The Myeloid Leukemia-associated Protein SET Is a Potent Inhibitor of Protein Phosphatase 2A*

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Two potent heat-stable protein phosphatase 2A (PP2A) inhibitor proteins designated I₁PP2A and I₂PP2A have been purified to apparent homogeneity from extracts of bovine kidney (Li, M., Guo, H., and Damuni, Z. (1995) Biochemistry 34, 1988–1996). N-terminal and internal amino acid sequencing indicated that I₂PP2A was a truncated form of SET, a largely nuclear protein that is fused to nucleoporin Nup214 in acute non-lymphocytic myeloid leukemia. Experiments using purified preparations of recombinant human SET confirmed that this protein inhibited PP2A. Half-maximal inhibition of the phosphatase occurred at about 2 nM SET. By contrast, SET (up to 20 nM) did not affect the activities of purified preparations of protein phosphatases 1, 2B, and 2C. The results indicate that SET is a potent and specific inhibitor of PP2A and suggest that impaired regulation of PP2A may contribute to acute myeloid leukemogenesis.

A number of defined chromosomal translocations occur in specific subtypes of myeloid leukemia indicating that these translocations play an important role in the process of leukemogenesis (1, 2). As a result of translocation, nearby oncogenes and other genes involved in the control of proliferation or differentiation can be activated through alterations in regulatory DNA sequences that leave the encoded protein intact (e.g. Myc) or through formation of fusion genes that encode chimeric proteins (e.g. Bcr-Abl, E2A-Pbx, and Pml-RAPα) (1, 2). However, although several of the chromosomal translocations and the resulting fusion genes that occur in leukemia have been identified, often the function of the individual proteins encoded by the fusion transcripts has not been determined (1, 2).

Protein phosphatase 2A (PP2A) is a large mammalian protein serine/threonine phosphatase that regulates diverse cellular processes (3–6). In cells, PP2A is thought to exist as heterotrimeric forms termed PP2Aα and PP2Aβ and composed of a catalytic C subunit and A and B (B, B’, or B”) subunits (3–6). A dimeric form of PP2A, termed PP2Aβ2, has also been isolated from numerous sources and is composed of the A and C subunits (3–6). However, this enzyme is thought not to exist in vivo because the missing B subunit may have been lost during the isolation procedures (3–6). Two forms of the A and C subunits exhibiting apparent Mr values of ~65,000 and 36,000 and 86% (7, 8) and 97% identity (9–13) in their predicted amino acid sequences, respectively, have been identified by molecular cloning methods. In addition, several distinct B subunits of apparent Mr, ~54,000, 55,000, 56,000, 72,000, and 130,000 have also been identified (e.g. see Refs. 7 and 14–17). Although the significance of the different A, B, and C subunits is not well understood, the distinct substrate specificities of the various trimeric PP2A forms appear to be conferred, at least in part, by the variable B subunit (3–6).

However, despite progress on the structure of PP2A, little information is available on the regulation of this enzyme. Nonetheless, evidence has emerged that PP2A is inactivated by phosphorylation (18, 19) and activated by methylation (20) of its C subunit. In addition, we recently purified to apparent homogeneity from extracts of bovine kidney two heat-stable and PP2A-specific inhibitor proteins designated I₁PP2A and I₂PP2A (21). These inhibitor proteins act noncompetitively and exhibit apparent Kᵢ values in the nanomolar range (21). The inhibitor proteins appear to be PP2A-specific because, in contrast to PP2A, they do not affect the activities of PP1, PP2B, and PP2C (21), the other major mammalian protein serine/threonine phosphatases (3–6). Furthermore, I₁PP2A and I₂PP2A did not affect the activities of 11 different protein kinases (21). Because the purified preparations exhibited distinct peptide patterns following cleavage with Staphylococcus aureus V8 protease, I₁PP2A and I₂PP2A may be the products of distinct genes (21). However, direct evidence for this possibility was not provided.

This study was undertaken to determine the identity of I₂PP2A on a firm basis. In this communication, we show that the purified bovine kidney preparations of I₂PP2A represent a truncated form of SET (22), a largely nuclear protein also termed PHAP-II (putative class II human histocompatibility leukocyte-associated protein II) (23) and TAF (template-activating factor) (24). In acute non-lymphocytic myeloid leukemia, the SET gene, which is located on chromosome 9q34 centromeric to c-abl and nup214, is fused to nup214 (also termed can) apparently as a result of translocation (22). This SET-Nup214 fusion gene encodes a 5-kilobase transcript that contains a single open reading frame reading frame predicting a chimeric SET-Nup214 protein of apparent Mr, ~150,000 (2, 22). The results suggest that fusion of SET with Nup214 in acute myeloid leukemia may impair the normal regulation of PP2A and contribute to leukemogenesis.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain MBP (25), PP1 and PP2B (26) and bovine kinase I₁PP2A (21), I₂PP2A (21), PP2A (26), PP2B (26), PP2C (26), and protamine kinase (27) were purified to apparent homogeneity as described. Human kidney polyadenylated RNA was from phosphatase 2C; MBP, myelin basic protein; Nup214, nucleoporin 214; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
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Clontech. Synthetic oligonucleotides were synthesized at Life Technologies, Inc. Other materials are given elsewhere (27–31).

$^{32}$P-Labeled MBP was prepared by incubation with the proteosome kinase (27) exactly as described (21). Assay of PP2A, PP2A$_2$, PP2A$_C$, PP1$_C$, PP2B, and PP2C was performed as reported (21, 26). One unit of phosphatase activity was defined as the amount of enzyme that catalyzes the release of 1 nmol of phosphate/min from MBP. To ensure linearity, the extent of phosphoryl group release was limited to <10%. Inhibitor protein activity was measured as described (21). One unit of inhibitor protein activity was defined as the amount of protein that inhibited 1 unit of PP2A$_C$ by 50% in the standard assay.

SDS-PAGE was performed in slab gels (12% acrylamide) with 0.1% SDS and a 0.5% agarose buffer, pH 8.3 (23). Protein bands were detected by staining with Coomassie Blue. Protein was determined as described (33).

Generation, Purification, and Sequencing of Tryptic Peptides—Aliquots (2 $\mu$g) of purified bovine kidney I$_{252}$ were subjected to SDS-PAGE, followed by electrophoretic transfer onto Immobilon-P membranes. After staining with Poncova S, bands corresponding to I$_{252}$A were cut out and subjected to overnight incubation at 37°C with N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (0.05 mg) as described (34). Tryptic peptides were purified by high pressure liquid chromatography reversed-phase chromatography on an Aquapore RP-300 column ($\times$250 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid. After washing with this solution, the column was developed with linear gradients from 0.15 to 1.0% trifluoroacetic acid to 0.08% trifluoroacetic acid containing 38.5% (v/v) acetonitrile in 30 min, followed by a linear gradient from 0.08% trifluoroacetic acid containing 38.5% acetonitrile to 0.08% trifluoroacetic acid containing 59.5% (v/v) acetonitrile in 10 min. Fractions (0.045 ml) were collected, and the absorbance at 214 nm was determined. The amino acid sequences of peptides eluting at about 38% (peptide I) and 48% (peptide II) acetonitrile in the N terminus of I$_{252}$A were determined using standard reagents for gas phase chemistry on an automated protein sequencer (Applied Biosystems) equipped with an on-line UV detector to identify phenylthiodyantion derivatives.

Generation of SET cDNA—First strand SET cDNA was generated at 42°C for 2 min and 10°C (22), pH 8.3, containing 50 mM KCl, 5 mM MgCl$_2$, 20 units of RNase inhibitor, 1 mM of each dNTP, 0.5 $\mu$g of human kidney polyadenylated RNA (Clontech), 50 pmol of the 3' SET-based oligonucleotide (GGATCCCTAGCTCCTCCTCCCT), and 2.5 units of murine leukemia virus reverse transcriptase (Perkin-Elmer). After heat denaturation at 99°C for 5 min, a 0.005-ml aliquot of the mixture was subjected to amplification by polymerase chain reaction with the primer set described (34). Amino Acid Sequencing—Amino acid sequences available at the PIR, SwissProt, and GenBank data bases indicated that peptides I and II were identical to residues 45–55 and 182–196 predicted for human SET, respectively (22). Sequences are underlined) with residues 45–55 and 182–196 predicted for human SET, respectively (22).

RESULTS AND DISCUSSION

Amino Acid Sequencing—To establish the identity of I$_{252}$A$, we set out to determine the amino acid sequence of this protein. Initially, the amino acid sequences of two tryptic peptides (LCNQASEEILK, peptide I) and (QHEEPESTFTDHD, peptide II), generated and purified as described under "Experimental Procedures," were determined. Comparison with amino acid sequences available at the PIR, SwissProt, and GenBank data bases indicated that peptides I and II were identical to residues 45–55 and 182–196 predicted for human SET, respectively (22).

Earlier, we reported (21) that the N-terminal amino acid sequence of I$_{252}$A (SDGADATSTK) showed 70% identity (differences are underlined) with residues 17–26 of SET (22). Because the purified preparations of bovine kidney I$_{252}$A exhibit an apparent $M_c$, $\sim$20,000 (21), whereas SET exhibits an apparent $M_c$, $\sim$38,000 (34), we estimated that I$_{252}$A may have been derived from SET by proteolysis possibly during the purification procedure and that the three amino acid differences noted in the N terminus may be species and/or tissue related. In this connection, it is pertinent that the human SET gene encodes two transcripts of 2.0 and 2.7 kilobases that result from the use of alternative polyadenylation sites. However, both transcripts contain identical open reading frames (2, 22).

Expression and Purification of SET—To test whether SET inhibits PP2A in a manner corresponding to I$_{252}$A, a cDNA coding for human SET was generated and placed into a pET-21a vector under the control of the T7lac promoter as described under "Experimental Procedures." Consistent with the possibility that SET inhibits PP2A, IPTG-induced expression of this cDNA in bacteria resulted in about a 10-fold increase in PP2A inhibitor activity as determined following poly-L-lysine-agarose chromatography (Fig. 1). Because of interference from an unidentified endogenous inhibitor(s), differences in PP2A inhibitor activity in bacterial extracts from control and IPTG-treated cells could not be distinguished. This (these) endogenous inhibitor(s) was (were) not a product(s) of the introduced cDNA because similar activity was detected in extracts of bacteria.
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Fig. 1. Induction of PP2A Inhibitor. Extracts from 500-ml cultures of control (A) and IPTG-treated (B) bacteria containing SET cDNA were prepared as described under "Experimental Procedures." Each extract was then applied onto a separate column (2.5 × 8.5 cm) of poly-L-lysine-agarose equilibrated with buffer B. Each column was washed with 500 ml of buffer B followed by buffer B containing 0.3 × NaCl. Inhibitor activity was recovered with buffer B containing 0.8 × NaCl. Fractions (3 ml) were collected, and PP2A inhibitor activity (●) was determined according to Li et al. (21). The absorbance at 595 nm (○) was determined according to Bradford (33) using 0.05-ml aliquots of the indicated fractions.

To more directly test the possibility that SET inhibits PP2A, a procedure was developed to purify the IPTG-induced PP2A inhibitor to apparent homogeneity from the bacterial extracts as described under "Experimental Procedures." This procedure was based on the one employed previously in the purification of I2PP2A from extracts of bovine kidney (21) and included chromatography of the extracts on poly-L-lysine-agarose, precipitation with ethanol, and gel permeation chromatography on Sephacryl S-200, followed by trichloroacetic acid and ethanol precipitation. The purified preparations of the recombinant protein consisted of a single Coomassie Blue staining polypeptide of apparent M, ~39,000 as estimated by SDS-PAGE (Fig. 2). N-terminal amino acid sequencing of these preparations (to the 10th residue) confirmed that the purified protein was SET. Typically, about 3 mg of this protein was obtained from a 500-ml culture of transformed cells.

Effect of SET on PP2A Activity—The effect of the purified recombinant SET preparations on PP2A activity was examined next. These preparations inhibited PP2A potently (Fig. 3). Half-maximal inhibition of the phosphatase occurred at about 2 nM (Fig. 3), similar to the potent inhibition obtained with purified preparations of bovine kidney I2PP2A (21). Previously, we showed that, by contrast to PP2A, purified preparations of PP1C, PP2B, and PP2C were unaffected by I2PP2A (21). Similar experiments revealed that recombinant human SET also exhibited little or no effect on the activities of PP1C, PP2B, and PP2C (Fig. 3). Together, these results indicate that SET is a potent and specific inhibitor of PP2A. Because the recombinant SET preparations inhibited PP2A, (Fig. 3) PP2A2 (not shown), and PP2A1 (not shown), SET is analogous to I2PP2A in that it appears to act by binding to the C subunit of the phosphatase.

Earlier studies suggested a role for SET as a transcriptional activator because it enhanced adenovirus core DNA replication in HeLa cell extracts (24). The molecular basis of this effect was not determined, although our results raise the possibility that it may have been a consequence of PP2A inhibition. A role for SET in antigen-mediated responses was also suggested because this protein appeared to bind to a peptide (CFI1KGLRK-SNAAERRGPL) patterned on an amino acid sequence present in the cytoplasmic C-terminal region of the DR2α chain of human histocompatibility class II receptor (23). However, the functional significance of this interaction and whether it occurs with the intact receptor were not determined. Based on the results presented herein, we recommend that SET be renamed I2PP2A to indicate its function and to distinguish it from I1PP2A, which is the product of a distinct gene.2 This revised nomenclature is used in the remaining discussion.

The results presented in this communication provide a firm basis for further characterization of the physiological role of I2PP2A. Tissue and species distribution studies indicate that, by analogy with PP2A (3–6), I2PP2A is ubiquitous (36), suggesting its potential importance for controlling the activity of the phosphatase in diverse cells and tissues. Interestingly, I2PP2A undergoes phosphorylation on unidentified serines in intact cells (36). However, how this phosphorylation affects I2PP2A activity and whether or not this regulation responds to extracellular stimuli is unknown. Further studies are also needed on the mechanism of action of I2PP2A. In this connection, the C terminus of I2PP2A (22) (and that of I1PP2A) is highly acidic (22), and deletion of this acidic tail (residues 224–277) abolished the stimulation by I2PP2A of adenovirus core DNA replication in HeLa cell extracts (24). However, whether deletion of the acidic tail abolishes the inhibition of PP2A by I2PP2A (and/or I1PP2A) remains to be determined. In this regard, it is pertinent that,

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The results presented in this communication suggest that the subunit and inhibit the phosphatase in transformed cells (40). The protein predicted by this gene fusion contains residues 1–270 of PP2A and thus contribute to leukemogenesis. The chimeric cytolic myeloid leukemia (22), may also lead to altered regulation of leukemic cells that express the I2 protein. Thus, it will be important to determine how, relative to normal cells, the activity of nuclear PP2A is affected in leukemic cells that express the I2P2AP2A-Nup214 fusion protein.

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