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Casein Kinase 2 Phosphorylates Recombinant Human Spermidine/Spermine N^1-Acetyltransferase on both Serine and Threonine Residues

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Casein kinase 2 purified from human erythrocyte cytosol has been found to phosphorylate human spermidine/spermine N^1-acetyltransferase (SSAT) expressed as a fusion protein in E. coli and purified to homogeneity with a specific activity similar to that reported for pure human SSAT. The amino acid sequence of the protein revealed not less than four phosphorylable residues, optimal target for protein kinase 2 phosphorylation being flanked by acid residues in position +1 and +3. Our results indicate that most ^{32}P-phosphate is taken up by Ser residues, as evidenced by HCl hydrolysis and electrophoresis and that the phosphorylation extent is modulated by the physiological polyamine concentration. Partial digestion with trypsin at a low concentration for less than one hour preferentially hydrolyzes Lys-Arg-Arg in position 141-143 of the SSAT suggesting that the Ser-phosphorylated residues are located in the C-terminus of the protein, probably Ser 146 and 149.

The polyamines putrescine, spermidine and spermine occur naturally as polycationic components of all eukaryotic cells, and are present in their cytoplasm, nuclei and mitochondria (1). Their precise role is still obscure, though they are regarded as involved in cell proliferation and differentiation (reviews in 2, 3), oncogene expression (4), oestrogen-receptor stabilization (5) and, in some cases, cell survival (6).

The intracellular concentration of polyamines in mammalian cells is controlled by a combination of carefully regulated enzymatic steps, including the biosynthetic enzymes ornithine decarboxylase and S-adenosylmethionine decarboxylase, and the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT).

SSAT is a cytosolic protein with a very short half-life. It is rapidly induced by a number of factors, including polyamines and their analogues, hormones, toxic agents (8) and hypoxia (9). The effect of this induction is to reduce spermidine and spermine content, thus preventing the accumulation of high and potentially toxic polyamine levels. Analysis of the human SSAT sequence (10) shows some residues, Thr_{10}, Thr_3, Ser_{146} and Ser_{149} in the target sequence recognized by protein kinase 2 (CK-2), ubiquitous tetrameric enzyme with two alpha (36 kDa) and two beta (24–28 kDa) subunits (review in 11). The increase of CK-2 content in the nucleus by effect of exposure to extracellular growth stimuli has been correlated with an increase of the nuclear polyamine concentration during the duplication phase (12, 13). Moreover it has

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Abbreviations: SSAT, spermidine/spermine N^1-acetyltransferase; CK-2, casein kinase 2; spm, spermine; spd, spermidine.

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been shown that polyamines promote translocation of CK-2 and that spermine is able to link the enzyme to the mitochondrial membrane, possibly by inserting CK-2 in the inner mitochondria compartment (13, 14).

It is not known whether CK-2 in its turn influences cell polyamine content. SSAT could be a good candidate.

The findings reported here indicate that CK-2 purified from human erythrocyte cytosol phosphorylates SSAT on both Ser and Thr residues in at least three main tryptic peptides and that this phosphorylation is influenced, though to a different extent, by spermine, spermidine and putrescine. It is inhibited by NaCl and heparin.

METHODS AND MATERIALS

Expression and purification of recombinant human SSAT. Recombinant human SSAT was produced in E. coli. The pET19b-SSAT expression vector was constructed by inserting the 516-base pair BamHI/BamHI fragment from pSATh1 (10) into the multiple cloning site of the pET19b vector. The insertion resulted in a twenty-six amino acid addition (MGHHHHHHHHHHSSGHIDDDDKHMLE) to the amino terminus. This extra arm contains 10 consecutive histidines and allows easy purification. The specific activity of the fusion protein was about the same as that reported for purified human SSAT (16). E.coli BL21(DE3) cells were transformed with pET19b-SSAT and grown in LB medium supplemented with ImM isopropyl-1-thio-beta-D-galactopyranoside and 100μg/ml ampicillin.

SSAT was expressed as about 8% of the total soluble protein. The enzyme was purified by a two-step procedure. First, immobilized metal affinity cromatography (IMAC) binding the recombinant oligohistidine stretch was used. Then the enzyme was further purified on a resin containing covalently bound spermine. Analysis by SDS-PAGE after Coomassie Blue staining revealed a homogeneous protein of about 22 kDa.

Purification of CK-2. CK-2 has been isolated from human erythrocyte cytosol (17). The DEAE-sepharose pooled second peak was subjected to Heparin-sepharose chromatography and eluted with 0.7 M NaCl. The symmetric peak was collected and further purified by P-cellulose chromatography and eluted with 0.6 M NaCl.

Assays. Ser/Thr phosphorylation was performed by incubating 8 μM SSAT or 50 μg casein at 30 °C for 8 min in 30 μl reaction mixture containing 50 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 20 μM (gamma-^{32}P)ATP (3×10^6 cpm/nmol) in the presence of CK-2 (2 units).

When the effect of spermine, spermidine, putrescine, NaCl and heparin respectively was assayed, increasing concentrations of each compound were added to the incubation mixture in the conditions described above. Reactions were stopped by addition of 2% sodium dodecylsulfate (SDS) and 1% beta-mercaptoethanol (final concentrations) followed by a 5 min treatment at 100 °C as described in (18).

The solubilized proteins were analyzed by 0.1% SDS/15% PAGE, essentially according to Laemmli (19). After electrophoresis, gels were stained with Coomassie Brilliant Blue according to Laemmli. Dried gels were then autoradiographed at −80°C with intensifying screens. One unit of CK-2 was defined as the amount catalyzing the incorporation of 1 pmol of ^{32}P into casein in 1 min under the above conditions. For the evaluation of the ^{32}P incorporated into SSAT, the radioactivity corresponding to the band of the protein was determined with an Instant Imager.

SSAT activity was assayed according to Persson and Pegg (20) by measuring radioactivity taken up by spermidine from ^{14}C-acetylCoA. One unit of SSAT represents 1 nmol of product/min at 30 °C.

Protein was measured by the method of Bradford (21).

^{32}P-peptide mapping of SSAT. ^{32}P-labeled SSAT obtained by incubation with CK-2 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 on polyvinylpyrrolidone-treated nitrocellulose pieces, as described in (22) and the resulting peptides were separated by two-dimensional thin-layer electrophoresis at pH 8.9 (40 min at 1000 v), followed by ascending chromatography in a solution containing 25% pyridine, 7.5% acetic acid, 37.5% butanol (23).

Incubations with a low trypsin concentration for 15, 30, 45 min were performed essentially according to (23), except that trypsin was 2 μg for a 30 μg SSAT sample, followed by 0.1% SDS-18% PAGE of the peptides removed by trypsin.

Synthetic peptide experiments. The synthetic peptide reproducing the aminoacid sequence from 10 to 22 of the fusion protein of SSAT was investigated by incubating increasing peptide concentrations with CK-2 in the phosphorylation conditions described above. The reaction was stopped as above, and the gel treatment was as described for the SSAT phosphorylation, except that the electrophoresis was performed in 0.1% SDS - 18% PAGE.

Phosphoaminoacid analysis. Radioactive samples of the SSAT protein phosphorylated as described above were eluted from the gels by blotting to nitrocellulose filters and tryptically digested as above. The resulting peptides were treated with 6 M HCl at 105 °C for 4 h. High-voltage paper electrophoresis was performed as described (24).

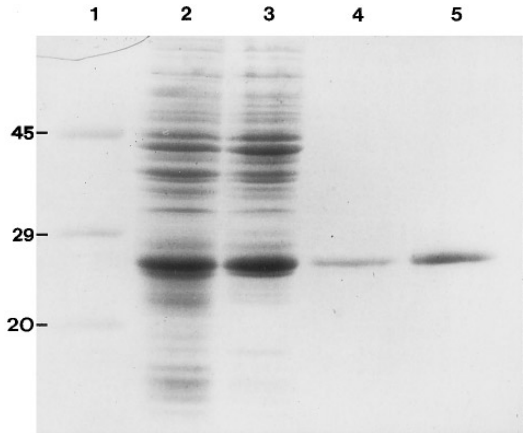

FIG. 1. Coomassie-stained gel of human recombinant SSAT after successive purification steps. Lane 1, molecular mass markers (KDa); lane 2, crude extract; lane 3, 20,000×g supernatant; lane 4, post-IMAC; lane 5, post-spermine-Sepharose.

Materials. The plasmid pET 19b and E.coli BL21(DE3) were obtained from Novagen (USA); 1-^{14}C-acetylCoA (58 mCi/mmol) and gamma-^{32}P-ATP (3000 Ci/mmol) from Amersham (UK); IMAC and sepharose from Pharmacia Biotech (Sweden). Spermine-sepharose was prepared according to the instructions of the Sepharose manufacturer. DEAE-sepharose, HeparinSepharose CL-6B, P-cellulose and all other biochemical reagents were purchased from Sigma (U.S.A.). The synthetic peptide $(H)_3$-$(S)_2$-G-H-I-$(D)_4$- was provided by Advanced Biotech Italia Srl.

RESULTS AND DISCUSSION

Purification of SSAT

As SSAT is normally found in very low amounts in mammalian cells, it has been obtained as a fusion protein by using a cDNA encoding human SSAT.

Bacteria transformed and grown as described under Methods were harvested by centrifugation, suspended in IMAC 5 buffer (20 mM Tris, 0.5 M NaCl, 10% glycerol, 5 mM imidazole, pH 7.9) and sonicated. After centrifugation, the supernatant was applied to an IMAC column. The enzyme was eluted with IMAC-EDTA buffer (the same as above, but with 100 mM EDTA instead of imidazole). The active fractions were pooled and loaded on a spermine-Sepharose column. SSAT was eluted with 30 mM spermidine in 50 mM Tris, 0.8 M NaCl, pH 7.5. The active fractions were concentrated by ultrafiltration. The enzyme was stored at −70°C in 50 mM Tris, pH 7.5. A summary of the purification procedure is given in Table 1. In the experiment shown, the enzyme was purified about 13-fold with a yield of 77%.

Fig. 1 shows the Coomassie blue stained gel (SDS-PAGE) of SSAT after successive purification steps. The final enzyme fraction gave a single band of about 22 kDa. This recombinant enzyme was purified as described in large amounts and used for the experiments.

In Vitro Phosphorylation of SSAT

CK-2 purified from human erythrocyte cytosol catalyzed ^{32}P-labeling of increasing SSAT concentrations (Fig. 2). Phosphorylation occurred on both Ser- and (less extensively) Thr-residues, as indicated by acid hydrolysis of the ^{32}P-SSAT (Fig. 3).

Phosphopeptide mapping of the ^{32}P-SSAT phosphorylated by CK-2 indicated that at least three main peptides are obtained by exhaustive trypsin treatment (Fig. 4). However, after a weak tryptic cleavage, which would involve the aminoacids at position 141-143 as the primary

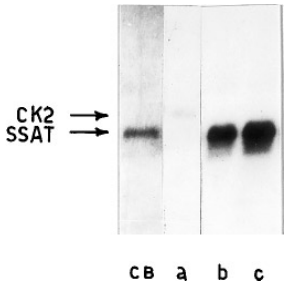

FIG. 2. ^{32}P-labeling pattern of CK2-catalyzed SSAT phosphorylation. SSAT (4 μM lane b, 8 μM lane c) was incubated at 30 °C for 8 min in the presence of CK-2 isolated from human erythrocyte cytosol (2 units) in the incubation mixture described under Methods. Lane a shows the autophosphorylation pattern of CK-2 incubated in the absence of SSAT. The samples were analyzed by 0.1% SDS-15% PAGE (see Methods). Lane CB shows the Coomassie blue stained gel corresponding to lane c. Autoradiograms were exposed for 24 h.

site digestion (25), only two bands were evident: after a few minutes, a higher molecular mass band, about 22 KDa, representing the whole protein, and a lower mass band, about 2 KDa, probably representing the C-terminus of the protein, were observed (Fig. 5, lane a). When the trypsin treatment time was increased to 30 and 45 min respectively, the higher molecular weight band disappeared completely and the other became more evident (Fig. 5, lane b and c) suggesting that phosphorylation mainly occurred in the C-terminal end of the protein.

To determine whether the introducing peptide is also phosphorylated, we used a 1.5 KDa peptide with the same sequence from 10 to 22 of the fusion protein. Increasing concentrations

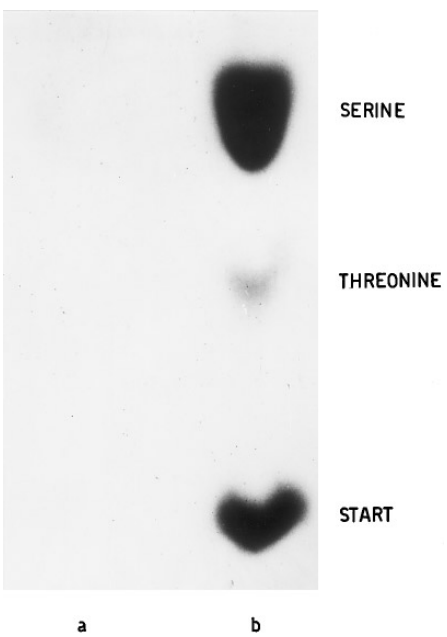

FIG. 3. Phosphoaminoacid analysis of SSAT ^{32}P-labeled by CK-2. A sample of auto-phosphorylated CK-2 (lane a) and of SSAT phosphorylated by CK-2 (lane b) (see Methods) were eluted by blotting to nitrocellulose filters and tryptically digested (see Methods). The resulting ^{32}P-peptides were treated with 6 M HCl at 105 °C as described under Methods.

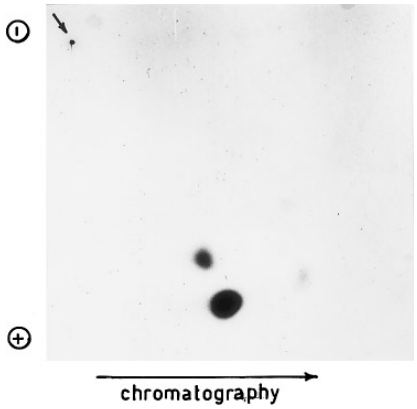

FIG. 4. Electrophoretic pattern of ^{32}P-peptides from exhaustively trypsin digested SSAT phosphorylated by CK-2. The sample of ^{32}P-labeled SSAT, subjected to electrophoresis as described under Methods, was blotted to nitrocellulose filters and exhaustively digested with trypsin for 4 h at 30 °C. The resulting ^{32}P-peptides were separated by two dimensional thin layer chromatography as described under Methods and autoradiographed for 36 h.

were incubated with CK-2 in the presence of (gamma-^{32}P)ATP. Fig. 6 shows that the lane corresponding to the greater peptide concentration (more than 30-fold the SSAT concentration used in the phosphorylation experiments) presents a negligible signal.

Investigation of the activation or inhibition of SSAT phosphorylation by CK-2 showed that spermine is the most effective polyamine. It slightly increased CK-2 activity at 0.1 mM concentration (9%), but gave total inhibition at 5 mM, with an I_{50} at 1.25 mM (Fig. 7). Spermidine and putrescine were much less effective, though their curve pattern was the same (Fig. 7). Moreover NaCl and heparin were strong inhibitors (not shown), as occurs for the substrates already known for CK-2. I_{50} is about 50 mM for NaCl and 30 ng/ml for heparine.

In conclusion, these results demonstrate for the first time that CK-2 is able to phosphorylate SSAT. This protein can therefore be added to the list of natural substrates of CK-2 (26).

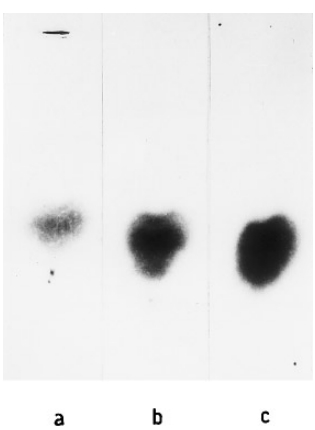

FIG. 5. Electrophoretic pattern of ^{32}P-peptides from weakly trypsin digested SSAT phosphorylated by CK2. ^{32}P-SSAT phosphorylated by CK-2 analyzed by 0.1%/SDS/15% PAGE and blotted to nitrocellulose filters was treated with trypsin (2 μg for each sample) for 15 (lane a), 30 (lane b) and 45 min (lane c) respectively. The resulting ^{32}P-peptides were separated by 0.1% SDS/18% PAGE. Autoradiograms were exposed for 72 h.

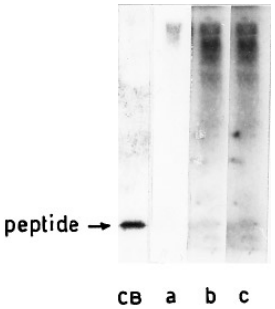

FIG. 6. Phosphorylation pattern of the synthetic peptide reproducing part of the fusion protein sequence of SSAT. Increasing synthetic peptide concentrations, 24 μM (lane b), and 240 μM (lane a and c) were incubated with CK-2 in the phosphorylation conditions described under Methods and analyzed by 0.1% SDS/18% PAGE. The upper signal in lane a, b and c represents CK-2 autophosphorylation pattern. Gels shown in lane b and c were subjected to a shorter time of Coomassie Blue treatment (about ten hours destaining) compared to gel in lane a, in order to minimize a possible loss of the peptide. Lane CB shows the Coomassie Blue stained gel, corresponding to lane c.

Among these are several proteins involved in gene expression and protein synthesis, and also components of signalling pathways, like IGF receptor, the regulatory subunit of cAMP-dependent protein kinase and of protein phosphatase-1, etc. Some key enzymes of metabolic pathways, such as acetylCoA carboxylase and ornithine decarboxylase are also phosphorylated by CK-2, although ''silently''. As SSAT is the regulatory enzyme of the pathway responsible for interconversion and/or catabolism of polyamines, the observed phosphorylation could have a role in modulating cellular content of these cations. This posttranslational modification could directly regulate the activity of SSAT by acting on the enzyme conformation or altering the ability of SSAT to interact with other proteins, regulatory molecules or the substrate itself. The last hypothesis is particularly attractive, since it has been shown that Glu_{152} and the carboxyl domain are involved in the polyamine substrate binding and Glu_{152} is part of the target sequence recognized by CK-2. Furthermore, the phosphorylation can modify the enzyme

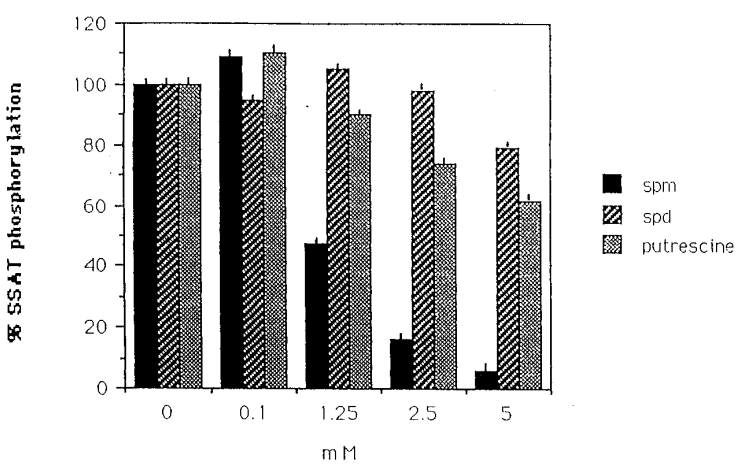

FIG. 7. Effect of increasing polyamine concentration on CK-2-catalyzed SSAT phosphorylation. Phosphorylation conditions were as described under Methods. Proteins were analyzed by 0.1% SDS/15% PAGE. Radioactivity was determined by an Instant Imager. The values reported are the means of at least three separate experiments (S.E. less than 15%).

turnover by affecting the rate of proteolytic cleavage. This has been shown to be decreased when Glu_{152} is bound to polyamine analogues (25).

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