Volume 291, number 1, 29–32 FEBS 10218 October 1991
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ADONIS 001457939100950U

Use of a monoclonal antibody (APE-1) directed against the conserved GTPEYLAPE motif present in protein kinases as an affinity purification reagent

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Received 9 July 1991; revised version received 2 August 1991

cDNA sequence studies have predicted the existence of protein kinases that have not been characterized at the protein level. This laboratory has previously shown that polyclonal rabbit antibodies directed against a highly conserved sequence motif, GTPEYLAPE, present in the catalytic domain of many protein kinases will react during immunoblots with several protein kinases containing the same or homologous motifs. In this report I describe the development of a mouse monoclonal antibody, designated APE-1, that reacts with the GTPEYLAPE motif. In addition, the use of this monoclonal antibody in the affinity purification of kinase activities from rabbit reticulocyte lysate is described. This approach may provide a general method for the purification of previously uncharacterized protein kinases that share regions which are homologous to the GTPEYLAPE motif. Refinements in this method may permit the rapid purification of nonabundant and/or rapidly inactivated protein kinases that have not been isolated using other approaches.

Protein kinase; eIF-4E; Affinity purification; Monoclonal antibody

1. INTRODUCTION

Most protein kinases contain a sequence that is homologous to the GTPEYLAPE motif in their catalytic domain [1,2]. Previous studies have shown that polyclonal antibodies can be raised against a synthetic oligopeptide containing the nine residue GTPEYLAPE motif [3]. Such polyclonal antibodies reacted during immunoblotting with several different protein kinases containing a homologous sequence and identified a 55 kDa protein in A431 cell plasma membrane vesicles that is a putative uncharacterized protein kinase. In this report I describe the development of a monoclonal antibody directed against the GTPEYLAPE motif and show that it can be used to purify protein kinase activities from a crude cell lysate. This or similarly designed monoclonal antibodies may provide an approach to isolating protein kinases or other enzymes that have only been detected at the cDNA level.

Abbreviations: cAK, cAMP dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; eIF-4E, the 25 kDa mRNA cap binding protein.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Keyhole limpet hemocyanin and Freund's adjuvants were from Cal Biochemical (San Diego, CA). EDC, sulfo-NHS and reacti-gel were from Pierce Chemical Co. (Rockford, IL). Alkaline phosphatase conjugated antibodies were from Accurate Chemical and Scientific Corp. (Westbury, NY). Reagents for sub-isotyping mouse monoclonal antibodies (kit EK-5050) were from HyClone Laboratories, Inc. (Logan, UT). All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Purified cAMP dependent protein kinase (bovine lung) was a generous gift from Jackie Corbin (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine). Protein A Sepharose was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ).

2.2. *Immunization*

The oligopeptide CGGGTPEYLAPEGGK was synthesized and purified as described elsewhere [3]. Purified peptide was crosslinked to KLH using EDC and sulfo-NHS as described in detail elsewhere [3]. A BALB/c mouse that had received intraperitoneal injections of 50 μg followed by 12–25 μg of crosslinked peptide emulsified in Freund's adjuvant for a total of 12 booster immunizations, including a final boost 3 days prior to fusion, was selected for hybridoma development. Serum from this animal reacted with purified cAK during immunoblots.

2.3. *Monoclonal antibody production*

Splenic lymphocytes were fused with P3X63-AG8.653 mouse myeloma cells (ATCC #CRL1580) at a ratio of 5 spleen cells per myeloma cell using polyethylene glycol [4,5]. Cells were plated onto ten 96-well culture plates in HAT medium without feeder layers. Hybridoma supernatants were assayed first for the production of immunoglobulin using a dot blot alkaline phosphatase ELISA assay [3,5]. Hybridoma supernatants were applied to nitrocellulose paper using a dot-blot template (Bio Rad), each well was washed with 200 μl of TBS (20 mM

Tris-HCl, pH 7.4, 154 mM NaCl) per well, the paper was removed from the apparatus and blocked with 5% nonfat dried milk with 1% normal goat serum in TBS for 2 h and then washed twice with TBS. The nitrocellulose was then probed with a rabbit anti-mouse IgG/IgM conjugated to alkaline phosphatase followed by NBT and BCIP substrates as described elsewhere [3].

Hybridoma supernatants that tested positive for the production of antibodies were assayed using nitrocellulose bound cAK, that contains the GTPEYLAPE sequence, using an alkaline phosphatase ELISA. Purified cAK catalytic subunit (200 ng with 2.5 μg bovine serum albumin) was applied to nitrocellulose paper using a dot-blot template (Bio-Rad). The nitrocellulose was washed, removed from the apparatus and blocked with 5% nonfat dried milk and washed again as described above. The nitrocellulose paper was re-inserted into the dot-blot apparatus and incubated with 100 μl of hybridoma supernatant per well. Following incubation with hybridoma supernatants the nitrocellulose was removed from the apparatus, washed, and bound mouse immunoglobulins identified by using a secondary rabbit anti-mouse IgG/IgM conjugated to alkaline phosphatase followed by NBT and BCIP substrates as described in detail elsewhere [3]. Hybridomas producing antibodies that reacted with nitrocellulose bound cAK catalytic subunit were subcloned using thymocyte feeder layers. The subcloned hybridomas were rescreened using nitrocellulose bound cAK. Hybridoma supernatants testing positive on this screen were then assayed for the ability to immunoprecipitate autophosphorylated [^{32}P]cAK catalytic subunit. In brief, hybridoma supernatants and [^{32}P]cAK were incubated for 1 h on ice in 1 ml of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.1% SDS. Protein A sepharose (10 μl packed volume) was added, the samples mixed for 1 h, protein A sepharose was washed at least 3 times and samples analysed by SDS-PAGE and autoradiography. A subcloned hybridoma, designated APE-1, identified in this manner was used for the subsequent studies. Mouse ascites containing monoclonal antibodies were produced using this hybridoma by standard techniques [5].

Monoclonal antibodies produced from the APE-1 hybridoma in culture and by the ascites method were sub-isotyped using a kit purchased from HyClone Laboratories, Inc. The detailed instructions provided by the manufacturer were followed. In brief, goat anti-mouse capture antibodies were applied to 96-well plates and kept at 4°C overnight, the wells were washed, monoclonal antibody samples and standards applied and incubated for 1 h at room temperature, the wells were washed, and sub-isotyping antiserum (rabbit anti-mouse) was applied followed by a 1 h incubation at room temperature, the wells were washed again and goat anti-rabbit IgG peroxidase conjugate was added. Following a 1 h incubation at room temperature wells were washed and citrate buffer containing 1% urea peroxide was added to develop incubations.

2.4. *Monoclonal antibody affinity chromatography*

The APE-1 monoclonal antibody was purified from mouse ascites using ammonium sulfate precipitation and anion exchange FPLC chromatography. Monoclonal antibody was crosslinked to reactigel (Pierce Chemical Co.) using the instructions provided by the manufacturer. Reticulocyte lysate (50 ml) was used as starting material and diluted in buffer to the following final concentrations: 50 mM Tris, pH 7.5 (4°C), 2 mM EDTA, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. This starting material was recirculated over a 1 ml bed volume column, overnight at 4°C at a flow rate of 10 ml/h. The column was then washed with 20 ml of buffer and proteins eluted in a stepwise manner using 2 ml fractions of buffer containing KCl at 0.1, 0.2, 0.3, 0.5, and 1 M concentrations. The fractions collected were concentrated in centricon-10 microconcentrators, diluted and reconcentrated to lower the final salt concentration to below 200 mM. Samples were then assayed for protein kinase activity in incubations containing 100 μM [γ-^{32}P]ATP using either casein, a crude preparation of human erythrocyte eIF-4E or no substrate as described in detail elsewhere [6]. Phosphoproteins were identified by SDS-PAGE and autoradiography as described elsewhere [6].

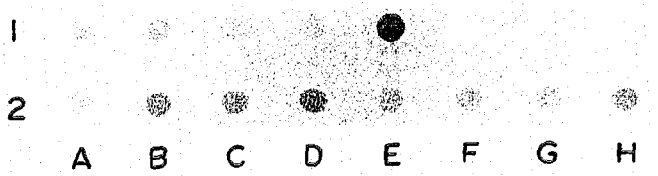

Fig. 1. Screening of hybridoma supernatants containing mouse antibodies by immunoblotting using purified cAK as antigen. Hybridoma supernatants containing mouse antibodies, as identified during the screening procedure described in section 2, were tested for their ability to react with purified bovine cAK bound to nitrocellulose. The immunoblots were done using 200 ng of cAK bound to nitrocellulose and an alkaline phosphatase ELISA as described in section 2. Mouse preimmune serum (1F) served as a negative control. Ascites from the immunized mouse used for the hybridoma fusion (1E) provided a positive control. Hybridomas producing the supernatants tested in 2B, 2C, 2D and 2H were subcloned for further analysis.

3. RESULTS AND DISCUSSION

The initial screening of hybridomas was for the production of IgG or IgM antibodies as described in section 2. A total of 80 hybridoma supernatants scored positive for the production of antibodies (data not shown). These supernatants were then tested for their ability to react with nitrocellulose-bound cAMP-dependent protein kinase as described in section 2. A total of 4 hybridomas produced antibodies that gave a clearly

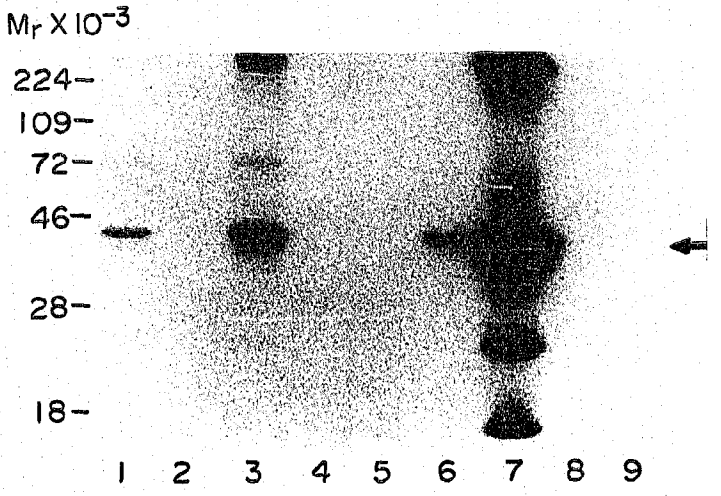

Fig. 2. Screening of hybridoma supernatants that reacted with nitrocellulose-bound cAK for the ability to immunoprecipitate [^{32}P]cAK catalytic subunit. [^{32}P]cAK was prepared by incubating purified catalytic subunit with [γ-^{32}P]ATP as described in section 2. Hybridoma supernatants were incubated with [^{32}P]cAK for 1 h, followed by protein-A sepharose for 1 h and washed 3 times in 'immunoprecipitation buffer'. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography as described in section 2. This figure shows an autoradiogram of some of the immunoprecipitates observed furing the screening procedure. The quantity of ^{32}P-labeled antigen used in each incubation is shown in lane 7 with the location of the cAK catalytic subunit indicated by an arrow. Controls using irrelevant hybridoma supernatant (lane 8) and mouse preimmune serum (lane 9) did not immunoprecipitate [^{32}P]cAK. The hybridoma producing the supernatant represented in lane 3 was designated APE-1 and used for subsequent studies.

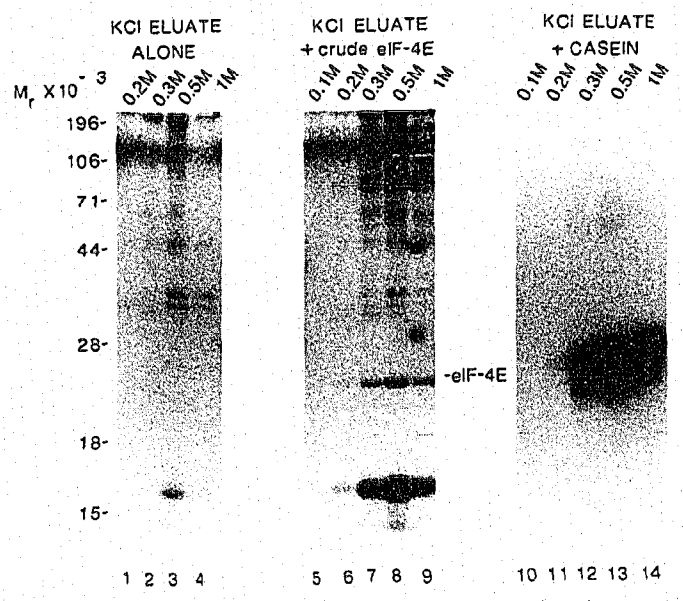

Fig. 3. Isolation of protein kinase activities from rabbit reticulocyte lysate using an APE-1 monoclonal affinity column. Rabbit reticulocyte lysate was used as starting material as described in section 2. After washing, the column proteins that remained bound were eluted using a stepwise KCl elution procedure. Fractions collected were concentrated as described in section 2, assayed for kinase activity and samples analyzed by SDS-PAGE and autoradiography. The figure shows phosphoproteins present when eluates were incubated alone (lanes 1–4), with crude eIF-4E (lanes 5–9), or with casein (lane 10–14). The autoradiogram for incubations containing casein represents a 6 h exposure while the others represent a 7 day exposure.

positive signal during immunoblotting with purified cAMP-dependent protein kinase (Fig. 1). These hybridomas were subcloned using standard methods and rescreened using nitrocellulose-bound cAK. Hybridomas testing positive in the cAK immunoblot assay were further screened for their ability to immunoprecipitate [^{32}P]cAK catalytic subunit as shown in Fig. 2. The subcloned hybridoma producing the supernatant capable of immunoprecipitating [^{32}P]cAK catalytic subunit as shown in Fig. 2 lane 3 was selected for further studies and designated APE-1.

Large quantities of the APE-1 monoclonal antibody were produced using the ascites method. Sub-isotyping of monoclonal antibodies produced in cultures and mouse ascites demonstrated that the monoclonal antibody was IgM (data not shown). Monoclonal antibodies were partially purified from mouse ascites and crosslinked to an agarose gel matris as described in section 2. Reticulocyte lysate was applied to the column, washed, and the KCl eluates were assayed for protein kinase activity using eIF-4E and casein as described in section 2. eIF- 4E was chosen as a substrate because relatively few protein kinases phosphorylate it and one diffult-to-purify enzyme remains to be isolated from rabbit reticulocytes [6,8]. Casein was chosen as a second substrate because it is phosphorylated by many protein kinases. The maximal kinase activity with regard to both substrates was eluted from the column with 0.5 M KCl (Fig. 3). The approximately 16 kDa phosphoprotein, seen in lanes 7–9 of Fig. 3, that follows the same pattern as phosphorylated eIF-4E, is most likely a proteolytic product of eIF-4E that has previously been observed as a phosphoprotein [8]. SDS-PAGE and silver staining of proteins eluted from the column demonstrated that relatively few proteins remained bound following the wash procedure, indicating that the column functioned with specificity (data not shown). However, additional studies are needed to identify which protein(s) are responsible for the kinase activities isolated. Previous studies with rabbit polyclonal anti-GTPEYLAPE antibodies have demonstrated their ability to react during immunoblotting with cAK (GTPEYLAPE), the γ subunit of phosphorylase kinase (GTPSYLAPE) and Ca^{2+}/calmodulin kinase II (GTPGYLSPE) [3]. However, both protein kinase C (GTPDYIAPE) and casein kinase I did not react [3]. The results reported here and the previously defined specificity of anti-GTPEYLAPE antibodies provide evidence that an additional protein kinase capable of phosphorylating eIF-4E, other than casein kinase I and protein kinase C, is present in reticulocyte lysate [3,6–8]. The APE-1 monoclonal antibody should prove useful in isolating this protein kinase from reticulocytes for further studies. In addition, it may also have applications in isolating protein kinases that are responsible for the stimulated phosphorylation of eIF-4E following treatment of cells with PDGF and EGF or from cells infected with viruses that alter the phosphorylation state of eIF-4E [9–11].

These results clearly demonstrate that a protein kinase(s) activity can be partially purified from a whole cell extract using an APE-1 monoclonal affinity column. Additional studies are being conducted to identify which proteins eluted from the column are the actual protein kinase(s) responsible for the activity observed. Although an additional purification step will most likely be required, the initial affinity isolation of enzymes using the APE-1 monoclonal antibody may provide an effective first purification step for nonabundant or rapidly inactivated protein kinases. Current efforts are being directed at the optimization of affinity purification procedures using the APE-1 monoclonal and the isolation of other APE monoclonals from hybridomas produced during the course of these studies. The approach of developing affinity purification reagents based solely on cDNA sequence information should permit the development of methods that can isolate families of uncharacterized proteins that share relatively small homologous structural motifs consisting of 9 or possibly fewer amino acids.

Acknowledgements: I thank Heather L. Panella and William H. Tettelbach for their able technical assistance and Terry Bellew for her expert assistance with the hybridoma fusion. This work was supported by NIH grant GM 40219 and The Department of Veterans Affairs.

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