A Monoclonal Antibody Which Identifies the Autophosphorylation Domain of Autophosphorylating Protein Kinase 500

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ABSTRACT

Autophosphorylating protein kinase 500 is a serine protein kinase expressed progressively with the steps of cellular transformation, approaching levels from 50- to 100-fold in the terminal stages of malignancy. The enzyme possesses a sharply restricted range of substrates: itself and a ribosomal protein with a molecular weight of 31,000 (S6). We report here on the characterization of a monoclonal antibody directed against the autophosphorylation domain of AUT-PK 500. The specificity of the antibody is evidenced by blockage of the enzyme phosphorylation without interfering with the native S6 or synthetic octapeptide (S6-1) serine residue phosphorylations. This represents an important step in identifying a probe that can be used to explore the structure and potential function of AUT-PK 500 in cellular transformation.

INTRODUCTION

AUT-PK 500 was originally purified from the rat adrenocortical carcinoma based on its abnormally high expression in this tumor (1). The expression of AUT-PK 500 is elevated 100-fold in this spontaneously occurring carcinoma, 50-60-fold in chemically induced rapidly growing hepatomas, 30-fold in chemically induced mammary carcinoma, 20-fold in a cultured hepatoma cell line, and 30-fold in a pituitary tumor (2). Since the moderate (4-7-fold) enzyme expression occurs in the early steps of neoplasia and there is no increased expression in fetal tissue or embryonic cells (2), these results indicate that AUT-PK 500 is a normal gene product, the expression of which is strikingly elevated during the process of neoplasia but is unrelated to the differentiation or developmental processes. Although we do not know if AUT-PK 500 overexpression is causally or coincidentally related to either cellular immortalization or carcinogenesis, the unique nature of AUT-PK 500 raises an intriguing question as to its potential role as a cellular transforming protein. Unlike other previously characterized cyclic nucleotide-dependent and -independent protein kinases, AUT-PK 500 is neither regulated by the cyclic nucleotides nor is its autophosphorylation stimulated by calcium or calcium-calmodulin (1). It is also distinct from other cyclic nucleotide-independent protein kinases such as casein kinase I and II (3, 4) and the protease-activated kinases I and II (5, 6), in that it phosphorylates its own serine residue and a single 40S ribosomal protein of M, 31,000, the well-characterized S6 ribosomal protein (7). Furthermore, AUT-PK 500 differs from most oncogene products and membrane growth factor receptors in that it is a cytoplasmic protein kinase which phosphorylates itself and the S6 ribosomal protein (1). The only amino acids phosphorylated in these two substrates is serine (1); however, it is possible that a threonine in an appropriate environment might be phosphorylated.

In order to delineate the potential role of autophosphorylation and S6 kinase activities of AUT-PK 500 in cellular transformation, we now report on the development of monoclonal antibody IIIC 3.3 against the autophosphorylation domain of the enzyme which can be used as a probe to dissect the autophosphorylation and S6 kinase activities of the protein kinase.

MATERIALS AND METHODS

Materials. Polyethylene glycol 1000, aminopterin, hypoxanthine, and thymidine were from Duchland Laboratories (Denver, PA); Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco (Grand Island, NY); Pristane mineral oil was from Aldrich (Milwaukee, WI). Protein A coupled to Sepharose CL-4B was obtained from Pharmacia (Piscataway, NJ). Goat IgG directed against mouse IgG was from Sigma Chemical Company (St. Louis, MO).

AUT-PK 500 was purified from rat adrenocortical carcinoma 494 according to the method of Ganguly et al. (1), and the membrane-bound ribosomes were prepared from postmitochondrial supernatants of rat liver homogenates as described by Ikehara and Pitot (8).

Culture Medium. Dulbecco's modified Eagle's medium was supplemented with penicillin (50 units/ml), streptomycin (50 ¡g/ml), and fetal bovine serum (10%).

Immunization and Cell Hybridization. Three BALB/c mice (5 weeks old) were each injected i.p. with 25 ¡g of purified AUT-PK 500 in 0.1 ml of 0.05 M PBS emulsified with an equal volume of Freund's complete adjuvant. Booster injections of 20 ¡g protein were given in the same way with incomplete adjuvant after 14 days and every 2 weeks thereafter for 2 months. 10 days after the last booster injection, the mice were bled and the sera checked for antibody titers. The mouse with the most satisfactory antibody titer received a final injection of 25 ¡g of antigen i.v., and the spleen was removed 3 days after the final injection. Mouse spleen cells (1 × 109) were fused with exponentially growing mouse Sp2/0 myeloma cells according to the method of Geffer et al. (9). The fusion products were diluted with 10 ml of Dulbecco's modified Eagle's medium containing 15% fetal bovine serum. 100 ¡l of this suspension were plated in 96-well Costar culture plates and incubated at 37°C in an atmosphere of 95% air and 5% CO2. On the day after cell fusion, 100 ¡l of 2× hypoxanthine/aminopterin/thymidine selective medium were added to each well. Following the appearance of hybridoma cell colonies, the culture fluids were tested for anti-AUT-PK 500 antibody production by ELISA (described below). The positive hybridomas were cloned twice by limiting dilution and the clones were expanded.

Cells from one of the positive clones selected for inhibition of autophosphorylation, were injected i.p. in BALB/c mice (8 × 106 cells/mouse) primed with Pristane to generate ascitic fluid. The IgG fraction of ascites was prepared by using a Protein A-Sepharose 4B column.

For characterization of antibody subclass, ascites fluid was fractionated on Protein A-Sepharose column according to the method of Ey et al. (10). Using buffers of decreasing pH, IgG1, IgG2a, and IgG2b could be sequentially eluted at pH 6.0-7.0, 4.5-5.0, and 3.5-4.0, respectively. Buffer solutions used were either 0.15 M sodium phosphate (pH 6.5-8.0) or 0.1 M sodium citrate/citric acid (pH 3.0-6.0) containing azide at 0.05%. The samples from column fractions were analyzed by SDS-polyacrylamide gel electrophoresis.

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5. The abbreviations used are: AUT-PK 500, autophosphorylating protein kinase 500; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris/saline, 20 mM Tris-Cl (pH 7.4) with 0.15 M NaCl; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

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ELISA for AUT-PK 500. Polyvinyl chloride microtiter plates (Costar) were coated with 100 µl of purified AUT-PK 500 (150 ng) in 20 mM Tris-HCl, pH 7.6, according to the method of Voller et al. (11). The wells serving as controls were coated only with Tris buffer. The nonspecific binding sites of all wells were blocked by 3% BSA. Hybridoma medium (100 µl) was added in duplicate to wells that had been incubated with AUT-PK 500 and also to wells incubated with Tris buffer, which served as a minus-antigen control.

The plates were incubated for 2 h at room temperature. The samples were then removed and the wells washed three times with 0.05 M PBS. To all the wells, 100 µl of goat-anti-mouse-IgG coupled to peroxidase (1:800 in 0.05 M PBS with 0.5% bovine serum albumin) were added and the plate incubated for 1 h at room temperature. The wells were washed thrice, and for the color reaction, 100 µl of the substrate (o-phenylenediamine 600 µM/ml in 0.1 M phosphate citrate buffer, pH 6.5, with 0.06% H₂O₂) were added to all the wells. The reaction was terminated after 15–20 min with 5 M H₂SO₄ and the absorbance was measured at 492 nm by a spectrophotometer.

Synthesis of S6-1 Octapeptide. The octapeptide Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (S6-1, Ref. 12) was synthesized by the Molecular Resource Center, University of Tennessee, Memphis, TN. The peptide was cleaved from the resin and deprotected by Immuno-Dynamics, Inc., La Jolla, CA. The peptide S6-1 was estimated by reversed-phase high-performance liquid chromatography to be approximately 90% pure.

Protein Kinase Assay. Protein kinase activity was measured by the incorporation of ³²P from [γ-³²P]ATP into an appropriate substrate or the enzyme itself. To determine the effect of monoclonal antibody on the phosphorylation of AUT-PK 500 and its substrate, 2 µg AUT-PK 500 were incubated with 5 µl of nonimmune mouse IgG and in the presence and absence of 5 µl of monoclonal antibody for 15 min at 4°C. Membrane-bound ribosomes (20 µg in 10 µl) were added to these mixtures, and the reactions were initiated by the addition of 20 µl of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 2.5 x 10⁻³ M [γ-³²P]ATP. The reactions were incubated at 37°C for 15 min, then terminated with 4X sample buffer containing 10% glycerol, 5.6% 2-mercaptoethanol, 3% SDS, and 0.0625 M Tris-HCl (pH 6.8). The phosphorylated proteins were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis and autoradiography as described (1).

Protein kinase assays using the synthetic octapeptide as substrate were performed in a reaction mixture (100 µl) containing 20 mM sodium phosphate, 10 mM MgCl₂, 1 mM dithiothreitol, 60 µM unlabeled ATP, 0.75 µCi [γ-³²P]ATP (3000 Ci/mmole) and 60 µM S6-1 at pH 7.4. The reaction was initiated by addition of 1 µg AUT-PK 500 and allowed to incubate for 30 min at 37°C. Then 40 µl of the mixture was spotted on a 2 x 2 cm squares of Whatman P-81 phosphocellulose filter paper and washed twice with Tris/saline containing 0.05% NaCl to obtain the antigen (10). The eluted fractions were neutralized by 1 M Tris-HCl (pH 8.5), concentrated, dialyzed against 0.05 M PBS. The resultant hybridoma cells were screened by ELISA for the presence of antibody against AUT-PK 500 AUTOPHOSPHORYLATION DOMAIN ANTIBODY.

Immunoprecipitation of AUT-PK 500. A postmitochondrial supernatant of a tumor homogenate was precipitated by 60% saturation with ammonium sulfate. The precipitate was dissolved in Tris-HCl buffer, iodinated as reported previously (2), divided into aliquots and incubated overnight at 4°C with either monoclonal antibody or nonimmune mouse IgG followed by a 16-h incubation with 10 µl of undiluted goat IgG directed against mouse IgG. The immune complex was precipitated by the addition of a 100-µl suspension of Protein A-Sepharose 4B beads followed by a 1-h incubation at room temperature and centrifugation for 10 min at 2000 rpm. The pellet was washed twice with 0.05 M PBS (pH 7.4) and the bound protein was dissociated by a 1-h incubation with 100 µl of sample buffer containing 10% glycerol, 5.6% 2-mercaptoethanol, 3% SDS, and 0.0625 M Tris-HCl (pH 6.8). After centrifugation, dissociated proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Radioactive bands were visualized by exposing the dried gels to Kodak XAR-5 film.

Western Blot. 5 g of adrenocortical carcinoma tissue was extracted as described (1) and subjected to 60% saturated ammonium sulfate precipitation. The precipitate was dissolved in 20 mM Tris-HCl (pH 7.4) and aliquots containing 40 µg and 80 µg protein were applied on 6% SDS-polyacrylamide gel and then subjected to Western blotting with monoclonal antibody IIC 3.3 according to the method of Towbin et al. (13) using goat anti-mouse IgG coupled to peroxidase to visualize the bound monoclonal antibody.

Antibody Affinity Column. The purified monoclonal IgG from ascites fluid was immobilized on the Protein A-Sepharose column. IgG (1 mg) in 0.1 M phosphate buffer (pH 8) was incubated with 0.3 g Protein A-Sepharose (1.2 ml of swollen gel) for 16 h at 4°C. The IgG-coupled gel was placed in a 3.0-ml Pharmacia column and equilibrated with 0.1 M phosphate buffer (pH 8.0). 1 ml of crude AUT-PK 500 extract from the DEA-cellulose step (1) was autophosphorylated (1) and then applied on the immunosorbent column and incubated for 3 h at room temperature. Elution was done with 0.1 M citrate buffer in a stepwise manner, first at pH 6.5 to elimate loosely bound proteins and then at pH 5.0 to obtain the antigen (10). The eluted fractions were neutralized with 1 M Tris-HCl (pH 8.5), concentrated, dialyzed against 0.05 M Tris-HCl (pH 7.5), examined by a 6% SDS-polyacrylamide gel electrophoresis, and analyzed by autoradiography. The same procedure was applied to an aliquot of the DEA-cellulose which had not been autophosphorylated. The pH 5.0 eluate from the protein A column was adjusted to pH 7.5, autophosphorylated, and examined by SDS-polyacrylamide gel electrophoresis and autoradiography as above.

RESULTS

Production of Monoclonal Antibodies. After 2 months of immunization with AUT-PK 500, the sera from BALB/c mice were examined by ELISA for the presence of antibody against the enzyme by using peroxidase-linked goat-anti-mouse antibody. Mouse spleen from a mouse with an antibody titer of 1:20,000 was removed and fused with the SP-2/0 mouse myeloma cells. The resultant hybrid cells were screened by ELISA and then cloned by limiting dilution. The strongly positive hybridomas yielding an absorbance at 492 nm of 0.2 units higher than the control in the ELISA were selected for cloning. Based upon the results of the ELISA, three subclone lines of hybridoma cells were selected for their ability to inhibit autophosphorylation and cloned twice and maintained as stable cell lines. Two of the monoclonal antibodies designated IIC 3.3 and IVD-8.1 were found to block the autophosphorylation of AUT-PK 500. While the third positive clone did not affect either the autophosphorylation or S6 kinase activity of the enzyme.

Anti-AUT-PK 500 antibodies were precipitated (by addition of ammonium sulfate to 40% saturation) from the culture fluid of hybrids IIC 3.3 grown in mass culture. The precipitate was resuspended in PBS, dialzyed against the same solution, and stored at −20°C. The hybrid cells were also injected into BALB/c mice where they produced ascites fluid. The fluid was collected from the peritoneal cavity with a syringe and the cells removed by centrifugation.

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The ascites fluid produced by IIIC 3.3 hybridoma was fractionated on Protein A-Sepharose column (Fig. 1) for characterization of antibody class and subclass. Using buffers of decreasing pH, it was observed that with pH 6.0 and pH 3.5, no IgG was detected in the eluate, whereas with pH 5.0, bands at M, 55,000 and 25,000, representing heavy and light chains of the IgG2a type antibody, were visible. The antibody showed approximately 98% purity.

Affinity of IIIC 3.3 Antibody for AUT-PK 500. Slot blot analysis revealed that the IIIC 3.3 antibody shows affinity for AUT-PK 500. AUT-PK 500 reacted with the monoclonal antibody in a dose-dependent fashion, the reaction intensity being increased with increased concentrations of AUT-PK 500 ruling out any nonspecific binding (Fig. 2). There was no reaction with nonimmune mouse IgG. Immunoprecipitation of $^{[125]}$I-AUT-PK 500 from a crude tumor extract further demonstrated that monoclonal antibody IIIC 3.3 is directed against an AUT-PK 500 epitope. No labeled protein was precipitated with nonimmune IgG, whereas a single iodinated protein band at M, 240,000 that comigrated with the partially purified $^{[125]}$I-AUT-PK 500 was obtained with the monoclonal antibody IIIC 3.3 (Fig. 3).

Western Blot Analysis Using Monoclonal Antibody IIIC 3.3. Western blot analysis using monoclonal antibody IIIC 3.3 of a crude extract of adrenocortical carcinoma 494 revealed a single immunoreactive band at M, 240,000 identical to the molecular weight of pure AUT-PK 500 in the same system (Fig. 4). An additional lane on the nitrocellulose sheet representing 80 ¿tg of extract was cut off and developed with nonimmune mouse IgG. No immunoreactive bands were detected.

Adsorption of AUT-PK 500 by Immobilized Monoclonal Antibody IIIC 3.3. A major fraction of AUT-PK 500 was bound to the Protein A-Sepharose column coupled to monoclonal antibody when crude AUT-PK 500 [DEAE-cellulose purification step (1)] previously autophosphorylated was applied to the column (Fig. 5A). Similarly, the antibody column step could effectively purify AUT-PK 500 to homogeneity from the rat adrenocortical carcinoma tissue postmitochondrial supernatants. An antibody-bound AUT-PK 500 could be eluted with 0.1 m citrate buffer (pH 5) as a single protein migrating at M, 240,000 along with the antibody near the dye band on a 6% SDS-polyacrylamide gel and visualized by autoradiography.

![Fig. 1. Characterization of the monoclonal antibody IIIC 3.3 using a Protein A-Sepharose column. After washing with PBS, the column was eluted with 0.1 m citrate buffer, pH 6 (lanes 2 and 3), pH 5.5 (lane 4), pH 5.0 (lanes 5 and 6), and pH 3.5 (lane 7). Samples of ascites fluid (lane 1) without purification were also included. Samples from column fractions were analyzed by SDS gel electrophoresis and visualized by Coomassie blue staining. Lane 8, molecular weight standards.](image)

![Fig. 2. Affinity of monoclonal antibody IIIC 3.3 for AUT-PK 500. In the immunoslot blot assay, between 15 and 480 ng of purified AUT-PK 500 were dotted on a nitrocellulose filter (lane B) and incubated with IIIC 3.3 antibody. As a control (lane A), a filter dotted with AUT-PK 500 in an identical way was incubated with buffer without antibody. Both filters were incubated with $^{[125]}$I-labeled anti-mouse antibody as detailed under "Materials and Methods." The autoradiogram was developed after 12 h of exposure to X-ray film.](image)

![Fig. 3. Immunoprecipitation of $^{[125]}$I-AUT-PK 500 from a 60% ammonium sulfate precipitation of tumor extract by IIIC 3.3 monoclonal antibody. Lane 1, 50 ¿l of iodinated partially purified AUT-PK 500 (DEAE-cellulose eluate); lane 2, immunoprecipitate of the crude extract with 4 ¿g of nonimmune mouse IgG; lane 3, immunoprecipitate of the crude extract after immunoprecipitation with 4 ¿g of monoclonal antibody. The partially purified enzyme and precipitated pellets were electrophoresed on a 6% SDS-polyacrylamide gel and visualized by autoradiography.](image)
AUT-PK 500 (Fig. 5B). In a separate experiment, a partially purified (DEAE-cellulose eluate) extract which had not been autophosphorylated was absorbed to the monoclonal antibody column and eluted as above. The eluate was neutralized, autophosphorylated, and subjected to SDS-polyacrylamide gel electrophoresis as above. Again, a single phosphorylated band at Mr 240,000 was detected (not shown).

Specific Blockage of the AUT-PK 500 Autophosphorylation by the Monoclonal Antibody IIIC 3.3. AUT-PK 500 catalyzes the phosphorylation of its serine residue(s) when incubated with [γ-32P]ATP, whereas in the presence of the monoclonal antibody, this reaction is inhibited (Fig. 6A). On the other hand, the monoclonal antibody does not affect the ability of the enzyme to catalyze the phosphorylation of its substrate S6 ribosomal protein (Fig. 6B). The synthetic octapeptide S6-1 which contains two serine residues is also phosphorylated by AUT-PK 500. Polyclonal antibody inhibits this phosphorylation. In contrast, the monoclonal antibody does not affect the AUT-PK 500-dependent phosphorylation of S6-1 (Fig. 7). There was no significant effect of preimmune rabbit serum or nonimmune mouse IgG on the phosphorylation of S6-1 by AUT-PK 500.

DISCUSSION

The present study describes the production of a monoclonal antibody which specifically inhibits autophosphorylation of AUT-PK 500. The authenticity of the antibody is attested by

Fig. 4. Western blot detection of AUT-PK 500 in a crude tumor extract using monoclonal antibody IIIC 3.3. Aliquots containing 40 and 80 μg of protein at the same purification step as in Fig. 3 were subjected to Western blotting using a 6% SDS-polyacrylamide gel and monoclonal antibody IIIC 3.3. Left lane, 40 μg of protein; right lane, 80 μg of protein. A third lane which also received 80 μg of protein was cut from the nitrocellulose sheet and developed with nonimmune mouse IgG revealed no bands.

Fig. 5. A, Adsorption of AUT-PK 500 by monoclonal antibody IIIC 3.3 affinity column. 1 ml of crude AUT-PK 500 [DEAE-cellulose purification step (1)] (lane 1) was applied on the antibody-bound Protein A-Sepharose column and the column with bound AUT-PK 500 eluted with 0.1 M citrate buffers. Lane 2, the flow-through from affinity column; lane 3, citrate wash at pH 6.5; lane 4, citrate wash pH 5; lane 5, standard molecular weight markers. The column fractions were analyzed on a 6% SDS-polyacrylamide gel. The gel was stained with Coomassie blue and destained with 7% acetic acid. B, 1 ml of the crude AUT-PK 500 [DEAE-cellulose purification step (1)] was autophosphorylated and then applied on the immunosorbent column. The eluted fractions at pH 5.0 and 6.5 were neutralized with 1 M Tris-HCl (pH 8.5), concentrated, dialyzed against 0.05 M Tris-HCl (pH 7.5), examined by a 6% SDS-polyacrylamide gel, and analyzed by autoradiography. Lane 1, 50 μg of crude AUT-PK 500; lane 2, citrate wash at pH 6.5; lane 3, eluted fraction at pH 5.0.
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Fig. 6. A, Specific blockage of AUT-PK 500 autophosphorylation by monoclonal antibody IIC 3.3. AUT-PK 500 (2 μg) was incubated in the presence and absence of monoclonal antibody (5 μl) and [γ-32P]ATP (2.5 × 10⁻⁷ M). The treated samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described under "Materials and Methods." Lane 1, AUT-PK 500; lane 2, AUT-PK 500 + the monoclonal antibody; lane 3, the monoclonal antibody. B, AUT-PK 500 (2 μg) was incubated in the presence of membrane-bound ribosomes (20–30 μg) and monoclonal antibody IIC 3.3 (5 μl) with [γ-32P]ATP (2.5 × 10⁻⁷ M). The samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described under "Materials and Methods." Lane 1, AUT-PK 500; lane 2, AUT-PK 500 + membrane-bound ribosomes; lane 3, AUT-PK 500 + membrane-bound ribosomes + IIC 3.3 antibody; lane 4, membrane-bound ribosomes; lane 5, IIC 3.3 antibody.

Fig. 7. Phosphorylation of the synthetic peptide, S6-1, by AUT-PK 500 in the presence of polyclonal antibody (1 μl) and monoclonal antibody (2 μl). The phosphorylation of S6-1 (60 μM) was measured in the presence and absence of the antibodies as indicated in the column labels. The reaction was initiated by the addition of 1 μg of AUT-PK 500 as described under "Materials and Methods." The results are means ± the standard deviation of three measurements. Column identification: S6-1, RRLSSLRA; E, AUT-PK 500; Ab, polyclonal anti-AUT-PK 500; MAb, monoclonal anti-AUT-PK 500.

AUT-PK 500 are blocked by the polyclonal anti-AUT-PK 500 antibody (1). In contrast, the monoclonal antibody blocks the autophosphorylation but not the AUT-PK 500-dependent phosphorylation of ribosome protein S6 (Fig. 6A). Higher concentrations, up to 100 μl, of the monoclonal antibody in the 100 μl reaction volume did not inhibit phosphorylation of ribosomal protein S6. To further delineate between the two enzyme activities (autophosphorylation and S6 kinase), the synthetic S6-1 octapeptide (Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala), corresponding to a portion of the S6 ribosomal protein phosphorylation domain (12) was scrutinized as a substrate of AUT-PK 500, it is a substrate and the phosphorylation was blocked by the polyclonal anti-AUT-PK 500 but not by the monoclonal antibody (Fig. 7). These results demonstrate the specificity of the monoclonal antibody against autophosphorylation of the enzyme. They further reveal that the antibody does not interfere with the catalytic site of AUT-PK 500 which phosphorylates the S6 ribosomal protein. Assuming the enzyme contains only one kind of catalytic site, it follows that the same catalytic site phosphorylates the autophosphorylation site(s) of the enzyme and the S6 kinase phosphorylation domain of the ribosomal protein. Since the autophosphorylation blockage does not stop the enzyme from phosphorylating ribosomal protein S6, the antibody results indicate that the enzyme self-phosphorylation is not apparently necessary for the AUT-PK 500 S6 kinase activity.

The fact that monoclonal antibody IIC 3.3 inhibits autophosphorylation without inhibiting substrate phosphorylation suggests that the monoclonal antibody binds to a site which includes or is close to the serine residue(s) which is autophosphorylated. However, the free serine hydroxyl appears not to

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be essential for binding since the monoclonal antibody affinity column selectively removed both phosphorylated and unphosphorylated AUT-PK 500 from partially purified tumor extracts.

Besides AUT-PK 500, several other serine protein kinases (cAMP-dependent, cGMP-dependent, protein kinase C) possess S6 kinase activity (14–19). Using polyclonal anti-AUT-PK 500 and the cAMP-dependent protein kinase heat-stable inhibitor probes, the AUT-PK 500 and cAMP-dependent S6 kinase activities are differentiated (1), however.

Another intriguing aspect of S6 phosphorylation is that certain membrane tyrosine protein kinase activators (insulin, epidermal growth factor, platelet-derived growth factor), when incubated with intact cells (Table 1, and “Discussion” of Ref. 19), induce phosphorylation of serine residues of the S6 ribosomal protein, although the activated growth factor receptors do not directly phosphorylate the ribosomal protein. Obviously, a cytosolic serine protein kinase is a mediator of membrane tyrosine protein kinase for the S6 phosphorylation. To understand the biochemical link between the transmembrane and cytosolic protein kinase requires the development of specific protein kinase probes. We believe the monoclonal antibody specific against the autophosphorylation domain of AUT-PK 500 is one of those probes which will contribute to the basic understanding of the potential role of AUT-PK 500 and other S6 kinases in cellular regulation and transformation.

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