Polyamine analogues inhibit the ubiquitination of spermidine/spermine *N*¹-acetyltransferase and prevent its targeting to the proteasome for degradation

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Spermidine/spermine *N*¹-acetyltransferase (SSAT), a key enzyme in mammalian polyamine catabolism, undergoes rapid turnover (half-life approx. 30 min) and is highly inducible in response to polyamine analogues such as bis(ethyl)spermine (BE-3-4-3), which greatly stabilize the enzyme. Rapid degradation of SSAT in reticulocyte lysates was preceded by formation of a ladder of ubiquitinated forms, and required the production of high-molecular-mass complexes with ubiquitin (HMM-SSAT-Ubs). Mutation of all 11 lysines in SSAT separately to arginine demonstrated that no single lysine residue is critical for its degradation *in vitro*, but mutant K87R had a significantly longer half-life, suggesting that lysine-87 may be the preferred site for ubiquitination. Mutations at the C-terminus of SSAT, such as

E171Q, resulted in marked stabilization of the protein, due to the lack of formation of the HMM-SSAT-Ubs. Addition of BE-3-4-3 prevented the accumulation of ubiquitin conjugates and the proteasomal degradation of wild-type SSAT. These results indicate that conformational changes brought about by the binding of polyamine analogues prevent the efficient polyubiquitination of SSAT, leading to a major increase in the amount of SSAT protein, and that alteration of the C-terminal end of the protein has a similar effect in preventing the productive interaction with an E2 or E3 component of the ubiquitin pathway.

Key words: *N*¹,*N*¹²-bis(ethyl)spermine, proteolysis, stabilization, ubiquitin.

INTRODUCTION

Spermidine/spermine *N*¹-acetyltransferase (SSAT) is a catabolic enzyme that plays a key role in the regulation of intracellular polyamine concentrations. It catalyses the *N*¹-acetylation of spermine and spermidine to form less charged derivatives that are either excreted from the cell or undergo further metabolism by polyamine oxidase to form spermidine or putrescine respectively [1]. Therefore, although polyamines are required for cell growth and differentiation, SSAT is thought to prevent overaccumulation of the higher polyamines from becoming toxic to the cell, and so maintains a balanced ratio of polyamines according to cellular needs [2].

SSAT is a highly inducible enzyme that responds to a wide range of stimuli, and this property is a major component in the regulation of polyamine homeostasis [1]. Endogenous SSAT activity is normally very low in cultured cells and tissues. Regulation of enzyme activity occurs via changes in the amount of enzyme protein, and these alterations are mediated through effects at several levels, including gene transcription, mRNA translation and protein turnover [3–6]. In the uninduced state, SSAT turns over very rapidly, although accurate measurements of the half-life are difficult to obtain because of the low level of the protein. Studies in which SSAT was expressed from a plasmid in stably transfected CHO cells indicated that SSAT has a half-life of approx. 30 min in the absence of inducers [7,29]. The rapid turnover of SSAT can also be readily demonstrated *in vitro* in reticulocyte lysate-derived systems [8]. In both systems, the degradation of SSAT is blocked by the proteasomal inhibitor MG132 (carbobenzoxycarbonyl-L-leucyl-L-leucyl-L-leucinal), indicating

that it is mediated via the proteasome, and in reticulocyte lysates the formation of ladders of high-molecular-mass bands, thought to be ubiquitin (Ub) conjugates, was demonstrated [7,29].

The known mammalian SSAT protein sequences show at least 95 % identity [1]. Despite its rapid turnover, SSAT has no PEST-rich sequence [9], although the C-terminal sequence MAT(A)EE has been shown to be required for rapid turnover, and may serve this function [8]. Purified SSAT has been reported to be phosphorylated by casein kinase II [10], but the stoichiometry and significance of this observation in terms of enzymic activity or stability is not known.

The most powerful inducers of SSAT known are polyamine analogues such as *N*¹,*N*¹²-bis(ethyl)spermine (BE-3-4-3) and *N*¹,*N*¹¹-bis(ethyl)norspermine (BE-3-3-3) [11,12]. Such analogues probably act by mimicking the ability of the natural polyamines, but are more potent due to a stronger interaction with the regulatory sites, and due to the fact that they are not themselves substrates for SSAT, so that their effect is not limited by SSAT-mediated degradation. BE-3-3-3 and BE-3-4-3 are effective anti-tumour agents in cell culture and animal models [13,14] and are currently undergoing clinical trials for this purpose. The induction of SSAT is clearly implicated in their anti-neoplastic activity, since cell lines resistant to these analogues do not induce SSAT, and restoration of SSAT in such cell lines restores sensitivity [15].

Exposure to BE-3-4-3 greatly increases the half-life of SSAT *in vivo* [5,6,15] and in reticulocyte lysate degradation systems [8]. Such stabilization is a major factor in the drug-induced increase in SSAT activity. These results suggest the possibility that a direct interaction between the analogue and the SSAT protein

Abbreviations used: SSAT, spermidine/spermine *N*¹-acetyltransferase; BE-3-4-3, *N*¹,*N*¹²-bis(ethyl)spermine; BE-3-3-3, *N*¹,*N*¹¹-bis(ethyl)norspermine; Ub, ubiquitin; Uba1, ubiquitin aldehyde; MG132, carbobenzoxycarbonyl-L-leucyl-L-leucyl-L-leucinal; HMM-SSAT-Ub, high-molecular-mass SSAT complexes with ubiquitin; FI and FII, fractions I and II respectively of rabbit reticulocyte lysate; DHFR, dihydrofolate reductase.

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brings about a conformational change that reduces the ability of the protein to serve as a substrate for the Ub/proteasome pathway.

Many short-lived proteins are substrates for degradation by the Ub/proteasome pathway [16,17]. Targeting for degradation by the 26 S proteasome occurs in a series of reactions that involve the initial covalent attachment of Ub to a free amino group of the protein substrate [18]. Additional Ub molecules are ligated to the modified substrate to generate a multi-Ub chain [19] that provides increased affinity of the targeted protein for the 19 S regulatory subunit of the proteasome [20]. Multiple chains of at least four residues are needed to provide this recognition [21]. The initial step in this cascade is catalysed by a Ub-activating enzyme (E1) that mediates the ATP-dependent activation of the C-terminal glycine of Ub, forming a thiol ester with E1. This activated Ub is transferred from E1 to a member of a family of Ub-conjugating (E2) enzymes that, usually in a complex with a Ub-protein ligase (E3), forms an isopeptide bond between Ub and the protein substrate [17].

In the present experiments, we have investigated the mechanism by which BE-3-4-3 prevents proteasomal degradation of SSAT. Our results suggest a mechanism by which polyamine analogues induce a conformational change in the wild-type protein that either masks a degradation signal recognized by the E2-E3 ligase complex or precludes the ubiquitination of specific lysine residues in SSAT by this complex. This mechanism is strikingly different from that which has been reported previously in studies of the ligand-mediated inhibition of proteolysis in rabbit reticulocyte extracts in which the target protein was dihydrofolate reductase (DHFR) [22,23]. The degradation of DHFR is inhibited by the binding of the inhibitor methotrexate, but the effect was not brought about by interference with ubiquitination, but with the unfolding of the substrate moiety that is necessary for degradation by the proteasome [23]. Our results therefore indicate a novel manner by which protein stabilization, leading to an increase in the cellular content of an important regulatory enzyme, can occur.

EXPERIMENTAL

Materials

Oligodeoxynucleotides were purchased from Gibco BRL (Grand Island, NY, U.S.A.). L-[³⁵S]Methionine (translation grade) was obtained from DuPont NEN (Boston, MA, U.S.A.). [1-¹⁴C]Acetyl-CoA (50 Ci/mol) was purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.). TNT T7 coupled rabbit reticulocyte lysate translation system and RNasin were from Promega (Madison, WI, U.S.A.). Rabbit reticulocyte lysate prepared from phenylhydrazine-treated New Zealand White rabbits for use in degradation assays was obtained from Cocalico Biologicals (Reamstown, PA, U.S.A.). MG132 was obtained from Calbiochem (San Diego, CA, U.S.A.). Ub aldehyde (Ubal), bovine K48R-Ub and bovine methylated Ub were purchased from Boston Biochem (Cambridge, MA, U.S.A.). Plasmid purification columns were from Qiagen (Chatsworth, CA, U.S.A.). ATP, creatine kinase, phosphocreatine, cycloheximide, Ub and isopropyl β -D-thiogalactopyranoside were purchased from Sigma (St. Louis, MO, U.S.A.). BE-3-4-3 and BE-3-3-3 were kindly provided by Dr Raymond J. Bergeron (University of Florida, Gainesville, FL, U.S.A.).

Preparation of mutants of SSAT

Selected residues in SSAT were mutated using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene, La

Jolla, CA, U.S.A.) as described previously [24]. Mutant plasmids were sequenced to confirm the presence of the desired mutation and to ensure the absence of secondary mutations. Alterations to Arg used either codon AGA or AGG, which are used for some of the residues in native SSAT. These changes did not introduce codons that are poorly translated.

Expression and degradation of SSAT

The plasmid pSAT9.3, containing the human SSAT cDNA cloned into the Bluescript vector, was used to express the protein from the T7 promoter using the rabbit reticulocyte TNT coupled transcription/translation system, as described previously [24]. The activity of the proteins synthesized in this way was determined by running parallel reactions using either unlabelled or ³⁵S-labelled methionine. Differences in the amount of protein produced were corrected by normalizing the activity measurements with the amount of ³⁵S-labelled protein synthesized, as determined by Phosphorimage analysis of the scanned gels. ³⁵S-labelled wild-type and mutant SSAT proteins were used as substrates for degradation in a standard 200 μ l (or fraction thereof) reticulocyte lysate assay that contained 40 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine, 0.05 mg/ml creatine kinase, 0.1 mM cycloheximide and 50 μ l of unfractionated rabbit reticulocyte lysate, unless stated otherwise. The TNT synthesized substrate constituted 2% (v/v) of the degradation assay. Assays were incubated at 37 °C, and 30 μ l aliquots were removed at the time intervals indicated in the Figures. Samples were mixed with SDS sample buffer, boiled for 5–7 min and resolved on 15% (w/v) polyacrylamide gels. The gels were fixed and dried, and the resulting images were scanned and quantified using a Molecular Dynamics Phosphorimager-SI and ImageQuaNT application software. Alternatively, TNT synthesized [³⁵S]SSAT was precipitated using ammonium sulphate to remove free [³⁵S]-methionine, and the pellet obtained after centrifugation (15000 g for 15 min) was washed, resuspended in buffer A (50 mM Tris/HCl, pH 7.5, 2.5 mM dithiothreitol and 0.1 mM EDTA) and washed repeatedly with 6 ml of buffer A during concentration using a Centricon-10 microconcentrator (Amicon). The resulting protein was used as a substrate for a standard degradation assay in which trichloroacetic acid was added to a final concentration of 5% (v/v) after timed incubation intervals and the resulting mixture was placed on ice for 15 min. The clear supernatant obtained after centrifugation (15000 g, 15 min) was used to monitor the acid-soluble radioactivity to determine the rate of SSAT degradation.

Preparation of rabbit reticulocyte fraction II (FII)

Rabbit reticulocyte lysate was fractionated on a column of DE52-cellulose (Whatman) pre-equilibrated with 3 mM potassium phosphate, pH 7.0, and 1 mM dithiothreitol. Unadsorbed material containing Ub and designated fraction I (FI) was collected, while FII was eluted from the column in 20 mM Tris/HCl buffer, pH 7.2, containing 0.5 M KCl and 1 mM dithiothreitol. The Ub-depleted FII was concentrated by ammonium sulphate precipitation and dialysed as described previously [25].

Expression of rat UBC4-1 isoform in *Escherichia coli*

A cDNA plasmid encoding the rat UBC4-1 Ub-conjugating enzyme [26] was generously donated by Dr Simon S. Wing (McGill University, Montreal, Canada). The pET-UBC4 plasmid

was expressed in B384 (DE3) lysogen *E. coli* (Novagen) in LB medium supplemented with 50 µg/ml ampicillin at 30 °C, and crude bacterial extracts were prepared after a 2 h induction with 1 mM isopropyl β-D-thiogalactopyranoside. Cell pellets were washed in ice-cold buffer A and the cells were lysed by sonication at 4 °C. The supernatant obtained after centrifugation of the bacterial lysate at 30000 *g* for 45 min was assayed for protein content [27] and used to supplement degradation assays, as described in the legends to the appropriate Figures.

Assay for SSAT activity

Proteins synthesized from plasmid in the TNT system in the presence of unlabelled methionine were diluted in buffer A and assayed for SSAT activity at 30 °C, as described previously [28]. A standard assay mixture contained 50 mM Tris/HCl, pH 7.8, 3 mM spermidine and 16 µM [1-¹⁴C]acetyl-CoA in a total volume of 100 µl.

RESULTS

Ub-dependent degradation of SSAT *in vitro*

Previous results have shown that *in vitro*-translated [³⁵S]-methionine-labelled SSAT undergoes ATP-dependent degradation in a rabbit reticulocyte lysate assay [8], and that the observed proteolysis can be inhibited both *in vitro* and in CHO cells by MG132 [29]. A ladder of complexes observed by

SDS/PAGE was formed from [³⁵S]SSAT on incubation with such reticulocyte lysates (Figure 1A). In order to confirm that these were SSAT-Ub conjugates, the degradation assay was performed in the presence of Ubal, an isopeptidase inhibitor that stabilizes protein-Ub conjugates [30,31]. Treatment with 0.5 µM Ubal stabilized the SSAT conjugates formed and decreased the rate of SSAT degradation (total radioactivity remaining after 60 min of incubation with 0.5 µM Ubal was 45% of the original, compared with 30% remaining in the no-treatment control) (Figure 1B). A parallel assay supplemented with 5 µM Ubal failed to form the high-molecular-mass SSAT complexes that are present at the top of the gels (referred to as HMM-SSAT-Ub conjugates). However, the pattern of SSAT conjugation could be restored upon addition of 0.2 µM Ub to the assay containing 5 µM Ubal, indicating that SSAT is a substrate for the ubiquitination pathway and that the ladder of conjugates corresponds to polyubiquitinated forms of the protein. Under these conditions, a substantial fraction of the SSAT was converted into high-molecular-mass SSAT conjugates.

Effects of polyamine analogues on the degradation of wild-type SSAT and E152Q-SSAT

Incubation of wild-type SSAT with 100 µM of the polyamine analogue BE-3-4-3 caused a marked stabilization of the protein in the rabbit reticulocyte lysate assay (Figure 2A, left lanes). There are two possible mechanisms by which BE-3-4-3 could stabilize SSAT. In the first case, BE-3-4-3 could alter the ability of the wild-type SSAT protein to undergo ubiquitination. Alternatively, the analogue may inhibit or interfere directly with a component of the Ub/proteasome pathway. To distinguish between these two possibilities, the ubiquitination of wild-type SSAT was compared with that of a mutant of SSAT in which glutamate-152 was substituted with glutamine (E152Q-SSAT). This mutation was shown previously to disrupt the binding of BE-3-4-3 to the protein [24].

The presence of BE-3-4-3 had little effect on the time course of ubiquitination or the stability of E152Q-SSAT (Figure 2A, right lanes). In contrast with the striking decrease in HMM-SSAT-Ub conjugates formed with wild-type SSAT, ubiquitination of the mutant E152Q-SSAT and its degradation were not attenuated greatly by BE-3-4-3 (Figure 2A). Figures 2(B) and 2(C) show an analogue dose-response experiment that included Ubal and MG132 in the reactions to monitor more clearly the accumulation of polyubiquitinated conjugates of wild-type or E152Q-SSAT. Increasing concentrations of BE-3-4-3 or BE-3-3-3 caused stabilization of the intact 20 kDa wild-type SSAT protein, with a dose-dependent decrease in accumulated HMM-SSAT-Ub conjugates (Figure 2B). In contrast, neither analogue prevented Ub-conjugate formation with the mutant E152Q-SSAT (Figure 2C), and there was only a marginal effect on ubiquitination of the 20 kDa E152Q-SSAT protein at an analogue concentration of 100 µM. These results suggest that BE-3-4-3 and BE-3-3-3 are unlikely to interact with or inhibit components of the ubiquitination pathway directly. Rather, it appears that binding of either analogue to wild-type SSAT may induce a conformational change in the protein that inhibits its ubiquitination and therefore prevents its targeting to the proteasome for degradation.

Effects of C-terminal mutations on the stability of SSAT

SSAT is a homodimer with a subunit size of 171 amino acids [32]. Mutation of the C-terminal residue in SSAT to generate a fully active E171Q mutant stabilized the protein against degradation

Figure 1 Effect of Ubal on the accumulation of ubiquitin complexes of SSAT

[³⁵S]SSAT was synthesized in the TNT-coupled transcription/translation system and used as a substrate in the degradation assay described in the Experimental section. Reactions were incubated at 37 °C in the absence or presence of the indicated concentration of Ubal, supplemented or not with 0.2 µM Ub. At the times shown, 30 µl aliquots were removed and analysed by SDS/PAGE (A) and Phosphorimage analysis (B), as described in the Experimental section. Molecular-mass markers are shown on the left in kDa in (A). In (B), 'input' is defined as the sum of the 20 kDa intact SSAT protein and the HMM-SSAT-Ub conjugates at zero time.

Figure 2 Effects of BE-3-4-3 on the degradation and ubiquitination of wild-type SSAT and E152Q-SSAT

Assays were carried out as described in the legend to Figure 1. In (A), ^{35}S -labelled wild-type SSAT or the E152Q mutant protein was used as a substrate for a time course of degradation in the absence or presence of $100\ \mu\text{M}$ BE-3-4-3 added to the reaction at zero time. Panels (B) and (C) show a dose-response study in which reactions were incubated with the indicated concentration of polyamine analogue in the presence of $5\ \mu\text{M}$ Ubal, $50\ \mu\text{M}$ MG132 and $0.1\ \text{mM}$ Ub. Shown are the results of reactions containing ^{35}S -labelled wild-type SSAT (B) and ^{35}S -labelled E152Q-SSAT (C). Molecular-mass markers are shown on the left in kDa.

in the reticulocyte lysate system [8]. There was almost a complete absence of Ub conjugates when mutant E171Q-SSAT was substituted for wild-type SSAT (Figure 3). A similar lack of ubiquitinated complexes was observed with other C-terminal mutants, including E170Stop and +172A/173A (results not shown). These results suggest that an intact C-terminal sequence in SSAT may be required for a maximal rate of Ub conjugation to SSAT under the standard reaction conditions.

Reactions containing ^{35}S -labelled E171Q-SSAT were incubated in the presence of $0.5\ \mu\text{M}$ or $5\ \mu\text{M}$ Ubal to examine whether there was an increase in the steady-state level of any E171Q-Ub conjugates formed under these conditions. The presence of Ubal alone showed that some conjugation does occur and that it can be increased significantly when the reaction is

Figure 3 Comparison of the stability and ubiquitination of wild-type SSAT and E171Q-SSAT in unfractionated rabbit reticulocyte lysate

Assays were carried out as described in the legend to Figure 1 using ^{35}S -labelled wild-type SSAT or E171Q-SSAT protein as the substrate for degradation. Aliquots of $30\ \mu\text{l}$ were removed at the times indicated for SDS/PAGE analysis. Molecular-mass markers are shown on the left in kDa.

Figure 4 Effects of Ubal on the accumulation of Ub complexes of mutant E171Q-SSAT

Shown in (A) are assays processed as described in the legend to Figure 1 using ^{35}S -labelled E171Q-SSAT as a substrate for the degradation reaction. Reactions were incubated in the absence or presence of the indicated concentration of Ubal supplemented or not with $0.2\ \mu\text{M}$ Ub. Aliquots of $30\ \mu\text{l}$ were removed at the times indicated and resolved by SDS/PAGE. Molecular-mass markers are shown on the left in kDa. Panel (B) provides the results from Phosphorimage analysis, where 'input' is defined as the calculated volume of the sum of the $20\ \text{kDa}$ intact SSAT protein and the HMM-SSAT-Ub conjugates.

supplemented with Ub (Figure 4). However, the proportion of HMM-SSAT-Ub conjugates observed with wild-type SSAT under the same conditions was much greater than for E171Q-SSAT (compare Figure 4 with Figure 1). This suggests that the interaction of E171Q with the necessary E2 and E3 enzymes may be less efficient than for wild-type SSAT, and/or that any

Figure 5 Effects of crude rat UBC4-1 extract on ubiquitination of wild-type SSAT in FII

UBC4-1 was expressed from a pET-UBC4 plasmid in *E. coli* and crude extract was prepared as described in the Experimental section. ³⁵S-labelled wild-type SSAT was synthesized in the TNT system and added to a 120 μ l degradation mix that substituted FII (~ 2.5 mg/ml) for unfractionated lysate in the assay. Aliquots of 30 μ l were removed at the times indicated and resolved by SDS/PAGE. Molecular-mass markers are shown on the left in kDa. The Figure compares ubiquitination of SSAT in the presence of 0.1 mg/ml crude bacterial extract without UBC4-1 (lanes 1–3) and in the presence of 0.1 mg/ml UBC4-1 extract (lanes 4–12). The reactions shown in lanes 1–6 contained 0.2 μ M Ub; this was substituted in lanes 7–9 with 0.2 mM methylated Ub and in lanes 10–12 with 0.1 mM purified recombinant K48R-Ub.

conjugates formed are subject to rapid disassembly by Ubal-sensitive isopeptidases.

Ubiquitination of SSAT by FII of reticulocyte lysates requires factor(s) from FI

The rabbit reticulocyte lysate was passed over a column of DEAE-cellulose to collect unadsorbed FI and the bound FII that was eluted in high salt [33]. FII allows the study of the factors required for the ubiquitination and degradation of SSAT in a system that depends on adding back exogenous ATP and Ub [25].

Wild-type SSAT was not degraded in FII, with only limited ubiquitination and a complete absence of HMM-SSAT-Ub conjugates even after addition of Ub (Figure 5, lanes 1–3). However, the pattern of ubiquitination of wild-type SSAT observed previously in unfractionated reticulocyte lysate could be restored when FI was added back to FII (results not shown). Addition to FII of a crude extract of the rat Ub-conjugating enzyme UBC4-1 also restored the pattern of ubiquitination observed upon adding back FI, indicating that ubiquitination by FII is limited by the amount of such an E2 enzyme that is present in FI (Figure 5, lanes 4–6).

Reactions in which methylated Ub or a K48R mutant of Ub was substituted for Ub produced an altered pattern of SSAT conjugation (Figure 5, lanes 7–12). In both cases, the predominant species was the mono-Ub conjugate of SSAT. Very little of the higher forms was seen when K48R-Ub was used (Figure 5, lanes 9–12), but a weak ladder of higher-molecular-mass products was seen with methylated Ub. This may have been due to the presence of small amounts of unmethylated Ub in the preparation used or to the presence of small amounts of Ub or SSAT-Ub conjugates from the substrate synthesis reaction.

Table 1 Effects of lysine $\rightarrow$ arginine mutations in SSAT on activity and half-life

Wild-type SSAT and mutants were synthesized as both unlabelled and ³⁵S-labelled proteins and assayed for enzyme activity as described in the Experimental section. The half-life of each ³⁵S-labelled protein was determined after using each as a substrate in the degradation assay described in the Experimental section. Enzyme activity is shown as the mean of duplicate or triplicate assays. These values were normalized from triplicate gels obtained from a representative experiment in which all mutants were assayed at the same time. The values agree within $\pm 15\%$ of the mean. Individual half-life values determined from gels using at least four time points for each mutant in replicate experiments are shown. Note that the first values for each mutant were derived from a single experiment run under identical conditions using the same batch of reticulocyte lysate.

Position of mutation	Enzyme activity (% of wild type)	Half-life (min)
None	100	28, 29, 28
K3R	134	23, 18
K22R	149	17
K26R	120	25
K39R	105	21
K61R	102	21, 20
K87R	70	65, 59, 75
K111R	100	16, 13
K141R	35	14, 13
K158R	139	23, 25
K161R	98	21, 21
K166R	78	27, 22

These results provide evidence that there is a single preferred site for the addition of Ub to SSAT.

Degradation and ubiquitination of lysine $\rightarrow$ arginine mutants of SSAT

In order to investigate lysine residues in SSAT that could serve as acceptor sites for Ub, site-directed mutagenesis was carried out on each of the 11 lysine residues in SSAT. Each was changed individually to arginine. The SSAT protein was expressed in the TNT system and its enzymic activity was measured as described previously [24]. The half-life in the reticulocyte lysate degradation system was also measured (Table 1).

Comparison of the enzymic activities of the SSAT mutant proteins confirmed that, as expected, in most cases the conservative replacements with arginine had little effect on the enzymic activity and therefore are unlikely to greatly affect the overall structure of the native SSAT dimer (Table 1). One of these mutants, K141R, showed a major decrease in activity, and this was due to an increase in the apparent K_m for spermidine from ~ 50 μ M to ~ 390 μ M. This result is consistent with previous studies showing that amino acid residues close to this site are involved in substrate binding [24,32]. All other lysine $\rightarrow$ arginine mutants had K_m values comparable with that of the wild-type enzyme (30–60 μ M).

Only one of the lysine $\rightarrow$ arginine mutants, K87R, had a prolonged half-life, which was 65 min compared with the 28 min observed with wild-type SSAT (Table 1). The appearance of HMM-SSAT-Ub conjugates was clearly much less with this mutant SSAT than with wild-type SSAT (compare Figure 6A with Figures 1 and 2). Furthermore, when tested in the system described above in which the SSAT mutant proteins were added to FII supplemented with UBC4-1 and K48R-Ub to halt the ubiquitination reaction at the monomer conjugate, the K87R-SSAT clearly showed the least accumulation of HMM-SSAT-Ub conjugates (Figure 6B). In addition, a much greater percentage of the K87R mutant was unconjugated after 60 min compared

Figure 6 Degradation and ubiquitination of lysine → arginine mutants of SSAT

(A) Degradation of K87R-SSAT in an unfractionated reticulocyte lysate. Assays were carried out as described in the legend to Figure 1 using ^{35}S -labelled K87R-SSAT as the substrate. Aliquots were removed at the times indicated and resolved by SDS/PAGE as described in the Experimental section. (B) Formation of mono-ubiquitinated SSAT for each of the mutant SSAT proteins in the presence of K48R-Ub. Assays were carried out using the mutant SSAT proteins described in Table 1 synthesized in the presence of [^{35}S]methionine in the TNT system. The proteins were added to FI supplemented with UBC4-1 and 0.2 mM recombinant K48R-Ub, as in lanes 10–12 of Figure 5. The percentages of the zero-time 20 kDa SSAT protein present as either the unmodified 20 kDa band (no shading) or the mono-ubiquitinated band (grey shading) after a 60 min incubation are shown as a histogram in (C). Molecular-mass markers are shown on the left in kDa in (A) and (B).

with all other mutants (Figure 6C). These results suggest that lysine-87 may serve as a primary site for ubiquitination of SSAT in this system; however, in its absence, one or more other lysine residues may be used.

Proteasomal degradation of wild-type SSAT and selected mutants

The rapid degradation of wild-type SSAT and its stabilization in response to the binding of BE-3-4-3 was also demonstrated clearly by measuring the conversion of [^{35}S]SSAT into trichloroacetic acid-soluble radioactivity (Figure 7). Proteasomal degradation was determined as the difference between trichloroacetic acid-soluble radioactivity released in the absence compared with the presence of 50 μM MG132. There was a substantial release of radioactivity from wild-type SSAT, which was completely prevented by the presence of BE-3-4-3. The release of radioactivity from mutant E152Q-SSAT was similar to wild type, but, in this case, BE-3-4-3 was less effective in preventing release. There was virtually no conversion of radioactivity from the E171Q mutant into a trichloroacetic acid-soluble form (Figure 7). Release of trichloroacetic acid-soluble radioactivity from the K87R mutant was markedly lower than from the wild type, but was still observable, confirming that this mutant is more stable than the wild-type protein.

DISCUSSION

The present study provides direct *in vitro* evidence for involvement of the ubiquitination system in the conjugation and degradation of human SSAT (Figure 1) [8,34]. As much as 68% of the SSAT was converted into Ub conjugates in the presence of 5 μM Ubal, provided that the reticulocyte lysate system was supplemented with additional Ub (Figure 1). The deficiency in free Ub available for conjugation to SSAT in the presence of 5 μM Ubal is presumably due to a more complete inactivation of isopeptidase activity than at an inhibitor concentration of 0.5 μM . Isopeptidase activity would be expected to replenish the steady-state level of free Ub upon hydrolysis of polyUb chains generated after substrate proteolysis. Since this experiment uses an unpurified system in which SSAT is one of many substrates for the Ub pathway, it is likely that both substrate competition for Ub and inactivation of the Ub-regenerating system accounts for the decrease in HMM-SSAT-Ub conjugates formed under these conditions. The finding that SSAT was not degraded despite the increased ubiquitination observed in the presence of supplemental Ub may be due to inhibition of the proteasome by the accumulation of unanchored polyubiquitinated chains produced after proteolysis of unlabelled substrates.

The high rate of SSAT turnover in rabbit reticulocyte lysate under the conditions described is consistent with a half-life of

Figure 7 Proteasomal degradation of wild-type SSAT and selected mutants

³⁵S-labelled wild-type (wt) SSAT and selected mutants (E152Q, E171Q, K87R) were synthesized in the TNT system, and the proteins were precipitated by ammonium sulphate to remove free [³⁵S]methionine and concentrated as described in the Experimental section. An aliquot of ³⁵S-labelled protein corresponding to ~ 50 000 c.p.m. was added to a 200 μ l degradation assay and incubated at 37 °C. Aliquots of 30 μ l were removed from the reaction at the times indicated and precipitated with trichloroacetic acid (TCA) as described in the Experimental section. Parallel assays were done in the absence (DMSO vehicle) or presence of 50 μ M MG132, and the acid-soluble radioactivity released during each incubation was measured. Proteasomal-mediated degradation was determined by subtracting any radioactivity released in the presence of MG132 from that released in the presence of DMSO, and plotted as trichloroacetic acid-soluble radioactivity expressed as a percentage of total radioactivity in the assay aliquot at zero time.

approx. 30 min determined for SSAT in CHO cells. This estimated half-life was extended to > 300 min in cells treated concurrently with cycloheximide and MG132 [29], which lends physiological relevance to the data obtained in the cell-free system. The rapid turnover of SSAT is also consistent with its role in regulating the polyamine catabolic pathway. Ornithine decarboxylase, another short-lived enzyme controlling polyamine biosynthesis whose negative regulation by polyamines is inversely related to that of SSAT, is also targeted for degradation by the 26 S proteasome, but in a unique, Ub-independent manner by its association with antizyme [35].

With the exception of the transcriptional activator MyoD, in which the N-terminal residue serves as the Ub acceptor site [18], the ϵ -amino group of one or more internal lysine residues serves as the target acceptor site(s) for ubiquitination of endogenous substrates. To this end, we made single conservative mutations to arginine of all 11 lysine residues in SSAT in order to try to identify a potential Ub acceptor site. With the exception of the K141R mutant, in which the first residue in a KRR motif that makes up part of the active site is replaced [1,24,32], causing a significant increase in the K_m for spermidine, these mutations had little effect on SSAT activity. The K87R mutant, whose half-life was more than twice that of wild-type SSAT under the same conditions, was the only one that showed a significant increase in half-life. Although there was some formation of HMM-SSAT-Ub-conjugates with the K87R mutant, this did not compare with the abundance of polyubiquitinated wild-type SSAT or other lysine $\rightarrow$ arginine mutants. These results suggest that lysine-87 may be a preferred acceptor site for the initial Ub addition, although a secondary, less efficient site may be used when the primary site is mutated. Other examples of proteins in which lysine residues were mutated either singly or in clusters have illustrated that no single lysine is necessary for ubiquitination. These include the transcription factors c-Jun [36] and Gcn4 [37]. It has been suggested that, in some cases, any one of a number

of lysines can serve as a Ub acceptor site providing that they are sterically available to the relevant ubiquitination enzymes [38]. In contrast, the ubiquitination of I κ B α (inhibitor of nuclear factor κ B) involves two distinct lysine residues at positions 21 and 22 [39].

Fractionation of the reticulocyte lysate extracts suggests that the UBC4-1 enzyme can bring about the ubiquitination of human SSAT in FII (Figure 5). This enzyme is a 16.7 kDa ubiquitous rat isoform of mammalian UBC4/UBC5 enzymes that belongs to the Class I family of E2s [40]. The rat UBC4-1 and UBC4-testis isoforms [26,40] are identical in amino acid sequence with the human E2s UbcH5B and UbcH5C respectively [41]. In addition to their role as Ub carrier proteins, E2 enzymes may be involved in substrate recognition by conjugating substrates directly or, probably more commonly, through an E2-E3 complex [42]. A large family of E2 enzymes exists in which enzymes are classified according to similarity of structure, but differ in their properties and intracellular localization [43]. UBC4/UBC5 E2s were first described in *Saccharomyces cerevisiae*, where they mediate the selective degradation of short-lived and abnormal proteins [16].

Since FII is essentially devoid of free Ub, it was possible to use methylated Ub or K48R-Ub to examine the effect on the pattern of SSAT ubiquitination in FII supplemented with UBC4-1. In the presence of methylated Ub or K87R-Ub, the predominant species formed corresponded to the mono-conjugate (Figure 5). Methylated Ub should serve as a chain terminator, irrespective of the site in Ub used for the formation of additional conjugates. However, the lack of such conjugates with recombinant K48R-Ub suggests that the HMM-SSAT-Ub chains are assembled through lysine-48 of Ub, which is the linkage most commonly utilized by substrates destined for proteasomal degradation [44].

Polyamine analogues such as the bis(ethyl)spermine derivatives used in the present study were developed as an alternative to enzyme inhibitors of the polyamine pathway and are undergoing evaluation as cancer chemotherapeutic agents [45]. They were designed to mimic polyamines by down-regulating the synthesis of the natural polyamines through suppression of the biosynthetic enzymes, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, but cannot substitute for the natural polyamines in supporting cell growth [46]. An important effect of many of these analogues is their ability to induce SSAT activity, which leads to depletion of cellular polyamine pools through catabolism of spermine and spermidine. Although the mechanism responsible for this induction is not fully understood, at least part of the response is due to stabilization of the SSAT protein.

The results in Figure 2(A) demonstrate that SSAT is susceptible to Ub-mediated proteasomal degradation in rabbit reticulocyte lysate, but is largely protected from proteolysis when incubated in the presence of 100 μ M BE-3-4-3. This protection is due to the substantial decrease in the formation of HMM-SSAT-Ub conjugates in the presence of the analogue, and it is likely that a conformational change in the SSAT protein that occurs upon analogue binding inhibits the interaction with the Ub-conjugating system. The first part of this hypothesis, that analogue binding affects the conformation of SSAT, is consistent with previous results using purified recombinant SSAT. These showed that the native protein conformation is unstable and that enzyme activity is lost rapidly when incubated in buffer at 30 °C, but the loss in activity could be prevented completely by the addition of 2.5 μ M BE-3-4-3 from the start of incubation, or was reversed when the analogue was added after 30 min. In addition, the susceptibility of purified SSAT to digestion by trypsin was also prevented by co-incubation with the analogue, indicating that, upon binding to the protein, BE-3-4-3 converts it into a form that is resistant

to cleavage by trypsin [24]. The higher concentration of BE-3-4-3 required to bring about the change in SSAT conformation that imparts resistance to proteasomal degradation in the reticulocyte lysate degradation/ubiquitination assays studied in Figure 2 presumably reflects non-specific binding of the analogue to anionic components in the lysate.

The finding that BE-3-4-3 and BE-3-3-3 produce a dose-dependent stabilization of the 20 kDa SSAT protein band and a decrease in the accumulation of HMM-SSAT-Ub supports the second part of the hypothesis, which is that the alteration in the configuration of SSAT in response to binding of such polyamine analogues prevents a productive interaction with a Ub ligase complex.

It is likely that this conformational change involves the positioning of the C-terminal domain of SSAT. The C-terminal -MAT(A)EE domain of SSAT is important for activity and for polyamine-analogue-mediated protection of the protein from protease digestion [24]. Mutation of the extreme C-terminal residue in SSAT to form mutant E171Q (or the addition of two terminal lysine or alanine residues) results in the retention of SSAT activity, and each mutant is stable in reticulocyte lysates even in the absence of polyamine analogues [8]. The experiment described in Figure 3 shows that this stability also appears to be due to a failure to form HMM-SSAT-Ub conjugates. It would be very useful to identify a catalytically active but stable mutant of SSAT that could be used in the absence of polyamine analogues to study the effects of overexpression of high SSAT activity in cells. However, E171Q-SSAT clearly can form conjugates with Ub when incubated in the presence of Ubal and excess Ub (Figure 4), indicating that the stability observed in Figure 3 is likely to be due to a lower affinity for the enzymes necessary for the ubiquitination of SSAT. From this result, it seems unlikely that mutant E171Q would be a suitable candidate for overexpression of SSAT in cells.

Detailed studies of the ability of the folic acid analogue, methotrexate, to prevent the degradation of a modified DHFR N-end rule substrate have been carried out [22,23]. These showed that the binding of the drug does not inhibit ubiquitination, but instead prevents the unfolding of the substrate moiety that is necessary for degradation by the proteasome. Our results show that polyamine analogues stabilize SSAT by a different and unique mechanism in which it is the ubiquitination steps that are affected.

In summary, the stabilization of SSAT from its normal rapid turnover appears to be due to a conformational change in the protein after the non-covalent binding of the polyamine or analogue that decreases the ability of the protein to serve as a substrate for polyubiquitination, which is likely to occur at lysine-87. The initial step in this ubiquitination requires an E2 Ub-conjugating enzyme similar to rat UBC4-1. Determination of the three-dimensional structure of SSAT by X-ray crystallography and reconstitution of an *in vitro* system using purified components to try to pair the relevant E2 and E3 enzymes, which are in progress, will enable this novel system for the regulation of a key metabolic enzyme to be fully characterized and understood at the molecular level.

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