NH₂-terminal Modification of the Phosphatase 2A Catalytic Subunit Allows Functional Expression in Mammalian Cells*

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Brian E. Wadzinski†§, Bartholomew J. Eisfelder‡, Leonard F. Peruski, Jr.‡, Marc C. Mumby‡, and Gary L. Johnson‡$$

From the ‡Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206, the Department of Pharmacology, University of Colorado School of Medicine, Denver, Colorado 80262, and the §§Department of Pharmacology, University of Texas Southwest Medical Center, Dallas, Texas 75235

Functional expression of recombinant wild-type phosphatase 2A catalytic subunit has been unsuccessful in the past. A nine-amino-acid peptide sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein was used to modify the NH₂- and/or COOH-terminus of the phosphatase 2A catalytic subunit. Addition of the nine-amino-acid sequence at the NH₂-terminus allowed recombinant phosphatase 2A expression as a predominantly cytosolic phosphatase 2A enzyme. The 12CA5 monoclonal antibody that recognizes the nine-amino-acid hemagglutinin peptide sequence was used to immunoprecipitate the epitope-tagged phosphatase 2A catalytic subunit. Assay of the immunoprecipitated epitope-tagged phosphatase 2A demonstrated an okadac acid-sensitive dephosphorylation of [32P]histone H1 and [3H]myelin basic protein similar to that measured with the wild-type enzyme. Functional phosphatase activity could be demonstrated for the NH₂-terminal modified phosphatase 2A catalytic subunit following transient expression in COS cells or stable expression in Rat1a cells. In contrast, the COOH-terminal-modified phosphatase 2A catalytic subunit was very poorly expressed. The NH₂-, COOH-modified subunit, having the nine-amino-acid hemagglutinin peptide sequence encoded at both termini of the polypeptide, was also expressed as a functional phosphatase 2A enzyme. Thus, NH₂-terminal modification of the phosphatase 2A catalytic subunit results in a functional plasmid-expressed enzyme. The unique nine-amino-acid epitope-tag sequence also provides a method to easily resolve the recombinant phosphatase 2A from the endogenous wild-type gene product and related phosphatases expressed in cells.

Protein phosphorylation/dephosphorylation events are critically involved in the control of cell phenotype, growth, and differentiation (Cantley et al., 1991). The immediate signal produced by many growth factor receptors is stimulated by tyrosine kinase activity (Hunter, 1987). The major response observed following hormone/growth factor receptor-stimulated tyrosine kinase activity is dephosphorylation by phosphatase 2A (Scheidtmann et al., 1991); Virshup et al., 1989; Walter et al., 1990; Yang et al., 1991). The growth control machinery of the cell is basically overtaken by T antigens to allow uncontrolled cell growth and virus replication. Both SV40 and polyoma T antigens bind the phosphatase 2A enzyme (Mumby and Walter, 1991). In fact, the only known function in terms of stable protein interactions for SV40 and polyoma small T antigen is the binding of the phosphatase 2A enzyme (Pallas et al., 1990; Walter et al., 1990). One functional role of the small T antigen shown in vitro is the inhibition of SV40 large T antigen dephosphorylation by phosphatase 2A (Scheidtmann et al., 1991). Additionally, small T antigen was also shown to inhibit the phosphatase 2A catalyzed dephosphorylation of the p53 growth suppressor gene (Scheidtmann et al., 1991).

The study of Ser/Thr phosphatases in mammalian cells using genetic strategies for expression of recombinant proteins has been frustrating. This frustration has been due to problems inherent to expression of Ser/Thr phosphatases using standard gene transfer techniques. First, attempts to express functional phosphatase catalytic subunits in mammalian cells using gene transfer techniques have met with little success (Green et al., 1987). The expressed phosphatase polypeptides have been denatured and inactive. Second, the catalytic subunits of Ser/Thr phosphatases are highly conserved (Cohen, 1989, 1990; Wadzinski et al., 1990), and antibodies specific for different phosphatases and their isoforms have been lacking.

To overcome the problem of expressing functionally active phosphatase catalytic subunits, we hypothesized that the catalytic subunit polypeptide primary sequence would have to be modified. For this purpose, we began to manipulate the NH₂- and COOH-terminal regions of the phosphatase 2A catalytic subunit using mutagenesis strategies of the cDNA encoding
the human phosphatase 2A gene product (Arino et al., 1988). In this report, we demonstrate that addition of a nine-amino-acid peptide sequence at the NH2 terminus of the phosphatase 2A catalytic subunit is sufficient to stabilize the translation product in a functional form. An antibody recognizing the nine-amino-acid "epitope-tag" sequence can be used to resolve the plasmid-expressed gene product from endogenous phosphatases, including the wild-type phosphatase 2A enzyme. The epitope-tagged phosphatase 2A enzyme can be functionally expressed both transiently and stably in mammalian cells. Thus, for the first time, genetic strategies seem feasible to alter the expression and to study the functional properties of specific Ser/Thr phosphatases in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were obtained from New England BioLabs or United Biochemical Corp. *Thermus aquaticus* DNA polymerase (AmpliTaQ) was obtained from Perkin-Elmer Cetus. Oligonucleotide primers were synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer. Okadaic acid was purchased from Calbiochem (La Jolla, CA). The abbreviations used are: PCR, polymerase chain reaction; TPA, 12-0-tetradecanoylphorbol-13-acetate.

**Construction of Epitope-tagged PP2A cDNA—Oligonucleotides encoding the epitope sequence YPYDVPDYA were inserted in frame with the human liver phosphatase 2A cDNA (Arino et al., 1988) using the polymerase chain reaction (PCR).** A cDNA fragment encoding the NH2-terminal-tagged phosphatase 2A was generated by successive PCR and restriction digestion. The first PCR product was made using the antisense oligonucleotide 5'-TGG TTA AGT C TA ATC TGG AAC ATC ATA-3', and the first PCR product as template. The resulting PCR product was digested with the restriction enzymes HindIII and BstXI. This resulting cDNA fragment and the cDNA fragment of PP2A from the BstXI site to the HindIII site (5'-terminal of phosphatase 2A) were ligated into the HindIII cloning site of the mammalian expression vector pCW1-NEO (Wooll et al., 1988) to produce the NH2-terminal-tagged phosphatase 2A vector pCW1-NEO. Orientation and proper construction of all of the constructs was verified by restriction enzyme analysis.

**Transfection Procedure**—Clonal Rat1a cells or COS-1 cells (6 h post-transfection) were dislodged from the dish using trypsin, pelleted by centrifugation, and washed with phosphate-buffered saline. A cytosolic extract was obtained by repetitively passing cells in a low salt buffer (50 mM Tris, pH 7.4, 2 mM MgCl2, 1 mM EDTA, 0.02 units of aprotinin/ml, and 10 μg of leupeptin/ml) 2 times in a 25-gauge needle. The cytosolic extract was centrifuged for 15 min at 10,000 ×g to remove nuclei and cell debris. This extract was used for immunoprecipitation and immunoblotting. For subcellular fractionation, the broken cells were centrifuged 5 min at 2,000 ×g, and the crude nuclear pellet was washed once with the low salt buffer. The supernatants from the low speed centrifugation were collected and centrifuged at 100,000 ×g for 30 min. The supernatants (cytosolic extracts, C) were precipitated with trichloroacetic acid, washed with acetone, dried, and resuspended in Laemmli sample buffer. The nuclear (N) and 100,000 ×g pellets (P) were also solubilized in Laemmli sample buffer, boiled 10 min, centrifuged briefly and centrifuged 10 min at 10,000 ×g to remove nuclei and cell debris.

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bovine serum albumin and [³²P]histone H1 (2.5 x 10⁶ cpm/μg) or [³²P]myelin basic protein (1.4 x 10⁶ cpm/μg). Samples were incubated at 30 °C for varying times and then protein precipitated on ice with 20% trichloroacetic acid. For okadaic acid inhibition studies, 1 μl of 100 x okadaic acid concentrations in Me2SO was added to the phosphatase reaction mixture. Reactions were incubated for 30 min at 30 °C before precipitation with trichloroacetic acid. The precipitated protein was pelleted by centrifugation at 10,000 g for 15 min. The supernatants were collected and quantitated for [³²P] released by scintillation counting. Fractions from Mono-Tated protein was pelleted by centrifugation at 10,000 g for 15 min. The supernatants were collected and quantitated for [³²P] released by scintillation counting. Fractions from Mono-

RESULTS AND DISCUSSION

The difficulty encountered in expressing cloned Ser/Thr phosphatase 2A and related phosphatases has limited progress in analyzing the cellular functions of this important family of regulatory proteins. In a past report, recombinant phosphatase 2A expressed in mammalian cells has resulted in a denatured, inactive form of the polypeptide (Green et al., 1987). This problem is likely inherent to the improper folding of the protein during translation.

To analyze the expression and biological function of cloned phosphatase 2A, we initiated experiments that had two goals. First, to modify the primary sequence of phosphatase 2A at both the NH₂ and COOH terminus to determine if this would allow functional expression of the translation product; and second, to encode a unique antigenic epitope to discriminate the plasmid expressed phosphatase 2A from the endogenous phosphatase 2A catalytic subunit. Fig. 1 shows the strategy that was successful in accomplishing both goals. The nine-amino-acid epitope YDYPDVPDYA of the influenza hemagglutinin protein (Wilson et al., 1984) was encoded at either the NH₂, COOH, or both the NH₂ and COOH termini of phosphatase 2A. The chimeric cDNAs encoding the nine-amino-acid epitope and the full-length phosphatase 2A polypeptide were inserted into the pCW1-NE0 expression plasmid (Woon et al., 1988) and used for both transient and stable transfection analysis.

The plasmid expressed phosphatase 2A is uniquely antigenically epitope-tagged so it may be distinguished from the endogenous wild-type phosphatase 2A gene product. The epitope-tagged sequence is YDYPDVPDYA and is recognized by the 12CA5 monoclonal antibody. Fig. 2 shows the expression and fractionation of the epitope-tagged phosphatase 2A. In panel A the transient expression in COS cells and stable expression in Rat1a fibroblasts is shown. Transient expression in COS cells revealed that the NH₂-terminal (N²⁰-PP2A) and NH₂- and COOH-terminal (N²⁰-PP2A-C²⁰) fusion proteins were expressed with an M, of 38,000 and 40,000, respectively. Interestingly, the COOH-terminal epitope-tagged phosphatase 2A fusion protein (PP2A-C²⁰) was not detectable. In stable Rat1a transfectants, only the NH₂-terminal epitope-tagged phosphatase 2A fusion protein was detected. Multiple independent Rat1a clones demonstrated varying levels of N²⁰-PP2A expression. Assay of [³²P]histone H1 phosphatase activity in crude cell lysates of N²⁰-PP2A-expressing cells did not show a dramatic change in total histone dephosphorylation activity relative to wild-type cells (not shown). This is not unexpected given the levels of expression and the presence of high histone phosphatase activity found in the crude lysates. Many independent G418-resistant Rat1a clones were screened for expression of the PP2A-C²⁰ and N²⁰-PP2A-C²⁰ fusion proteins (not shown). No clones were isolated expressing the PP2A-C²⁰ fusion and very low level expression of the N²⁰-PP2A-C²⁰ fusion protein was observed with Rat1a transfectants. These findings indicated that modification of the phosphatase 2A COOH terminus appeared to diminish expression of the polypeptide, particularly in stable Rat1a cell transfectants. A similar lack of expression of the COOH-terminal epitope-tagged phosphatase 2A fusion protein was observed with stable transfectants of Chinese hamster ovary and NIH3T3 cells (not shown). In transient assays, the N²⁰-PP2A-C²⁰ fusion protein was expressed reproducibly at

![Fig. 1. Schematic diagram of the expression system for epitope-tagged phosphatase 2A.](image)

- **A.** Expression
- **B.** PP2A Distribution
- **C.** Epitope-tagged PP2A Distribution

**Fig. 2. Expression and distribution of phosphatase 2A and epitope-tagged phosphatase 2A.** COS-1 cells and Rat1a cells were transfected with pCW1-NEO plasmid without a cDNA insert (control) or with pCW1-NEO having a cDNA encoding the NH₂-terminal tagged (N²⁰-PP2A), the COOH-terminal tagged (PP2A-C²⁰), or double NH₂-/COOH-terminal tagged (N²⁰-PP2A-C²⁰) phosphatase 2A chimeras. Total cell extracts, crude nuclear extracts (N), cytosolic extracts (C), or high speed pellets (P) were prepared as described under “Experimental Procedures” and the samples resolved by SDS-PAGE (10% acrylamide). Proteins were transferred to nitrocellulose and probed with 12CA5 monoclonal antibody (A and C) or anti-phosphatase 2A monoclonal antibody (B) followed by rabbit anti-mouse IgG and [¹²⁵I] Protein A as described under “Experimental Procedures.” The endogenous phosphatase 2A, N²⁰-PP2A, and N²⁰-PP2A-C²⁰ phosphatase chimeras behaved as 36-, 38-, and 40-kDa polypeptides, respectively.
greater levels than that in stable transfectants. The results are consistent with NH2-terminal addition of the nine-amino-acid epitope allowing the functional expression of the recombinant phosphatase 2A fusion protein, whereas addition of the same sequence to the COOH terminus of phosphatase 2A does not allow for expression of the phosphatase 2A translation product.

Panel B of Fig. 2 shows the relative subcellular distribution of the endogenous wild-type phosphatase 2A catalytic subunit in nuclear and high-speed pellet and supernatants of lysed COS and Rat1a cells. When cells are lysed in the absence of detergents by passage through a 26-gauge needle the majority of the phosphatase 2A catalytic subunit is found in the high-speed supernatant. Comparatively low levels are found in the nuclear and high-speed pellet fractions. Thus, the phosphatase 2A enzyme is primarily a soluble protein.

The plasmid expressed NH2-terminal and NH2- and COOH-terminal epitope-tagged phosphatase 2A fusion proteins have similar fractionation profiles when transiently expressed in COS cells (Fig. 2, panel C). Similarly, the NH2-terminal epitope-tagged phosphatase 2A fusion behaved primarily as a soluble cytoplasmic protein when stably expressed in Rat1a cells (panel C). These findings demonstrate that the recombinant NH2-terminal epitope-tagged phosphatase 2A fusion proteins were soluble when expressed in mammalian cells and fractionated using differential centrifugation essentially identical to the endogenous wild-type enzyme.

Fig. 3 shows the fractionation by Mono Q-FPLC of the wild-type endogenous phosphatase 2A and NH2-terminal epitope-tagged phosphatase 2A fusion protein. Using protein kinase C-phosphorylated [32P]histone H1 as a phosphatase 2A substrate (Jakes and Schlender, 1988), it is readily apparent that the elution profile of the NH2-terminal epitope-tagged phosphatase 2A fusion protein is virtually identical to the wild-type endogenous phosphatase 2A enzyme. The two overlapping peaks of phosphatase activity observed in the control and transfected cells is related to the partial resolution of the heterotrimeric phosphatase 2A complex (first eluting peak, fractions 11–12) versus the heterodimeric phosphatase 2A enzyme (second eluting peak, fractions 12–15). This finding is based on the Mono Q elution profiles for purified heterodimeric (AC) complex and the heterotrimeric phosphatase 2A enzyme, which has the C subunit associated with the A and B regulatory subunits (Kamibayashi et al., 1991). Comparing the Mono Q elution profiles of purified AC and ABC forms of phosphatase 2A with the elution profile of NH2-terminal epitope-tagged phosphatase 2A, indicated that the NH2-terminal epitope-tagged phosphatase 2A eluted in both peaks corresponding to the two enzyme forms (not shown). This finding indicates that the NH2-terminal epitope-tagged fusion protein associates with the phosphatase 2A regulatory subunits.

By using the 12CA5 antibody, it was also possible to immunoprecipitate the epitope-tagged phosphatase 2A fusion proteins (Fig. 4). The NH2- and double NH2- and COOH-terminal phosphatase 2A fusion proteins were shown to be active in immunoprecipitates from lysates of the appropriate COS or Rat1a cell transfectants. Immunoprecipitation of functional epitope-tagged phosphatase 2A could be accomplished from both crude lysates and following fractionation on Mono Q columns. It was found that the phosphatase 2A activity was significantly increased when the epitope-tagged phosphatase 2A fusion protein was released from the antibody complex using the YPYDVPDYA antigenic peptide, indicating the free enzyme was more active than when complexed to the antibody. The basis for the increased activity is unclear but may be related simply to substrate accessibility.

The NH2-terminal phosphatase 2A fusion protein released from the immune complex with peptide epitope was subsequently resolved by SDS-PAGE in parallel to serial dilutions of purified phosphatase 2A catalytic subunit (not shown). The proteins were then immunoblotted with a phosphatase 2A antibody raised against the COOH-terminal 14 amino acids of phosphatase 2A, which will equally recognize the wild-type and NH2-terminal epitope-tagged proteins. Using this method to determine the relative amount of NH2-terminal phospa-
these findings indicate that the soluble character, enzymatic purified phosphatase 2A catalytic subunit. Cumulatively, wild-type catalytic phosphatase 2A subunit. These characteristics of the NH2-terminal epitope-tagged phosphatase 2A are virtually indistinguishable from the endogenous activity, chromatographic behavior, and okadaic sensitivity the antibody-bead complex with 25 ng of purified transfected with control plasmid without a phosphatase 2A cDNA to the phosphatase 2A polypeptide had little effect on its cloned phosphatase 2A catalytic subunit may involve changes in protein folding during translation. Why NH2-terminal modification of the phosphatase 2A catalytic subunit permits functional expression relative to COOH-terminal modification is obviously unknown, but its utility is apparent. The epitope-tagged phosphatase 2A, for the first time, provides functional expression and detection of recombinant serine/threonine phosphatases. This has not been accomplished in mammalian cells with any other procedure for this set of gene products. The expressed fusion protein is easily resolved from endogenous cellular phosphatases by immunoprecipitation, and the antigenic epitope peptide can be used to dissociate the enzyme from the antibody complex. This strategy may have general utility for the study of other phosphatases as well. In fact, any phosphatase for which a cDNA has been isolated can be epitope-tagged and tested for expression in mammalian cells.

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**FIG. 4.** Phosphatase activity of immunoprecipitated Nw-PP2A polypeptide. COS-1 cells (A) and Ratla cells (B and C) transfected with control plasmid without a phosphatase 2A cDNA insert (○) or plasmids having cDNAs encoding Nw-PP2A-Cneo (▲) or Nw-PP2A (■) were immunoprecipitated with 12CA5 monoclonal antibody followed by protein A-Sepharose. Protein was eluted from the antibody-bead complex with 25 μM YPYDVPDYA peptide. The WPi-PP2A polypeptide was immunoprecipitated from WPi-PP2A-expressing and control cells treated with 12CA5 antibody and protein A-Sepharose. The washed immunoprecipitates were incubated with the YPYDVPDYA peptide to release the epitope-tagged phosphatase 2A, for the first time, provides functional expression relative to COOH-terminal modification of the phosphatase 2A catalytic subunit permits functional expression relative to COOH-terminal modification is obviously unknown, but its utility is apparent. The epitope-tagged phosphatase 2A, for the first time, provides functional expression and detection of recombinant serine/threonine phosphatases. This has not been accomplished in mammalian cells with any other procedure for this set of gene products. The expressed fusion protein is easily resolved from endogenous cellular phosphatases by immunoprecipitation, and the antigenic epitope peptide can be used to dissociate the enzyme from the antibody complex. This strategy may have general utility for the study of other phosphatases as well. In fact, any phosphatase for which a cDNA has been isolated can be epitope-tagged and tested for expression in mammalian cells.

**FIG. 5.** Okadaic acid inhibition of the Nw-PP2A enzyme activity. The Nw-PP2A polypeptide was immunoprecipitated from Ratla cells stably expressing the NH2-terminal epitope tagged phosphatase 2A (■). G418-resistant Ratla cells transfected with pCWl-neo plasmid (●) without the Nw-PP2A cDNA were used for control immunoprecipitations. Lysates from Nw-PP2A-expressing and control cells were treated with 12CA5 antibody and protein A-Sepharose. The washed immunoprecipitates were incubated with the YPYDVPDYA peptide to release the epitope-tagged phosphatase 2A enzyme. The dephosphorylation of [32P]histone H1 (A) or [32P]myelin basic protein (B) was then determined in the presence of the indicated concentrations of okadaic acid as described under "Experimental Procedures."
Epitope-tagged Phosphatase 2A

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