Biochemical and Biophysical Research Communications **290,** 463–468 (2002)
doi:10.1006/bbrc.2001.6204, available online at http://www.idealibrary.com on IDEAL®

Phosphorylation of Recombinant Human Spermidine/Spermine N^1-Acetyltransferase by CK1 and Modulation of Its Binding to Mitochondria: A Comparison with CK2

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Received November 28, 2001

Cytosolic spermidine/spermine acetyltransferase (SSAT) catalyzes the acetylation of the N^1-propylamino groups of spermine and spermidine. The enzyme has a very short half-life and is rapidly induced by various stimuli. Once acetylated, these polyamines are subjected to the action of polyamine oxidase, which, besides initiating polyamine catabolism, may produce reactive oxygen species that in turn trigger modifications in subcellular compartments such as mitochondria. The present work evaluates the ability of the cAMP-independent Ser/Thr-protein kinase CK1 to phosphorylate SSAT. Results demonstrate that SSAT is phosphorylated by CK1, in sites distinct from those phosphorylated by CK2. Moreover, both phosphorylation processes are involved in the uptake of SSAT into rat liver mitochondria. Although CK2 is less effective than CK1 in phosphorylating SSAT, CK2 phosphorylation is much more powerful in preventing binding of SSAT to mitochondrial structures. These results suggest the involvement of CK1- and CK2-mediated SSAT phosphorylation in regulating the contents of polyamines and SSAT itself within subcellular compartments and implicate SSAT and polyamines as indirect modulators of progression through the cell cycle.

Key Words: **SSAT; phosphorylation; CK1; CK2; mitochondria.**

Cytosolic SSAT selectively catalyses acetylation of the primary amino groups of the aminopropyl moieties of spermidine and spermine. By removing a positive charge, acetylation decreases the electrostatic binding energy of spermidine and spermine, and may therefore promote the displacement of the two polyamines from their binding sites. Acetylation of polyamines thus seems suitable for their efflux from cell to external fluids and for transport from one cellular compartment to another—for example, from the cell nucleus into the cytoplasm (1, 2). It has also been observed that acetylation reduces polyamine transport in mitochondria (unpublished results).

SSAT has a very short half-life (3) and is rapidly induced by several stimuli, including hormones (4), toxic agents (3), hypoxia (5) and cytokines (6).

Recent studies using HCT116 cells demonstrate that SSAT induction is regulated at both transcriptional and post-transcriptional levels by conditions which arrest cell growth, both mechanisms being affected by endogenous polyamine contents (7).

The recently described polyamine analogue N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazeundecane (CPEN Spm), which represents a new class of antitumor agents, promotes superinduction of SSAT in susceptible cell types, and has been demonstrated to induce apoptosis (8).

Another class of polyamines, represented by DE-333, also induces SSAT and promotes cell cycle progression and apoptosis (9).

Previous studies have shown that SSAT is phosphorylated by CK2 *in vitro* (10). The amino acid sequence of SSAT also contains probable target sites for CK1, a ubiquitous monomeric enzyme able to phosphorylate Ser/Thr residues in a wide variety of substrates (11). CK1 appears to be responsible for the regulation of certain enzymes, such as protein phosphatase-1 (12) and glycogen synthase (13), and has been shown to phosphorylate hormone receptors [e.g., the insulin receptor (13)].

Abbreviations used: CK, casein kinase; RLM, rat liver mitochondria; MAO, monoamine oxidase; PAO, polyamine oxidase; SSAT, spermidine/spermine acetyltransferase.

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0006-291X/02 $35.00

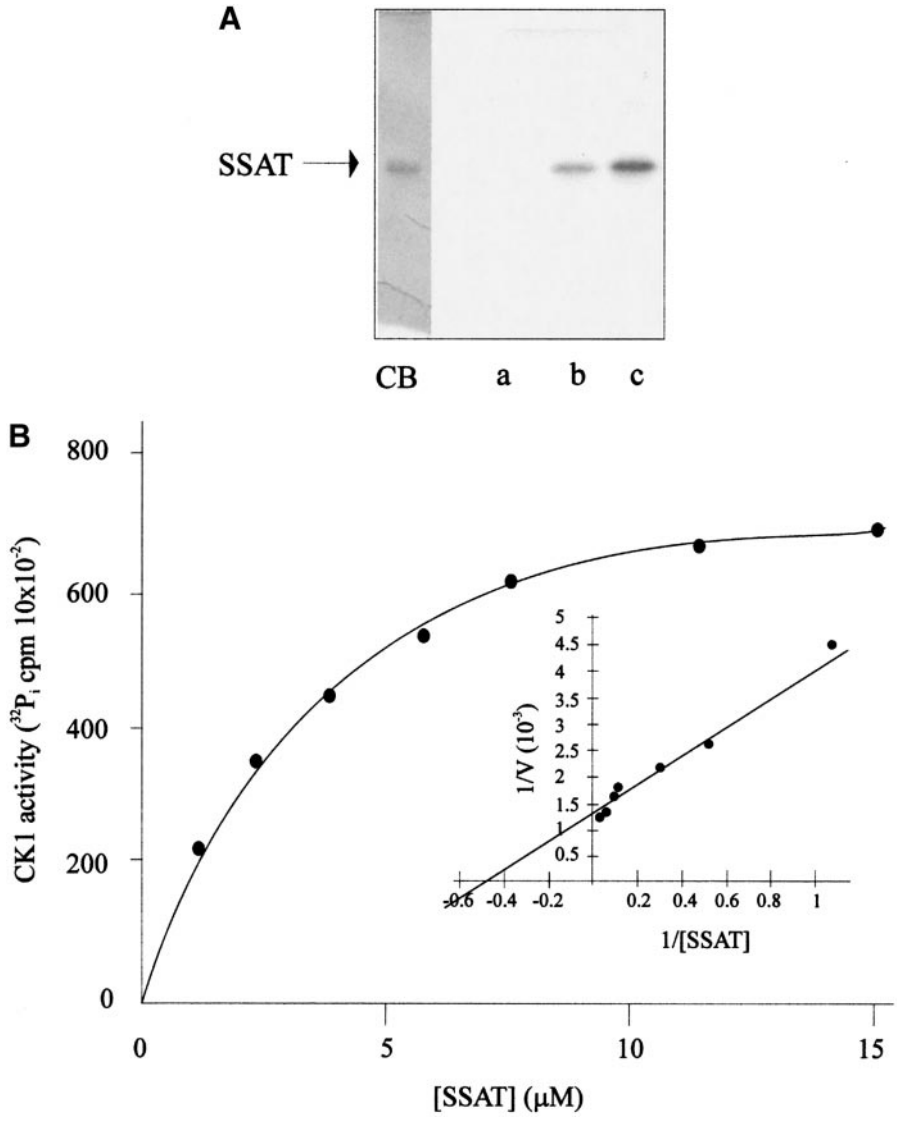

FIG. 1. Pattern of CK1-catalyzed SSAT phosphorylation (A) and K_m determination (B). (A) SSAT (4 μM, lane b; 8 μM lane c) incubated at 30°C for 8 min in the presence of CK1 isolated from human erythrocyte cytosol (2 units) in incubation mixture (see Materials and Methods). Lane a, autophosphorylation pattern of CK1 incubated in absence of SSAT. Samples analyzed by 0.1% SDS;/15% PAGE (see Materials and Methods). Lane CB, Coomassie-blue stained gel corresponding to lane c. Autoradiograms were exposed for 24 h. (B) Activity assay carried out as described under Materials and Methods; various amounts of SSAT were present. (Inset) Double-reciprocal plot of the same data.

All these observations prompted us to investigate whether CK1 is able to phosphorylate SSAT to the same extent and under the conditions previously shown for CK2 and to inquire into the possible physiological significance of SSAT phosphorylation, at both the level of its activity and its subcellular location.

In this regard, it must be emphasized that polyamines are actively transported by energized mitochondria (14–16); and have a modulatory effect on pyruvate dehydrogenase activity (17). The possibility that SSAT is taken up by mitochondria would provide new insights into the role of polyamines in these organelles.

The present paper demonstrates that CK1 is able to phosphorylate SSAT, primarily on Ser residues; tryptic peptides containing these serine(s) are distinct from those phosphorylated by CK2. Although Ser/Thr phosphorylation by CK1 or CK2 does not seem to affect the activity of SSAT, it clearly alters binding of enzyme mitochondrial membranes.

MATERIALS AND METHODS

Purification of enzymes. CK1 was isolated from human erythrocyte cytosol (18) by DEAE–Sepharose chromatography. The first pooled peak was subjected to heparin–Sepharose chromatography

and eluted with 0.4 to 0.5 M NaCl. The symmetric peak was collected and further purified by P-cellulose chromatography and eluted with 0.5 to 0.6 M NaCl. CK2 and SSAT were obtained as described in Ref. (10).

Phosphorylation assays. Ser/Thr phosphorylation was performed by incubating recombinant human SSAT (various amounts) (or 25 μg casein) at 30°C for 8 min in a 30-μl reaction mixture containing 50 mM Tris/HCl buffer (pH 7.5), 10 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (3×10^6 cpm/nmol), and 1 unit of either CK1 or CK2, purified by human erythrocyte cytosol.

Reactions were stopped by the addition of 2% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol (final concentrations), followed by 5-min treatment at 100°C (10).

The solubilized proteins were analyzed by 0.1% SDS/12% PAGE according to Laemmli (19) and visualized by staining with Coomassie brilliant blue (19).

One unit (U) of CK1 or CK2 was defined as the amount catalyzing the incorporation of 1 pmol ^{32}P into casein in 1 min, under the above conditions.

The amount of ^{32}P incorporated into SSAT was quantified by scanning the Coomassie blue-stained gels using a Packard Instant Imager.

SSAT activity assay. SSAT activity was assayed according to Persson and Pegg (20) by measuring the radioactivity taken up by spermidine from ^{14}C-acetylCoA, one unit of SSAT being defined as the amount of enzyme acetylating 1 nmol of spermidine/min at 30°C.

^{32}P-peptide mapping of SSAT. ^{32}P-labeled SSAT obtained by incubation with CK1 was resolved by SDS–PAGE as described above, transferred electrophoretically to nitrocellulose filters, and located by autoradiography. The phosphorylated band was excised and tryptically digested, and the resulting peptides were separated by two-dimensional electrophoresis/thin layer chromatography (10).

Incubations with low trypsin concentrations were performed for 15 and 45 min, as described in Ref. (10).

Phospho-amino acid analysis. Radioactive samples of the SSAT protein phosphorylated were electrotransferred from gels to nitrocellulose filters and tryptically digested, as described above. The resulting peptides were treated with 6 M HCl at 105°C for 4 h and separated by high-voltage paper electrophoresis, as described (21).

Mitochondrial preparations. Rat liver mitochondria were isolated from rats by conventional differential centrifugation in a buffer containing 250 mM sucrose and 5 mM Hepes, 1 mM EDTA (pH 7.4) (22). EDTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as a standard (23).

Binding of SSAT to rat liver mitochondria. ^{32}P-phosphorylated SSAT, obtained as described above, and control SSAT, prepared in the presence of 55°C-pretreated CK1 or CK2 (inactivated enzymes), were incubated with intact rat liver mitochondria (1 μg SSAT/40 μg RLM) at 0°C for 10 min. Samples were then microfuged; pellets were washed once in Hepes buffer, pH 7.4, containing 300 mM sucrose, and supernatants were recovered and subjected to SDS–PAGE, as described above. Proteins were electrotransferred to nitrocellulose and immunostained with anti-SSAT antibody (24) followed by an HRP-labeled secondary antibody and detection by enhanced chemiluminescence (ECL, Amersham).

RESULTS

The results reported in Fig. 1 demonstrate that CK1 is able to phosphorylate SSAT, as previously reported for CK2 (10), the reaction exhibiting an apparent K_m value of about 2 μM (inset in Fig. 1). The same result is also obtained with CK2 (results not shown).

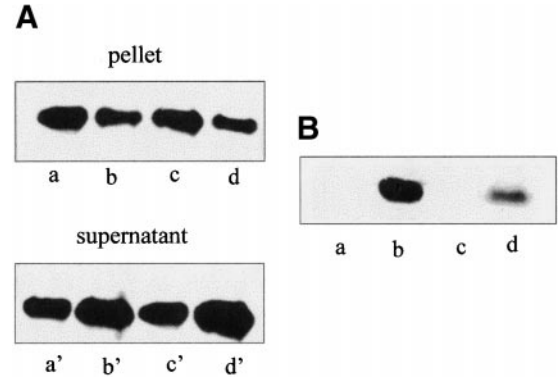

FIG. 2. Binding of unphosphorylated and CK1- or CK2-phosphorylated SSAT to rat liver mitochondria (A) and correlation of binding with phosphorylation level by CK1 and CK2 (B). (A) Immunoblot of SSAT of mitochondrial pellets (lanes a–d) and corresponding supernatant fractions (lanes a′–d′). Forty micrograms of RLM was incubated with 1 μg of SSAT and either 55°C-inactivated CK1 (2 U) (lanes a and a′) and active CK1 (lanes b and b′) or 55°C-inactivated CK2 (2 U) (lanes c and c′) and active CK2 (lanes d and d′) under conditions described under Materials and Methods. (B) Autoradiography of CK1- and CK2-phosphorylated SSAT. One microgram of SSAT was incubated as indicated under Materials and Methods either with 55°C-inactivated CK1 (2 U) (lane a) and active CK1 (lane b) or 55°C-inactivated CK2 (2 U) (lane c) and active CK2 (lane d).

This posttranslational modification may have physiological relevance and affect SSAT function either directly, by modulating the catalytic activity of the enzyme, or indirectly, by affecting its binding properties to intracellular structures, thus preventing or facilitating contact with its target substrate.

In regard to the catalytic activity of SSAT, no difference was noted in the ability of the phosphorylated or unphosphorylated enzyme to acetylate spermidine in an *in vitro* assay (data not shown).

As shown in Fig. 2A, unphosphorylated SSAT is readily sequestered by intact RLM, at a ratio of about 0.4–0.5 μg/40 μg intact RLM (compare lanes a with inactive CK1, and c with inactive CK2, with the respective supernatant containing unbound SSAT in lanes a′ and c′). When RLM are incubated in the presence of CK1-phosphorylated (lane b) or CK2-phosphorylated (lane d) SSAT, the amount of phosphorylated SSAT (P-SSAT) bound to mitochondria corresponds to only 50 and 30%, respectively, of the bound nonphosphorylated enzyme, as also indicated by the counterbalanced increase in the corresponding supernatants (lanes b′ and d′).

It is noteworthy that the decreased ability of phosphorylated SSAT to associate with RLM appears to be kinase-specific, as CK2-P-SSAT is less readily bound than CK1-P-SSAT (compare lanes b and d with lanes b′ and d′).

That this effect is kinase-specific is demonstrated by the autoradiograms of Fig. 2B in which the phosphor-

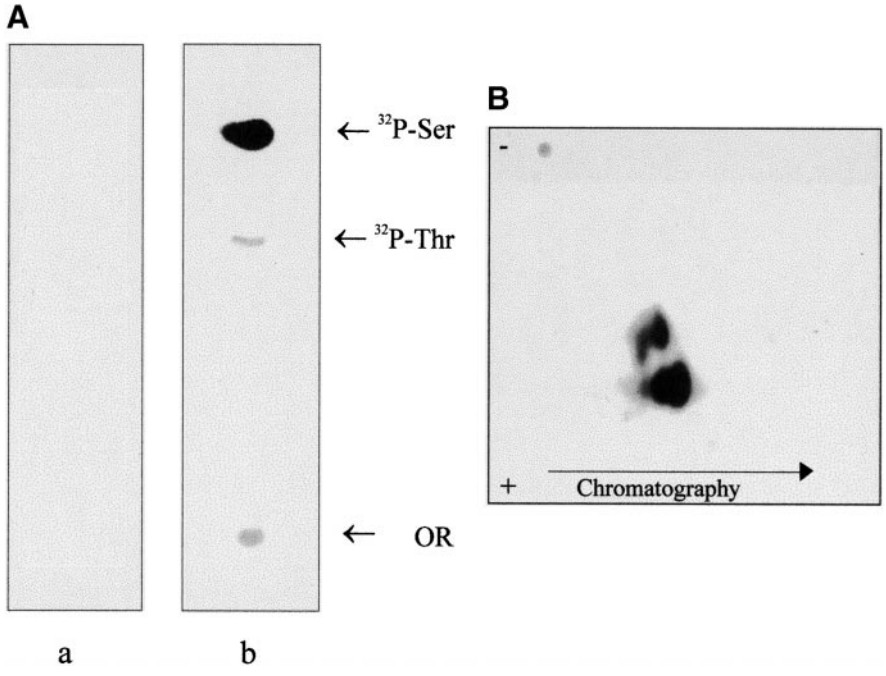

FIG. 3. Phospho-amino acid analysis of SSAT ^{32}P-labeled by CK1 (A) and electrophoretic patterns of ^{32}P-peptides from exhaustively trypsin-digested SSAT phosphorylated by CK1. (A) Samples of autophosphorylated CK1 (lane a) and SSAT phosphorylated by CK1 (lane b) (see Materials and Methods) were eluted by blotting to nitrocellulose filters and tryptically digested (see Materials and Methods). Resulting ^{32}P-peptides were treated with 6 M HCl at 105°C, as described. (B) Samples of ^{32}P-labeled SSAT subjected to electrophoresis were blotted to nitrocellulose filters and exhaustively digested with trypsin for 4 h at 30°C. Resulting ^{32}P-peptides were separated by two-dimensional thin-layer chromatography and autoradiographed for 36 h.

ylation level of SSAT by CK2 (lane d) is greatly reduced when compared with that obtained with CK1 (lane b).

These results give rise to the hypothesis that CK1 and CK2 phosphorylate different sites on SSAT. Phospho-amino acid analysis of ^{32}P-SSAT revealed that serines represent the main targets of CK1 with only a minor ^{32}P-Thr spot detected (Fig. 2B), as already reported for CK2-phosphorylated SSAT (10).

In addition, the electrophoretic patterns of tryptic ^{32}P-peptides of SSAT phosphorylated by CK1 (Fig. 3B) are completely different from those of SSAT phosphorylated by CK2 (10).

Mild tryptic digestion of SSAT phosphorylated by CK1 or CK2 (Fig. 4), i.e., to limit cleavage to the primary site of digestion at SSAT residues 141–143 (25), yields different ^{32}P-labeled bands. After a 15-min trypsin treatment, only two bands, one having a low molecular mass, were evident from CK2 phosphorylated SSAT (lane a), as also previously reported (10). Instead, phosphorylation by CK1 produces the same labeled bands as CK2, and a new, different, band having a low molecular mass (lane b). The higher molecular mass bands observed in both lanes refer to whole phosphorylated SSAT.

When trypsin treatment time was increased to 45 min, the higher molecular mass bands disappeared completely for both CK2 and CK1 treatment, indicating total digestion of SSAT. Both CK1 and CK2 P-SSAT exhibit increased labeling of their low-molecular-mass bands (lanes c and d, respectively) with respect to 15-min digestion.

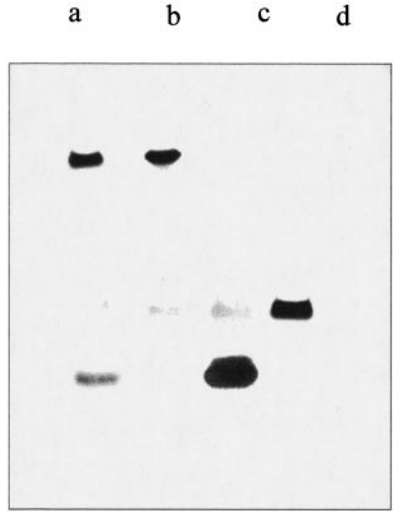

FIG. 4. Electrophoretic patterns of ^{32}P-peptides obtained by limited trypsin digestion of SSAT phosphorylated by CK1 and CK2. Two-microgram samples of SSAT ^{32}P-labeled by CK1 (lanes a and c) or CK2 (lanes b and d) were subjected to SDS–PAGE, transferred to nitrocellulose, and treated with trypsin (2 μg for each sample) for 15 min (lanes a and b) or 45 min (lanes c and d). Resulting ^{32}P-peptides were separated by 0.1% SDS/18% PAGE. Autoradiograms were exposed for 72 h.

In this regard, it should be emphasized that the new band identified by CK1 (with the lowest molecular mass) shows a stronger increase in phosphorylation than the other band, suggesting that most of the CK1-P-SSAT target sites are not located in proximity to residues 141–143, as evidenced for CK2, but in distinct peptides which do not overlap with the CK2 sites (10).

DISCUSSION

A previous study (10) demonstrated the ability of SSAT to undergo CK2 phosphorylation, probably on C-terminal serine residue(s), thus adding SSAT to the list of CK2 substrates (11).

The present study revealed that CK1 can also phosphorylate SSAT and gives new insights into the possible role of this kinase in the pathway of SSAT-related polyamine catabolism.

CK1 phosphorylates SSAT to a much greater extent than CK2 (Fig. 2B), and at distinct target sites, as revealed by ^{32}P-peptide mapping (Fig. 3B) and by comparison of patterns obtained after trypsin treatment (Fig. 4).

However, CK2 is much more effective in modulating P-SSAT binding behavior to RLM than CK1, suggesting that it is the specific phosphorylated target residue rather than the overall level of phosphorylation (i.e., negative charges) that is responsible for preventing binding of SSAT to organelles.

According to this hypothesis, CK1 phosphorylates a larger number of Ser (rather than Thr) residues than CK2, but these are less important than the CK2 targets in modulating SSAT uptake into RLM.

The uptake of SSAT by RLM represents a very important event connected with the catabolic pathway of polyamines in these organelles. In fact, as previously reported (26) and also confirmed by our preliminary results, besides PAO, also monoamine oxidase (MAO) is able to oxidize acetylated polyamines (the reaction products of SSAT activity to form aldehydes and H_2O_2). The resulting reactive oxygen species can induce the permeability transition of mitochondrial inner membranes when the organelles are accumulating large amounts of Ca^{2+}. Results of many studies (27) have firmly established that this phenomenon is closely connected to the pathway leading to apoptosis (28, 29).

As a result of an increase in CK2 gene transcription, CK2 activity increases during the early phase of the cell cycle (30). Therefore, SSAT phosphorylation may represent an important mechanism directing this enzyme to a cellular compartment accessible to proteasomal degradation. Such downregulation of SSAT activity would maintain polyamines at levels sufficient for cell cycle progression.

Alternatively, the introduction of negative charges on the C-terminal end of SSAT by phosphorylation may directly control its binding to proteasome, as previously suggested (31).

In conclusion, the reduced uptake of phosphorylated SSAT into mitochondria may represent a regulatory mechanism involved in polyamine catabolism.

ACKNOWLEDGMENTS

We thank Miss Carla Munari for technical assistance and Mr. Giovanni Di Falco for secretarial work.

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