Properties of the Spermidine/Spermine N\textsuperscript{1}-Acetyltransferase
Mutant L156F That Decreases Cellular Sensitivity to the Polyamine Analogue N\textsuperscript{1}, N\textsuperscript{11}-Bis(ethyl)norspermine

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Properties of a mutant form of spermidine/spermine \( N^1 \)-acetyltransferase, L156F (L156F-SSAT), that is present in Chinese hamster ovary cells selected for resistance to the polyamine analogue \( N^1, N^11 \)-bis(ethyl)norspermine (BE 3-3-3) were investigated. Increased \( K_m \) values, decreased \( V_{\text{max}} \) values, and decreased \( k_{\text{cat}} \) values with both polyamine substrates, spermidine and spermine, indicated that L156F-SSAT is an inferior and less efficient acetyltransferase than wild-type SSAT. Transfection of L156F-SSAT into C55.7Res cells indicated that cellular SSAT activity per nanogram of SSAT protein correlated well with the \textit{in vitro} data and was also \(-20\) fold less for the mutant protein than for wild-type SSAT. Increased expression of L156F-SSAT was unable to restore cellular sensitivity to BE 3-3-3 despite providing measurable basal SSAT activity. Only a 4-fold induction of L156F-SSAT activity resulted from the exposure of cells to the polyamine analogue, whereas wild-type SSAT was induced \(-300\)-fold. Degradation studies indicated that BE 3-3-3 cannot prevent ubiquitination of L156F-SSAT and is therefore unable to protect the mutant protein from degradation. These studies indicate that the decreased cellular sensitivity to BE 3-3-3 is caused by the lack of SSAT activity induction in the presence of the analogue due to its inability to prevent the rapid degradation of the L156F-SSAT protein.

The natural polyamines putrescine, spermidine, and spermine are essential for normal growth and cell proliferation (1–4). It has also been demonstrated that polyamine synthesis is increased in many tumors and tumor-derived cell lines (3). The polyamine pathway was therefore thought to be an ideal target of chemotherapeutic intervention designed to disrupt abnormal tumor cell growth (1, 5). However, the initial attempts to exploit this target using inhibitors of the polyamine synthetic enzymes ornithine decarboxylase and \( S \)-adenosylmethionine decarboxylase produced disappointing results \textit{in vivo} because of the compensatory cellular uptake of polyamines to fulfill growth requirements (6, 7). To circumvent this problem, structural polyamine analogues were designed using the rationale that if the therapeutic agent closely resembled the essential natural compound, then the cellular self-regulatory mechanisms controlling uptake and synthesis might remain intact, but the growth-affecting functions might be disrupted (5, 8, 9).

Some of the first polyamine analogues synthesized and tested as antiproliferative agents were bis(ethyl) analogues of spermine, BE 3-4-3, BE 3-3-3, and BE 4-4-4-4 (5). Since that time, a wide variety of structural polyamine analogues have been synthesized with modifications to the natural polyamine structures that include small symmetrical terminal substituents, large unsymmetrical terminal substituents, increased or decreased internal carbon chain lengths, the introduction of sites of unsaturation in the internal carbon chains, and even the linking together of two or more of these altered structures (5, 10, 11). Results from testing in cell systems also have been widely varied and, unfortunately, few clear cut structure/function relationships have been discernible. Despite that, several of the polyamine analogues have shown therapeutic promise, and BE 3-3-3 is now in phase II clinical trials with multiple tumor types (12). However, to actively design better chemotherapeutic agents it is necessary to understand which actions are essential to the cytotoxicity, and that is something that has been difficult to state with certainty for the polyamine analogues.

Investigating the mechanisms by which cells become resistant to drugs is one approach to determining the mechanism of action of the drug. We have found that a point mutation in the SSAT gene led to a loss of sensitivity to BE 3-3-3 in the C55.7Res cell line, which was selected for resistance to polyamine analogues (13). It had been demonstrated that there is an apparent correlation between the cytotoxic effects of some of the bis(ethyl)polyamine analogues and the induction of SSAT activity (14, 15), but it had not been shown that this was integral to the cytotoxicity of the analogue. We now report the detailed characterization of the mutant L156F-SSAT protein both \textit{in vitro} and in C55.7Res cells. The results demonstrate that this single amino acid change has ramifications for the enzyme activity and binding with both natural polyamine substrates, spermidine and spermine, and that the mutation renders the protein a less efficient acetyltransferase than the wild-type SSAT protein. It is also now demonstrated that the L156F change reduces interaction of the polyamine analogue with the SSAT protein and that this is directly linked to the

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\textsuperscript{1} The abbreviations used are: BE 3-4-3, \( N^1, N^{11} \)-bis(ethyl)spermine; BE 3-3-3, \( N^1, N^{11} \)-bis(ethyl)norspermine; SSAT, spermidine/spermine \( N^1 \)-acetyltransferase; wtSSAT, wild-type SSAT; CHENSpm, \( N^1 \)-ethyl-\( N^{11} \)-(cyclohexyl)methyl)-4,8-diazaundecane; BE 4-4-4-4, 1,19-di-(ethylamino)-5,10,15-triazanodacene; MTT, 3-(4,5-Dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide; CHO, Chinese hamster ovary; CMV, cytomegalovirus; Ub, ubiquitin; Ubal, ubiquitin aldehyde; IPTG, isopropyl \( \beta \)-thiogalactopyranoside.
EXPERIMENTAL PROCEDURES

Materials—[1-14C]acetyl-CoA (63 Ci/mol) was obtained from ICN Biochemicals (Costa Mesa, CA). LipofectAMINE Plus and Reagent, and gentamicin were purchased from Invitrogen. BE 3-4-3 and BE 3-3-3 were kindly provided by Dr. Raymond Bergeron (University of Florida, Gainesville, FL). Putrescine, MTT, IPTG, ATP, creatine kinase, phosphocreatine, cycloheximide, and ubiquitin were purchased from Sigma. Oligonucleotides were synthesized by Invitrogen or the Pennsylvania State University’s *γ* College of Medicine oligonucleotide core facility (Hershey, PA). EasyTag<sup>TM</sup> L-[<sup>3</sup>HS]methylamine was purchased from PerkinElmer Life Sciences. Rabbit reticulocyte lysate prepared from phenylhydrazine-treated New Zealand White rabbits was obtained from Cocalico Biologicals (Reamstown, PA) for use in the degradation studies. Ubal was purchased from Boston Biochem (Cambridge, MA). Talon Superflow metal affinity resin was obtained from Clontech. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). MG132 was obtained from Calbiochem. PD-10 desalting columns were purchased from Bio-Rad.

Cell Culture—The CHO cell line C55.7 (16), a kind gift from Dr. Immo Scheffler (University of California at San Diego, La Jolla, CA), and its derivatives were maintained in minimum essential α-minimal essential (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA), 100 µM putrescine, 100 units/ml penicillin, and 100 units/ml streptomycin. Cultures were incubated at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere and passaged every 5–7 days to maintain exponential growth. For all experiments, concentrated solutions of BE 3-3-3 and BE 3-4-3 (10 mM and 1 mM, respectively, in water, stored at −20 °C) were diluted with medium to the desired concentrations.

Transfection of pCMV-SSAT—The transfection of pCMV-L156F-SSAT into C55.7Res cells was accomplished using LipofectAMINE Plus reagent according to manufacturer’s instructions.

Growth Inhibition Assay—Exponentially growing cells were plated in 96-well plates<sup>1</sup> in 100 µl of medium per well in a 96-well plate. After a 12–18 h period for the cells to attach, 200 µl of medium containing 1.5 × the desired final drug concentration was added. Cells were incubated in the absence or presence of at least six drug concentrations for 144 h, at which time the medium was aspirated, and 100 µl of 5 mg/ml MTT (Sigma) was added. The cells were aspirated, and 100 µl of 50% EtOH in Me<sub>2</sub>SO (v/v) was added to each well. After 20 min, the A<sub>570</sub> (a value directly proportional to the number of viable cells (17)) was determined using a Bio-Rad plate reader. IC<sub>50</sub> values were determined from plots of the percentage of A<sub>570</sub> of untreated control cells versus the logarithm of the drug concentration. Each complete experiment was performed at least twice.

Analysis of SSAT Activity—Exponentially growing cells were plated in triplicate in 10<sup>4</sup> cells/cm². Following attachment, the medium was changed, and cells were incubated for the desired time. SSAT activity was determined in cell extracts by an assay that measures the incorporation of radioactivity from [1-<sup>14</sup>C]acetyl-CoA into [1-<sup>14</sup>C]acetyl-putrescine in 10 min at 30 °C as described previously (18). A standard assay mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM spermidine, and 12.7 µM [35S]methionine in a total volume of 100 µl. Using purified enzymes, the standard assay was linear to at least 2 ng of wtSSAT and 20 ng of L156F-SSAT. For assays to determine K<sub>m</sub> and V<sub>max</sub>, 0.5 ng of purified wtSSAT and 8 ng of purified L156F-SSAT were incubated with nine different substrate concentrations (S<sub>i</sub>) ranging from 1 µM to 3 mM for spermidine and 0.1 µM to 1 mM for spermine. K<sub>m</sub> values were determined from the linear regression of plots of [S]/[SSAT activity versus [S]] (r<sup>2</sup> ≥ 0.993 for all regression plots). V<sub>max</sub> values were determined from the exponential curve fit of plots of SSAT activity versus [S]. Values of h<sub>max</sub> were determined as described by Forshay (19).

Analysis of Intracellular Polyamine Content—Cells were plated as described for SSAT activity determination and then harvested and extracted with 10% (v/v) trichloroacetic acid. Aliquots were assayed for polyamine content using ion-paired, reversed-phase high performance liquid chromatography and post-derivatization with o-pthalaldehyde as described previously (20).

Western Analysis—Proteins present in cell extracts were resolved by SDS-PAGE using a 15% gel. Electrotransfer to polyvinylidene difluoride membrane (Micron Separations Inc., Waterborough, MA) was followed by hybridization with a polyclonal anti-SSAT antibody (prepared as described previously (21)) and detection using the Vistra Western blot detection kit (Amersham Biosciences). An Amersham Biosciences fluorimager model 595 and ImageQuant application software (Molecular Dynamics, Sunnyvale, CA) were used for visualization and quantitation.

Structure of Plasmids—The Chameleon double-stranded site-directed mutagenesis kit (Stratagene) was used to change the nucleotide sequence CTO codong for leucine 156 of human wtSSAT cDNA in the plasmid pSAT9.3 (14) to TTC coding for phenylalanine. The primers used were P-GGTTTGGGATCCAAGATCGAC (nucleotides to achieve the mutation are shown in bold type) and the KpnI primer included in the Chameleon kit. The resulting construct was termed pSAT9.3L156F, and the SSAT coding region was sequenced to confirm the presence of the correct codon for phenylalanine 156 and the absence of any 2° mutations.

The plasmid pSAT9.3L156F was then used as a template for PCR to introduce a BamHI restriction site 5′ of the initiation codon (5′-CAGGGGCTTGATCCAAAAAGGAGA-3′) (the BamHI site is shown in bold type) and to change the existing BamHI codon downstream of the stop codon to a unique Nhel site (5′-CTAGAACACTGCTGCTAGCCGGGCTG-3′) (the Nhel site is shown in bold type). The Expand high fidelity PCR System (Roche Molecular Biochemicals) was used according to manufacturer’s instructions. The PCR product was digested with BamHI and Nhel enzymes and then ligated into the pCMV-Neo-Bam vector digested with the same enzymes, as described previously (22). The SSAT coding region of the resulting plasmid, termed pSSAT-L156F-SSAT, was sequenced to confirm that no secondary mutations were introduced during plasmid construction.

To construct a plasmid containing the cDNA coding for a His-tagged SSAT-L165F mRNA, the BamHI/Nhel fragment described above was digested with Spal and HindIII. The Spal/HindIII fragment was then ligated into the same sites of the pQE30SSAT plasmid containing the SSAT cDNA sequence with a His tag sequence present at the amino-terminal end. The resulting plasmid, termed pQE30L156FSSAT, was sequenced to confirm that the desired construct had been obtained.

The plasmid pSAT9.3L156F was also used as a template for PCR to make l156F/E170stop-SSAT and l156F/MATEEAA-SSAT, which has two additional alanine residues added to the carboxyl terminus. The amplification primer used to construct the l156F/MATEEAA-SSAT plasmid contains the gene for the His tag sequence present at the amino-terminal end. The resulting plasmid, termed pQE30L156FSSAT, was sequenced to confirm that the desired sequence had been obtained.

Expression and Degradation of SSAT and L156F-SSAT in Vitro—<sup>35</sup>S-labeled wtSSAT and L156F-SSAT proteins were synthesized in vitro from the T7 promoters of the pSAT9.3 and pSAT9.3L156F plasmids, respectively, using the T<sub>T</sub> coupled transcription/translation system (Promega, Madison, WI).

The standard 200-µl assay for degradation studies contained 50 µl of rabbit reticulocyte lysate, 40 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 0.5 mM ATP, 10 mM phosphocreatine, 0.05 mg/ml creatine kinase, 0.1 mM cycloheximide, and <sup>35</sup>S-labeled protein produced by the T<sub>T</sub> reaction used as substrate at 1–3% (v/v). Some of the degradation assays also included one or more of the following: 0.1 mM BE 3-3-3, 0.1 mM MG132, 5 µM Ubal, and 0.15 mM Ub. Assays were incubated at 37 °C, and 30–µl aliquots were removed at the desired times. Those aliquots were then mixed with SDS sample buffer, boiled 5 min, and electrophoresed on 3% (v/v) gels. The resultant bands were exposed to imaging screens, and, following an appropriate period of time, the resulting images were viewed and quantitated using an Amersham Biosciences PhosphorImager SI and ImageQuant application software.

Protein Purification—The His-tagged wtSSAT and L156F-SSAT proteins were purified essentially as described previously (23) but with the following modifications. Protein expression was induced in the bacterial culture by the addition of IPTG to a final concentration of 0.01 µM, and following harvest and cell lysis, the soluble cell fraction was loaded onto a gravity flow column packed with Talon Superflow metal affinity resin.

RESULTS

Activity of Purified L156F-SSAT—Site-directed mutagenesis as described under “Experimental Methods” was used to change the nucleotide sequence of human SSAT to code for resistance to the analogue that is exhibited by the C55.7Res variants of this cell line.
phenylalanine instead of leucine at amino acid 156. Although this mutation was originally identified in the Chinese hamster SSAT mRNA, we chose to study the human SSAT because, between the human and Chinese Hamster sequences, there is 96% homology in the 171 amino acids making up the SSAT monomer, and this mutation occurs in a completely conserved region of the protein. Additionally, many previous studies relevant to SSAT activity and interactions with the analogues have been conducted using the human SSAT protein. Purified His-tagged proteins were used to compare the kinetic parameters of the mutant human L156F-SSAT protein with that of the human wtSSAT with the two natural polyamine substrates spermidine and spermine. L156F-SSAT $K_m$ values are increased by 4.0-fold and 9.9-fold, respectively, for spermidine and spermine as compared with the wild-type SSAT (Table I), indicating that the affinity of the mutant enzyme for both of the natural substrates is significantly reduced. The $K_m$ values reported here for wtSSAT are in agreement with SSAT $K_m$ values in the literature, which range from 55–140 $\mu$M for spermidine and 5–60 $\mu$M for spermine (1). The parameter $k_{cat}$, a measure of turnover of substrate to product at the active site of the protein, indicates that significantly less product is produced by the L156F-SSAT than by the wild-type protein over any given time period by a given amount of protein (Table I). An overall estimate of enzyme efficiency can be determined from the $k_{cat}/K_m$ value, which can be considered a specificity constant. The $k_{cat}/K_m$ values indicate that the efficiency of the mutant protein is significantly reduced, because the values for L156F-SSAT are 56 and 78 times lower with spermidine and spermine substrates, respectively, than those for the wtSSAT (Table I).

**Activity of L156F-SSAT in Cells**—To raise the activity of L156F-SSAT in C55.7Res cells to levels that were significantly above the limit of detection of the SSAT activity assay and could be studied, pCMV-L156F-SSAT was transfected into the C55.7Res cells as described under “Experimental Procedures.” Only 6 of the 87 C55.7Res+L156F clones selected following transfection exhibited SSAT activity of $>100$ pmol/min/mg protein, and the SSAT activity values of those clones ranged from 106–186 pmol/min/mg protein. These values are considerably lower than the 650–1900 pmol/min/mg protein that we obtained previously from 13 of the 44 CHO clones transfected with pCMV-wtSSAT (22). Western analysis indicated that the seven C55.7Res+L156F clones exhibiting the highest SSAT activities expressed levels of SSAT protein ranging from 0.6–2.4 times that of wtSSAT clone 43, which has basal SSAT activity of $\sim1800$ pmol/min/mg protein (data not shown). These results suggest that the lack of SSAT activity was not a result of failure of the C55.7Res+L156F cells to express significant amounts of the L156F-SSAT protein.

Analysis of Western blots of purified SSAT protein and cell extracts was used to determine the amount of SSAT protein in the extracts of cells expressing wtSSAT or L156F-SSAT. That value was then used to calculate the SSAT activity per nanogram of SSAT protein (with spermidine as the substrate) in the same cell extracts. Table II indicates that the L156F-SSAT activity in the cell extracts was $>17$ times lower than that of the wtSSAT activity, which agreed well with the data obtained for the purified proteins where L156F-SSAT activity was 14-fold less than that of wtSSAT. The cellular activities for both L156F-SSAT and wtSSAT with spermidine substrate also correlated well with the $V_{max}$ values determined for the purified enzymes (1.9 and 26.0 pmol/min/mg protein, respectively).

**Effects of Expression of L156F-SSAT Activity in C55.7Res Cells**—It was of interest to determine whether the increased expression of L156F-SSAT that brought basal SSAT activity to a measurable level similar to or greater than that of the analogue-sensitive cells could restore cellular sensitivity to BE 3-3-3. The two C55.7Res+L156F clones that exhibited the highest basal SSAT activity were used to test the sensitivity to BE 3-3-3 by determining the IC$_{50}$ values compared with C55.7 and C55.7Res cells. The results, shown in Fig. 1, indicated that both C55.7Res+L156F clones were nearly as resistant to BE 3-3-3 as was the C55.7Res cell line. Therefore, increased expression of the L156F-SSAT protein that raised basal SSAT activity to levels comparable or slightly higher than normal basal SSAT activity from wtSSAT protein was unable to restore cellular sensitivity to the polyamine analogue.

The results of the BE 3-3-3 sensitivity experiments indicate that, even though there was measurable basal SSAT activity in the L156F-SSAT-transfected cells, in the presence of the polyamine analogue the activity of the mutant SSAT enzyme did not reach levels that would result in increased toxicity, suggesting that BE 3-3-3 is unable to induce activity of L156F-SSAT. To test this hypothesis, SSAT activity was measured in cells of C55.7Res+L156F-SSAT clone 1 following 48 h of exposure to several concentrations of BE 3-3-3 and compared with that of C55.7 and C55.7Res cells. The results, shown in Fig. 2, indicated that BE 3-3-3 concentrations of up to 100 $\mu$M failed to induce L156F-SSAT more than 3.8 times, whereas 25 $\mu$M at BE 3-3-3 resulted in $\sim300$-fold induction of the wtSSAT of the C55.7 cells.

BE 3-4-3 and CPENSpm are polyamine analogues that also have been demonstrated to cause significant induction of SSAT, whereas CHENSpm causes only minimal increases in SSAT activity. C55.7Res cells exhibit cross-resistance to both BE 3-4-3 and CPENSpm but are as sensitive to CHENSpm as C55.7 cells (data not shown). Because it is likely that the

### Table I

**Kinetic parameters for purified L156F-SSAT and wtSSAT proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ $\mu$M</td>
<td>$k_{cat}$ s$^{-1}$</td>
</tr>
<tr>
<td>L156F-SSAT</td>
<td>229.6</td>
<td>0.63</td>
</tr>
<tr>
<td>wtSSAT</td>
<td>57.5</td>
<td>8.7</td>
</tr>
</tbody>
</table>

### Table II

**SSAT protein amounts and specific activities in cells transfected with L156F-SSAT or wtSSAT**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SSAT (ng)/mg protein in 50 $\mu$g cell extract</th>
<th>SSAT activity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C55.7Res-L156F clone 1</td>
<td>94</td>
<td>0.9</td>
</tr>
<tr>
<td>C55.7Res-L156F clone 2</td>
<td>22</td>
<td>1.5</td>
</tr>
<tr>
<td>WtSSAT cl 43</td>
<td>44</td>
<td>25.8</td>
</tr>
</tbody>
</table>
Properties of SSAT Mutant L156F

leucine to phenylalanine amino acid change of the mutant SSAT protein causes a conformational change in this protein as compared with the wild-type SSAT, it is possible that individual analogues might exhibit different responses with the mutant protein. Therefore, we compared SSAT activity of cells of C55.7Res + L156F-SSAT clone 1 and wtSSAT clone 43 in the absence or presence of the four polyamine analogues (Fig. 3). It should be noted that the basal SSAT activity of the wtSSAT clone 43 cells is elevated to ~1800 pmol/min/mg protein as compared with the ~15 pmol/min/mg protein in the C55.7 cells, and, therefore, even though the induced SSAT activity reached as high as ~35,000 pmol/min/mg protein, the fold induction over untreated at ~20 is less than the ~300-fold observed for the C55.7 cell line as shown in Fig. 2. The results indicated that, with the analogues that highly induced wtSSAT activity (13-20-fold induction), L156F-SSAT activity was induced, at most, 4-fold with BE 3-3-3 and <1.5-fold for BE 3-4-3 and CPENSpm. Although CHENSpm resulted in much less induction of the wtSSAT protein (at most 3-fold), the induction of the L156F-SSAT was still even less (at most 1.4-fold).

Effects of BE 3-3-3 on the Degradation of L156F-SSAT in Vitro—Because the L156F mutation was suppressing induction of SSAT activity by BE 3-3-3, it was logical to investigate the effect of the analogue on degradation of the mutant protein. Shown in Fig. 4 are the time courses of degradation in vitro of the two SSAT proteins in the absence or presence of 1 mM BE 3-3-3. The wtSSAT protein is rapidly degraded over the 90 min period in the absence of BE 3-3-3; however, when the analogue is present there is almost complete stabilization and lack of degradation over the same time period (Fig. 4A). This is reflected in the half-life measurements of the proteins as calculated from regression analysis of the plots of SSAT protein amounts versus time under each condition. The half-life of the untreated wtSSAT protein was 22 min, whereas it was greatly extended to ~144 h when the protein was exposed to BE 3-3-3. In contrast to this behavior, the L156F-SSAT protein was rapidly degraded regardless of whether BE 3-3-3 was present or not (Fig. 4B). The half-life of the untreated mutant protein, at 15 min, was similar to that of the wtSSAT; however, the L156F-SSAT half-life was not significantly increased (18 min) when the protein was exposed to BE 3-3-3.

The degradation in vitro of L156F/E170stop-SSAT and L156F-SSAT with two additional alanine residues added to the carboxyl terminus (L156F/MATEEAA-SSAT) was also investigated. Both of these modifications to the carboxyl-terminal end of the L156F-SSAT resulted in stabilization of the protein even in the absence of polyamine analogue (Fig. 4, C and D). The half-life measurements for the untreated proteins, at 105 min for L156F/E170stop-SSAT and 154 min for L156F/MATEEAA-SSAT, were significantly increased over that of the untreated wtSSAT at 22 min. Exposure of the double mutants to BE 3-3-3 had no additional effect.

We also investigated the ability of BE 3-3-3 to block ubiquitination of the L156F-SSAT protein. The rabbit reticulocyte degradation system used for the studies described above was used again, but with the modification that ubiquitin aldehyde and MG 132 were added to the reticulocyte lysate to prevent breakdown of polyubiquitinated SSAT protein by inhibiting polyubiquitin chain hydrolysis and proteasomal degradation. This allowed resolution on the polyacrylamide gel of the subsequent visualization of ubiquitinated species of the L156F-SSAT protein present in aliquots from the degradation assay. It can be seen in Fig. 5, that there was no difference between the
pattern of ubiquitination obtained for the untreated L156F-SSAT protein and that observed following the addition of BE 3-3-3 to the assay system. These results indicated that the polyamine analogue failed to prevent ubiquitination of the mutant SSAT protein in vitro. This was in contrast to results reported by Coleman and Pegg (24) for the wtSSAT protein, where 100 μM BE 3-3-3 prevented formation of SSAT-Ub conjugates and caused stabilization of the wtSSAT protein.

Effects of BE 3-3-3 on the Degradation of Cellular L156F-SSAT Activity—To determine whether the effect of BE 3-3-3 on the mutant SSAT protein was the same in the cell system as that observed in vitro, the C55.7Res+L156F-SSAT clone 1 was used for half-life measurements of both cellular L156F-SSAT protein and activity (Fig. 6). Regression analysis of the plots of L156F-SSAT band volume from the Western blot versus time was used to calculate the half-life of the cellular protein in the absence or presence of BE 3-3-3 (Fig. 6A). The results indicated that there was rapid degradation of cellular L156F-SSAT protein despite the presence of the polyamine analogue and that the protein half-life was not increased over that of the untreated control (24.1 and 24.9 min half-life for untreated and BE 3-3-3-treated cells, respectively). Measurements of SSAT activity of the same cell extracts indicated a similar lack of BE 3-3-3 prolongation of enzyme activity, as the half-life of L156F-SSAT activity was 17.1 min in untreated cells and 18.1 min in those exposed to BE 3-3-3 (Fig. 6B). The activity of wtSSAT expressed in C55.7Res cells was, as expected, short-lived in the absence of BE 3-3-3 (21 min half-life) and stabilized by the polyamine analogue (248 min half-life) (Fig. 6B). These results corroborated the in vitro degradation results (Fig. 4).

DISCUSSION

The first definitive evidence that induction of SSAT activity was an integral part of the actions of BE 3-3-3 and similar polyamine analogues was the selection of the analogue-resistant C55.7Res cell line that exhibited decreased basal SSAT activity and a lack of SSAT induction upon exposure to the analogue (13). Restoration of wtSSAT activity in that cell line also restored sensitivity to BE 3-3-3 and provided a solid link between the altered SSAT activity and the resistance to the analogue. A point mutation was identified in the SSAT mRNA of the analogue-resistant C55.7Res cells that results in an amino acid change from leucine to phenylalanine at position 156 of the SSAT protein. Although previous studies (25–28) had examined the effects of mutations on activity and degradation of the SSAT protein, all of that work was carried out in vitro with the SSAT protein to which the mutation of interest had been introduced. The fact that the L156F-SSAT has arisen within a cell system and replaced the wtSSAT within those cells selected to be resistant to polyamine analogues has provided us with a unique opportunity to address several previously unanswerable questions.

At the time of selection of the C55.7Res cell line and identi-
fication of the mutation in the SSAT mRNA, it was uncertain whether or not the mutant mRNA could be translated to protein within the cell. The transfection of human L156F-SSAT cDNA into the C55.7Res cells and the subsequent detection of L156F-SSAT protein in those cells have now confirmed that the mutant SSAT mRNA can be translated. Levels of expression of the mutant SSAT protein that were attained were similar to those previously achieved when wtSSAT cDNA was transfected into CHO cells, and a comparison of the basal activity of the two proteins demonstrated that the activity of L156F-SSAT was significantly lower than that of the wtSSAT.

The explanation of the cause of the decreased cellular SSAT activity of the C55.7Res cells was provided by the determination of the kinetic parameters of the purified L156F-SSAT as compared with those of the wtSSAT. With both of the natural substrates, spermidine and spermine, \( V_{\text{max}} \) values were increased, \( K_m \) values were decreased, and rate of turnover of substrate to product was significantly reduced for L156F-SSAT. These data indicate that the protein interactions with the natural substrates are altered and that this mutation has rendered the enzyme an inferior and less efficient acetyltransferase than the wtSSAT. This suggests that the single amino acid change in the SSAT sequence has significant effects on the protein conformation that decrease the ability of the enzyme and substrate to come together to efficiently carry out the reaction to form the acetylated polyamines. The observed increases in \( K_m \) values and decreases in \( V_{\text{max}} \) with spermidine and spermine substrates are consistent with the effects reported by Coleman et al. (25, 26) of different mutations induced in the same region of the SSAT sequence. The mutation of amino acid 152 from glutamic acid to either glutamine or lysine or the mutation of amino acid 155 from arginine to alanine caused a reduction in activity and an increase in \( K_m \) with spermidine and spermine substrates (25, 26). These demonstrations that a single amino acid change can have profound effects on the ability of SSAT to interact with its natural substrates help to explain why the region of the SSAT protein around residue 156, where the leucine to phenylalanine mutation occurred, is fully conserved across all of the known mammalian species where the SSAT sequence has been deduced.

It is important to note that, although the basal SSAT activity of the L156F-SSAT in the C55.7Res cells is 17 to 28-fold lower than that of wtSSAT in the parental cells, there is no significant difference in the growth of the untreated cells. As the rate-limiting enzyme of polyamine catabolism, SSAT functions to help maintain the steady state level of total cellular polyamines and prevent high levels of spermidine and spermine that could be toxic to cells (1, 29). SSAT can rapidly respond to physiological changes that modify the distribution and/or total levels of cellular polyamines, and it can aid redistribution by the conversion of spermine to spermidine and/or spermidine to putrescine as well as decrease total cellular polyamines, because the acetylated forms of spermidine and spermine are more readily excreted from the cell. Under normal physiological conditions, SSAT levels in cells are very low, suggesting that little SSAT activity is required to maintain polyamine homeostasis, perhaps due to the numerous regulatory mechanisms that control ornithine decarboxylase and S-adenosylmethionine decarboxylase levels. It is therefore consistent that the 17–28-fold reduction in SSAT activity in the C55.7Res cells does not appear to have any effect on normal cellular growth or functioning of the untreated cells. It is also known that SSAT activity becomes more important in response to stimuli that alter normal physiological conditions. Other than the polyamines spermine and spermidine themselves, other stimuli that have been demonstrated to induce SSAT activity and thus invoke its role in maintaining polyamine homeostasis include natural hormones and growth factors such as corticosteroids, estradiol, and catecholamines, compounds known to cause toxicity such as carbon tetrachloride and dialkylamines, and a variety of compounds used as anticancer drugs, such as adriamycin, 5-fluorouracil and methotrexate, as well as the polyamine analogues (1). Therefore it is also consistent that the effects of the L156F-SSAT mutation are evident under conditions of BE 3-3-3-treatment where SSAT would be an important response factor in attempting to restore normal polyamine homeostasis and maintain cell health. It has recently been suggested that there may be an additional cellular system contributing to polyamine homeostasis through the back conversion of spermine to spermidine (30), but more data will be needed to understand the nature of and relative importance of such a system. However, it is clear that the SSAT response is important to the action of BE 3-3-3 and similar polyamine analogues, because the inability to reach high levels of enzyme activity results in resistance to the analogue.

It was unclear in the original studies whether the basal SSAT activity in the C55.7Res cells was just so low that the enzyme was induced by the analogue, but the levels were not high enough to result in toxicity even following induction, or whether the L156F-SSAT could not be induced by BE 3-3-3. Because, as shown above, it is now clear that the activity of the mutant enzyme is reduced with the natural polyamine sub-

![Fig. 6. Effects of BE 3-3-3 on half-life of cellular SSAT activity and protein.](http://www.jbc.org/)

Cells of C55.7Res + L156F-SSAT clone 1 were grown for 48 h, 200 μM cycloheximide was added to stop protein synthesis, and cells were harvested at the times shown for Western analysis (A) and assay of SSAT activity (B) as described under “Experimental Procedures.” Values shown in panel A are from quantitation of a Western blot of single samples from a representative experiment. SSAT activity values shown in panel B are mean ± S.D. (n = 3) of a representative experiment.
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strates, this is an important issue. The current studies have made it certain that increasing basal SSAT activity to levels normally observed within cells without restore the capability to induce high levels of SSAT activity in response to BE 3-3-3 is insufficient to restore sensitivity to the analogue. Expression of wtSSAT protein in the C55.7Res cells also restored the ability to induce the SSAT activity upon analogue treatment. However, the C55.7Res + L156F-SSAT clones that exhibited basal levels of SSAT at least equivalent to that of parental C55.7 cells were still resistant to BE 3-3-3, because SSAT activity was not increased more than 4-fold upon challenge with the analogue. Failure to induce the activity of the mutant enzyme was not limited to BE 3-3-3, as BE 3-4-3 and CPENSpm, two related analogues known to highly induce SSAT activity, also produced only minimal increases in the activity of L156F-SSAT. Therefore, the L156F mutation not only affects the interaction of the natural substrates with SSAT, but also decreases the ability of the polyamine analogues to bind to the SSAT protein and cause induction of the protein and activity.

The current studies have also demonstrated that the failure of BE 3-3-3 to induce L156F-SSAT activity results from the inability of the analogue to protect the mutant protein from rapid degradation as it does the wild-type SSAT (22). The short half-life of the SSAT protein is one of the characteristics that allows it to function effectively in the regulation of polyamine homeostasis, because the rapid adjustment of cellular SSAT activity can be achieved through synthesis or degradation of the enzyme. Coleman and Pegg (24) have recently demonstrated that BE 3-3-3 protects the SSAT protein by interfering with the ubiquitination necessary to target the protein for proteasomal degradation. The current data supports the conclusion that the conformational change brought about by the L156F mutation of the SSAT protein prevents the interaction between the analogue and the protein that is necessary to prevent the ubiquitination of SSAT. There were no differences observed in the polyubiquitination patterns of the L156F-SSAT in the absence or presence of BE 3-3-3 in vitro, and these data, coupled with the failure of the analogue to increase the cellular half-life of either the protein or activity, suggest that it is this specific lack of ability to interfere with SSAT ubiquitination that results in resistance to the polyamine analogue. The stabilization of L156F-SSAT by mutation of the carboxyl terminus of the protein is consistent with the results of Coleman and Pegg for wtSSAT (24) and also supports the conclusions stated above. If the conformational change resulting from the L156F mutation merely caused the protein to be so altered that it was recognized as a misfolded protein and thus rapidly degraded, it is unlikely that the mutant protein would respond as wtSSAT does to the additional mutation of the carboxyl-terminal end. Therefore, it seems more likely that it is the alteration of the specific interaction of the protein with the polyamine analogue that is resulting in the failure to shield the protein from ubiquitination. The fact that the toxicity of the analogue is attenuated when the half-life is not lengthened and that the activity thus does not increase to high levels indicates that this ability to protect the SSAT protein from degradation is one of the properties of the analogue that is essential for its toxic action. Resistance to chemotherapeutic agents is one of the major limitations encountered in their clinical use. Because BE 3-3-3 is now in phase II clinical trials, it is not premature to consider whether resistance such as was selected in the C55.7Res cells could occur with therapeutic use of the polyamine analogue. A recent report on a Phase I clinical trial using BE 3-3-3 reported the peak plasma levels achieved at the MTD to be 17 µM BE 3-3-3 (12). Therefore, the 10 µM concentration of the polyamine analogue used to select for the C55.7Res resistance to BE 3-3-3 is a clinically relevant concentration as is the method of short exposure to the drug followed by a period of recovery. The fact that the observed mechanism of resistance to BE 3-3-3 is the result of a mutation that does not alter the normal SSAT function in the absence of analogue drastically enough to limit its occurrence suggests that the potential exists that other relatively innocuous mutations may be present in the SSAT gene in tumors that are targets of polyamine analogue use. The inherent genetic instability of transformed tissues and tumors would suggest that this is more than a hypothetical concern. As long as such mutations do not reduce SSAT activity to critical levels that interfere with polyamine homeostasis under normal physiological conditions, there would be no selection pressure against their propagation, and the effects would only become important upon exposure to the polyamine analogue, which would be rendered less effective.

REFERENCES
Properties of the Spermidine/Spermine \( \text{N}^\text{1} \)-Acetyltransferase Mutant L156F That Decreases Cellular Sensitivity to the Polyamine Analogue \( \text{N}^\text{1,\text{N}^\text{11}} \)-Bis(ethyl)norspermine

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