p300-Mediated Acetylation Stabilizes the Th-Inducing POK Factor

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The lineage-specifying factor Th-inducing POK (ThPOK) directs the intrathymic differentiation of CD4 T cells. Although the regulation of ThPOK at the transcriptional level has been extensively studied, specific posttranslational modifications regulating the activity of ThPOK have not been addressed. In this paper, we show that ThPOK is an unstable protein that is more readily degraded in CD8 T cells compared with CD4 T cells. Among the various proteins that bind ThPOK, acetyltransferase p300 specifically promotes the acetylation of ThPOK at K210, K216, and K339, outcompeting ubiquitination, thereby stabilizing the protein. In CD4 T cells, attenuation of p300-mediated acetylation promotes the degradation of ThPOK. In contrast, mutation of lysines 210, 216, and 339 to arginines stabilizes ThPOK and enhances its ability to suppress the expression of CD8 molecule and cytotoxic effectors in CD8 T cells. Our results reveal an essential role of p300-mediated acetylation in regulating the stability of ThPOK and suggest that such regulation may play a part in CD4/CD8 lineage differentiation.


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Abbreviations used in this paper: CHX, cycloheximide; DP, double positive; IB, immunoblotting; IP, immunoprecipitation; MG-132, carboxenzoxy-Leu-Leu-leucinal; shRNA, short hairpin RNA; siRNA, small interfering RNA; SP, single positive; ThPOK, Th-inducing POK; ThPOK-TT, T7-tagged ThPOK; ThPOK-TG, ThPOK transgenic line; Ub, ubiquitin; WT, wild-type.

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Materials and Methods

Cell culture and transfection

HEK293T and Plat-E or Jurkat cells were maintained in DMEM or RPMI 1640 in the presence of 10% FBS. HEK293T cells were transfected by calcium phosphate precipitation, as previously described (18). Transfection of Jurkat cells with DNA or small interfering RNA (siRNA) was performed by electroporation using a Gene Pulser X cell apparatus (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s instructions. Plat-E cells were transfected using the FuGene-HD Transfection Reagent (Roche, Indianapolis, IN).

Plasmids and Abs

The plasmids HA-p300 and dominant negative mutant HA-p300DN were purchased from Upstate Biotechnology (Lake Placid, NY). FLAG-p300 was a gift from Dr. P.L. Puri (Telethon Institute, Rome, Italy). FLAG-Gcn5 and FLAG-PCAF were purchased from Addgene (Cambridge, MA). FLAG and T7-tagged ThPOK full-length and deletion mutants were constructed in the mammalian expression vectors p3FLAG-CMV-10 and pcDNA3-T7. The various deletion mutants of p300 (aa 1–672, 623–1193, 1069–1459, 1410–1942, and 1893–2414) were generated by PCR and cloned into p3FLAG-CMV-10. Lysine-to-arginine (K/R) mutants of ThPOK were generated by PCR-based site-directed mutagenesis. The pCMV-driven retroviral bicistronic expression vector pMX-IRES-EGFP was used for retroviral infection. ThPOK, ThPOKR, or H1-driven p300 short hairpin RNA (shRNA) was subcloned upstream of IRES and EGFP. For expression of T7- or FLAG-tagged ThPOK variants by retrovirus, the T7 or FLAG sequence was first amplified by PCR and inserted downstream of the CMV promoter. The ThPOK variants were then subcloned.

Stimulation and staining of T cells were performed using the following mAbs from BD Biosciences Pharmingen (San Diego, CA): anti-TCR (H57-597), anti-CD28 (37.51), anti-CD4 (RM4-5), and anti-CD8a (53-6.7). Anti-HA (Y-11) and anti-p300 (sc-585) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-T7 was from Novagen (Madison, WI). Anti-FLAG (M2) and anti–β-actin were from Sigma-Aldrich (St Louis, MO). Anti-acetyl lysine rabbit polyclonal Ab was from ImmuneChem Pharmaceuticals (Burnaby, British Columbia, Canada). Anti-human ThPOK Ab was from Abcam (Cambridge, U.K.). To generate anti-mouse ThPOK Ab, a DNA fragment encoding ThPOK (aa 152–351) was cloned into pGEX-4T-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ), and the GST-tagged fusion proteins of ThPOK were expressed, purified, and used as Ags to immunize mice for generation of anti-ThPOK polyclonal Abs according to standard protocols.

Mice

C57BL/6, AND, and OT-1 TCR transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). ThPOK transgenic line C8 was kindly provided by Dr. Remy Bosselut (National Cancer Institute, Bethesda, MD). The ThPOK transgenic line (ThPOK-TG) was generated in our laboratory by expression of ThPOK under the control of the human CD2 promoter.
promoter and locus control region in the transgenic expression vector p29A2 (5). Mice were maintained in a specific pathogen-free facility and analyzed between 4 and 8 wk of age. Animal experimentation protocols were approved by Institutional Animal Care and Use Committee of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

**T cell preparation, purification, and staining**

Single-cell thymocyte or splenocyte suspensions were prepared and stained as described (19). Cell fluorescence was acquired, typically for $1 \times 10^5$ cells, on a two-laser FACSCalibur (BD Biosciences, San Jose, CA) with four-decade logarithmic amplification and was analyzed with FlowJo software (Tree Star, Ashland, OR). Live cells were identified by forward light scatter and propidium iodide gating. CD4+ and CD8+ T cells were purified with CD4 Cell Positive Isolation Kit (Miltenyi Biotec, Auburn, CA) or Dynal Mouse CD8 Cell Negative Isolation Kit (Invitrogen, Carlsbad, CA), respectively. Thymocytes from C57BL/6 mice, AND, and OT-1 TCR transgenic mice or retrovirus-infected splenocytes were sorted using a FACSAria (BD Biosciences). Cell purity was $>90\%$, as assessed by surface staining and flow cytometry.

**ThPOK complex purification**

To purify the ThPOK protein complex, HEK293T cells were transfected with plasmid expressing T7-tagged ThPOK (ThPOK-T7). At 2 d after transfection, cells were harvested and lysed in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol) with a proteinase inhibitor mixture (P8340; Sigma-Aldrich). Cell lysates were immunoprecipitated with anti-T7 agarose (Novagen). The eluted proteins were subjected to SDS-PAGE and stained by colloidal blue. The visible protein bands were excised and subjected to trypsin digestion followed by mass spectrometry analysis.

**Immunoprecipitation and immunoblot analysis**

Cells were lysed on ice for 30 min in lysis buffer with protease inhibitor mixture, and the lysates were cleared by centrifugation. The resulting supernatants were immunoprecipitated with anti-T7 agarose, anti-FLAG beads (A2220; Sigma-Aldrich), or appropriate Abs plus protein A/G-Sepharose beads (sc-2003; Santa Cruz Biotechnology) at 4˚C. After washing, 2X sample loading buffer was added to the immunoprecipitates. Samples were subjected to immunoblot analysis with the indicated Abs and HRP-conjugated secondary Ab (Santa Cruz Biotechnology).

**Protein acetylation in vitro**

FLAG-p300 (1 µg) and GST-ThPOK (2 µg) protein was incubated in 50 µl acetyltransferase assay buffer with or without 10 µM acetyl CoA (Sigma-Aldrich) at 30˚C for 45 min on a rotating platform, as previously described (20). Samples were then subjected to immunoblot analysis with anti-ThPOK, anti-p300, or anti-acetyl lysine Abs.

**FIGURE 3.** ThPOK interacts with p300. A. The T7-tagged ThPOK protein complex was immunoprecipitated with anti-T7 agarose from HEK293T cells, as described in Materials and Methods. Specific bands were analyzed by mass spectrometry, and p300 peptide sequences were identified. B, HEK293T cells were transfected with HA-p300 or ThPOK-T7, or both, as indicated. Immunoprecipitation (IP) with anti-HA Ab coupled to protein A/G–agarose (to pull down HA-p300) was performed, followed by immunoblotting with the indicated Abs. C, p300 was immunoprecipitated from thymocyte lysates from C57BL/6 mice with anti-p300 Ab. ThPOK and p300 protein levels were determined by immunoblotting with anti-ThPOK and anti-p300 Ab, respectively. D, upper panel, Schematic representation of ThPOK and the deletion variants (ThPOK-1 to ThPOK-5). N-terminal BTB-POZ domain (black box) and the four C-terminal zinc fingers (gray box) are indicated. Lower panel, HA-p300 was expressed in HEK293T cells with FLAG-tagged ThPOK or its deletion variants, as indicated. Immunoprecipitation was performed with anti-HA Ab coupled to protein A/G–agarose (to pull down HA-p300) was performed, followed by immunoblotting with the indicated Abs. E, upper panel, Schematic representation of the C terminus of ThPOK and the deletion variants. Lower panel, HA-p300 was expressed in HEK293T cells with FLAG-tagged C-terminal region of ThPOK (F-348–544) or the deletion variants, as indicated. Immunoprecipitation and immunoblotting were performed as described in D. F, upper panel, Schematic representation of p300 and the deletion variants (p300-1 to p300-5). Lower panel, ThPOK-T7 was expressed in HEK293T cells with FLAG-tagged p300 deletion variants, as indicated. Immunoprecipitation was carried out with anti-FLAG agarose (to pull down FLAG-p300 variants). Immunoblot analysis was performed with the indicated Abs. The asterisk indicates IgH.
RNA interference

The sequence targeting p300 mRNA used in siRNA- or shRNA-mediated gene silencing was 5'-GGGCGTCTAACCATCTC-3'.

Real-time quantitative PCR

RNA was extracted and quantified as described (18). RNA was reverse transcribed to cDNA with SuperScript III First-Strand Kit (Invitrogen). The mRNA levels of ThPOK were assessed relative to HPRT mRNA levels by real-time RT-PCR (Rotor gene 6000; Corbett Life Science/Quagen, Valencia, CA) with SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan). Primers for real-time PCR were as follows: HPRT (5'-CTCTGCTG-GATTACATTAAAGCACC-3'); ThPOK (5'-CTGCTGCTCTACTGGTAATA-3'; 5'-CTTCTCTGCTGTAAGGAT-3'); perforin (5'-CCGCATCTCGGCCTTAC-3'; 5'-TCGGG-CTTCTGCTCTCCTCA-3'); granzyme B (5'-AGGAGAAGACCCAGCAAG-3'; 5'-CAACACCAACCATAGCAC-3'); IFN-γ (5'-AGTGCCATAAGTG-TGGAA-3'; 5'-CTTAACTTGGCAATCTC-3').

Retrovirus transduction

Transduction with retrovirus was performed as described (21). In brief, Plat-E cells were transfected with pMX-ires-EGFP or the indicated retroviruses. Transduced lineage cells were collected from cultured medium 60 h after transfection. At 24 h after activation, the T cells were incubated with retroviral supernatant containing 20 μg polybrene (Chemicon International, Billerica, MA) and centrifuged at 1200 × g for 2 h at 30°C. After removal of the viral supernatants, cells were cultured with fresh medium and subsequently used for the indicated experiments.

Results

The stability of ThPOK differs in CD4 and CD8 lineage cells

Consistent with previous reports (4, 5), the ThPOK protein can be detected in the intermediate CD4^CD8^low thymocytes from either AND (MHC class II-restricted) or OT-1 (MHC class I-restricted) TCR transgenic mice, and the protein level of ThPOK was statistically higher in AND thymocytes compared with OT-1 thymocytes (Fig. 1A–C). However, treatment with the proteasome inhibitor carbobenzoxy-Leu-Leu-leucinal (MG-132) noticeably reduced this difference (~1.5 times), suggesting that ThPOK stability differs in these two kinds of cells (Fig. 1A–C).

CD4^CD8^low subsets from both AND and OT-1 mice were then treated with cycloheximide (CHX) to block protein synthesis. Interestingly, the ThPOK protein from OT-1 thymocytes was degraded more rapidly than that from AND cells (Fig. 1D, 1E). This finding implies that the ThPOK protein is more stable in MHC class II-restricted CD4^CD8^low subsets than in MHC class I-restricted subsets. To further examine whether ThPOK stability is differently regulated in CD4 and CD8 T cells, retroviral-mediated expression of T7-tagged ThPOK was performed, followed by CHX treatment. The results show that T7-tagged ThPOK protein is degraded more rapidly in CD8 T cells (Fig. 1F, 1G). In support of the above observation, ThPOK-TG, in which ThPOK was moderately expressed compared with a previous ThPOK transgenic line C8 (5), was still able to generate CD8 SP cells (Fig. 2A, 2B). Moreover, the ThPOK protein was accumulated in CD4 SP but eliminated in CD8 SP cells, whereas the exogenous ThPOK mRNA was similarly expressed in both CD4 SP and CD8 SP cells from the transgenic mice (Fig. 2C, 2D).

Taken together, these data suggest that ThPOK is an unstable protein and is more stabilized in CD4 lineage cells than in CD8 lineage cells.

ThPOK associates with p300

To identify factors involved in the regulation of ThPOK stability, immunoprecipitation was performed. ThPOK-T7 was expressed in HEK293T cells, and anti-T7 agarose was used to immunopurify the ThPOK protein complex from the cell lysates. Proteins eluted from anti-T7 agarose were subjected to SDS-PAGE and visualized with colloidal blue staining. Specific bands were subjected to tandem mass spectrometry, and the peptide sequences of seven proteins were unambiguously detected (Fig. 3A, Table I). Interestingly, we detected p300, an acetyltransferase that catalyzes the acetylation

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein Name</th>
<th>Peptide Sequence</th>
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<tr>
<td>1</td>
<td>Histone acetyltransferase p300 (REFSEQ:NP_001420)</td>
<td>KCFNEIQGESVSLGDDPSQPQTTINKEQFSKR, KLGLOLDLESNQQAATQTQPSDSSR, KLGQYFRQBEQDPMGLCYGCCDQQEER, KLEFSPQTLCCGYKQ, RAQTAGLYGVSDDNPPQVKE, KEQSQLTATQTRT, KFICISDLRA, KIDLTLNRL</td>
</tr>
<tr>
<td>2</td>
<td>PremRNA-processing-splicing factor 8 (REFSEQ:NP_006436)</td>
<td>KGVIQVYDLGQDGQGMSRV, KISLQAIIQLVLRK, RIMGNIDASLIVERI, RVASVSQNAISVAASQNIART, KAGTDDPSHMTGPSQAASCLDLNVTVR, KAKRYPANOQNTIPPTSRS, KAQNMQASSLLELNLYFRT, KPAFEPEDQYVQFTPLOCQDDPERQ</td>
</tr>
<tr>
<td>3</td>
<td>Nuclear pore complex protein Nup155 (REFSEQ:NP_004289)</td>
<td>KDGQIFEFFERQ, KELLITDLPDNRK, KGAITDLGQGELRAPIQSNLAKY, KLYQHEINLFSK, KDDLLNYLTEGKRM, KEKKKNLSVIDLVLPLKH, KFICEQDHNQFLDLRL, KIKHELNMTCEPVTQPPKPER, KAFKEDHLQQRH, RAVLAACSHYFKK, RQSQFQBCPVCHKI, RTQCLEYRT</td>
</tr>
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<td>7</td>
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<td>KAFKEDHLQQRH, RAVLAACSHYFKK, RQSQFQBCPVCHKI, RTQCLEYRT</td>
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Table I. Identification of the proteins that specifically presented in the ThPOK-T7 sample by mass spectrometry
of various proteins, including p53, Runx3, and histones (22). Because acetylation has been reported to regulate the stability of many proteins (23, 24), p300 was chosen for further study. We first examined the interaction between ThPOK and p300. HA-tagged p300 (HA-p300) and ThPOK-T7 were coexpressed in HEK293T cells. ThPOK-T7 was detectable in the HA-p300–precipitated fraction rather than in the control fraction, confirming the interaction of the proteins (Fig. 3B). Further experiments show that ThPOK also coprecipitates with p300 in thymocytes (Fig. 3C), indicating that the endogenous ThPOK–p300 complex also forms in thymocytes.

To gain more insight into the p300–ThPOK complex, the regions of ThPOK and p300 responsible for interaction were mapped. A series of constructs encoding ThPOK deletion variants (ThPOK-1 to ThPOK-5) fused with FLAG tags were coexpressed with HA-p300 in HEK293T cells. Coimmunoprecipitation shows that the C-terminal domain (residues 348–544) of ThPOK, which contains four zinc fingers (residues 340–58), binds to p300 (Fig. 3D). This domain was further divided into two fragments: aa 348–404 and aa 404–544, containing the first or last two zinc fingers, respectively. The fragment aa 348–404 rather than aa 404–544 associates with p300 (Fig. 3E). Subsequent mapping of the interaction region in p300 showed that the region spanning aa 1410–1942, which contains a portion of the HAT domain, is involved in the interaction with ThPOK (Fig. 3F).

**p300 acetylates ThPOK in vivo and in vitro**

Because ThPOK interacts with acetyltransferase p300, we were curious regarding whether ThPOK is modified by acetylation under certain circumstances. Endogenous ThPOK in Jurkat cells was immunoprecipitated with anti-ThPOK Abs, followed by immunoblotting with Abs specific for acetylated lysines. Acetylation of ThPOK was detectable and the acetylation level increased upon trichostatin A treatment in a concentration-dependent manner (Fig. 4A, 4B). To further confirm the acetylation of ThPOK, all acetylated proteins in thymocytes or splenocytes from C57BL/6 mice were pulled down with anti-acetyl lysine Abs. ThPOK was present in the acetylated fractions, indicating its acetylation in both thymocytes and splenocytes in vivo (Fig. 4C, 4D).

We next determined whether p300 is involved in the acetylation of ThPOK. HEK293T cells were cotransfected with ThPOK and several acetyltransferase-expressing constructs, including p300,

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** ThPOK is acetylated by p300 in vivo and in vitro. A, In Jurkat cells, levels of acetylated ThPOK were determined by immunoprecipitation with rabbit IgG or anti-ThPOK Ab, followed by immunoblotting with an anti-acetyl lysine Ab. ThPOK protein levels were determined by immunoblotting with anti-ThPOK Ab. B, Jurkat cells were treated with 3 μM trichostatin A for the indicated times before harvest. Levels of acetylated ThPOK and total ThPOK were determined as described in A, C, and D. Levels of acetylated ThPOK were determined in the thymocytes from C57BL/6 mice (C) and the splenocytes from C57BL/6 mice (D) by immunoprecipitation with rabbit IgG or anti-acetyl lysine Ab, followed by immunoblotting with anti-ThPOK Ab. E, T7-tagged ThPOK was expressed in HEK293T cells with p300, p300DN, PCAF, or Gcn5, as indicated. Levels of acetylated ThPOK were determined by immunoprecipitation with an anti-T7 Ab, followed by immunoblotting with an anti-acetyl lysine Ab. ThPOK, p300, p300DN, PCAF, and Gcn5 were detected by immunoblotting with the indicated Abs. F, Jurkat cells were electroporated with control or p300 siRNA. At 72 h following transfection, ThPOK was immunoprecipitated, and equal amounts of ThPOK from the pellet fraction were loaded; levels of acetylated ThPOK were determined by immunoblotting with anti-acetyl lysine Ab. ThPOK, p300, p300DN, PCAF, and Gcn5 were detected by immunoblotting with the indicated Abs. G, HA-p300 and FLAG-ThPOK were coexpressed in HEK293T cells with increasing concentrations of FLAG-tagged ThPOK-aa 348–404 (F-348–404) (left panel) or ThPOK-aa 398–544 (F-398–544) (right panel). Immunoprecipitation with rabbit IgG or anti-acetyl lysine Ab, followed by immunoblotting with anti-ThPOK Ab. H, T7-tagged ThPOK was expressed in HEK293T cells with p300, p300DN, PCAF, or Gcn5, as indicated. Levels of acetylated ThPOK were determined by immunoprecipitation with an anti-T7 Ab, followed by immunoblotting with an anti-acetyl lysine Ab. ThPOK, p300, p300DN, PCAF, and Gcn5 were detected by immunoblotting with the indicated Abs. I, The acetylation of GST-ThPOK by p300 was measured by in vitro acetylation assay and detected by immunoblotting with anti-acetyl lysine Ab.
PCAF, and Gcn5. Robust acetylation of ThPOK was detected when p300 was cotransfected, whereas PCAF and Gcn5 failed to induce a change (Fig. 4E). Importantly, the dominant negative mutant of p300 (HA-p300DN) failed to acetylate ThPOK, indicating that the enhanced acetylation of ThPOK is dependent on the acetyltransferase activity of p300 (Fig. 4E). The role of p300 was further elucidated by siRNA-mediated knockdown. Loss of p300 expression noticeably reduced the acetylation of ThPOK in Jurkat cells (Fig. 4F).

The results in Fig. 3E show that the small fragment of ThPOK (aa 348–404) interacts with p300. Overexpression of the FLAG-tagged aa 348–404 fragment (F-348–404), but not the aa 398–544 fragment (F-398–544), dose dependently outcompeted the binding of full-length ThPOK with p300 (Fig. 4G). Thus we used aa 348–404 as a competitor to study the importance of p300 in the acetylation of ThPOK. The T7-tagged aa 348–404 fragment (348–404-T7), full-length ThPOK, and p300 were coexpressed in HEK293T cells. As aa 348–404 expression increased, the acetylation of full-length ThPOK decreased (Fig. 4H, left). In contrast, the fragment aa 398–544 (398–544-T7), which did not affect ThPOK–p300 complex formation, failed to inhibit the acetylation of ThPOK (Fig. 4H, right).

To determine if p300 is sufficient to acetylate ThPOK, an in vitro approach was used. Purified ThPOK protein was acetylated by the p300 protein in the presence of substrate acetyl-CoA (Fig. 4I). Taken together, these data demonstrate that ThPOK is directly and specifically acetylated by p300.

Lysine residues 210, 216, and 339 in ThPOK are targeted for p300-mediated acetylation

ThPOK belongs to the POK family of transcription factors, members of which are characterized by two conserved motifs: a regulatory POZ/BTB domain in the N-terminus involved in interaction with other transcription factors, and four zinc finger domains in the C terminus (9). To map the acetylated lysines in ThPOK, several deletion mutants were coexpressed with p300 in HEK293T cells and acetylation assays were performed. Deletion of the middle domain (ThPOK-M and ThPOK-C) containing aa 144–348 abolishes the acetylation of ThPOK, whereas absence of the N-terminal domain containing the POZ/BTB domain had no effect (ThPOK-ΔN). Thus, the residues acetylated by p300 lie in the ThPOK-M domain (Fig. 5A). This conclusion was further confirmed by mutation analysis. Point mutations of all five lysines in the middle domain (K207, K210, K216, K339, and K343) to arginine resulted in elimination of the acetylation of ThPOK in the presence of p300 (Fig. 5B, 5D).

To identify the precise sites of acetylation, ThPOK mutants with lysine to arginine (K/R) substitutions in the middle domain were tested for p300-dependent acetylation in HEK293T cells. Single mutation of any of the five lysines did not dramatically affect the acetylation of ThPOK, suggesting acetylation occurs on multiple lysine residues (Fig. 5B, 5D). Interestingly, the combinatorial mutation of K210, 216, and 339 remarkably reduced the acetylation of ThPOK, indicating acetylation occurs on these three lysines (Fig. 5C, 5E). To further specify the acetylation site, all double mutations derived from the three lysines (K210, 216, and 339) were analyzed by acetylation assay. The double mutants show partial reduction of acetylation, suggesting that all three lysines are involved in the acetylation of ThPOK (Fig. 5C, 5E). Therefore, the ThPOK-K210/216/339R (ThPOK'R) mutant represents the minimum combination of mutations that abrogates the acetylation of ThPOK. In vitro assays show that ThPOKR was not acetylated by p300, confirming the importance of the three lysines in p300-mediated acetylation of ThPOK (Fig. 5F). Importantly, ThPOKR still interacts with full-length p300, indicating that the absence of acetylation is not due to the lack of interaction (Fig. 5G).

**FIGURE 5.** Mapping the acetylation sites of ThPOK. *A.* Upper panel, Schematic representation of ThPOK and its deletion variants. Lower panel, ThPOK-T7, either WT or its deletion variants, was expressed in HEK293T cells in the absence or presence of p300-HA. Levels of acetylated ThPOK or its deletion variants were determined by immunoprecipitation with anti-T7 agarose, followed by immunoblotting with anti-lysyl eure Ab. B and C, HA-p300 was expressed in HEK293T cells with T7-tagged WT ThPOK or its lysine mutants, as indicated. Levels of acetylated ThPOK or the indicated lysine mutants were determined as described in A, D and E. Quantification of the acetylation level of ThPOK or its lysine mutants in B and C. Data are the mean of three separate experiments. Error bars indicate SD. F, The acetylation of GST-ThPOK or the K210/216/339R mutant (GST-ThPOK'R) by p300 was measured by in vitro acetylation assay, as described in Fig. 4I. G, HA-p300 was expressed in HEK293T cells with ThPOK-T7 or the K210/216/339R mutant (ThPOK'R-T7). Immunoprecipitation with anti-T7 agarose was performed, followed by immunoblotting with the indicated Abs.
p300-mediated acetylation stabilizes ThPOK

We then traced the functional consequence of acetylation after confirming that p300 acetylates ThPOK at K210, 216, and 339. One particularly important observation is that when coexpressed with p300, the protein level of ThPOK was increased (Fig. 4E). Another interesting phenomenon is that the acetylation of ThPOK in CD4+ CD8low subsets from AND mice is stronger compared with those from OT-1 mice (Fig. 6A). Moreover, when T7-tagged proteins are expressed in CD4 and CD8 T cells, the wild-type (WT) ThPOK, but not ThPOKR, in CD4 T cells is more strongly acetylated than in CD8 T cells (Fig. 6B). All these observations indicate that p300-mediated acetylation may stabilize ThPOK. To determine whether this is true, cells were cotransfected with vectors encoding ThPOK and p300, and the protein level of ThPOK was monitored for different time durations. Interestingly, coexpression of p300, but not p300DN, dramatically reduced the degradation of ThPOK, indicating that p300-mediated acetylation of ThPOK enhances its stability (Fig. 6C, 6D; compare lanes 4–6 and 7–9 with lanes 1–3 of Fig. 6C). The proteasome inhibitor MG-132 blocked the degradation of ThPOK, indicating that ThPOK is degraded by the ubiquitin–proteasome pathway (Fig. 6C, 6D). To determine whether p300-mediated acetylation affects the ubiquitination of ThPOK, a ubiquitination assay was performed in HEK293T cells. Accumulation of ubiquitinated ThPOK was observed with MG-132 treatment and p300, but not p300DN, substantially attenuating the ubiquitination of ThPOK (Fig. 6E).

We then compared p300 mRNA by real-time PCR analysis and found that it was more abundant in CD4 SP than in CD8 SP cells (Fig. 6F). This result indicates a positive correlation between p300 expression and the acetylation of ThPOK. To further examine the role of p300-mediated acetylation in regulating the stability of ThPOK, retrovirus-mediated shRNA knockdown of endogenous p300 was performed in T cells. In p300 silenced cells, the basal protein level of endogenous ThPOK was noticeably reduced (Fig. 6G) and decreased more rapidly in the presence of CHX, compared with control cells (Fig. 6G, 6H). These data suggest that the
stability of ThPOK is dependent on p300 in T cells. In support of this observation, retrovirus-mediated expression of ThPOK-aa 348–404 (F-348–404), rather than ThPOK-aa 398–544 (F-398–544), reduced the acetylation of ThPOK (Fig. 6I) and decreased its half-life in the presence of CHX (Fig. 6J, K). Together, these data demonstrate that p300-mediated acetylation is essential for regulating the stability of ThPOK by inhibiting its degradation through the ubiquitin-proteasome pathway. In CD4 T cells, ThPOK is highly acetylated and stabilized, whereas the contrary is true in CD8 T cells.

Acetylation of ThPOK competes with ubiquitination

Two possibilities exist regarding how acetylation regulates the stability of ThPOK (25). First, acetylation and ubiquitination may occur on different lysine residues, and acetylation inhibits ubiquitination indirectly by affecting the exposure of certain lysines to a ubiquitin ligase or by preventing recruitment of the ubiquitin ligase. In this case, mutation of the lysines for acetylation would increase the ubiquitination of ThPOK and reduce the stability. Second, acetylation and ubiquitination may occur at the same lysine residues (K210, 216, and 339), and acetylation blocks ubiquitination by competing with the ubiquitination sites. Therefore, mutation of K210, 216, and 339 would simultaneously block acetylation and ubiquitination and stabilize ThPOK. To elucidate the two possibilities, we compare the protein level of WT ThPOK with that of ThPOKR. In the absence of p300, the protein level of WT ThPOK was relatively low and dramatically increased in the presence of p300 (Fig. 7A, lanes 1 and 2, and 7B). Interestingly, protein levels of ThPOKR, the mutant that could not be acetylated, were high without p300, and p300 expression did not further increase the levels (Fig. 7A, last two lanes, and 7B). WT ThPOK underwent degradation, whereas ThPOKR was stable after CHX treatment in the time durations monitored (Fig. 7C, 7D). Furthermore, accumulation of ubiquitinated WT ThPOK, but not ThPOKR, was observed (Fig. 7E). Therefore, these results indicate that ubiquitin is conjugated to K210, 216, or 339 of ThPOK, where acetylation occurs.

To determine whether ThPOK was ubiquitinated on single or multiple sites within K210, 216, and 339, the protein level of single or double mutants in the absence or presence of p300 was determined.

FIGURE 7. Acetylation stabilizes ThPOK by competition with ubiquitination. A, ThPOK-T7, either WT or the indicated mutants, was expressed in HEK293T cells in the absence or presence of p300-HA. At 24 h following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of WT ThPOK or the indicated mutants were determined by immunoblotting with anti-T7 Ab. B, Quantification of ThPOK protein levels in A. Data are the mean of three separate experiments. Error bars indicate SD. C, ThPOK-T7 or ThPOKR-T7 was expressed in HEK293T cells. At 24 h following transfection, cells were treated with 50 μg/ml CHX for the indicated time. Levels of ThPOK were determined by immunoblotting with anti-T7 Ab. D, Quantification of ThPOK protein levels in C. E, Ubiquitin was coexpressed in HEK293T cells with ThPOK-T7 or ThPOKR-T7. At 48 h following transfection, the cells were treated with 10 μM MG-132 for 5 h and then harvested for immunoprecipitation. Ubiquitination was detected by anti-ubiquitin Abs. Results shown are representative of three independent experiments.

FIGURE 8. Stabilized ThPOK possesses better inhibiting ability of CD8 and cytotoxic effector genes. A, CD4-depleted splenocytes were transduced with control, ThPOK, or ThPOKR retroviral supernatants. At 48 h after transduction, cells were analyzed for GFP positivity, as well as CD4 or CD8 surface expression by flow cytometry. B, Expression of perforin, granzyme B (Gran B), IFN-γ, and ThPOK(R) genes was analyzed by RT-PCR in sorted GFP + CD8 T cells shown in A. Data are the mean of three separate experiments. Error bars indicate SD.
Single or double mutation yielded partially increased protein levels in the absence of p300 compared with WT ThPOK (Fig. 7A, 7B), the extent of which correlated with the number of K to R mutations. The protein level was further elevated by p300. Thus, like acetylation, ubiquitination also occurs on multiple sites on K210, 216, and 339.

Together, these results suggest that K210, K216, and K339 are targeted for both acetylation and ubiquitination. The p300-mediated acetylation of K210, K216, and K339 stabilizes ThPOK by antagonizing ubiquitin conjugation.

The stabilized mutant ThPOKR possesses a better repression activity than does ThPOK

In the periphery, ectopic expression of ThPOK in mature CD8 T cells attenuates the expression of CD8 molecule and cytotoxic effectors (21). This assay was introduced in our study to test whether stabilized ThPOK is more potent than WT ThPOK. CD8 T cells were sorted and transfected with GFP-based retrovirus containing the pCMV-driven WT ThPOK or ThPOKR expression cassette as well as GFP. The GFP-positive cells were analyzed by FACS. As shown in Fig. 8A, CD8 expression was reduced in WT ThPOK-transfected CD8 T cells and further decreased in ThPOKR-transfected CD8 T cells. Real-time PCR amplification of reverse-transcribed cDNA from GFP-positive cells also showed that ThPOKR transfection inhibited the expression of cytotoxic genes, including perforin, granzyme B, and IFN-γ, to a greater extent than did WT ThPOK (Fig. 8B). Noticeably, the transcription of WT ThPOK and that of ThPOKR are similar (Fig. 8B). Therefore, stabilized modification of ThPOK enhances its ability to inhibit the expression of CD8 and cytotoxic effectors in CD8 T cells.

Discussion

CD4 lineage cells maintain a consistently higher level of ThPOK protein than do cells of the CD8 lineage. Our study demonstrated that this is achieved by regulation also at the posttranslational level, besides at the transcriptional level, as previously reported (7, 13, 26). In CD4 cells, p300-mediated acetylation of ThPOK enhances its stability by antagonizing ubiquitin conjugation on the same lysine residues. In contrast, ThPOK protein was less acetylated and ThPOK degradation was substantially enhanced in CD8 cells. Our data are the first report of acetylation as a posttranslational modification of ThPOK and imply that such regulation may play an important role in thymic T cell differentiation.

Acetylation is one of the posttranslational modifications that occur on nonhistone and histone substrates and coordinates many cellular processes, such as cell growth, differentiation, and apoptosis (23, 27). Protein acetylation is catalyzed by a wide range of acetyltransferases, of which the best characterized is p300, CBP, and PCAF (28). p300 has been reported to interact with and acetylate several transcription factors in the immune system, such as Runx3 (29), GATA-3 (30), and Foxp3 (31). In this study, we found that p300 could also acetylate ThPOK, another key NF in the immune system. p300 specifically acetylates ThPOK, thus stabilizing and potentiating its function. Our data, together with other reports (30, 31), imply that p300-mediated acetylation of ThPOK may play an essential role in regulating several processes in the immune system, including CD4/CD8 differentiation, T cell homing, and regulatory T cell generation.

Ubiquitination is another well-known posttranslational modification, in which single or multiple molecules of ubiquitin are covalently conjugated to a target protein (32). Our results show that ThPOK is also ubiquitinated and is likely degraded by the proteasome pathway. Ubiquitination is regulated by other modifications, including phosphorylation and acetylation (33). Phosphorylation of a certain protein leads to ubiquitination by providing a negatively charged environment that favors ubiquitin to conjugate. In contrast, the acetyl group is not charged and added on the lysine group where ubiquitin also occurs. The effect is either to alter the conformation of the protein to change the sensitivity to ubiquitination or to block ubiquitination directly by competing with ubiquitin conjugation. In this study, we showed that both acetyl groups and ubiquitin can be linked to lysine 210, 216, and 339 of ThPOK and that acetylation protects ThPOK from ubiquitin-mediated degradation by outcompeting ubiquitination in CD4 T cells. It remains unknown where and how this degradation occurs and which ubiquitin ligase (E3) is involved in this pathway. Further studies are needed to answer these questions.

After T cell precursors enter the thymus, they progress through a series of developmental stages before they become either MHC class I-restricted CD8 T cells or MHC class II-restricted CD4 T cells (34). The transcription factor ThPOK promotes CD4 lineage commitment, whereas Runx3 and Runx1 are required for CD8 T cell development (7, 26). The transcription of the ThPOK gene is substantially upregulated from CD69+ DP to CD4+CD8low stage and is maintained at high levels in CD4 SP thymocytes, but not in CD8 SP cells (7, 9). It has been reported that Runx complexes could substantially repress ThPOK transcription, although this is not sufficient for full ThPOK silencing (13, 14). One interesting question that remains to be answered is why the ThPOK level increases further in class II-restricted CD4 cells but returns to baseline in class I-restricted CD8 cells, given that ThPOK can promote the transcription of ThPOK. In this paper, we show that posttranslational modification may contribute to the establishment of the differential expression pattern of ThPOK in thymic subsets. The ThPOK protein is more stable in CD4 lineage cells than in CD8 cells; thus the positive feedback loop controlling ThPOK expression is eventually generated in CD4 lineage cells, but not in CD8 cells. Interestingly, this is achieved, at least partially, by acetylation-mediated stabilization.

In conclusion, we demonstrate that the ThPOK protein is acetylated by p300, which protects ThPOK from ubiquitin-dependent degradation. Our studies provide novel insights into the posttranslational modifications of ThPOK and suggest that this regulation may play an important role in CD4/CD8 lineage decision and T cell function.

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Disclosures

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