ZAP 70, a key enzyme involved in the proximal signaling events during T cell activation, in the modulation of RXRE- and RARE-dependent activation in T lymphocytes. Surprisingly, ZAP 70-negative Jurkat T cells showed considerable loss of both RXRE- and RARE-mediated transactivation as compared with wild type Jurkat cells. In addition, ZAP 70-negative cells failed to exhibit normal protein kinase C θ and calcineurin-induced transcriptional activity. ZAP 70-negative cells that were reconstituted with active ZAP 70 regained the transactivation function, whereas cells expressing kinase-dead form of ZAP 70 failed to do so. Defective transcriptional activation was also observed in actively proliferating human peripheral blood T lymphocytes in which RNA interference was used to induce loss of ZAP 70 expression. In addition, an Lck-deficient Jurkat cell line that cannot efficiently activate ZAP 70 was also found defective in RXRE-mediated transcription. Finally, RNA interference-induced loss of ZAP 70 or Lck protein in Jurkat cells resulted in significant decrease in the RXRE-dependent activation. Together, these results suggest a novel functional role for ZAP 70 in nuclear receptor-driven transactivation in T lymphocytes.

**Experimental Procedures**

Following antigen binding, T cell antigen receptor (TCR) activates an array of intracellular signaling cascades (1–4). One of the earliest events is the activation of Src family of protein-tyrosine kinases (PTK) Lck and/or Fyn that phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR. This creates the docking site for an SH2 domain containing PTK called ZAP 70. ZAP 70 binds to two phosphotyrosine residues of individual ITAMs and is subsequently phosphorylated and activated by Lck and/or Fyn (5, 6). These signaling events allow recruitment and activation of a number of other signaling proteins at the TCR followed by activation of downstream signaling pathways. ZAP 70 plays a critical role in the normal T cell signaling and positive and negative selection of thymocytes. ZAP 70 knock-out mice and patients with mutations in ZAP 70 have severe combined immune-deficiency syndrome phenotypes and other abnormalities (7–10).

Retinoid X receptors (RXRs) are members of nuclear receptor superfamily that also include retinoic acid receptor (RAR), thyroid hormone receptor (TR), and vitamin D receptor (VDR) (11–15). RXRs function as ligand-dependent transcription factors by homodimerization or through heterodimerization with other members of the retinoid and steroid receptor family of transcription factors. The physiological role of RXRs in T lymphocyte function is not well understood. We have recently found that T cell activation signals induce loss of transcriptional activation mediated by nuclear receptors via activation of mitogen-activated protein kinase signaling pathways and induction of receptor-corepressor interaction (16, 17). In addition, activated PKCβ was found to synergize with calcineurin to induce transcriptional activation mediated by these receptors (18). However, the role of proximal TCR signaling events and the activation of PTK in the modulation of retinoid receptor-dependent transcription is not known. Here we have shown that ZAP 70-negative Jurkat cells, T cells manipulated to express low levels of ZAP 70, or cells that cannot efficiently activate ZAP 70 exhibit loss of retinoid receptor-mediated transcriptional suggesting that ZAP 70 is essential for normal retinoid receptor function in T cells.

**Cells and Other Reagents**—T lymphocyte leukemia Jurkat cell line (clone E6-1) and Lck-deficient Jurkat cell line J.CaM1.6 were obtained from American Type Culture Collection (Manassas, VA). ZAP 70-negative Jurkat cell line P116, P116 cells stably expressing c-myc-tagged wild type ZAP 70 (P116/pWT), and P116 cells stably expressing c-myc-tagged kinase-dead ZAP 70 mutant (K369R) (P116/pDK) (19) were kindly provided by R. L. Wange (National Institutes of Health, Baltimore). These cells were maintained in RPMI 1640 medium (BioWhittaker, Frederick, MD) supplemented with 10 mM HEPES buffer, 2 mM l-glutamine, 60 μg/ml gentamicin, and 10% fetal bovine serum (HyClone, Logan, UT). Human peripheral blood mononuclear cells, obtained by lymphapheresis of healthy donors, were purified by Ficoll density gradient centrifugation. Purified peripheral blood mononuclear cells were treated with phytohemagglutinin and interleukin-2 for 2 days in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The cells were washed to remove phytohemagglutinin and maintained in interleukin-2. In this report, these cells will be referred to as proliferating peripheral blood T (PBT) cells. PBT cells were 98% CD3-positive as monitored by flow cytometry. 9-cis-Retinoic acid (9-CRA) and all-trans-retinoic acid were from Sigma and were used at 1 μM. ZAP 70 and Lck antibodies were from BD Biosciences, and RARα, RXRα, and c-myc tag antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

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2 The abbreviations used are: TCR, T cell antigen receptor; PTK, protein-tyrosine kinase; RNAi, RNA interference; DA, dominant active; PKC, protein kinase C; CN, calcineurin; RAR, retinoic acid receptor; RXR, retinoid X receptor; ITAM, immunoreceptor tyrosine-based activation motif; TR, thyroid hormone receptor; TRE, TR element; VDR, vitamin D receptor; VDRE, VDR element; PBT, peripheral blood T; 9-CRA, 9-cis-retinoic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethylpropane-1,3-diol; MES, 4-morpholineethanesulfonic acid; siRNA, small interfering RNA.

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FIGURE 1. **Defective RXRE- and RARE-dependent transactivation** in ZAP 70-negative P116 cells. A, ZAP 70-negative P116 and wild type Jurkat cells were transfected with 5.0 μg of reporter plasmids in the presence or absence of receptor expressing plasmid or ligand. Cells were harvested after 36 h for luciferase measurement as described under “Experimental Procedures.” B, cytoplasmic (C) and nuclear (N) extracts of WT Jurkat, P116, P116/pWT, and P116/pDK cells were subjected to Western blot analysis using antibodies to ZAP 70, c-myc tag, RARα, and RXRα. Arrows inside the figure indicate nonspecific bands observed with RARα and RXRα antibodies in the cytoplasmic extracts. C, ZAP 70-negative P116 and wild type Jurkat cells were transfected with 5.0 μg of TKCRBP11-Luc or TKRARE-Luc plasmid in the presence or absence of 2.5 μg of the indicated plasmid constructs. Cells were harvested after 36 h for luciferase activity measurement as described under “Experimental Procedures.”
Western Blot—Nuclear and cytoplasmic isolations were performed with the NE-PER Nuclear and Cytoplasmic kit from Pierce as per the manufacturer’s instructions. Protein extracts were electrophoresed in a 10% NuPAGE BisTris gel using NuPAGE MES-SDS running buffer (Invitrogen) and transferred to a polyvinylidene difluoride membrane using XCell Blot Module (Invitrogen). The protein was detected using the fluorophore-labeled secondary antibodies and Odyssey Infrared Imaging System (Li-cor Biotechnology, Lincoln, NE).

Transfections and RNA Interference (RNAi)—RXRE-, RARE-, TRE-, and VDRE-driven transcriptional activation was studied by transfection using luciferase based reporter plasmids TKCRBP1I-Luc, TKRARE-Luc, TKDR4-Luc, and TKDR3-Luc, respectively, as described previously (17). The TKCRBP1IM-Luc plasmid containing RXRE mutant sequence (17) was used as RXR specificity control. Human glyceraldehyde-3-phosphate dehydrogenase promoter that lacks an RXRE or RARE site was isolated by PCR and cloned into pGL3-Luc basic vector (Promega, Madison, WI). Plasmids expressing human RXRα, RARα, TRα, and VDR were as described (17). pC6F vector, pCEFL, dominant active protein kinase Cα (DA-PKCα), and dominant active calcineurin (DA-CN) have been described previously (18). The psX/ZAP70 plasmid obtained from Dr. R. L. Wange (National Institutes of Health, Baltimore) was used as template to isolate ZAP 70 cDNA by PCR for cloning in pcDNA4/HispMaxTOPO vector (Invitrogen). ZAP 70 and Lck SMARTpool siRNAs were purchased from Upstate Biotechnology (Lake Placid, NY). Jurkat and Jurkat-derived cells were transfected by electroporation using a Gene Pulser II (Bio-Rad) at 0.250 kV and 975 microfarads as described (18). PBT cells were electroporated using a T cell Nucleofection kit from Amaxa Biosystems (Cologne, Germany) according to the manufacturer’s instructions. Transfected cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum and harvested at the indicated time periods, and luciferase activity was determined using Luciferase Assay System (Promega). Luciferase activity was normalized to protein concentrations in the extracts. The values in the figures represent the mean of two to three independent experiments with standard error calculated for each value.

RESULTS AND DISCUSSION

Defective RXR- and RAR-mediated Transactivation in ZAP 70-negative Jurkat Cells—Our earlier reports have shown that Jurkat cells have functional RXR- and RAR-dependent transcriptional machinery that can be regulated by a number of signaling pathways (16–18). To dissect the role of ZAP 70 kinase in modulating RXR and RAR-dependent gene activation, we used a ZAP 70-negative cell line named P116. This cell line has been generated from wild type Jurkat E6 cells by mutagenesis using the frameshift mutagen, ICR-191 (19). Wild type Jurkat cells and P116 cells were transfected with TKCRBP1I-Luc reporter, which binds RXR either as homodimer or heterodimer with RAR, in the presence or absence of RXRα expressing plasmid and 9-CRA. Surprisingly, P116 cells were considerably defective in supporting transcription from TKCRBP1I-Luc plasmid as compared with the wild type Jurkat cells. This defect was observed with endogenous as well as exogenously expressed RXRα and was independent of the presence or absence of the 9-CRA (Fig. 1A). We also tested TKRARE-Luc reporter plasmid, containing DR5 element from RARβ2 promoter specific for RXR/RAR heterodimerdependent transcription. Similar to TKCRBP1I-Luc, there was a marked reduction in the transcription driven by TKRARE-Luc plasmid in P116 cells as compared with wild type Jurkat cells. As a control, luciferase activity driven by glyceraldehyde-3-phosphate dehydrogenase promoter, which lacks an RXRE or RARE site, did not show any significant reduction in P116 cells. Little or no luciferase activity was observed when cells were transfected with TKCRBP1I-M-Luc, a plasmid containing mutations in RXRE site, indicating the specificity of retinoid receptor-dependent activation assay. Under similar conditions reporter
activities driven by TKDR3-Luc and TKDR4-Luc, specific for RXR/VDR- and RXR/TR-mediated activation respectively, were not reduced in P116 cells as compared with wild type cells. Together, these results indicate that ZAP 70 expression is essential for RXR-dependent transcriptional activation either as homodimer or heterodimer with RAR but is not crucial for transcriptional activation involving heterodimerization with TR and VDR. This selectivity for RAR containing heterodimers is striking and is indicative of specific role of ZAP 70 to modulate retinoid receptor function in T cells.

RXRα and RARα are two major isoforms of RXRs and RARs in T cells mostly located in nuclear compartments. To exclude the possibility that the loss of retinoid receptor activity in P116 cells is due either to the loss of expression of one or both of these receptor proteins or export to cytoplasmic compartment, we investigated whether wild type Jurkat and P116 cells express comparable levels of RARα and RXRα proteins in their nuclear compartments. Western blot analysis (Fig. 1B) indicates that wild type Jurkat and P116 cells express similar levels of RARα and RXRα protein in the nuclear compartments. Furthermore, RARα and RXRα proteins were not detected in cytoplasmic compartments. These results indicate that the absence of ZAP 70 does not alter the localization of receptor proteins in the cell and instead suggest the role of ZAP 70 in a post-translational mechanism that regulates receptor activity in Jurkat cells.

FIGURE 3. RNAi-induced loss of ZAP 70 expression attenuates RXRE-dependent transcription in PBT and Jurkat cells. PBT and Jurkat cells were transfected with 5.0 μg of TKCRBPⅡ-Luc plasmid in the presence or absence of 5 μM ZAP 70 specific siRNA or scrambled RNA as described under “Experimental Procedures.” The extracts were subjected to Western blot analysis (A) and luciferase activity measurement (B) as described under “Experimental Procedures.”

FIGURE 4. Defective RXRE-dependent transactivation in Lck-deficient cells. Lck-negative JCam1.6 and wild type Jurkat cells were transfected with 5.0 μg of TKCRBPⅡ-Luc plasmid in the presence or absence of 2.5 μg of RXRα plasmid or 9-CRA. Cells were harvested after 36 h for luciferase measurement as described under “Experimental Procedures” (A). Jurkat cells were transfected with 5.0 μg of TKCRBPⅡ-Luc plasmid in the presence or absence of 5 μM Lck-specific siRNA or scrambled RNA as described under “Experimental Procedures.” The extracts were subjected to Western blot analysis (B) and luciferase activity measurement (C) as described under “Experimental Procedures.”
P116 cells have been reported to be defective in PKC\(\theta\) signaling (20). We have previously shown that expression of DA-CN and DA-PKC\(\theta\) synergistically induce RXRE-dependent transcription in Jurkat cells (18). We next investigated whether this PKC\(\theta\) and CN synergism can be observed in P116 cells. Wild type Jurkat cells and P116 cells were transfected with TKCRBPII-Luc or TKRARE-Luc plasmid in the presence or absence of pCEFL vector or pCEFL/DA-PKC\(\theta\) or a combination of pCEFL vector and DA-CN or pCEFL/DA-PKC\(\theta\) and DA-CN plasmids. We expected that the expression of DA-PKC\(\theta\) and DA-CN would circumvent the need for ZAP 70 expression to support RXRE- and RARE-mediated activation in P116 cells. Surprisingly, P116 cells exhibit considerably low DA-PKC\(\theta\)/DA-CN-induced receptor-dependent activation contrary to wild type Jurkat cells that showed a robust DA-PKC\(\theta\) and DA-CN synergism (Fig. 1C). These results indicate that defective PKC\(\theta\) signaling due to lack of ZAP 70 expression in P116 cells may not alone be responsible for low transcriptional activity in these cells. ZAP 70 (possibly nuclear ZAP 70) may play an additional role independent of PKC\(\theta\) and CN in modulating RXRE- and RARE-mediated activity.

Since similar data were obtained with RXRE and RARE containing reporter vectors subsequent experiments have been performed with TKCRBPII-Luc vector alone.

**Enzymatically Active ZAP 70 Expression Reconstitutes RXRE-mediated Transactivation in P116 Cells**—To determine whether defective RXRE signaling in ZAP 70-negative P116 cells can be corrected by the expression of enzymatically active ZAP 70, we studied P116 cells transiently expressing ZAP 70 protein and P116 cells stably expressing the enzyme. P116 cells were transfected with TKCRBPII-Luc plasmid in the presence or absence of pcDNA4/HisMaxTOPO vector or ZAP 70 expressing pcDNA4/HisMaxTOPO/ZAP 70 plasmid (Fig. 2A). Transfection of P116 cells with ZAP70 expressing plasmid reconstituted RXRE activity significantly as compared with cells transfected with vector (Fig. 2B) indicating that expression of ZAP 70 is important for normal RXRE activity. These data also excludes the possibility that lower
reporter activity in P116 cells may have partly been due to the lower transfection efficiency of these cells as compared with wild type cells.

Previous studies have shown that P116 cells that stably express wild type ZAP 70 regain most of the phosphorylation events whereas mutant ZAP 70 reconstituted cells fail to do so (19). We investigated whether P116 cells that stably express wild type ZAP 70 or catalytically inactive mutant (K369R) of ZAP 70 were able to support RXRE-dependent activation. Transfection of P116/pWT cells with TCRβPII-Luc plasmid yielded activation levels that closely approached the levels seen in wild type Jurkat cells. In contrast, transfection of P116/pDK cells with TCRβPII-Luc plasmid yielded activation levels that were similar to the levels obtained with P116 cells (Fig. 2C). We next studied the DA-PKCθ/DA-CN synergism-induced RXRE activity in enzymatically active ZAP 70 reconstituted P116 cells and P116 cells that were reconstituted with catalytically inactive ZAP 70. Fig. 2C shows that contrary to catalytically inactive ZAP 70 reconstituted P116 cells that showed low PKCθ/CN-induced transcriptional activity, enzymatically active ZAP 70 reconstituted cells regained most of PKCθ/CN-induced the RXRE-dependent transcription. Together, these results demonstrate that PTK activity of ZAP 70 can correct the defects in basal as well as PKCθ/CN-induced RXRE-dependent signaling in P116 cells unlike catalytically inactive ZAP 70 that fails to restore receptor activity in these cells.

RNAi-induced Loss of ZAP 70 Expression Interferes in RXRE-dependent Transactivation—To extrapolate the data obtained with P116 cells to a more physiologic model for T cell function, we used normal human PBT cells in which ZAP 70 expression was partially knocked down by RNAi. siRNA duplexes specific for human ZAP 70 mRNA were introduced into PBT cells using a Nucleofection kit from Amaxa Biosystems. This method of transfection resulted in more than 75% loss in ZAP 70 protein expression in these cells (Fig. 3A). To determine the role of ZAP 70 in RXRE-dependent function in PBT cells, they were transfected with TCRβPII-Luc in the presence or absence of ZAP 70-specific siRNA or a nonspecific scrambled RNA. The results show that ZAP 70 specific siRNA considerably attenuated RXRE-mediated transcription whereas scrambled RNA did not have significant effect on the transcription (Fig. 3B). We also used RNAi to knock down nearly 90% of ZAP 70 protein expression in Jurkat cells (Fig. 3A). Transfection of Jurkat cells with TCRβPII-Luc in the presence of ZAP 70 specific siRNA resulted in a significant down-regulation of RXRE-dependent transcription in these cells, whereas scrambled RNA did not show significant effect on transcription (Fig. 3B). RNAi data further confirm the essential role of ZAP 70 in mediating RXRE activity in Jurkat cells and indicate that ZAP 70-dependent RXRE regulation is also operative in normal human peripheral blood T cell pool.

Lck-deficient Jurkat Cells Are Defective in RXRE-dependent Transcription—In addition to being important for binding of ZAP 70 to ITAM, Lck is required for phosphorylation and activation of ZAP 70 (1–4). We considered the possibility that the lack of Lck expression could compromise RXRE-dependent transcription due to the lack of active ZAP 70. Wild type Jurkat cells and Lck-deficient JCam 1.6 cells were transfected with TCRβPII-Luc plasmid in the presence or absence of RXRα expressing plasmid and 9-CRA. The results (Fig. 4A) indicate a considerable decrease in RXRE activity in JCam 1.6 cells as compared with wild type Jurkat cells. These data show that Lck is essential for the RXRE-mediated transcription. Additional evidence that Lck plays an important role in RXRE-mediated transcription was obtained by experiments involving RNAi-induced loss of Lck expression in Jurkat cells. Jurkat cells transfected with Lck-specific siRNA duplexes resulted in about 75% loss of Lck protein (Fig. 4B). Transfection of Jurkat cells with TCRβPII-Luc in the presence or absence of Lck-specific siRNA or nonspecific siRNA showed that while nonspecific siRNA had no effect on the RXRE activity, Lck-specific siRNA attenuated RXRE-dependent transcription (Fig. 4C).

The role of nuclear receptor mediated gene expression in the normal functioning of T cells has remained elusive. It is increasingly becoming clear that epigenetic mechanisms play an important role in defining the retinoid receptor-dependent function during T cell signaling (Fig. 5). It is, however, not clear how overlapping signaling intermediates modulate either activation or inhibition of receptor function resulting in outcomes as diverse as cell division, proliferation, development, stress response, homeostasis, and apoptosis. In T cells ZAP 70 is not only present in cytoplasm (for translocation to TCR following T cell activation), but the protein is also localized in the nucleus (Ref. 21 and Fig. 1B) where its function is unknown. Whether cytoplasmic or nuclear ZAP 70 plays a role in modulating RXRE and RARE mediated transcription remains to be seen. RXRs have a number of tyrosine phosphorylation sites that are important for their transcriptional activity in Jurkat cells. However, ZAP 70 failed to phosphorylate recombinant RXRα in direct phosphorylation studies (data not shown). Whether ZAP 70 is involved in the phosphorylation RXRα remains to be studied. ZAP 70-mediated phosphorylation of RARα may play essential role in the transcriptional activity of this receptor. ZAP 70 is essential for phosphorylation and activation of number of downstream protein kinases (5, 6, 22) and phosphatases (23) in addition to its essential role in the translocation of PKCθ to the lipid rafts during the formation of functional immune synapse. We have previously shown that RXRE activity is modulated by PKCθ, CN, and mitogen-activated protein kinase signaling pathways (16, 18). RXRE and RARE transactivation may involve modulation of one or all of these signaling pathways by ZAP 70. The finding that the expression of a combination of DA-PKCθ and DA-CN failed to restore RARE and RXRE activity in P116 cells indicates that ZAP 70 regulation of retinoid receptor activity may also involve PKCθ-independent pathways.

Deficient transcriptional activation in J.CaM1.6 cells provides further evidence for the important role of proximal signaling events, involving ZAP 70 and its upstream kinase Lck, in the retinoid receptor signaling in T cells. This study, however, does not rule out the possibility that Lck could modulate receptor activity independent of ZAP 70. In summary, we report a novel function of ZAP 70 in human T cells that links proximal signaling events with transcriptional machinery selectively driven by retinoid receptors in the nucleus. Further studies are needed to understand the mechanism of such selectivity and the physiological importance in T cell function.

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