



3-12-2003

Characterization of a gene encoding two isoforms of a mitochondrial protein upregulated by cyclosporin A in activated T cells

Laurent Mascarell
Institut Pasteur

Rodolphe Auger
Université Paris-Sud

Andres Alcover
Institut Pasteur

David M. Ojcius
Université Paris, dojcius@pacific.edu

Thomas Jungas
Université Paris

Follow this and additional works at: <https://scholarlycommons.pacific.edu/dugoni-facarticles>

 [next page for additional authors](#)

Part of the [Biochemistry Commons](#), [Immunity Commons](#), [Immunology of Infectious Disease Commons](#), and the [Medical Immunology Commons](#)

Recommended Citation

Mascarell, L., Auger, R., Alcover, A., Ojcius, D. M., Jungas, T., Cadet-Daniel, V., Kanellopoulos, J. M., & Truffa-Bacchi, P. (2003). Characterization of a gene encoding two isoforms of a mitochondrial protein upregulated by cyclosporin A in activated T cells. *Journal of Biological Chemistry*, 279(11), 10556–10563.
DOI: [10.1074/jbc.M313770200](https://doi.org/10.1074/jbc.M313770200)
<https://scholarlycommons.pacific.edu/dugoni-facarticles/76>

This Article is brought to you for free and open access by the All Faculty Scholarship at Scholarly Commons. It has been accepted for inclusion in All Dugoni School of Dentistry Faculty Articles by an authorized administrator of Scholarly Commons. For more information, please contact mgibney@pacific.edu.

Authors

Laurent Mascarell, Rodolphe Auger, Andres Alcover, David M. Ojcius, Thomas Jungas, Veronique Cadet-Daniel, Jean M. Kanellopoulos, and Paolo Truffa-Bacchi

Characterization of a Gene Encoding Two Isoforms of a Mitochondrial Protein Up-regulated by Cyclosporin A in Activated T Cells*

Received for publication, December 16, 2003

Published, JBC Papers in Press, December 18, 2003, DOI 10.1074/jbc.M313770200

Laurent Mascarell,^{a,b,c} Rodolphe Auger,^{c,d,e} Andres Alcover,^{f,g} David M. Ojcius,^{h,i}
Thomas Jungas,^{h,i} Véronique Cadet-Daniel,^a Jean M. Kanellopoulos,^{d,e,j}
and Paolo Truffa-Bachi^{a,k}

From the ^aUnité de Biologie des Populations Lymphocytaires and ^fUnité de Biologie des Interactions Cellulaires URA CNRS 2582, Institut Pasteur, 75724 Paris cedex 15, France, ^bUniversité Paris 7, Institut Jacques Monod, UMR 7592 CNRS, 2 place Jussieu, 75721 Paris cedex 5, France, and ^dInstitut de Biochimie et Biophysique Moléculaire et Cellulaire, Université Paris XI, 91405 Orsay cedex, France

Cyclosporin A (CSA) is an immunosuppressor used in organ transplantation. A recent proteomic analysis has revealed that activation of T cells in the presence of CSA induces the synthesis of hundreds of new proteins. Here we used representational difference analysis to characterize some of the corresponding induced genes. After cDNA bank screening we focused on one of these genes, which we named CSA-conditional, T cell activation-dependent (*CSTAD*) gene. This gene produces two mRNAs resulting from alternative splicing events. They encode two proteins of 104 and 141 amino acids, *CSTADp-S* and *CSTADp-L*, for the short and long forms, respectively. FK506 had the same effect as CSA, whereas rapamycin did not affect the level of *CSTAD* gene expression, demonstrating that inhibition of the calcineurin activation pathway is involved in *CSTAD* gene up-regulation. CSA also led to overexpression of *CSTAD* in mice immunized in the presence of CSA, confirming the *in vitro* analysis. Microscopic and cytofluorimetric analysis of cells expressing green fluorescent protein-tagged *CSTADp-L* and *CSTADp-S* showed that both proteins colocalize with mitochondrial markers and depolarize the mitochondrial transmembrane potential without causing release of cytochrome *c*, apoptosis, or necrosis. Both *CSTADp* isoforms are sensitive to proteinase K, implying that they are located in the mitochondrial outer membrane. These data reveal a new mechanism of action for CSA, which involves up-regulation of a gene whose products are sorted to mitochondria and depolarize the mitochondrial membrane.

T cell activation and proliferation result in a large reshuffling of the gene transcription program; many genes become activated, whereas others are silenced (1). This process is highly coordinated to ensure the acquisition of optimal effector functions and cell survival (2). Genes encoding different proto-oncogenes are induced immediately, whereas genes encoding cell growth and differentiation factors are induced later; finally, genes involved in cell division are transcribed at even later times. T cell proliferation can be prevented by immunosuppressive drugs such as cyclosporin A (CSA),¹ a molecule successfully used in clinics to prevent graft rejection. CSA interferes with T cell proliferation by blocking the transcription of a set of genes encoding growth and differentiation factors (for review, see Refs. 2 and 3). The transcription of these genes is regulated by a Ca²⁺-dependent pathway involving the activation of calcineurin. This serine phosphatase stimulates dephosphorylation of the nuclear factor of activated T cells (NFAT) (4). Dephosphorylated NFAT migrates to the nucleus where in conjunction with AP-1 it activates IL-2 gene transcription (5). CSA binds to various members of the cyclophilin protein family and forms a complex with calcineurin, preventing its activation. Thus dephosphorylation of NFAT is prevented, IL-2 is not produced, and T cells do not proliferate (6, 7). However, other biochemical pathways such as the Ras/Raf pathway responsible for AP-1 formation are not affected by CSA (8, 9). Finally, the stability of AP-1, a factor expressed transiently during activation, is increased in T cells activated in the presence of CSA (10).

CSA therapy does not induce tolerance toward graft antigens, and discontinuation of therapy is associated with graft loss due to rejection. Thus, patients need to be treated with the immunosuppressor during their entire life span. Deleterious effects such as nephrotoxicity, hypertension, symptomatic hyperuricemia, and hyperlipidemia are associated with this treatment. More damaging, CSA-treated patients display an increased incidence of lympho-proliferative disorders and skin cancers, a common and dreaded complication (11, 12). Different reports suggest that therapy with immunosuppressors can specifically stimulate the transcription of genes that may contribute to the onset of complications arising from therapy. In par-

* This work was supported by funds from the Institut Pasteur and the CNRS (URA 1961). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^b Present address: Unité de Biologie des Régulations Immunitaires, INSERM Unité 0352, Institut Pasteur, Paris 75724 cedex 15, France.

^c Both authors made equal contributions to this work.

^e Supported by grants from the Fondation pour la Recherche Médicale and the Association pour la Recherche sur le Cancer.

^g Supported by the Ligue Contre le Cancer.

ⁱ Supported by the Fondation pour la Recherche Médicale, Université Paris 7 and National Institutes of Health Grant R01 AI054624.

^j To whom correspondence may be addressed: Laboratoire D'activation Cellulaire et Transduction des Signaux, IBBMC, CNRS-UMR 8619, Université Paris XI, 91405 Orsay cedex, France. E-mail: Jean.Kanellopoulos@ibbmc.u-psud.fr.

^k To whom correspondence may be addressed: Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris cedex 15, France. E-mail: ptbachi@pasteur.fr.

¹ The abbreviations used are: CSA, cyclosporin A; *CSTAD*, CSA conditional, T cell activation-dependent gene; NFAT, nuclear factor of activated T cells; RDA, representational differences analysis; EDG-1, sphingosine 1-phosphate receptor; GFP, green fluorescence protein; ConA, concanavalin A; IL, interleukin; RT, reverse transcription; VDAC, voltage-dependent anion channel.

ticular, CSA increases transcription of the transforming growth factor β gene, suggesting that CSA may induce tumors in part due to overexpression of this immunosuppressive cytokine (13, 14). The finding that CSA up-regulates gene expression in cells from different tissues irrespective of their activation state suggests that calcineurin may be the ubiquitous target, which could then affect gene expression either negatively or positively.

The effects of CSA on gene up-regulation were extended recently by a proteomic analysis of newly synthesized proteins in T cells activated in the absence or presence of CSA (15). Remarkably, more than 100 proteins that are not found in resting or activated T cells were induced when stimulation was carried out in the presence of CSA. To identify the corresponding genes, we have now performed representational difference analyses (RDA) under the same conditions. Among the up-regulated genes we have identified a gene that we have named *CSTAD*, for CSA-conditional, T cell activation-dependent gene. *CSTAD* encodes two proteins of 104 and 141 amino acids that are localized in mitochondria. Although overexpression of the short isoform perturbs mitochondrial morphology and depolarizes the inner mitochondrial membrane, cytochrome *c* was not released, and cells did not undergo apoptosis nor necrosis. Finally, *CSTAD* up-regulation is also observed in mice immunized in the presence of CSA, suggesting that the phenomenon of CSA-induced gene up-regulation may be implicated in some of the deleterious effects of CSA treatment.

MATERIALS AND METHODS

Mice and Immunization—Five- to eight-week-old C57BL/6 female mice were obtained from IFFA-CREDO (L'Arbresle, France). Six- to eight-week-old female mice expressing a transgenic T cell receptor ($V\beta 3J\beta 1.2$ and $V\alpha 11J\alpha 84$) specific for the 88–104 peptide of pigeon cytochrome *c*-major histocompatibility complex II I-E^k complex (16) were bred at the Centre de Distribution, Typage & Archivage animal (Orléans, France).

Reagents—The synthetic peptide (Neosystem, Strasbourg, France) was dissolved in balanced salt solution (5 mg/ml) and kept at -80°C . Tetramethylrhodamine ethyl ester and MitoTracker Orange (CMXRos) were obtained from Molecular Probes (Eugene, OR). The mouse monoclonal anti-cytochrome *c* antibody (clone 7H8.2C12) was from Pharmingen. The antibody against HSP-60 was a gift from Dr. S. Susin. Texas Red-coupled anti-mouse IgG1 antibody was from Southern Biotechnology (Birmingham, AL). For cell culture experiments, CSA, kindly supplied by Novartis France, was dissolved in absolute ethanol. FK506 was a gift from Fujisawa Healthcare Inc. (Deerfield, IL). Rapamycin and DABCO (1,4-diazabicyclo(2.2.2)octane) were purchased from Sigma. For immunizations, CSA was mixed in olive oil (10 mg/ml) and kept at room temperature. CSA (2 mg) was administered intraperitoneally 1 day before and concomitantly with peptide injection (150 μg intravenously in the tail). A control group of mice received the peptide alone, and another group received olive oil alone.

T Cell Activation—T cells were purified as previously described (10). Briefly, spleen cells ($4 \times 10^6/\text{ml}$) were incubated with 75 μg of wheat germ agglutinin for 10 min and loaded on top of a tube containing 8 ml of balanced salt solution containing 50% fetal calf serum. Twenty minutes later non-agglutinated T cells were recovered from the top of the tube, centrifuged, and incubated with 0.2 M *N*-acetyl-D-glucosamine (Sigma) for 5 min to remove excess WGA. After 2 washes, the purity of the T cells was analyzed by fluorescence-activated cell sorter (90–95% of the recovered cells were CD3⁺). The T cell-enriched fraction responds to ConA-stimulation similarly to the unfractionated cells. Cells were cultured in 2 ml (2×10^6 cells/ml) in complete RPMI (RPMI 1640, Invitrogen) supplemented with 2 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ streptomycin, 50 units/ml penicillin, 5% heat-inactivated fetal calf serum, 5×10^{-5} M 2-mercaptoethanol. Two $\mu\text{g}/\text{ml}$ ConA (Sigma) were added at the beginning of the culture. CSA (1 $\mu\text{g}/\text{ml}$), FK506 (0.1 $\mu\text{g}/\text{ml}$), or rapamycin (0.1 $\mu\text{g}/\text{ml}$) were added to one group of cells. Non-activated cells and CSA-treated non-activated cells were cultured for comparison. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and recovered at different times.

cDNA Synthesis and RDA—Cells were collected at 4 and 24 h Quick-Prep® micro mRNA purification kit (Amersham Biosciences) according

to the manufacturer's instructions. Double-stranded cDNA was obtained with the Superscript Choice system for cDNA synthesis (Invitrogen). Testers were prepared from 3 μg of mRNA of ConA-activated CSA-treated T cells obtained after 4- and 24-h cultures. The driver was prepared from 3 μg of mRNA of ConA-activated T cells (4 and 24 h) and non-activated spleen cells. RDA was performed as described previously (17, 18). After three cycles of subtraction and amplification, subtraction products were digested by DpnII and cloned into the BamHI site of pUC18 plasmid. The cloned cDNA fragments were sequenced with M13–20 primer using the ABI PRISM® BigDye™ terminator cycle sequencing kit (PerkinElmer Life Sciences) and then processed on a 3700 DNA analyzer (Applied Biosystems). The nucleotide sequences were analyzed using the BLASTN program and the NCBI GenBank™ data base.

Analysis of the *CSTAD* Clone—The 5' end of the *CSTAD* cDNA clone was obtained by rapid amplification of cDNA ends using the GeneRacer kit (Invitrogen). The cDNA was prepared according to the manufacturer's instructions using total RNA of ConA-activated CSA-treated thymocytes. The PCR was performed with gene-specific primers in combination with the supplied GeneRacer 5' primer. The rapid amplification of cDNA ends product was gel-purified, cloned with TOPO TA cloning kit (Invitrogen) and then sequenced. The 3' end of the cDNA was determined by homology analysis using the GenBank™ data base. The complete sequence of the *CSTAD* clone was confirmed by sequencing the UUGC1M0246K22F genomic clone obtained from Dr. D. Dunn (University of Utah Genome Center, Salt Lake City, UT). Genomic organization was determined by analysis of nucleotide sequences in the mouse genome data base.

RT-PCR Analysis—Total RNA was isolated from various tissues, purified T cells were isolated by the acid guanidinium method (19), and cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's protocol using random hexanucleotide primers. The PCR primers are: β -actin, 5'-CACGATTTCCTCTCAGC and 5'-GCACCACACCTTCTACAA; IL-2, 5'-TGCTGACTCATCATCGA and 5'-AGGATGGAGAATTACAG; synaptotagmine-2-like, 5'-GGGTCATTTTAGCCAAAGC and 5'-CAAACAAAACCCAAACAAA; α -E-integrin, 5'-TGCAGTTCATGACCAGGACA and 5'-AGCTGACCACCAACATCTCCC; EDG-1, 5'-CTGTGGGCCCTCTCTCA and 5'-CGGCGCATCTCCTTGTTGGT; *CSTAD*-L, 5'-ATGGGCACGCTTGTGTCA; *CSTAD*-C, 5'-TGAGCCAGGTCACCCAAGAGATG; *CSTAD*-S, 5'-GCCCCGAGAACTGCTGAGAT.

PCR was performed for 30–40 cycles (95°C , 30 s; 55°C , 30 s; 72°C , 30 s). After electrophoresis on a 1.5% agarose gel the PCR products were visualized using a Vilber Lourmet transilluminator and the Biocart program.

Real-time PCR to Monitor Gene Expression—The differentially expressed genes were analyzed by real-time PCR using the above-mentioned primers. Total RNA was isolated, and cDNA was synthesized as described above. Real-time PCR was conducted with a SYBR green kit (PE Biosystems, Warrington, UK) according to the manufacturer's specifications using 3 mM MgCl_2 and 50 pmol of primer per reaction. The amount of each gene was normalized to a housekeeping gene (β -actin).

Plasmids, Transfections, and Fluorescence Microscopy—Plasmid constructs of full-length *CSTAD*-short and *CSTAD*-long fused to green fluorescent protein (GFP) cDNA were generated by cloning PCR products of the *CSTAD* genes in-frame with GFP into pcDNA3.1/CT-GFP-TOPO (Invitrogen) according to the manufacturer's instructions. Human fibrosarcoma 11.1 (U1A) cells were grown in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ streptomycin, 50 units/ml penicillin, pyruvate, and 10% heat-inactivated fetal calf serum. Cells were plated at 5×10^4 on glass coverslips in 24-well plates 16 h before transient transfection. Transient transfections using 2 μg of plasmid per well were performed by calcium phosphate (125 mM). GFP fluorescence was monitored 24 h after transfection. Mitochondrial localization was assessed by fluorescence microscopy using MitoTracker or monoclonal antibodies against mouse cytochrome *c* or hsp-60. MitoTracker was added at 100 nM before cell fixation, and cells were incubated for 10 min at 37°C . Cells were then fixed with 4% formaldehyde for 10 min at room temperature. Fixed cells that had not been treated with MitoTracker were incubated with anti-cytochrome *c* or anti-Hsp monoclonal antibodies and revealed with Texas Red-coupled anti-mouse IgG1 antibodies. For cytochrome *c* detection, permeabilization was carried out using 0.05% saponin as previously described (20). For HSP-60 detection, cells were permeabilized with 0.1% SDS as described (20). Coverslips were mounted onto glass slides using Mowiol mounting medium and 2.5% DABCO (1,4-diazabicyclo(2.2.2)octane). Confocal microscopy analysis was carried out using a Zeiss LSM-510 microscope and software. Z-series optical

sections were performed at 0.5- μ m increments. Green and red fluorescence emissions were collected separately to avoid fluorescence passage from one channel to the other.

Cytofluorimetry Analysis of Mitochondrial Transmembrane Potential and Cytochrome *c* Release—To measure changes in transmembrane potential ($\Delta\Psi$), 11.1 (U1A) cells were first transfected with the C-terminal GFP constructs of CSTAD-S and CSTAD-L. Twenty-four hours after transfection the cells were loaded with 150 nM tetramethylrhodamine ethyl ester in phosphate-buffered saline, 1% bovine serum albumin for 15 min at 37 °C. After the loading period the cells were rinsed with phosphate-buffered saline/bovine serum albumin and resuspended in 15 nM tetramethylrhodamine ethyl ester in phosphate-buffered saline/bovine serum albumin at room temperature. The $\Delta\Psi$ of cells was measured by cytofluorimetry (FL3), and GFP-positive cells were identified simultaneously on FL1. As a positive control for membrane depolarization, cells were incubated with 1 μ M staurosporine overnight.

Cytochrome *c* release was determined by cytofluorimetry using antibodies against cytochrome *c*, as previously described (21). Cell death was measured by cytofluorimetry after double-staining cells with propidium iodide and annexin V (22); both adherent cells and cells in suspension were collected for analysis.

Subcellular Fractionation—The cells used in these studies, 293 T cells, are derived from a human renal epithelial cell line that was transfected by an adenovirus E1A gene product. The cells were grown in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 50 μ g/ml streptomycin, 50 units/ml penicillin, 1 mM pyruvate, and 10% heat-inactivated fetal calf serum. Cells were plated at a density of 2×10^6 cells/10-cm dish the day before transfection. The cells were transiently transfected with 15 μ g of plasmid per dish using calcium phosphate. Two days after transfection the cells were collected, resuspended in isotonic mitochondrial buffer (MB: 250 mM sucrose, 10 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 1 mM EGTA, pH 7.4), and lysed by 30–40 strokes with a Dounce homogenizer. The homogenate was centrifuged at $1000 \times g$ for 5 min at 4 °C to eliminate nuclei and unbroken cells. The supernatant was divided into nine aliquots and centrifuged at $10,000 \times g$ for 15 min at 4 °C to obtain mitochondria-enriched pellets. The pellets were resuspended in MB supplemented with or without 1% Triton X-100 and treated with proteinase K (1–10 μ g/ml) on ice for 30 min. After boiling for 10 min in the presence of SDS loading buffer, the reaction mixtures were analyzed by SDS-PAGE and Western blotting.

Western Blot Analyses—The mitochondrial fractions were used for detection of VDAC, CSTAD-GFP fusion protein, and cytochrome *c*. The samples were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked with 3% nonfat milk in Tris-buffered saline containing 0.2% Tween 20 for 1 h at 37 °C. Blots were immunostained with rabbit anti-VDAC polyclonal antibodies (Oncogene Research Products, San Diego, CA) at a 1:2,000 dilution, rabbit anti-GFP polyclonal antibodies (Oncogene) at 1:2,000 dilution, and mouse anti-cytochrome *c* monoclonal antibody (Pharmingen) at 1:500 dilution at 4 °C overnight and probed with a 1:10,000 dilution of goat anti-rabbit IgG or goat anti-mouse IgG antibodies coupled to horseradish peroxidase. Specific bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences).

RESULTS

CSA Treatment Alters the Transcription of Genes in Activated T Cells—Two RDAs were performed on T cells activated for 4 and 24 h by ConA in the presence or absence of CSA, as described under "Materials and Methods." Fifty clones from each RDA were sequenced; readable nucleotide sequences were obtained for 30 and 23 clones for the 4-h and the 24-h incubations, respectively, and the sequences were analyzed using the BLASTN program and the GenBankTM data base. Primers specific for the clones obtained were designed using the Oligo® program (NBI). The effect of CSA on the level of expression of these genes was tested by RT-PCR on splenic T cells activated with ConA for 4 and 24 h in the presence or absence of CSA. Non-activated cells and cells cultured in the presence of CSA alone served as controls. Fig. 1 shows the level of mRNA accumulation for the four genes whose expression had been altered by the immunosuppressor, as detected by RDA. As expected, induction of the IL-2 gene was prevented by the immunosuppressor (10), attesting to the effectiveness of the CSA treatment. Incubation of cells with CSA alone had no effect on IL-2

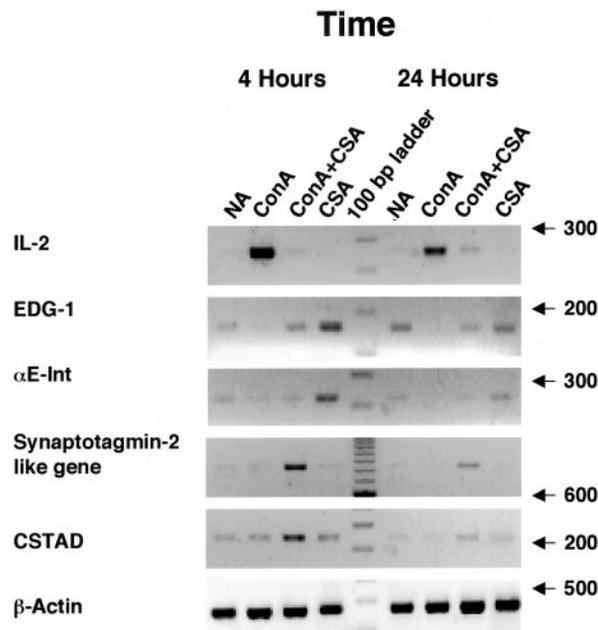


FIG. 1. T cell activation in the presence of CSA results in gene up-regulation. Non-activated spleen cells (NA) or spleen cells activated with ConA in the absence (ConA) or presence of CSA (ConA+CSA) were cultured for 4 and 24 h. A group of cells was incubated with CSA alone (CSA). After T cell purification and RNA extraction, PCR was performed with primers specific for IL-2, α -E-integrin (α -E-Int), EDG-1, synaptotagmin-2-like, and CSTAD (CSTAD-L and CSTAD-C), described under "Materials and Methods." The amplified material was resolved by gel electrophoresis, and the images were collected using a Vilber Lourmet transilluminator. β -Actin served as an internal standard for the quality and quantity of the mRNA preparation. The data are representative of five independent experiments.

gene expression but strongly increased the accumulation of mRNA encoding EDG-1 and α -E-integrin (Fig. 1).

Expression of the synaptotagmin-2-like gene was barely detected in resting T cells, and its level was barely modified by T cell activation. In contrast, gene expression was up-regulated when activation was carried out in the presence of CSA (Fig. 1). The mRNA encoding the gene that we have named CSA-conditional, T cell activation-dependent (CSTAD) was detected in non-activated T cells, but activation did not modify its amount. However, activation in the presence of CSA resulted in a large increase in the mRNA levels (Fig. 1). We, therefore, focused subsequently on the characterization of the products of this gene.

Genomic Localization and Tissue Distribution of CSTAD—Rapid amplification of cDNA ends was performed to obtain the complete sequence of the CSTAD cDNA, revealing a sequence of 901 nucleotides. Alignment analyses with the GenBankTM data base showed that the CSTAD cDNA shares 785 nucleotides at the 3' end with a Riken cDNA clone of 816 bases encoding a gene with unknown function located on mouse chromosome 2 (GenBankTM accession number AK020589; Gene ID, ENSMUSESTG00000040463). However, the two cDNAs differ in their 5' end region by a stretch of 31 nucleotides, which are unique to the Riken clone, and by a sequence of 116 bases that are present exclusively in the CSTAD cDNA. The localization of the CSTAD cDNAs was determined from GenBankTM data base analysis (Fig. 2A).

Two exons had been defined in the Riken clone. The coding sequences and the translation products are represented in Fig. 2B. The Riken and CSTAD cDNAs, thus, represent two alternative splice variants of the CSTAD gene. We named them CSTAD-S (identified by Riken) for the short and CSTAD-L (identified by RDA) for the long isoform, respectively. These

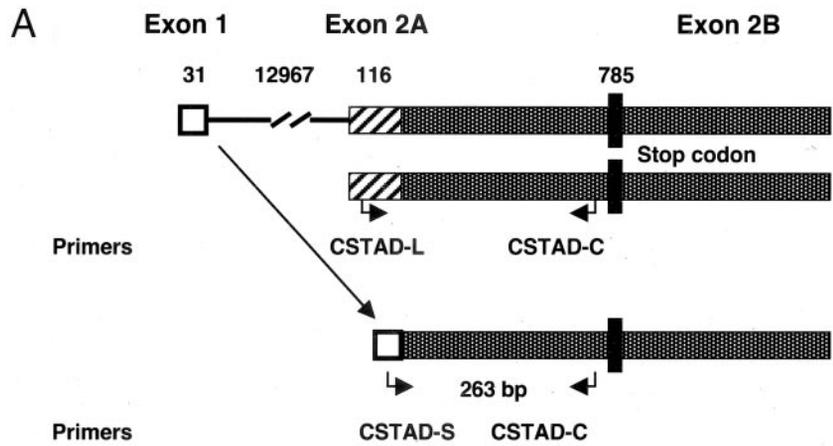
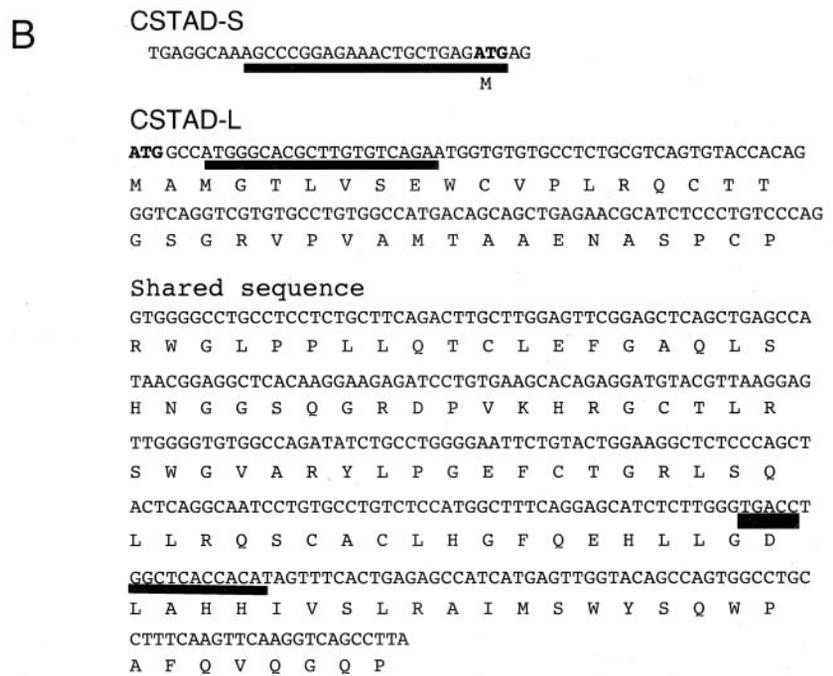


FIG. 2. Organization of the *CSTAD* gene. *A*, organization of the *CSTAD* gene as derived from the alignment of the sequences of the *CSTAD* and Riken (AK020589) clones with the mouse GenBank™ data base. The position of the primers used for the amplification of the *CSTAD*-L and -S isoforms is indicated by the arrows. *B*, unique and shared nucleotide and amino acid sequences of the short and long isoforms. The specific primer sequences are *underlined*.



cDNA encode two proteins of 104 (CSTADp-S) and 141 amino acids (CSTADp-L).

Up-regulation of the *CSTAD* gene could change the expression level of both the long and short *CSTAD* forms. This was assessed by PCR using the specific primers shown in Fig. 2A. Both *CSTAD*-L and *CSTAD*-S mRNAs increased in T cells activated for 4 and 24 h in the presence of CSA (Fig. 3A).

Real-time PCR was used to quantify the amount of both mRNAs (Fig. 3B). The data are presented as the ratio of the amount of *CSTAD*-L and -S in T cells activated in the presence of CSA to the amount of mRNA for the other experimental groups. The ratio of *CSTAD*-L and -S in CSA-treated cells *versus* non-activated cells is also shown. Both the *CSTAD*-L and *CSTAD*-S isoforms increased by a factor of 4–5 at 4 h compared with T cells activated by ConA. Similar increases were found for the other conditions (Fig. 3). At 24 h *CSTAD* increased by a factor of 10–13 compared with ConA-activated T cells. However, the absolute amount of the *CSTAD* isoforms decreased at 24 h (Fig. 3, panel A).

We next investigated the tissue distribution of the two alternative splice variants of *CSTAD*. All tissues tested positive for the two alternative splice variants, but the relative expression of the isoforms varied considerably. *CSTAD*-L is abundant in

the intestines, thymus, and skin (Fig. 4), whereas *CSTAD*-S is highly represented in the skin but is less abundant in the thymus and is barely detectable in the intestines.

CSA Treatment during Immunization Increases the Expression of *CSTAD* in Mice—Because a polyclonal activator induced *CSTAD* overexpression *in vitro*, we next evaluated whether *CSTAD* expression may change during physiological conditions *in vivo*. For this purpose we used transgenic mice expressing a T cell receptor specific for the pigeon cytochrome *c* peptide (16), whose immunization with the pigeon cytochrome *c* peptide leads to the expression of a number of cytokine-encoding genes (23). The mice were treated with CSA 1 day before and on the day of immunization, as previously described (23). As shown in Fig. 5, the efficiency of CSA-induced immunosuppression is attested by the blockade of the IL-2 gene. Expression of *CSTAD*-L and *CSTAD*-S was measured in mice treated with CSA alone or after immunization with the pigeon cytochrome *c* peptide with or without CSA. Mice were sacrificed 6 h after immunization, and the T cells were purified. The level of expression of the two *CSTAD* isoforms is shown in Fig. 5. Although the two alternative splice variants were detected in untreated or immunized mice, they were both up-regulated after immunization in the presence of CSA. This demonstrates

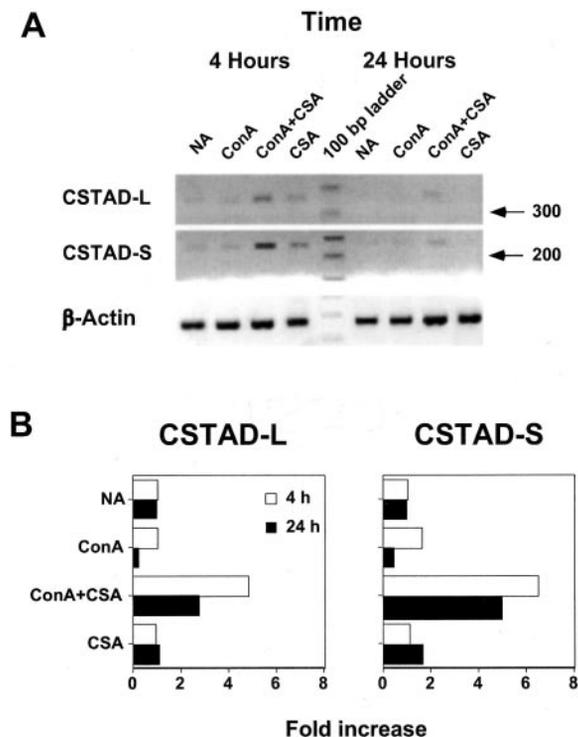


FIG. 3. Estimation of CSTAD-L and CSTAD-S mRNA accumulation by real-time PCR. A, cells activated in the presence or absence of CSA, as described in the legend of Fig. 1, were collected at 4 and 24 h. After T cell purification RNA was prepared, and PCR was performed with primers specific for CSTAD-L and CSTAD-S cDNA, as described under "Material and Methods." Gels and analyses were performed as indicated in the legend of Fig. 1. The data are representative of four independent experiments. B, real-time PCR using primers specific for each *CSTAD* isoform was performed as described under "Material and Methods" on the experimental groups described above. The results are expressed as fold increase of the CSTAD-L and -S isoforms in non-activated (NA) or activated T cells (ConA) or in T cells activated in the presence of CSA (ConA+CSA) or in T cells cultured in the presence of CSA (CSA). The data are representative of three independent experiments.

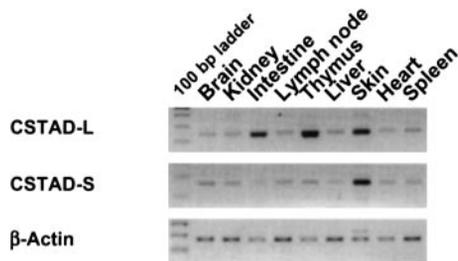


FIG. 4. Tissue distribution of the CSTAD-L and -S isoforms. RNA was prepared from different tissues as described under "Materials and Methods." PCR was performed using primers specific for the long and short isoforms. The amplified material was resolved by gel electrophoresis, and the images were collected using a Vilber Lourmet transilluminator. The data are representative of three independent experiments. β -Actin served as an internal standard for the quality and quantity of the mRNA preparation.

that *CSTAD* gene transcription in T cells activated in the presence of the immunosuppressor may be physiologically relevant.

Blockade of the Calcineurin Activation Pathway Increases *CSTAD* Expression—Two other immunosuppressors, FK506 and rapamycin, were used to ascertain whether the up-regulation of the *CSTAD* gene is linked to the inhibition of the calcineurin pathway. FK506, like CSA, acts on calcineurin, whereas rapamycin interferes with activation due to IL-2 binding to its high affinity receptor (for review, see Refs. 24 and 25).

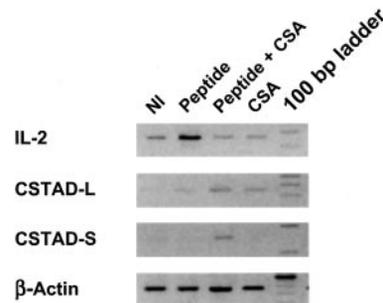


FIG. 5. The *CSTAD* gene is up-regulated in immunosuppressed, immunized mice. A group of transgenic mice expressing a T cell receptor specific for the cytochrome *c* peptide (88–104) was immunized with the cytochrome *c* peptide alone (Peptide) or with CSA (Peptide + CSA). Non-immunized (NI) and CSA-treated (CSA) mice were used as controls. Spleen were collected 6 h after peptide administration, and RT-PCR for each isoform was performed as described in the legend of Fig. 1. The data are representative of three experiments.

Splenic T cells were activated for 4 h in the presence or absence of CSA, FK506, or rapamycin. Control cells received the immunosuppressors but without activation. At the end of the stimulation, RT-PCR was carried out using *CSTAD-L*- and *CSTAD-S*-specific primers. As shown in Fig. 6, the IL-2 gene encoding mRNA was induced in T cells activated by ConA in the presence or absence of rapamycin but not in the presence of CSA or FK506, demonstrating the specificity of the immunosuppression. mRNA encoding for *CSTAD-S* and *CSTAD-L* accumulated in large amounts when activation was carried out in the presence of CSA or FK506. In contrast, rapamycin had no effect, strongly suggesting that the blockade of the calcineurin pathway is involved in up-regulation of the *CSTAD* gene.

***CSTAD-S* and *CSTAD-L* Colocalize with Mitochondrial Markers**—To identify the cellular localization of the *CSTAD* gene products, the cDNAs corresponding to the two isoforms were fused with the GFP gene. N-terminal and C-terminal GFP constructs were made. After transient transfection in mouse 3T3 fibroblasts, only the C-terminal construct gave a reproducible, high level of expression of the fused genes. Twenty-four hours after transient transfection the cells were analyzed by confocal microscopy. Both long and short *CSTAD* proteins were found in discrete subcellular organelles that morphologically resemble mitochondria (results not shown). To avoid competition with endogenous *CSTAD* proteins, a human fibroblast cell line, 11.1 (U1A), was then used to confirm mitochondrial localization, since an orthologous *CSTAD* gene was not found in the human genome. When mitochondria were labeled with MitoTracker, which accumulates in active mitochondria, or antibodies against cytochrome *c* or hsp-60, the *CSTAD*-GFP proteins were found to co-localize with the three mitochondrial markers (Fig. 7). However, better co-localization was observed between *CSTAD*-GFP and MitoTracker or hsp-60 (Fig. 7, A, B, D, and E) than with cytochrome *c* (Fig. 7, C and F).

***CSTAD-S* and *CSTAD-L* Are Localized to the Outer Membrane of Mitochondria**—To determine the mitochondrial localization of *CSTAD-S* and *CSTAD-L*, we transiently transfected 293T cells with the plasmids encoding these isoforms fused to GFP. Subcellular fractions enriched in mitochondria were isolated by differential centrifugation (26) and treated with increasing concentrations of proteinase K as described under "Materials and Methods." As shown in Fig. 8 (a typical experiment of three), the bands immunostained with the anti-GFP antibodies are degraded at 10 μ g/ml proteinase K, whereas in the Triton X-100-solubilized mitochondria the *CSTAD*-GFP fusion proteins are fully digested at 5 μ g/ml. Interestingly, the VDAC2 and VDAC1 proteins, which reside on the outer membrane of the mitochondria, are also degraded in the same range

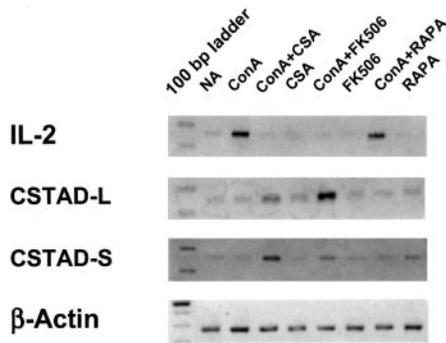


FIG. 6. Accumulation of mRNA encoding the long and short *CSTAD* isoforms depends on the blockade of the calcineurin pathway. Spleen cells were activated with ConA either alone (*ConA*) or in the presence of CSA (*ConA+CSA*), FK506 (*ConA+FK506*), or rapamycin (*ConA+RAPA*). Non-activated cells were cultured with CSA (*CSA*), FK506 (*FK506*), or rapamycin (*RAPA*). Four hours after activation the cells were recovered, and T cells were purified. RT-PCR was performed using primers specific for the short and long isoforms, and the amplified material was analyzed as described in the legend of Fig. 1.

of proteinase K concentrations as the *CSTAD* proteins. Conversely, cytochrome *c*, which is a marker for the intramembrane space, is not cleaved by the protease, except when the mitochondria are solubilized in detergent (Fig. 8). Thus, these experiments strongly suggest that the *CSTAD* proteins are localized on the outer membrane of mitochondria.

***CSTAD-S* and *CSTAD-L* Depolarize the Mitochondrial Membrane Potential**—After a 24-h transfection with the GFP constructs of *CSTAD-S* and *CSTAD-L* both isoforms were shown to decrease the transmembrane potential ($\Delta\Psi$) across the mitochondrial inner membrane (Fig. 9), although *CSTAD-S* was more effective than *CSTAD-L*. Among cells expressing GFP, more than half of the cells transfected with *CSTAD-S* had depolarized membranes compared with almost one-fifth for cells transfected with *CSTAD-L*.

Mitochondrial membrane polarization often leads to cytochrome *c* release from mitochondria. However, neither the short nor the long isoform led to release of cytochrome *c* into the cytosol (data not shown). Consistent with these results, we also observed that overexpression of either isoform does not lead to cell death of transfected cells. Neither apoptosis (as measured by annexin V labeling) nor necrosis (assayed by propidium iodide labeling) was observed in transfected cells (data not shown).

DISCUSSION

We have previously reported that activation of T cells in the presence of CSA induces the synthesis of a large number of proteins (15). However, the proteomic analysis of the newly synthesized polypeptides did not permit their characterization, an essential step in understanding the mechanisms underlying the deleterious effects associated with CSA therapy. Use of the RDA technique allowed us to identify genes that are modulated in activated T cells by the immunosuppressor either negatively or positively. Among these genes we have presently focused on the CSA-conditional, T cell activation-dependent gene, *CSTAD*.

The *CSTAD* cDNA obtained in the present study is 901 nucleotides long. It possesses an open reading frame beginning with an ATG at position 1, a stop codon at position 426, and a potential polyadenylation site at position 880. This cDNA shares 784 nucleotides with a Riken cDNA clone belonging to a gene of unknown function(s) located on mouse chromosome 2 (Ref. 27, accession number AK020589). The RDA and the Riken clones were named *CSTAD-L* and *-S* for the long and short isoform, respectively. They share a common sequence of 784

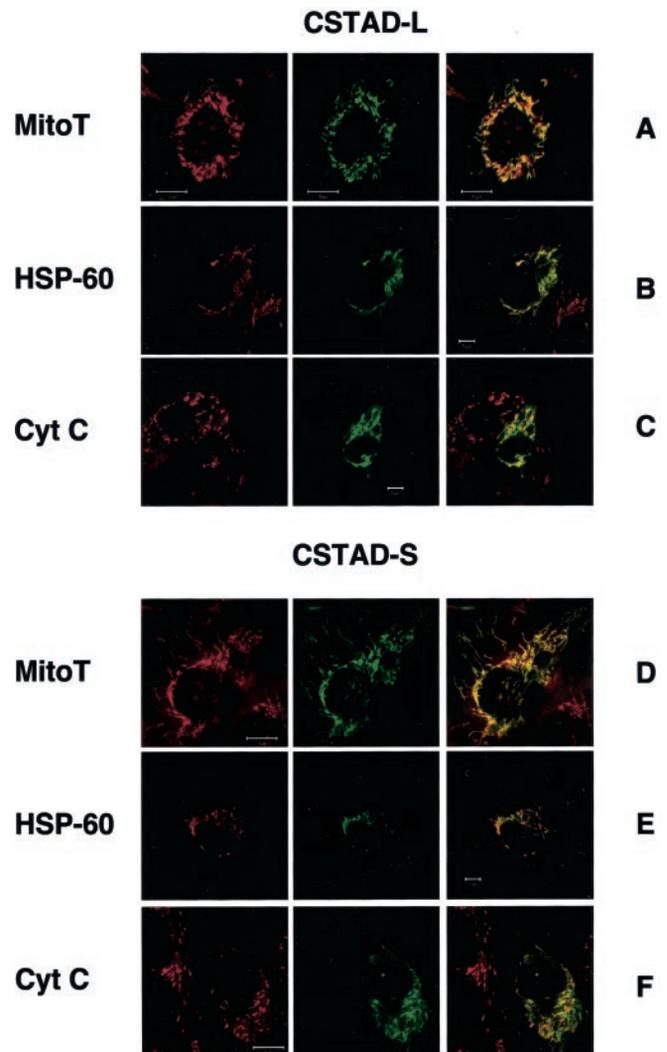


FIG. 7. Mitochondrial localization of *CSTAD-L* and *CSTAD-S* GFP-fused proteins. Human fibroblasts were transfected with a plasmid encoding either the long (upper panels) or short (lower panels) *CSTAD* isoform linked to the GFP gene. Twenty four hours later cells were fixed, and mitochondria were stained using MitoTracker (*MITO*, A and D), anti-hsp-60 (B and E), or anti-cytochrome *c* (*Cyt c*) monoclonal antibodies (C and F) followed by confocal microscopy analysis, as described under "Material and Methods." A medial confocal optical section is shown. Left column, *CSTAD-L*-GFP or *CSTAD-S*-GFP (green); center column, MitoTracker, cytochrome *c* or hsp-60 labeling (red); right column, overlay of green and red fluorescence.

nucleotides located in the 3' region and differ in their 5' region by a sequence of 116 nucleotides, which is unique to *CSTAD-L*, and by a sequence of 31 nucleotides, which is found exclusively in *CSTAD-S*. The *CSTAD-S* cDNA is encoded by two exons separated by an intron of 13,084 bases (gene ID ENSMUSESTG00000040463). The 116 nucleotides that are unique to the *CSTAD-L* cDNA are located immediately upstream of the *CSTAD-S* second exon and are, thus, situated in this intervening sequence. A third alternative splice variant was predicted in the project Ensembl (www.ensembl.org) from the gene structure using a combination of calculations (Exonerate, Blast, and Est-Genome). PCR amplification using primers located in exon 1 and exon 2 of this presumed cDNA did not produce any amplified material from resting, activated, or immunosuppressed T cells, thus excluding the existence of such an isoform in these cells. From our present data and the structure of the Riken cDNA clone we propose the organization of the *CSTAD* gene illustrated in Fig. 2. The "Riken" or *CSTAD-S* variant originates from the splicing and joining of

FIG. 8. CSTAD-S and CSTAD-L isoforms reside on the outer membrane of mitochondria. Mitochondrial preparations from 293 T cells transiently transfected with CSTAD-S or CSTAD-L were treated with various concentrations of proteinase K in the absence (–) or presence (+) of Triton X-100 at 4 °C for 30 min. The samples were analyzed by Western blot with rabbit anti-GFP polyclonal antibodies (*upper panels*), rabbit anti-VDAC polyclonal antibodies (*middle panels*), and mouse anti-cytochrome *c* (Cyt *c*) monoclonal antibody (*lower panels*).

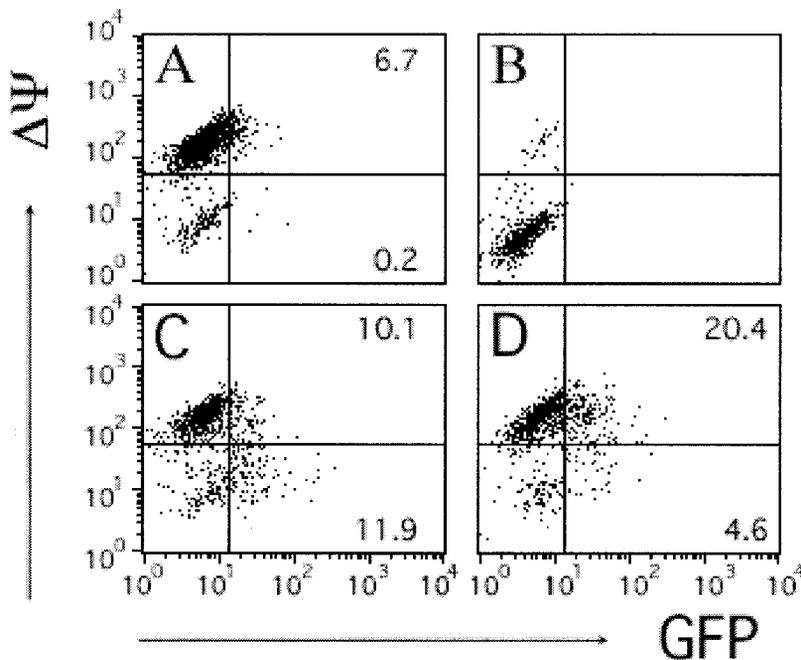
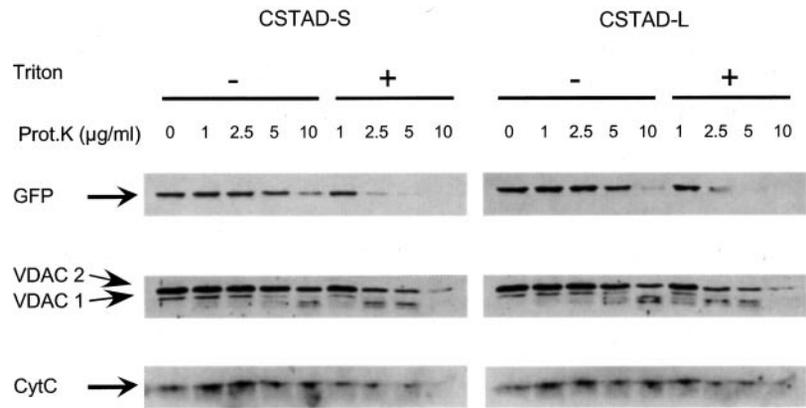


FIG. 9. Overexpression of CSTAD leads to depolarization of the mitochondrial transmembrane potential. Cells were transfected with a construct encoding GFP alone (A) or the short (C) and long (D) isoforms of CSTAD co-expressing GFP. As a control (B), untransfected cells were treated with staurosporine. The transmembrane potential ($\Delta\Psi$) was measured using tetramethylrhodamine ethyl ester, which accumulates in active mitochondria but not in depolarized mitochondria. By cytofluorimetry, both transfected (GFP-expressing) cells and $\Delta\Psi$ were measured simultaneously. The numbers in each quadrant refer to the percentage of cells in each quadrant.

exon 1 and 2B, whereas the CSTAD-L variant derives from the direct transcription of the sequence encoding exon 2A and 2B. CSTAD encodes two proteins of 104 and 141 amino acids. The corresponding sequences do not share obvious homology with other proteins in the data bank and do not contain potential glycosylation or phosphorylation sites.

The CSTAD gene is localized on mouse chromosome 2 (2A3) between the growth factor-independent 1B (*gfi-1B*) and the carboxyl ester lipase (*cel*) genes. These genes (*Gfi-1B* and *Cel*) have orthologues on human chromosome 9 (9q34.13 and 9q34.3). However, an orthologue of CSTAD was not found in the human gene bank data base. In addition, PCRs using the murine CSTAD-S and CSTAD-L primers did not yield amplified material on cDNA from Jurkat cells (data not shown), suggesting that CSTAD has no counterpart in humans; nevertheless, the presence of a functional homologue of CSTAD in humans cannot be excluded.

Up-regulation of CSTAD, as measured by the real-time PCR of the short and long form, is of the order of 5–12-fold in T cells activated in the presence of CSA compared with ConA-activated or non-activated T cells. This increased mRNA accumulation is observed 4 and 24 h after activation, although the relative amount of the mRNAs is larger at 4 h than at 24. No major differences in the relative quantity of the long and short isoforms were detected, suggesting that activation in the presence of CSA does not alter the mechanism(s) involved in the

maturation of CSTAD mRNA precursors. However, the expression of the two alternative splice variants may be differentially regulated, as illustrated by the differences in the levels of CSTAD-L and CSTAD-S mRNA in different tissues. Both isoforms are detected in all the organs tested, and although CSTAD-L and -S are highly represented in the skin, the long isoform is predominantly abundant in the intestine and the thymus, whereas the short form is expressed at higher levels in brain and kidney. It is interesting to note a correlation between the high level of CSTAD-L mRNA accumulation and the rapid cell turnover of the cells in skin, thymus, and intestine.

CSTAD up-regulation is also observed in mice immunized in the presence of CSA. A low level of CSTAD mRNA accumulation is found in splenic T cells of naive or peptide-immunized mice. CSA treatment alone had no effect on the relative abundance of the two alternative splice variants. Immunization in the presence of CSA resulted in a substantial increase of both isoforms. One may, therefore, conclude that CSTAD gene up-regulation by the immunosuppressor is of physiological relevance in mice.

The inhibition of calcineurin activity by the CSA-cyclophilin complexes has been well characterized. The inhibition prevents the dephosphorylation of NFAT, precluding its migration to the nucleus, a mandatory step for the transcription of growth and differentiation genes necessary for the proper effector function of T cells (7, 28). FK506, another immunosuppressor, also in-

hibits calcineurin, and activation in its presence also induces *CSTAD* gene up-regulation. In contrast, rapamycin, which interferes with a late signal originating from the occupancy of the IL-2 receptor, does not induce *CSTAD* overexpression. Taken together, these data show that inhibition of calcineurin in activated T cells up-regulates *CSTAD* gene expression, suggesting that, depending on its activation/inactivation status, this enzyme could be involved both in positive and negative control of gene expression. Even though activation is required for CSA to modify the expression of the *CSTAD* gene *in vitro* or *in vivo*, CSA by itself up-regulates α -E-integrin and EDG-1 gene transcription (Fig. 1). Similar CSA-induced gene up-regulation was also reported for IL-6 and transforming growth factor- β in a variety of cells (29–31). Whether the up-regulation of these genes is independent from the activation of a non-classical calcineurin pathway remains to be determined.

The mechanisms of protein import into the different mitochondrial compartments (the inner or outer membrane, the intermembrane space, or matrix) are being investigated extensively by a number of laboratories, which has led to the identification of targeting signals located in the N-terminal end of mitochondrial-located proteins (for review, see Ref. 32). However, none of the targeting signals described until now has been found in either the long or short forms of *CSTAD*. Internal signals have also been described (33, 34), but these are also absent from both *CSTAD* isoforms. Nevertheless, many mitochondrial import signals still remain to be identified, and it is still possible that a heretofore uncharacterized signal may be responsible for targeting of *CSTAD* to mitochondria.

cDNAs encoding *CSTAD* fused to GFP were transfected in a human cell line, and their localization was analyzed by confocal microscopy. A human cell line was chosen for most of the localization studies since *CSTAD* is not found in humans, excluding interference between transfected *CSTAD* and an orthologous protein. Both *CSTAD* isoforms were sorted predominantly to mitochondria, as the corresponding GFP-tagged proteins co-localized with the mitochondrial markers MitoTracker, cytochrome *c*, and hsp-60. Both *CSTAD*-S and *CSTAD*-L isoforms reside on the outer membrane of mitochondria, since they are cleaved by proteinase K. As a positive control, VDAC proteins, which are known to be expressed on the surface of this organelle, is also degraded by the protease (Fig. 8).

Interestingly, more than 50% of the cells transfected with the short isoform show a depolarization of the inner mitochondrial membrane, whereas this is the case of only 20% of the cells transfected with the long isoform. In both groups the depolarization was not accompanied by the release of cytochrome *c*, excluding an apoptotic effect of the *CSTAD* proteins. Finally, no apoptosis nor necrosis of transfected cells was observed.

Calcineurin, in addition to NFAT, also dephosphorylates and activates BAD (35), a pro-apoptotic member of a family of proteins anchored to the outer membrane of the mitochondria. This process is blocked by CSA and FK506. CSA also inhibits apoptosis through its binding to cyclophilin D, a mitochondrial protein located exclusively in the matrix (36, 37) and involved in the mitochondrial permeability transition (38, 39). Thus, the inhibition by CSA of calcineurin and its binding to cyclophilin D, although acting at different levels of cell physiology, prevent or retard cell apoptosis. *CSTAD*, a gene induced by CSA during T cell activation, produces two proteins that localize to mitochondria and provoke membrane depolarization, but consistent

with the anti-apoptotic properties of CSA acting at other levels, *CSTAD*-induced mitochondrial membrane depolarization does not lead to apoptosis. This finding reveals the presence of a new mitochondria-dependent signaling pathway stimulated by CSA treatment.

Acknowledgments—We thank J. Ragimbeau for advice in cell labeling and Dr. F. Huetz for constructive discussions, A. Lim for helpful advice, and Dr. S. Susin for the anti-Hsp-60 and anti-cytochrome *c* antibodies. Dr. D. Dunn University of Utah, Utah Genome Center, kindly provided the genomic clone. We thank E. Perret and P. Roux from the Plate-Forme d-Imagerie Dynamique at the Pasteur Institute for technical help with confocal microscopy.

REFERENCES

- Teague, T. K., Hildeman, D., Kedl, R. M., Mitchell, T., Rees, W., Schaefer, B. C., Bender, J., Kappler, J., and Marrack, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12691–12696
- Ullman, K. S., Northrop, J. P., Verweij, C. L., and Crabtree, G. R. (1990) *Annu. Rev. Immunol.* **8**, 421–452
- Rao, A. (1995) *J. Leukocyte Biol.* **57**, 536–542
- Rao, A., Luo, C., and Hogan, P. G. (1997) *Annu. Rev. Immunol.* **15**, 707–747
- Jain, J., McCaffrey, P. G., Valge-Archer, V. E., and Rao, A. (1992) *Nature* **356**, 801–804
- Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., and Crabtree, G. R. (1989) *Science* **246**, 1617–1620
- Jain, J. N., McCaffrey, P. G., Miner, Z., Kerppola, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993) *Nature* **365**, 352–355
- Mattila, P. S., Ullman, K. S., Fiering, S., Emmel, E. A., McCutcheon, M., Crabtree, G. R., and Herzenberg, L. A. (1990) *EMBO J.* **9**, 4425–4433
- Jain, J., Loh, C., and Rao, A. (1995) *Curr. Opin. Immunol.* **7**, 333–342
- Bemer, V., and Truffa-Bachi, P. (1996) *Eur. J. Immunol.* **26**, 1481–1488
- Thiel, G., Bock, A., Spondlin, M., Brunner, F. P., Mihatsch, M., Ruffli, T., and Landmann, J. (1994) *Transplant. Proc.* **26**, 2493–2498
- Stewart, T., Tsai, S. C., Grayson, H., Henderson, R., and Opelz, G. (1995) *Lancet* **346**, 796–798
- Hojo, M., Morimoto, T., Maluccio, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., and Suthanthiran, M. (1999) *Nature* **397**, 530–534
- Khanna, A. K., Cairns, V. R., Becker, C. G., and Hosenpud, J. D. (1999) *Transplant* **67**, 882–889
- Mascarell, L., Frey, H., Michel, F., Lefkowitz, I., and Truffa-Bachi, P. (2000) *Transplant* **70**, 340–348
- Kaye, J., Hsu, M.-L., Sauron, M.-E., Jameson, S. C., Gascoigne, R. R. J., and Hedrick, S. M. (1989) *Nature* **341**, 746–749
- Hubank, M., and Schatz, D. G. (1994) *Nucleic Acids Res.* **22**, 5640–5648
- Frazer, J. K., Pascual, V., and Capra, J. D. (1997) *J. Immunol. Methods* **207**, 1–12
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Roumier, A., Olivo-Marin, J. C., Arpin, M., Michel, F., Martin, M., Mangeat, P., Acuto, O., Dautry-Varsat, A., and Alcover, A. (2001) *Immunity* **15**, 715–728
- Bellosillo, B., Villamor, N., Lopez-Guillermo, A., Marce, S., Bosch, F., Campo, E., Montserrat, E., and Colomer, D. (2002) *Blood* **100**, 1810–1816
- Perfettini, J. L., Gissot, M., Souque, P., and Ojcius, D. M. (2002) *Methods Enzymol.* **358**, 334–344
- Mascarell, L., and Truffa-Bachi, P. (2002) *Immunol. Lett.* **14**, 137–143
- Ho, S., Chipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., Nourse, J., and Crabtree, G. R. (1996) *Clin. Immunol. Immunopathol.* **80**, S40–S45
- Rovira, P., Mascarell, L., and Truffa-Bachi, P. (2000) *Curr. Med. Chem.* **7**, 1–20
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) *Science* **275**, 1129–1132
- Kawai, J., et al. (2001) *Nature* **409**, 685–690
- McCaffrey, P. G., Perrino, B. A., Soderling, T. R., and Rao, A. (1993) *J. Biol. Chem.* **268**, 3747–3752
- Morton, R. S., and Dongari-Bagtzoglou, A. I. (1999) *J. Periodontol.* **70**, 1464–1471
- Williamson, M. S., Miller, E. K., Plemons, J., Rees, T., and Iacopino, A. M. (1994) *J. Periodontol.* **65**, 895–903
- Khanna, A., Kapur, S., Sharma, V., Li, B., and Suthanthiran, M. (1997) *Transplantation* **63**, 1037–1039
- Neupert, W. (1997) *Annu. Rev. Biochem.* **66**, 863–917
- Pfanner, N., Hartl, F., Guiard, B., and Neupert, W. (1987) *Eur. J. Biochem.* **1**, 289–293
- Smagula, C., and Douglas, M. (1988) *J. Biol. Chem.* **263**, 6783–6790
- Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999) *Science* **284**, 339–343
- Connern, C. P., and Halestrap, A. P. (1994) *Biochem. J.* **302**, 321–324
- Johnson, N., Khan, A., Virji, S., Ward, J. M., and Crompton, M. (1999) *Eur. J. Biochem.* **263**, 353–359
- Bernardi, P. (1996) *Biochim. Biophys. Acta* **1275**, 5–9
- Halestrap, A. P., Connern, C. P., Griffiths, E. J., and Kerr, P. M. (1997) *Mol. Cell. Biochem.* **174**, 167–172

**Characterization of a Gene Encoding Two Isoforms of a Mitochondrial Protein
Up-regulated by Cyclosporin A in Activated T Cells**
Laurent Mascarell, Rodolphe Auger, Andres Alcover, David M. Ojcius, Thomas Jungas,
Véronique Cadet-Daniel, Jean M. Kanellopoulos and Paolo Truffa-Bachi

J. Biol. Chem. 2004, 279:10556-10563.

doi: 10.1074/jbc.M313770200 originally published online December 18, 2003

Access the most updated version of this article at doi: [10.1074/jbc.M313770200](https://doi.org/10.1074/jbc.M313770200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 39 references, 10 of which can be accessed free at
<http://www.jbc.org/content/279/11/10556.full.html#ref-list-1>