Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 10620–10625, September 1999
Biochemistry

Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3

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Communicated by Hans H. Ussing, University of Copenhagen, Copenhagen, Denmark, May 12, 1999 (received for review February 25, 1999)

ABSTRACT **Signal transducers and activators of transcription (STATs) are rapidly phosphorylated on tyrosine residues in response to cytokine and growth factor stimulation of cell surface receptors. STATs hereafter are translocated to the nucleus where they act as transcription factors. Recent reports suggest that serine phosphorylation of STATs also is involved in the regulation of STAT-mediated gene transcription. Here, we studied the role of serine/threonine phosphatases in STAT3 signaling in human antigen-specific CD4$^+$ T cell lines and cutaneous T cell lymphoma lines, expressing a constitutively activated STAT3. We show that an inhibitor of protein phosphatases (PPs) PP1/PP2A, calyculin A, induces (*i*) phosphorylation of STAT3 on serine and threonine residues, (*ii*) inhibition of STAT3 tyrosine phosphorylation and DNA binding activity, and (*iii*) relocation of STAT3 from the nucleus to the cytoplasm. Similar results were obtained with other PP2A inhibitors (okadaic acid, endothall thioanhydride) but not with inhibitors of PP1 (tautomycin) or PP2B (cyclosporine A). Pretreatment with the broad serine/threonine kinase inhibitor staurosporine partly blocked the calyculin A-induced STAT3 phosphorylation, whereas inhibitors of serine/threonine kinases, such as mitogen-activated protein kinase-1 extracellular-regulated kinase-kinase, mitogen-activated protein p38 kinase, and phosphatidylinositol 3-kinase, did not. In conclusion, we provide evidence that PP2A plays a crucial role in the regulation of STAT3 phosphorylation and subcellular distribution in T cells. Moreover, our findings suggest that the level of STAT3 phosphorylation is balanced between a staurosporine-sensitive kinase(s) and PP2A.**

STATs (signal transducers and activators of transcription) are latent cytoplasmic transcription factors that upon activation translocate into the nucleus where they activate target genes (reviewed in ref. 1). At present, seven STATs have been cloned, all of which have an Src homology 2 domain near their carboxyl terminus and a tyrosine residue near position 700 (e.g., Y705 in STAT3). Upon ligation, cytokine and growth factor receptor-associated Janus kinases (JAKs) become activated, possibly by transphosphorylation and/or autophosphorylation. Once activated, JAKs phosphorylate the receptor on key tyrosine residues, which leads to recruitment of STAT proteins, which in turn are tyrosine-phosphorylated by JAKs. Phosphorylated STAT proteins homodimerize or heterodimerize through reciprocal Src homology 2-phosphotyrosine interactions and translocate to the nucleus where they bind specific DNA elements and regulate transcriptional activity of target genes (reviewed in refs. 1–3).

STATs also are serine-phosphorylated in response to ligation of many cytokine and growth factor receptors (reviewed in ref. 4). The major site for serine phosphorylation in STAT1 and STAT3 is residue 727 (5), allthough additional serine phosphorylation sites have been proposed (6). Serine phosphorylation of STAT proteins modulate the DNA binding and/or transcriptional activity. Thus, it was reported that serine phosphorylation of STAT proteins is required for maximal tyrosine phosphorylation, DNA binding, and/or transcriptional activity (7–9). In contrast, serine phosphorylation also has been shown to inhibit tyrosine phosphorylation of STAT proteins (6, 10, 11), whereas other findings indicate that serine phosphorylation of STAT proteins has no effect on tyrosine phosphorylation or DNA binding (7, 12–14). It is likely that the net effect of serine phosphorylation of STAT proteins depends on the type of extracellular stimulus, the cell type, and the activation status of the cell in question. In several STAT proteins (STAT1, STAT3, and STAT4) the serine residue 727 is located within a known mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) consensus phosphorylation site (pro-x-ser/thr-pro) (15), and MAPK/ERKs have been shown to be responsible for serine-727 phosphorylation of STAT proteins in several cell systems (6, 10–12, 16, 17). However, other findings indicate that non-ERK/MAPKs also are involved in serine phosphorylation of STAT proteins (6, 13, 14, 18, 19). Therefore, serine phosphorylation of STATs might be controlled by several kinases, including known and as-yet-unidentified serine kinases.

Four major classes of serine/threonine-specific protein phosphatases (PPs) have been described. These include PP1, PP2A, PP2B (calcineurin), and PP2C, with PP2B and PP2C being Ca^{2+} dependent, whereas PP1 and PP2A are not. PP1 and PP2A are widely distributed in the cytoplasm of mammalian cells and have been reported to be involved in signaling pathways, controlling cell proliferation, and participating in the activity of a variety of protein kinases and/or PPs, which themselves are key regulators of cell function (reviewed in ref. 20). Circumstantial evidence suggests that PP2A might be

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Abbreviations: STAT, signal transducer and activator of transcription; PP, protein phosphatase; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; hSIE, high-affinity sis-inducible element; pIRE, palindromic IFN regulatory element; OA, okadaic acid; CA, calyculin A; ETA, endothall thioanhydride; TAU, tautomycin; CyA, cyclosporine A; ICAM-1, intercellular adhesion molecule-1; GAS, gamma IFN activation site.

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directly involved in the regulation of STAT signaling. Thus, purified PP2A dephosphorylates STAT3 on serine residues *in vitro* (9) and inhibits the formation of serine-dependent STAT1/IFN-γ activation factor-DNA complexes *in vitro* (8). To address whether PP2A is involved in regulation of STAT3 serine phosphorylation *in vivo*, we examined the effect of PP inhibitors on the function and phosphorylation of STAT3 in cytokine-sensitive, human $CD4^+$ T cell lines and cutaneous T cell lymphoma lines, which express constitutively tyrosine-phosphorylated and activated STAT3 proteins.

MATERIALS AND METHODS

Cells. Antigen-specific human $CD4^+$ T cell lines were obtained from healthy donors and have been described (21). Cutaneous tumor T cell lines were established from skin biopsies from a patient with mycosis fungoides and have been described (22, 23). In this study we used two different cell lines: one early culture (My-la 3675) that depends on the cytokine IL-2 and one long-term culture (My-la 2059) that grows independently of cytokines.

Antibodies and Other Reagents. Antibodies against human PP2A (P47720), STAT3 (S21320), and STAT5 (S21520) were from Transduction Laboratories (Lexington, KY). Antibodies against human STAT3 (K-15, #sc-483) and ERK 2 (K-23, #sc-153) were from Santa Cruz Biotechnology. Antibodies against human STAT3 (#9132), STAT3 phospho-tyrosine 705 (#9131L), STAT3 phospho-serine-727 (#9134L), and phospho-thr202/tyr204 p42/44 MAPK (#9106) were from New England Biolabs. FITC- and horseradish peroxidase-conjugated secondary antibodies against mouse Ig (#P0260) and rabbit Ig (#P0217) were from Dako. Calyculin A (CA), okadaic acid (OA), endothall thioanhydride (ETA), tautomycin (TAU), and staurosporine were purchased from Alexsis (Läufel-Fingen, Switzerland). Cyclosporine A (CyA) was from Sandoz Pharmaceutical, MAPK/ERK kinase (MEK) inhibitor (PD98059) was purchased from New England Biolabs, and H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) was from Biomol (Plymouth Meeting, PA). Oligonucleotides used were: IL-2Rα gamma IFN activation site (GAS)d (5′-TTTCTTCTAGGAAGTACC-3′), IL-2Rα GASp (5′-ACATTTCTGATAATAGAATT-3′) (24), pIRE (palindromic IFN regulatory element) (5′-AGCTTAGGTTTCCGGGAAAGCAC-3′) (25), and hSIE (high-affinity sis-inducible element) (5′-GTCGACATTTCCCGTAAATCGTCGA-3′) (26).

Protein Extraction, Immunoprecipitation, and Western Blotting. After treatment with or without inhibitors for indicated times, the cells (3×10^6 cells/experiment for whole-cell lysates and 20×10^6 for immunoprecipitation) were rapidly pelleted, and the reaction was stopped by lysing the cells in ice-cold lysis buffer [1% NP-40/20 mM Tris·HCl, pH 8.0/137 mM NaCl/10% glycerol with the following inhibitors: 1 mM PMSF (in DMSO), 5 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μl/ml indoleacetic acid, 10 mM NaF]. Preparation of cytoplasmic/nuclear extracts, immunoblotting, immunoprecipitation, and oligonucleotide purification were conducted as described (22, 27). Blots were evaluated by using enhanced chemiluminescence according to the manufacturer's manual (Amersham Pharmacia).

^{32}P Radiolabeling of Cells and Purification of ^{32}P-Labeled STAT3. Cells (40×10^6/experiment) were washed two times in phosphate-free buffer (PFB; 140 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/5.5 mM glucose/20 mM Tris·HCl, pH 7.35) at room temperature. Cells were resuspended in phosphate-free medium (PFM; GIBCO/BRL, no. 11877–032) and incubated for 45 min at 37°C. Cells were spun down ($500 \times g$) and resuspended in 8 ml of PFM supplemented with 1.5 mCi of [^{32}P]orthophosphate (Amersham Pharmacia, PBS11) and incubated for 3 hr at 37°C. CA or CyA were added, and cells were incubated for an additional 1 hr. Cells hereafter were spun down ($500 \times g$) and washed three times in ice-cold PFB wash buffer [1 part (wt/wt) PFB/10 mM EDTA/5 mM EGTA/10 mM NaF/1 mM Na_3VO_4], and then lysed in phosphorylation lysis buffer (1% Triton X-100/10 mM Tris·HCl, pH 7.5/150 mM NaCl/10 mM EDTA/5 mM EGTA/10 mM NaF/1 mM Na_3VO_4/1 mM PMSF) on ice for 30 min. ^{32}P-labeled STAT3 was immunoprecipitated with 15 μl of anti-human STAT3 pAb as described above.

Thin-Layer Electrophoresis of Phosphoamino Acids. Immune complexes were boiled in 25 μl of SDS-sample buffer [0.06 M Tris·HCl, pH 6.8/10% glycerol/2% (wt/wt) SDS/1% DTT/0.002% bromphenol blue) for 5 min. They then were loaded and run on Precast 12% Tris-glycine gels (EC6005, NOVEX, San Diego) according to standard minigel procedures for the Novex XCell II Mini-Cell (E19001) system. SeeBlue PreStained Standards (NOVEX) were used as molecular weight markers. Resolved profiles and molecular weight markers were electrophoretically transferred from SDS/PAGE gel onto poly(vinylidene difluoride) (PVDF) membrane according to the NOVEX Western transfer apparatus instructions for the NOVEX blot module (E19051), and subjected to autoradiography overnight without a screen. ^{32}P-labeled proteins of interest were cut out from the PVDF membrane and incubated in 6 M HCl for 2 min at 100°C. The samples then were vortexed followed by hydrolysis at 110°C for 60 min and dried with a flow of nitrogen. The samples then were added to 10 μl of H_2O and run on 20×20-cm cellulose k-2F plates (60 mA, 55 min) in the presence of *o*-phosphoamino acid markers. The markers were visualized by spraying the plate with ninhydrin and *o*-phtalaldehyde, the latter procedure showing selective fluorescence of phosphotyrosine in UV light. The labeled phosphoamino acids were detected by autoradiography.

Confocal Laser Scanning Microscopy. Cells were incubated with or without inhibitors as indicated before fixation (1% paraformaldehyde, PBS). Intracellular staining for STAT3 using a STAT3 pAb (#9132) was performed as described (28). Confocal laser scanning microscopy was performed with a Zeiss CLSM 310. An argon laser (488 nm) was used for FITC fluorescence and a HeNe laser (543 nm) for transmitted light interference contrast. Objective was 40×/numerical aperture 1.2 water immersion.

RESULTS

CA Induces Serine Phosphorylation of STAT3. To address whether PP1/PP2A play a role in the regulation of STAT3 phosphorylation, T cells were incubated with CA for 30 min at 37°C in a humidified atmosphere, and STAT3 serine phosphorylation in total cell lysates was analyzed by Western blotting with an anti-STAT3 phosphoserine-727 antibody. As shown in Fig. 1*A Upper*, CA induces a concentration-dependent increase of serine-phosphorylated STAT3. $CD4^+$ T cell lines express large amounts of a high molecular weight isoform of STAT3, STAT3α, and low amounts of a low molecular weight isoform, STAT3β (27), and as shown in Fig. 1*A Upper*, CA induced an increased serine phosphorylation of both isoforms. CA, however, did not induce an increase in the total amount of STAT3 (Fig. 1*A Lower*), suggesting that inhibition of PP1/PP2A induced an increase in the ratio of phosphoserine STAT3 to total STAT3. The increase in phosphoserine STAT3 was paralleled by a gradual decrease in electrophoretic mobility of STAT3, which was most prominent at 80 nM of CA (Fig. 1*A Lower*). Higher concentrations of CA did not induce a further decrease in electrophoretic mobility or increase in serine phosphorylation of STAT3 (data not shown). A similar effect of CA on STAT3 serine phosphorylation and electrophoretic mobility was observed in cutaneous T cell lymphoma lines (see below). To confirm our findings

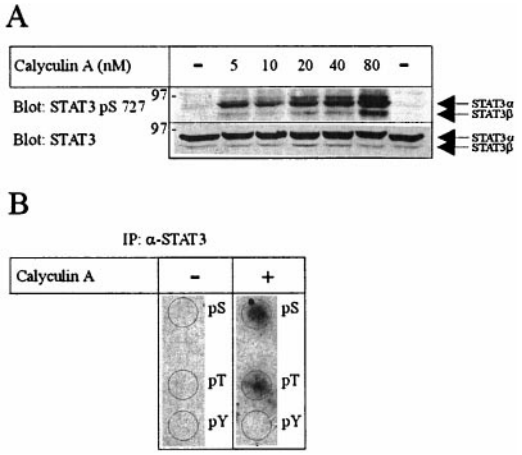

FIG. 1. (*A*) CA induces serine phosphoryaltion of STAT3 *in vivo*. Resting, antigen-specific human CD4$^+$ T cells were incubated with increasing concentrations of CA (5–80 nM) for 60 min, lysed in lysis buffer, applied to SDS/PAGE as described in *Materials and Methods*, and immunoblotted with anti-STAT3 pS727 (*Upper*), stripped and reblotted with anti-STAT3 (*Lower*). (*B*) Amino acid analysis of ^{32}P-orthophosphate-labeled STAT3. ^{32}P-labeled proteins of interest, representing STAT3 proteins from cutaneous T lymphoma cells incubated with or without CA for 60 min, were cut out from the poly(vinylidene difluoride) membrane, hydrolyzed in 6 M HCl, and separated by thin-layer electrophoresis. The ^{32}P-labeled phosphoamino acids were detected by autoradiography as described in *Materials and Methods.*

with the anti-STAT3 phosphoserine-727 antibody, T lymphoma cells were loaded with ^{32}P-orthophosphate and treated with or without CA before immunoprecipitation of STAT3. Immune-purified proteins were separated on a 12% Tris-glycine gel and subjected to autoradiography as described in *Materials and Methods*. Phosphoamino acid analysis of the immune-purified STAT3 from ^{32}P-labeled cells showed that CA induced a profound serine phosphorylation of STAT3 (Fig. 1*B*). In addition, CA induced threonine (but not tyrosine) phosphorylation of STAT3 (Fig. 1*B*). As shown in Fig. 2*A*,

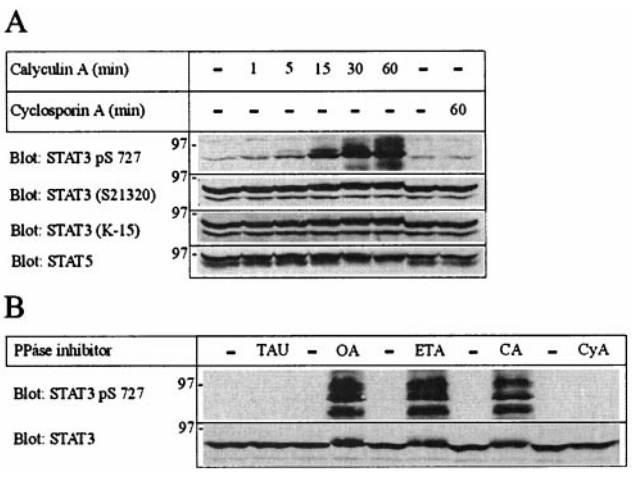

FIG. 2. (*A*) Inhibition of PP1/PP2A induces rapid (<1 hr) serine phosphorylation of STAT3. T lymphoma cells were incubated with CA (80 nM) or CyA (400 ng/ml) for 5–60 min, lysed, applied to SDS/PAGE, and immunoblotted with anti-STAT3 pS 727 pAb, stripped and reblotted with anti-STAT3 (S21320), anti-STAT3 (K-15), and anti-STAT5. (*B*) PP2A-specific, but not a PP1-specific, inhibitor, induced STAT3 serine phosphorylation. T lymphoma cells were incubated in either TAU (500 nM), OA (500 nM), ETA (100 μM), CA (80 nM), or CyA (400 ng/ml) for 60 min, lysed in lysis buffer and applied to SDS-PAGE, and immunoblotted with anti-STAT3 pS727 (*Upper*) and stripped, and reblotted with anti-STAT3 (*Lower*).

CA-induced serine phosphorylation of STAT3α and STAT3β was rapid (1–5 min), reaching a maximum after 60 min (Fig. 2*A*, *Top*). Incubation with CA also resulted in a time-dependent decrease in the electrophoretic mobility of STAT3α and STAT3β (Fig. 2*A*, *Middle*). However, the change in electrophoretic mobility of STAT3α was significantly larger than that of STAT3β (Fig. 2*A*, *Middle*), suggesting that STAT3α is more heavily serine/threonine-phosphorylated than STAT3β. Because both isoforms of STAT3 were phosphorylated on serine-727 (Fig. 2*A*, *Top*), these findings suggest that CA induces phosphorylation of additional serine (or threonine) residues. To address the question of whether or not PP1/PP2A inhibition by CA had an effect on other STAT proteins and/or other serine/theronine kinase substrates, the membranes were stripped and reblotted with an antibody that recognizes two related STAT5 proteins, STAT5a and STAT5b. As shown in Fig. 2*A*, *Bottom*, CA induced an decrease in the mobility of STAT5b. In contrast, CA did not induce a change the mobility of STAT5a (Fig. 2A, *Bottom*), TYK2, STAT4, and phospholipase C-1γ (data not shown). We only observed an induction of STAT serine phosphorylation when CA was added to intact, living cells. In contrast, when added to cell lysates, CA had no effect on STAT phosphorylation even at concentrations (400 nM) that inhibited PP1/PP2A activity by 98% (data not shown).

PP2A, But Not PP1 and PP2B, Inhibitors Induces Serine Phosphorylation of STAT3. CyA, a potent inhibitor of serine/theronine phosphatases PP2B (29, 30), had no effect on STAT3 serine phosphorylation (Fig. 2*A*, lane 8 from left) and electrophoretic mobility of either STAT3α or STAT3β (Fig. 2*A*, lane 8 from left). Thus, even at concentrations (800 ng/ml) that blocked CD3 mAb-induced proliferation, CyA had no effect on either PP1/PP2A activity or STAT3 serine phosphorylation, and CyA did not modulate the CA response (data not shown). To address whether PP1, PP2A, or both were involved in the regulation of STAT3 serine phosphorylation, we took advantage of inhibitors with different PP specificity: (*i*) OA and ETA, both of which predominantly inhibit PP2A (31–33), (*ii*) TAU, which predominantly inhibits PP1 (31, 33), (*iii*) CA, a potent inhibitor of both PP1 and PP2A (31), and (*iv*) CyA, a selective inhibitor of PP2B (29, 30). As shown in Fig. 2*B Upper*, CA, OK, and ETA induced comparable levels of STAT3 serine phosphorylation and a corresponding decrease in the electrophoretic mobility of STAT3 (Fig. 2*B Lower*). In contrast, the PP1 inhibitor, TAU, did not induce serine phosphorylation or a decreased electrophoretic mobility of STAT3 (Fig. 2*B*) at concentrations that blocked cytokine-mediated proliferation (data not shown). Taken together, these findings suggest that PP2A, but not PP1 and PP2B, is involved in the regulation of STAT3 serine phosphorylation *in vivo*. This conclusion agrees with the recent observation that PP2A, but not PP1, dephosphorylates serine-phosphorylated STAT3 *in vitro* (34).

Staurosporine Inhibits CA-Induced Serine Phosphorylation of STAT3. Several cytokines induce STAT3 serine phosphorylation via the MEK-MAPK(ERK) pathway (6, 10–12, 16, 17). Because the PP2A inhibitor, OK, is a potent activator of MAPK(ERK) (11), we asked whether CA induced serine phosphorylation of STAT3 via the MEK-MAPK pathway. As shown in Fig. 3*A*, both CA and OK induced activation of MAP (p42/44 ERK) and serine phosphorylation of STAT3. As expected, the MEK inhibitor PD98059 almost completely blocked CA-induced p42/44 ERK activation (Fig. 3*A*, lane 2 from left versus lane 1). Surprisingly, PD98059 had no effect on CA-induced serine phosphorylation of STAT3. Likewise, PD98059 strongly inhibited OK-induced ERK/MAPK activation but not induction of STAT3 serine phosphorylation, suggesting that ERK/MAPKs are not involved in CA/OK-induced STAT3 phosphorylation. H7, a broadly reacting serine/threonine kinase inhibitor, also has been shown to

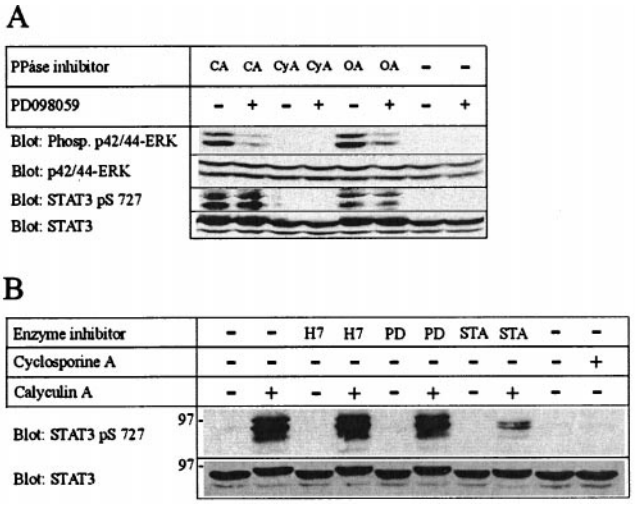

FIG. 3. (*A*) PD98059 inhibits CA-induced activation of ERK p42/44, but not CA-induced STAT3 serine phosphorylation. T lymphoma cells were preincubated with or without a MEK inhibitor: PD98059 at 50 μM for 60 min, before incubation with CA (80 nM), OA (500 nM), or CyA (400 ng/ml) for 60 min, lysed and applied to SDS/PAGE, and immunoblotted with anti-phospho-p42/44 ERK, stripped and reblotted with anti-p42/44 ERK, anti-STAT3 pS727, or anti-STAT3. (*B*) Inhibition of staurosporine-sensitive kinases inhibits CA-induced serine phosphorylation of STAT3. In parallel experiments antigen-specific human $CD4^+$ T cells were preincubated in either medium H7 at 200 μM, MEK inhibitor PD98059 at 50 μM (PD), or staurosporine (STA) at 100 nM for 60 min before incubation in medium, the PP1/PP2A-specific inhibitor CA at 80 nM or the PP2B-specific inhibitor CyA at 400 ng/ml for an additional 60 min. Cells were lysed and applied to SDS/PAGE as described in *Materials and Methods*, and immunoblotted with anti-STAT3 pS727 (*Upper*) and stripped and reblotted with anti-STAT3 (*Lower*).

inhibit cytokine-induced serine phosphorylation of STAT3 (6, 13). However, preincuabtion with H7 had no effect on CA-induced serine phosphorylation of STAT3. In contrast, preincubation with the broad serine/threonine kinase inhibitor staurosporine (35) profoundly inhibited CA-induced serine phosphorylation of STAT3α and STAT3β (Fig. 3*B* and data not shown). Staurosporine had no significant effect on the level of constitutively tyrosine-phosphorylated STAT3 (data not shown). Preincubation with inhibitors of serine/threonine kinases such as phosphatidylinositol 3-kinase (wortmannin, LY294002) and p38-MAPK (SB203580) had no effect on CA-induced phosphorylation of STAT3 (data not shown).

CA Modulates the Function and Subcellular Distribution of STAT3. As mentioned earlier, STAT3 is constitutively tyrosine-phosphorylated in certain cutaneous T lymphoma cells (22, 36), and as shown in Fig. 4*A*, CA induced a time-dependent inhibition of the level of tyrosine-phosphorylated STAT3 whereas CyA had no effect on STAT3 phosphorylation (Fig. 4*A*, *Top*, lane 8 from left). The CA-induced decrease in phosphotyrosine STAT3 was observed after a lag period of 30–60 min and always was preceded by an increase in phosphoserine STAT3 (Fig. 4*A*, *Middle*). It previously was shown (22) that the constitutively tyrosine-phosphorylated STAT3α proteins isolated from cutaneous T lymphoma cells bind oligonucleotide sequences corresponding to the GASd and GASp elements in the IL-2Rα promoter, the hSIE element in the c-*fos* promoter, and the pIRE element in the intercellular adhesion molecule-1 (ICAM-1) promoter, all of which contain a STAT3 binding motif (reviewed in ref. 37). As shown in Fig. 4*B*, CA almost completely inhibited the binding of STAT3α to the GASd and GASp probes. Likewise, CA induced a profound inhibition of STAT3α binding to the hSIE and pIRE probes (Fig. 4*B*). In contrast, CA had only a weak effect on the binding of STAT3β to the hSIE and ICAM-1 probes (Fig. 4*B*, lanes 6 and 8 from left), suggesting that the two STAT3 isoforms might be regulated differently by PP2A.

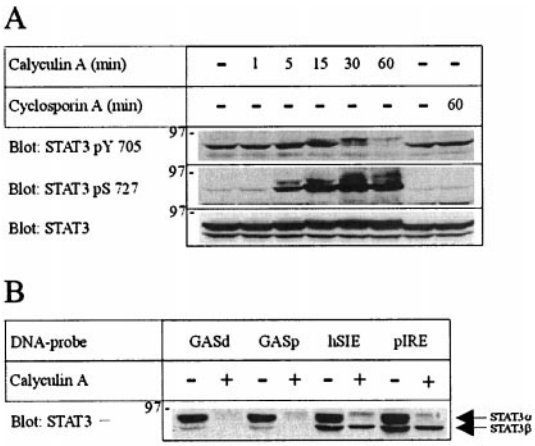

FIG. 4. CA inhibits the function of STAT3. (*A*) CA-induced serine phosphorylation of STAT3 precedes decrease in phospho-tyrosine STAT3. T lymphoma cells were incubated with CA (80 nM) or CyA (400 ng/ml) for 1–60 min, lysed, applied to SDS/PAGE, and immunoblotted with anti-STAT3 pY705 (*Top*), stripped and reblotted with anti-STAT3 pS727 (*Middle*), or anti-STAT3 (*Bottom*). (*B*) Inhibition of PP2A by CA inhibits DNA binding of STAT3 from T lymphoma cells. Cytoplasmic extracts from T lymphoma cells incubated with or without CA (80 nM) for 60 min were analyzed by affinity purification of STAT proteins by using biotinylated DNA probes [IL-2Rα (GASd or GASp)], c-*fos* (hSIE), or ICAM-1 (pIRE)]. STAT/DNA complexes were analyzed by Western blotting with anti-STAT3 mAb.

As shown in Fig. 5*A Top*, STAT3 is widely distributed in T lymphoma cells, and most notably, high levels of STAT3 expression is observed in the nucleus. CA induced a profound change in the subcellular distribution of STAT3. Thus, after CA treatment, STAT3 was found almost exclusively in the cytoplasm, whereas virtually no STAT3 was seen in the nucleus (Fig. 5*A*, *Middle*). In contrast, CyA had no significant effect on the subcellular distribution of STAT3 (Fig. 5*A*, *Bottom*). The CA-induced change in the subcellular distribution of STAT3 was observed as early as 5 min (data not shown), i.e., simultaneously with the induction of STAT3 serine/threonine phosphorylation but before the decrease in STAT3 tyrosine phosphorylation (Fig. 4*A*, *Top* vs. *Middle*). To confirm these findings, T lymphoma cells were treated with or without inhibitors as above before cell fractioning and Western blotting with anti-Stat3 antibodies. As shown in Fig. 5*B*, CA triggered strong serine phosphorylation of STAT3 in the cytoplasm and an almost complete disappearance of STAT3 from the nuclear fraction whereas CyA did not induce serine phosphorylation and redistribution of STAT3 (Fig. 5*B*).

DISCUSSION

In the present study we provide evidence that PPs play an important role in the regulation of STAT3 phosphorylation, subcellular distribution, and DNA binding. Thus, we show that CA, a potent inhibitor of both PP1 and PP2A, induced serine-727 phosphorylation of STAT3, whereas CyA, a potent inhibitor of PP2B, did not. Two structurally different PP2A inhibitors, OK and ETA, also induced a profound serine phosphorylation of STAT3, whereas the PP1 inhibitor, TAU, did not. Furthermore, PP2A inhibitors induced a shift in the electrophoretic mobility of STAT5b but not of STAT5a, TYK2, STAT4, and unrelated proteins such as phospholipase C-γ1, suggesting that the effect on STAT3 and STAT5b was highly specific. In addition to the effect on serine phosphorylation, PP2A inhibitors induced threonine phosphorylation of STAT3. It is unknown whether threonine phosphorylation

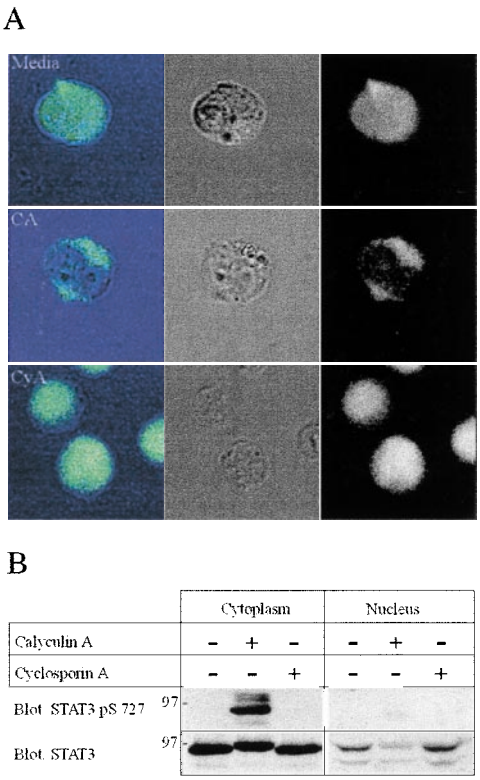

FIG. 5. Inhibition of PP2A modulates the subcellular distribution of STAT3. (*A*) Confocal laser scanning microscopy analysis of STAT3 location in T lymphoma cells incubated with solvent (media, *Top*), 80 nM CA (*Middle*), or 400 ng/ml CyA (*Bottom*). Cells were fixed in 1% paraformaldehyde in PBS and stained with anti-STAT3 (#9132) followed by confocal laser scanning microscopy analysis as described in *Materials and Methods*. (*Right*) Green fluorescence. (*Center*) Blue interference contrast. (*Left*) combination of both. (*B*) Serine phosphorylation inhibits nuclear translocation of STAT3. Cytoplasmic and nuclear extracts from T lymphoma cells incubated in medium CA (80 nM) or CyA (400 ng/ml) for 60 min were prepared as described in *Materials and Methods* and subsequently immunoblotted with anti-STAT3 pS727 (*Upper*) and stripped and reblotted with anti-STAT3 (*Lower*).

modulates the function of STAT3, but in a recent study, Chung *et al.* (6) observed that epidermal growth factor-induced threonine phosphorylation of STAT3 in COS cells transiently expressing STAT3. It therefore is possible that threonine phosphorylation plays a regulatory role in STAT3 signaling. In contrast to the effect on serine and threonine phosphorylation, CA did not induce phosphorylation on tyrosine residues. On the contrary, CA profoundly inhibited tyrosine phosphorylation of STAT3 in T lymphoma cells. Our observation that the decrease in STAT3 tyrosine phosphorylation was preceded by an increase in serine-727 phosphorylation coordinates well with the recent reports that ERK-MAPK-induced phosphorylation of serine-727 reduced tyrosine phosphorylation of STAT3 (6, 11). Because STAT3 is constitutively phosphorylated on tyrosine residues, and because the turnover of phosphotyrosine STAT3 is slow in these cells (ref. 22; M.N., unpublished observations), the decrease in tyrosine phosphorylation might not be caused by an inhibition of *de novo* phosphorylation of STAT3 by tyrosine kinases. Instead, PP2A inhibitors might induce tyrosine dephosphorylation of STAT3 via a direct or indirect activation of protein tyrosine phosphatases (PTPs). Others have hypothesized that serine phosphorylation triggers a decrease in tyrosine phosphorylation of STAT3 via an unidentified negative feedback mechanism involving PTPs (10), and the present finding that CA-induced serine phosphorylation of STAT3 always preceded a decrease in tyrosine phosphorylation is compatible with this hypothesis.

Because tyrosine phosphorylation is a prerequisite for DNA binding activity of STAT proteins, it is possible that the decreased binding of STAT3α to the GASd and GASp probes was caused by a decrease in tyrosine phosphorylation of STAT3α. It was a repeated observation that STAT3α binding to the hSIE and ICAM-1 probes was profoundly inhibited by PP2A inhibitors, whereas the binding of STAT3β was not, suggesting that the two isoforms of STAT3 are regulated differently by PP2A. Because STAT3α enhances the transcription of the ICAM-1 gene, whereas STAT3β inhibits it (25), it makes sense that the two STAT3 isoforms are regulated differently.

The physiological role of STAT3 serine phosphorylation is still controversial. As mentioned earlier, serine phosphorylation has been implicated in both positive and negative regulation of STAT proteins, and several kinases have been implicated in these complex regulatory events (6, 7, 10–14). Our findings suggest that PP2A, directly or indirectly, also plays a crucial role in the regulation of both serine/threonine phosphorylation and subcellular distribution of STAT3. It is unknown at present how inhibitors of PP2A induce serine and threonine phosphorylation of STAT3. Inhibitors of PP2A has been shown to induce activation of ERK/MAPKs (11), and ERK/MAPKs are responsible for cytokine-induced serine phosphorylation of STAT3 in several models (6, 10–12, 16, 17). Our observation that PD98059 almost completely blocked CA- and OA-induced activation of p42/44 ERK without affecting the induction of phosphoserine STAT3 strongly suggest that STAT serine phosphorylation was not mediated via the MEK-MAP(ERK) pathway. Instead, our findings show that inhibitors of PP2A trigger serine phosphorylation of STAT3 via a staurosporine A-sensitive pathway. PP2A may function as a negative regulator of an as-yet-unidentified, staurosporine-sensitive, STAT3 serine/threonine kinase. According to this hypothesis, inhibition of PP2A triggers an activation of this kinase, which in turn phosphorylates STAT3. Because PP2A (but not PP1) recently was shown to dephosphorylate serine-phosphorylated STAT3 *in vitro* (34), it is also possible that serine-phosphorylated STAT3 is a substrate for PP2A *in vivo* and that the level of STAT3 serine phosphorylation is balanced between serine phosphorylation by a constitutively, activated, staurosporine-sensitive kinase and dephosphorylation by PP2A.

It has been proposed that serine phosphorylation of STAT proteins takes place in the nucleus after binding to DNA (12). In our model, serine-phosphorylated STAT3 is almost exclusively found in the cytoplasm and not in the nucleus. Confocal laser scanning microscopy indicated that CA treatment induced a rapid change in subcellular distribution of STAT3. Because the redistribution from the nucleus to the cytoplasm coincided with the induction of serine/threonine phosphorylation (and not with the decrease in tyrosine phosphorylation) we hypothesize that PP2A inhibition by CA and OK induces serine phosphorylation of nuclear STAT3 proteins, which subsequently are exported to the cytoplasm.

In conclusion, the present study provides evidence that PP2A plays an important role in the regulation of STAT3 phosphorylation, subcellular distribution, and DNA binding activity in $CD4^+$ T cell and T lymphoma cells. Because cytokines such as IL-2 induce a transient drop in PP2A activity, which coincides with an increase in serine phosphorylation of STAT3 (38), it is possible that PP2A also is involved in the regulation of cytokine-induced STAT3 signaling.

We thank Dr. Craig W. Reynolds (Biological Response Modifiers Program, National Cancer Institute, Bethesda, MD) for the generous gift of rIL-2 and Gitte Kølander for technical assistance. This work was supported in part by The Danish Research Councils, Danish Biotech-

nological Center for Cellular Communication, Danish Allergy Research Center, Novo Nordic Foundation, Becketts Fond, Danish Medical Associations Research Foundation, The Danish Cancer Research Foundation (Dansk Kræftsforsknings Fond), The Danish Cancer Society (Kræftens Bekæmpelse), Købmand i Odense Johann og Hanne Weimann f. Seedorff's Legat, The Carlsberg Foundation, and University of Copenhagen.

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