Photoaffinity Labeling and Mass Spectrometry Identify Ribosomal Protein S3 as a Potential Target for Hybrid Polar Cytodifferentiation Agents*

(Received for publication, February 24, 1999)

Yael Webb‡§, Xianbo Zhou‡, Lang Ngo§, Virginia Cornish‡, Joachim Stahl¶, Hediye Erdjument-Bromage, Paul Tempst‡, Richard A. Rifkind§, Paul A. Marks§, Ronald Breslow‡, and Victoria M. Richon**

From the ‡Department of Chemistry, Columbia University, New York, New York 10027, the §Program of Cell Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, the ¶Max Delbrück Center for Molecular Medicine, 13122 Berlin-Butz, Germany, and the ||Program of Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

The ability of a novel class of hybrid polar compounds (HPCs) to induce differentiation and consequent cessation of proliferation of transformed cells has led to their development as potential chemotherapeutic agents in the treatment of cancer. Suberoylanilide hydroxamic acid (SAHA) is a prototype of a family of hydroxamic acid based compounds (SAHA-like HPCs) that can, at micromolar concentrations, induce a variety of transformed cell lines to differentiate. The mechanism of action of the HPCs is not entirely understood. Searching for a cellular target of the SAHA-like HPCs, we synthesized a photoaffinity labeling reagent structurally based on SAHA, and probed for SAHA-binding proteins in murine erythroleukemia (MEL) cells. Photoaffinity labeling in cell free extracts identified a 32-kDa protein (p32) that was specifically labeled by the photoaffinity reagent. Cell fractionation assays localized p32 to the P100 fraction. p32 was partially purified and identified by mass spectrometry as the 40 S ribosomal protein S3. Expression of epitope-tagged S3 in bacterial lysates followed by photoaffinity labeling confirmed its specific labeling. Identification of a cytodifferentiation agent target may shed light on the mechanism by which the SAHA-like HPCs exert their antitumor effects.

Hybrid polar compounds have been previously identified as potent inducers of differentiation in murine erythroleukemia (MEL) cells. The ability of a novel class of hybrid polar compounds (HPCs) to induce differentiation and consequent cessation of proliferation of transformed cells has led to their development as potential chemotherapeutic agents in the treatment of cancer. Suberoylanilide hydroxamic acid (SAHA) is a prototype of a family of hydroxamic acid based compounds (SAHA-like HPCs) that can, at micromolar concentrations, induce a variety of transformed cell lines to differentiate. The mechanism of action of the HPCs is not entirely understood. Searching for a cellular target of the SAHA-like HPCs, we synthesized a photoaffinity labeling reagent structurally based on SAHA, and probed for SAHA-binding proteins in murine erythroleukemia (MEL) cells. Photoaffinity labeling in cell free extracts identified a 32-kDa protein (p32) that was specifically labeled by the photoaffinity reagent. Cell fractionation assays localized p32 to the P100 fraction. p32 was partially purified and identified by mass spectrometry as the 40 S ribosomal protein S3. Expression of epitope-tagged S3 in bacterial lysates followed by photoaffinity labeling confirmed its specific labeling. Identification of a cytodifferentiation agent target may shed light on the mechanism by which the SAHA-like HPCs exert their antitumor effects.

* These investigations were supported in part by Grant CA-0974823 (to P. A. M. and R. A. R.) from the National Cancer Institute, Grant GM18754–35A1 (to R. B.) from the National Institute of Health, Grant BDI-9420123 (to P. T.) from the National Science Foundation, Core Grant P30 CA08748 from the National Cancer Institute, and grants from the Japan Foundation for the Promotion of Cancer Research Fund and the Dewitt Wallace Fund for Memorial Sloan-Kettering Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Box 86, Memorial Sloan Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

† The abbreviations used are: MEL, murine erythroleukemia; HPC, hybrid polar compound; SAHA, suberoylanilide hydroxamic acid; HMBA, hexamethylene bisacetamide; THF, tetrahydrofuran; NRDB, non-redundant data base; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PIPES, 1-piperazinemedi-ethanesulfonic acid; JaFIS, injection adaptable fine i source; HDAC, histone deacetylase; PCR, polymerase chain reaction; MALDI, matrix-assisted laser-desorption/ionization; TOF, time-of-flight; reTOF, reflec-
room temperature for 2 h, 1.13 mmol of 2,6-diiodo-4-aminoaniline in 20 ml of THF were added dropwise through an addition funnel and the reaction was stirred at room temperature overnight. Then the THF was evaporated in vacuo and the residue was partitioned between CHCl₃/H₂O. The organic layer was evaporated, and the product was purified by silica gel chromatography (5% MeOH/CHCl₃), to yield 0.337 g (57%) of 4. 1H NMR: δ 1.2 (m, 4H), 1.5 (m, 4H), 1.5 (m, 2H), 2.3 (t, 2H), 2.3 (t, 2H), 3.6 (s, 3H), 4.84 (s, 2H), 7.9 (s, 2H), 9.6 (s, 1H).

Suberic Acid (4-Amino-3,5-diiodoaniline) Amide, 5—A solution of 0.62 mmol of 4 and 3.17 mmol of LiOH·H₂O in 30 ml of 3:1 MeOH/H₂O was heated to 60 °C, until TLC showed no presence of the starting material. The MeOH was removed in vacuo, and the aqueous residue was diluted, filtered, and acidified to pH 5 with 1 M HCl. The resulting precipitate was filtered and recrystallized from MeOH to afford 0.2655 g (82.7%) of 5. 1H NMR: δ 1.2–1.3 (m, 4H), 1.4–1.6 (m, 4H), 2.2 (m, 4H), 4.8 (s, 2H), 7.9 (s, 2H), 9.6 (s, 1H), 12.0 (s, 1H).

Suberic (O-TBDPS) Hydroxamic Acid (4-Amino-3,5-diiodoaniline) Amide, 6—A solution of 0.616 mmol of carbonyl diimidazole in 10 ml of THF was added to a solution of 0.385 mmol of 5 in 20 ml of THF, and the reaction was stirred overnight at room temperature. Then, 0.914 mmol of H₂NO-TBDPS were added, and the reaction was stirred at room temperature overnight. The THF was removed, and the reaction mixture was purified over silica gel (10% MeOH/CHCl₃), then by preparative TLC, using the same solvent mixture. The product was washed with ether to afford 0.202 g (53%) of 6. ¹H NMR: δ 1.1 (s, 9H), 1.0–1.2 (m, 4H), 1.2–1.4 (m, 2H), 1.4–1.5 (m 2H), 1.9 (t, 2H), 1.9 (t, 2H), 4.8 (s, 2H), 7.3–7.4 (m, 6H), 7.6–7.7 (m, 4H), 7.9 (s, 2H), 9.8 (s, 1H), 10.6 (s, 1H).

Suberic Hydroxamic Acid (4-Amino-3,5-diiodoaniline) Amide, 7—A solution of 77 mg of 6 in 15 ml of THF was chilled to 0 °C, and 0.2 ml of a 25% solution of tetrabutyl ammonium fluoride in THF were injected. The solution was stirred for 5 h, then washed with ether, filtered, and acidified to pH 5 with 1 M HCl. The resulting precipitate was filtered and recrystallized from MeOH to afford 0.495 mg (97%) of 7. 1H NMR: δ 1.2–1.3 (m, 4H), 1.4–1.6 (m, 2H), 1.9 (t, 2H), 2.2 (t, 2H), 4.8 (s, 2H), 7.9 (s, 2H), 9.6 (s, 1H), 12.0 (s, 1H).

Suberic Hydroxamic Acid (4-Aminoaniline) Amide, 8a—Compound 8a was synthesized by catalytic hydrogenation of 7, using the above procedure.
**TABLE I**

| Name     | Structure |
|----------|-----------|
| SAHA     | ![SAHA structure](image) |
| [^H]498  | ![[^H]498 structure](image) |
| 498      | ![498 structure](image) |
| HMBA     | ![HMBA structure](image) |

*Photoaffinity labeling reagent is a structural analog of the parent compound SAHA.*

Specific activity 4 Ci/mmol.

Sulferic Hydroxamic Acid (4-Azidoaniline) Amide, 498—Compound 498 was synthesized from 8a using the above procedure for the synthesis of [^H]498, without the addition of 8.

**Cell Culture**

MEL DS19/Se9 cells, derived from 745A cells, were maintained in minimal essential medium supplemented with 10% fetal calf serum, penicillin, and streptomycin and incubated at 37 °C in 95% air, 5% CO₂ atmosphere. Cultures were initiated at a cell density of 1 × 10⁶ cells/ml, and all experiments were performed with cells in logarithmic growth phase. Induction of differentiation, cell density, and benzidine reactivity were determined as described (12).

**Preparation of Whole Cell Extracts**

An aliquot of 1.6 × 10⁷ MEL cells was washed twice with phosphate-buffered saline and lysed with 1 ml of lysis buffer (250 mM NaCl, 50 mM Heps/KOH, pH 7.0, 0.1% Nonidet P-40, 50 mM NaF, 5 mM EDTA, 0.1 mM Na₃VO₄, 50 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin) at 0 °C for 30 min. The lysed cells were centrifuged at 14,000 rpm (Beckman 5402 microcentrifuge) for 15 min. The supernatant was stored at -80 °C and used as whole cell extracts.

**Cell Fractionation by Differential Centrifugation**

Cell fractionation was performed according to the method previously described by van’t Hof et al. (13) with modifications. Briefly, 2 × 10⁸ MEL cells were washed twice with phosphate-buffered saline and suspended in 1 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 0.2 mM MgCl₂, 0 mM CaCl₂) at 0 °C for 15 min, then Dounce homogenized (1 stroke). After the addition of 200 µl of 1.25 mM sucrose and 2 µl of 0.5 mM EDTA, the mixture was centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant (S1) was removed. The pellet (P1) was resuspended in 1 ml of a mixture of 0.25 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and centrifuged at 1,000 × g for 10 min at 4 °C. The combined S1 supernatants were centrifuged at 10,000 × g for 10 min at 4 °C (SS-34 rotor). The supernatant (S10) was centrifuged for 45 min at 100,000 × g in a Beckman TL-100 desktop ultracentrifuge (TLA 100.2 rotor) for 1 h at 4 °C, and the supernatants were removed. For Triton X-100 extraction, PT fractions were resuspended in 10 mM Tris-HCl, pH 8.0, for further analysis, or solubilized in SDS sample buffer. For Zwittergent 3-16 extraction, the supernatants (S₁₀) were precipitated with 10% trichloroacetic acid, washed twice with ice-cold ether, and solubilized in SDS sample buffer. The samples were subjected to SDS-PAGE as described above. For mass spectrometric analysis, after electrophoresis, proteins were transferred to a nitrocellulose membrane and stained with Amido Black.

**Protein Digestion**

The 32-kDa band was excised from the nitrocellulose blot and processed for internal sequence analysis as described (14, 15). Briefly, in situ digestion was done using 0.1 µg of trypsin (modified sequencing grade; Promega, Madison, WI) in 10 µl of 100 mM NH₄HCO₃ (supplemented with 0.5% Zwittergent 3-16) for 2 h at 37 °C. The resulting peptide mixture was then loaded onto a 2-µl bed volume of Poros 50 R2 (PerSeptive, Framingham, MA) reversed-phase beads (sized to be between 40 and 60 µm, and slurry packed into an Eppendorf gel-loading tip), washed with 20 µl of 5% MeCN, 0.1% formic acid, and stepwise eluted in 4 µl of 16% (and then with 4 µl of 30%) MeCN, 0.1% formic acid; the two resulting fractions are designated “16% pool” and “30% pool.”

**Mass Spectrometry**

Each peptide pool was analyzed twice by matrix-assisted laser-desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), in the presence and absence of peptide calibrants (15). Aliquots (0.5 µl) were deposited on the probe surface, mixed with α-cyano-4-hydroxycinnamic acid solution (MALDI-Quality; Bruker-Daltonics, Bil-

![Fig. 2 Induction of differentiation of MEL cells.](image)

Cells were cultured with no inducer (C), SAHA (2.5 µM), or 498 (3.5 µM) for 5 days, after which the percentage of benzidine-positive staining cells was determined.

(E[N²HANCE, NEN Life Science Products), dried, and mounted on x-ray film for fluorography.

**Detergent Extraction**

For Triton X-100 extraction, P100 fractions were incubated for 30 min at 4 °C with Csk buffer (10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 mM sucrose, 10 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µM PMSF) at a final protein concentration of 5 mg/ml. For Zwittergent 3-16 extractions, Triton X-100-resistant fractions (PT₁₀) were incubated for 30 min at 4 °C with extraction buffer (20 mM Tris-HCl, pH 8.0, 0.1% Zwittergent 3-16, 150 mM NaCl, 5 mM NaF, 1 mM EDTA, 100 µg/ml aprotinin, 10 µM leupeptin, 1 µM PMSF) at a final protein concentration of 5–1 mg/ml. The samples were centrifuged at 100,000 × g for 1 h at 4 °C, and the supernatants were removed. For Triton X-100 extraction, PT fractions were resuspended in 10 mM Tris-HCl, pH 8.0, for further analysis, or solubilized in SDS sample buffer. For Zwittergent 3-16 extraction, the supernatants (S₁₀) were precipitated with 10% trichloroacetic acid, washed twice with ice-cold ether, and solubilized in SDS sample buffer. The samples were subjected to SDS-PAGE as described above. For mass spectrometric analysis, after electrophoresis, proteins were transferred to a nitrocellulose membrane and stained with Amido Black.

**Photoaffinity Labeling in Cell-free Extracts**

Cell preparations were incubated in 10 mM Tris-HCl, pH 8.0, with or without nonradioactive 498 for 1 h at 4 °C. [^H]498 was added to the final indicated concentration with the final protein concentration at 2 mg/ml. The samples were incubated for an additional 2.5 h, followed by 20 min of UV irradiation at 4 °C (Rayonet photochemical reactor lamp, Southern New England Ultraviolet Co., maximum light intensity at 253.7 nm). The UV irradiation experiments were carried out by maintaining the distance of 5 cm between the sample and light source. After irradiation, the samples were subjected to protein purification or solubilized in SDS sample buffer and subjected to SDS-PAGE. Gels were normalized for protein unless otherwise stated. Gels were enhanced by exposure to X-ray film.
lerica, MA) on the plate, and allowed to dry at room temperature; calibrants were diluted from concentrated stocks and mixed to yield 12.5 fmol of each per 0.2-μl volume of the same solvent prior to mixing with the analytes. MALDI-TOF mass spectra were acquired on a REFLEX III (Bruker-Franzen, Bremen, Germany) instrument equipped with a 337-nm nitrogen laser, a gridless pulsed-extraction ion source, and a 2-GHz digitizer. The instrument was operated in reflector mode; 25-kV ion acceleration, 26.25-kV reflector, and 21.4-kV multiplier voltages were used. Ion extraction was done 200 ns after each laser irradiance by pulsing down the source extraction lens to 17.7 kV from its initial 25-kV level to give appropriate time-lag focus conditions at the detector. Spectra were obtained by averaging multiple signals; laser irradiance and number of acquisitions (typically 100–150) were operator-adjusted to yield maximal peak deflections, derived from the digitizer as TOF data and displayed in real time as mass spectra using a SPARC station 5 (Sun Microsystems, Mountain View, CA). After recalibration with internal standards, monoisotopic masses were assigned for all prominent peaks, and a peptide mass list generated.

Electrospray ionization (ESI) MS was done on an API 300 triple quadrupole instrument (PE-SCIEX; Thornhill, Canada), modified with an injection adaptable fine ionization source (JaFIS) as described (16). Needle voltage ranged from 600 to 1350 V depending on the application. The voltages for the orifice and the curtain plate were set at 5 and 350, respectively. Q1 scans were collected using a 0.5-atomic mass unit step size, and a 3-ms dwell time over a mass range from 400 to 1400 atomic mass units; scans were averaged for statistical analysis, and Q1 resolution was set such that the charge state of singly, doubly, and triply charged ions could be ascertained. For operation in the MS/MS mode, Q1 was set to transmit the complete isotopic envelope of the parent. All spectra were averaged with a 0.5-Da step size and a 3-ms dwell time for 5 min over the mass range of the singly charged m/z. Q3 resolution was set such that the charge state of the fragment ions could be distinguished. Collision energies, as well as collision assisted dissociation gas pressures, were optimized individually for each peptide as to obtain the best MS/MS spectra.

Selected, “major” mass values (combined from the 16% and 30% peptide pools, but restricted to 900 atomic mass units ≤ m/z ≤ 3,000 atomic mass units) from the MALDI-TOF experiments were arbitrarily
taken to search a protein non-redundant data base (NRDB; European Bioinformatics Institute, Hinxton, UK) using the PeptideSearch (17) algorithm. A molecular mass range of up to 300 kDa was covered, with a mass accuracy restriction of 30 ppm or better, and a maximum of one missed cleavage site allowed per peptide. After a tentative identification was made, as many as possible of the experimental masses were fitted to the listed sequence (monoisotopic mass values), allowing for maximal 0.1-Da discrepancy. MS/MS spectra from the ESI triple quadrupole analyses were inspected for uninterrupted y<sub>0</sub> ion series using the “find higher Aas” routine of the BioToolbox (PE-SCIEX) software; the resultant information (2–6-amino acid partial sequence, plus corresponding precursor and fragment ion masses) was semi-automatically transferred, by way of a custom AppleScript (Apple Computer, Cupertino, CA), to the SequenceTag (18) program and used as a search string, with a 2-Da mass error restriction. In case less than three y<sub>0</sub> ions could be tagged, this limited information was taken (together with precursor ion mass) to search the data base using the PepFrag protein identification program from the PROWL resource (19).<sup>2</sup> Any protein identification thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data; this also allowed the discrimination of true from false positives in case more than one protein was retrieved.

**Construction of FLAG-tagged Ribosomal Proteins S3 in Bacteria**

The mouse ribosomal protein S3 cDNA was obtained by reverse transcriptase-PCR using total cellular RNA isolated from MEL cells. Reverse transcriptase-PCR, RNA isolation, and molecular cloning were carried out according to Ausubel <i>et al.</i> (20). The primers are designed according to DNA sequences for S3 in GenBank (Accession no. X76772) with additional sequences at 5′ end (lowercase letters below) that

---

<sup>2</sup>The PepFrag protein identification program from the PROWL resource is available via the World Wide Web (http://prowl.rocketefeller.edu/PROWL/pepfdragch.html).
contain restriction enzyme sites (HindIII for the 5′ end primers and BglII for the 3′ end primers). The sequences of these primers are as follows: 5′ end primer, 5′-ccc aag ctt ATG GCG GTG CAG ATT TCC-3′; 3′ end primer, 5′-ata aga tct CCA GAT GCA GCT CGC CAA GAC-3′. PCR products were ligated into pGEM-T vector (Promega) and verified by DNA sequencing. Inserts from clones with the correct DNA sequences were released from the vector by HindIII and BglII digestion, gel-purified, and ligated into HindIII- and BglII-digested pFLAG-MAC vector (Kodak). Transformation, confirmation of FLAG fusion junction, preparation of bacterial lysates, and analyses of FLAG-S3 fusion protein were done according to manufacturer’s instruction (Kodak). Bacterial lysates containing no fusion protein and FLAG-S3 were subjected to photoaffinity labeling as described above.

**Immunoblotting Analysis**

Photoaffinity-labeled samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Expression of S3 was detected by rabbit polyclonal anti-S3 antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (21). The signal was detected by the enhanced chemiluminescence system (Pierce).

**RESULTS**

**Design of Photoaffinity Labeling Reagent [3H]498—** We designed a photoaffinity labeling reagent structurally based on SAHA (Table I). The phenyl azide moiety was chosen as the photoactivatable group for two reasons. (a) The utility of phenyl azides as photoaffinity labeling reagents is well known and documented (22). Upon photolysis at 254 nm, azido groups generate a highly reactive nitrene that can bind to many cellular components. (b) The photoaffinity labeling reagent maintains its activity as a potent inducer of differentiation. We have previously synthesized a SAHA analog (498; Table I), and found that incorporation of an azido group in the para position of the benzene ring does not interfere with the differentiation activity (Fig. 2). Tritium labeling was chosen over other isotopes since bulky substituents such as iodine resulted in a marked decrease in the potency of the inducer.

**Synthesis—** The synthesis of [3H]498 (Fig. 1) involved introduction of tritium by catalytic dehalogenation of suberic hydroxamic acid (4-amino-3,5-ditritioaniline) amide (7), followed by conversion of the amine to an azide. Radiolabeling had to precede azidation because of the instability of the azide group to the reduction conditions used to introduce tritium.

Diodiation of 4-nitroanilene 1 with iodine monochloride (23) gave rise to 2,6-diodo-4-nitroaniline 2, which was reduced with SnCl2 (24) to yield 2,6-diodo-4-aminonoanilne 3. Activation of suberic acid monomethyl ester with carboxyldiimidazole followed by coupling to 3 gave rise to the ester 4. The ester was hydrolyzed with LiOH to yield the carboxylic acid 5 (25). The t-butylidihyphenylsilyl protected hydroxamic acid 6 was prepared by activation of 5 with carbonyl diimidazole, followed by reaction with the silyl protected hydroxylamine. Deprotection with tetrabutylammonium fluoride gave rise to hydroxamic acid 7. Compound 7 was catalytically tritiated with [3H2/Pd-C in methanol/H2O/Na2CO3 to yield 8 (specific activity 40 Ci/mmol). Compound 8 was diluted 10-fold with nonradioactive analog 8a, diazotized, and treated with sodium azide to yield suberic hydroxamic acid (4-azido-3,5-ditritioaniline) amide ([3H]498, specific activity 4 Ci/mmol).

**Induction of Differentiation—** At 3.5 μM, 498 induces on the average 50% of the cells to differentiate (Fig. 2). In addition, cell growth is inhibited by more than 50% (data not shown). In comparison, SAHA induces on the average about 70% of the cells to differentiate at 2.5 μM (Fig. 2), inhibiting cell growth by more than 50% (data not shown). Our preparation of [3H]498 was found to have a level of activity similar to that of the nonradioactive analog 498 (data not shown).

**Photoaffinity Labeling Studies in Cell-free Extracts—** We asked initially whether [3H]498 specifically binds any cellular proteins. We separated specific from nonspecific interactions by competition with excess nonradioactive analog 498. Whole cell lysates were prepared from MEL cells, incubated with [3H]498 in the presence or absence of nonradioactive 498 and irradiated. Protein samples were solubilized in SDS sample buffer, separated by SDS-PAGE, and visualized by fluorography. We observed (Fig. 3A) one specifically radiolabeled protein of 32 kDa (p32) and several nonspecifically labeled proteins. Furthermore, we specifically labeled p32 in the T24 human bladder cancer cell line and the ARP 1 human myeloma cell line (data not shown). SAHA-like HPCs induce T24 cells to differentiate (7) and ARP 1 cells to undergo apoptosis (7). Thus p32, has been identified as a SAHA-like HPC-binding protein.

To determine the cellular distribution of p32, we prepared P10, P100, and S100 fractions from MEL cells and assayed for the presence of p32. Fig. 3B shows that p32 localizes to the P100 fraction, and is the most abundant protein detected by the...
label in this fraction. To establish that binding to p32 occurs at the same concentration range at which SAHA and \(^{3}H\)498 induce differentiation, we followed the specific binding of \(^{3}H\)498 to p32 by incubating P100 fractions with different concentrations of the compound in the presence or absence of excess 498. \(^{3}H\)498 can be detected specifically bound to p32 in a dose-dependent manner (Fig. 3C), and at the concentration in which it is active as an inducer of differentiation. Furthermore, we performed a dose-response competition experiment, by incubating P100 fractions with 5 \(\mu\)M \(^{3}H\)498 and varying the concentration of 498. The labeling of p32 by \(^{3}H\)498 can be inhibited in a dose-dependent manner with increasing concentrations of 498 (Fig. 3D).

**Protein Purification and Mass Spectrometric Analysis**—We partially purified p32 by sequential extraction taking advantage of solubility differences between p32 and other cellular components. P100 fractions were extracted with buffer containing 1% Triton X-100, partitioning p32 into the detergent-resistant fraction (PTx). PTx fractions were extracted with buffer containing 0.1% Zwittergent 3-16, partitioning p32 into the detergent soluble fraction (STx + zw) (Fig. 4A). Silver staining (Fig. 4B) confirmed the partial purification of p32.

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and stained with Amido Black. The band corresponding to p32 was excised from the blot and subjected to MALDI-reflexion TOF (reTOF) MS and continuous flow ESI (JaFIS) triple quadrupole MS/MS (Fig. 5). Both types of MS analyses independently identified p32 as the 40 S ribosomal protein S3.

**Photoaffinity Labeling of Ribosomes and of FLAG-S3 in Bacteria**—To further confirm the identity of p32, we labeled a preparation of ribosomes from dog pancreas (26). p32 was specifically labeled in this preparation (Fig. 6). Moreover, p32 was the most abundantly labeled band detected in the ribosome preparation. To confirm that S3 is labeled by \(^{3}H\)498, we expressed FLAG-S3 in bacteria. Photoaffinity labeling of bacterial lysates revealed that S3 is specifically labeled by \(^{3}H\)498 (Fig. 7A). Western blots using S3 antiserum (21) (Fig. 7B) confirmed the expression of S3 in the bacteria. These results suggest that S3 is the SAHA-binding protein that was detected in cell-free extracts.

**DISCUSSION**

We have previously reported on a class of HPCs that are highly potent inducers of differentiation and/or apoptosis in transformed cells compared with the prototype HPC, HMBA (2). Hydroxamic acid-based HPCs, of which SAHA is the prototype, have been shown to induce differentiation of MEL cells and other transformed cell lines at micromolar concentrations.

The strict structural requirements that define a high potency inducer of differentiation led us to hypothesize that there is a specific receptor to which these agents bind in the cell. We have used a photoaffinity labeling reagent, structurally based on SAHA, in a search for SAHA-binding proteins. Using this approach, we have identified the ribosomal protein S3 as a target of the SAHA-like HPCs. The identification of a SAHA-binding protein provides evidence of a direct physical interaction between the SAHA-like HPCs and this cellular target.

S3 is located on the external surface of the 40 S ribosomal subunit and plays an important role in protein synthesis (21). It contributes to the domain where translation is initiated; it can be cross-linked to initiation factors eIF2 (27) and eIF3 (28), and is directly involved in ribosome-mRNA-aminooacyl tRNA interactions during translation (29).

A growing body of evidence indicates that individual ribosomal proteins and changes in their expression, participate in and modulate a variety of cellular activities (30, 31). Several recent reports implicate ribosomal proteins and other components of the translational machinery as regulatory mediators of growth, proliferation, and neoplastic changes (32–36).

Ribosomal protein S3 appears to possess apurinic/apyrimidinic endonuclease activity that strongly implicate it in DNA repair functions (32, 37–39). It contains an activity that cleaves 8-oxoguanine residues and 2,6-diamino-4-hydroxy-5-formamidoypyrimidine residues of damaged DNA (38), an associated apurinic/apyrimidinic lyase activity that cleaves phosphodiester bonds via a \(\beta,\gamma\) elimination (37), and a DNA deoxyribo- phosphodiesterase activity that removes sugar-phosphate residues from DNA substrates containing 5′ and 3′ incised apurinic/apyrimidinic sites (39).

Deregulation of the DNA repair machinery by an S3-related mechanism may contribute to tumorigenesis. Xeroderma pigmentosum group D patients, who show an increase in the frequency of sunlight-induced skin carcinomas and malignant melanomas (40), have been shown to lack an endonuclease activity that has been identified as S3 (32). Moreover, S3 mRNA is overexpressed in colorectal cancer (41).

Recently we reported that SAHA-like HPCs (but not HMBA), are potent inhibitors of histone deacetylase (HDAC) activity (7). The inhibition of the enzyme has been demonstrated both in cell-free extracts and in intact cells. Using photoaffinity labeling, we have recently obtained preliminary results suggesting that there is a direct and specific binding interaction in cell-free extracts between the SAHA-like HPCs and purified HDAC. Direct binding of HPCs to HDAC may contribute to the mechanism by which hydroxamic acid-based HPCs induce cell differentiation.

The identification of more than one potential target for the HPCs suggests that active agents may exert effects on more than one biological pathway in inducing terminal differentiation. The hypothesis that inducer-mediated commitment to terminal differentiation is a multistep process is supported by earlier studies (42, 43) with HMBA. We have shown (42) that dexamethasone (an inhibitor of inducer-mediated MEL cell differentiation) did not suppress early events in the process of commitment, but inhibited later steps involving initiation of cell division and globin mRNA synthesis. In other studies, Nomura et al. (43) reported that erythroid differentiation in MEL cells is a synergistic result of at least two distinctive and fundamentally different intracellular reactions: one originating from the inhibition or cessation of DNA replication and the other involving a transmembrane reaction triggered by inducing agents such as HMBA or dimethyl sulfoxide (another known inducer of differentiation in MEL cells). It is therefore plausible that SAHA targets more than one protein to induce MEL cell differentiation. The identification of S3 and HDAC as targets for the HPCs may allow the rational design of more potent compounds that will be effective in treating cancer. Further studies of these targets may contribute to the understanding of the control of cell proliferation and differentiation.

**Acknowledgments**—We thank Dr. Martin Wiedmann and Dr. Klaus Van Leyen for helpful discussions and for kindly providing ribosome preparations. We are grateful to Lynne Lacomis, Mary Lui, Anita Grewal, and Scott Geromanos for help with mass spectrometric analysis, and to Matthias Mann for the Pep tideSearch and SequenceTag programs.

**REFERENCES**

1. Breslow, R., Jursic, B., Yan, Z. F., Friedman, E., Leng, L., Ngo, L., Rifkind, R. A., and Marks, P. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5542–5546
2. Richon, V. M., Webb, Y., Merger, R., Sheppard, T., Jursic, B., Ngo, L., Civoil, F., Breslow, R., Rifkind, R. A., and Marks, P. A. (1996) Proc. Natl. Acad. Sci.

---

\(^{3}\) Y. Webb and V. Richon, unpublished observations.
