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# Development and characterization of polyclonal antibodies against a conserved sequence in the catalytic domain of protein kinases

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Using a synthetic oligopeptide (CGG**GTPEYLAPE**GGK) crosslinked to keyhole limpet hemocyanin we have raised polyclonal rabbit antibodies against a 9 residue homologous region found in the catalytic domain of most protein kinases. These antibodies reacted during Western immunoblotting with cAMP dependent protein kinase catalytic subunit, phosphorylase kinase $\gamma$ subunit and calcium calmodulin dependent protein kinase II which have homologous sequences of GTPEYLAPE, GTPSYLAPE and GTPGYLSPE, respectively. Five other protein kinases did not react with anti-GTPEYLAPE antibodies during Western immunoblotting. Affinity-purified antibodies were able to detect as little as 50 ng of cAMP dependent protein kinase and 200 ng of $Ca^{2+}$/calmodulin dependent protein kinase II. Immunoblotting of A431 cell plasma membrane vesicles indicated the presence of an approximately 55 kDa protein that contains the conserved sequence and is likely to be a protein kinase. Antibodies directed against conserved sequences present in protein kinases, or possibly other enzymes, may be useful in identifying previously uncharacterized enzymes at the protein level.

cAMP dependent protein kinase; Phosphorylase kinase; $Ca^{2+}$/calmodulin kinase II; A431 cell

## 1. INTRODUCTION

Recent studies have demonstrated that homologous regions exist in the catalytic domains of both serine/threonine and tyrosine protein kinases [1,2]. Among these regions is the sequence GTPEYLAPE that is found in the catalytic domain region of cAMP dependent protein kinases [3,4]. A homologous sequence similar to this, differing by 1–7 residues, is found in all other protein kinases for which sequence data have been reported [2]. Preliminary studies done with cDNA libraries suggest that previously unidentified protein kinases exist that contain this sequence [2]. The greatest variance in this sequence occurs at the fourth, fifth and sixth residues. In order to develop reagents that might identify new serine/threonine kinases at the protein level, possibly provide simplified affinity purification methods for some nonabundant or difficult to purify protein kinases and develop probes to possibly determine the function of the GTPEYLAPE sequence, we have raised polyclonal antibodies against a synthetic oligopeptide containing this sequence. We report here that antibodies against this short, well-conserved, homologous region can be raised in rabbits. In addition, we describe the method for developing such antibodies and the initial characterization of their ability to identify several protein kinases containing the same or similar sequences by Western immunoblotting methods.

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*Abbreviations:* EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; sulfo-NHS, *N*-hydroxysuccinimide; KLH, keyhole limpet hemocyanin; PBS, Dulbecco's phosphate-buffered saline; NBT, 4-nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate

## 2. MATERIALS AND METHODS

### 2.1. *Reagents*

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Reagents used for the synthesis of oligopeptides were from Peninsula Laboratories, Inc. (Belmont, CA). Nitrocellulose was from Bio-Rad (Richmond, CA). NBT and BCIP were from Promega (Madison, WI). Alkaline phosphatase conjugated goat anti-rabbit IgG was from Accurate Chemical and Scientific Corp. (Westbury, NY). Keyhole limpet hemocyanin and Freund's adjuvants were from Cal Biochemical (San Diego, CA). EDC and sulfo-NHS were from Pierce Chemical Co. (Rockford, IL). Purified protein kinases used in these studies were the generous gifts of other investigators. Casein kinase I, cGMP dependent and myosin light chain kinase were from Edwin Krebs. Phosphorylase kinase and calcium calmodulin dependent protein kinase II were from Thomas Soderling. Casein kinase I was from Neil Osheroff. Protein kinase C was from Robert Bell and cAMP dependent protein kinase catalytic subunit from Jackie Corbin. A431 cell plasma membrane vesicles were the generous gift of Stanley Cohen [5].

### 2.2. *Oligopeptide synthesis and crosslinking to KLH*

The oligopeptide CGG**GTPEYLAPE**GGK was synthesized using a Bechman model 990 peptide synthesizer [6,7]. The peptide was desalted and purified as described elsewhere [7]. Composition of the

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peptide was verified by amino acid analysis using a Water Associates Pico-tag amino acid analyzer. The oligopeptide was covalently crosslinked to KLH after previously described methods [8,9]. KLH (120 mg) was dissolved in 2 ml of 50 mM sodium phosphate, pH 7.4, and dialyzed overnight against 3 exchanges (2 liters) of 50 mM sodium phosphate, pH 7.4. The KLH was then dialyzed against at least 5 exchanges (2 liters) of 5 mM sodium phosphate, pH 7.4, over 3 days. Precipitated protein was removed by centrifugation at 20000 rpm in a Sorvall SS34 rotor for 15 min. The supernatant was stored at 4°C. Crosslinking reactions contained 350 $\mu$g of peptide (adjusted to approximately pH 7.4 with 0.02 N NaOH), 350 $\mu$g of KLH, 25 $\mu$l of 1.2 M EDC (in 5 mM sodium phosphate, pH 7.4), 20 $\mu$l of 100 mM sulfo-NHS (in deionized $H_2O$), 20 $\mu$l of 5 mM sodium phosphate, pH 7.4, and sufficient Milli-Q deionized $H_2O$ to yield a final volume of 300 $\mu$l. EDC was added to start reactions. Final incubations were sealed in 500 $\mu$l microfuge tubes, mixed and incubated overnight at room temperature.

### 2.3. *Immunization procedure and purification of monospecific antibodies*

Two female New Zealand white rabbits were immunized by multiple s.c. injections of 50 $\mu$g of peptide (60 $\mu$l of the reaction described above) suspended in 1 ml of a 50% emulsion of Freund's complete adjuvant in PBS. Booster immunizations were given s.c. at 5 week intervals with the same quantity of crosslinked peptide homogenized in Freund's incomplete adjuvant. One of the two rabbits developed antibodies that reacted to cAMP dependent protein kinase catalytic subunit in Western immunoblots. Once immune serum reacted in the screening assay at a dilution of 1:1000, serum was collected for affinity purification. Peptide affinity columns were made using Pierce AminoLink coupling gel following the directions of the manufacturer. Monospecific antibodies were purified by applying immune serum diluted with 9 vols of 1 M Tris, pH 8.0, to the column, washing with 16 column vols of 0.1 M Tris, pH 8.0, and eluting bound antibodies with 0.1 M glycine, pH 3.0. Fractions (0.5 ml) were collected in 50 $\mu$l of 1 M Tris, pH 8.0, and samples analyzed by SDS-PAGE and Coomassie staining. Fractions containing antibodies were concentrated, diluted with PBS and reconcentrated using Centricon 30 (Amicon) concentrators. Affinity-purified antibodies were stored at 4°C with 0.02% sodium azide.

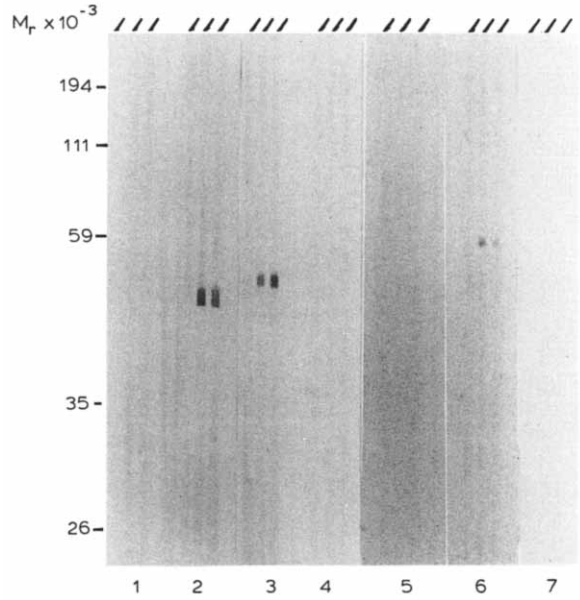


Fig. 1. Western immunoblot of purified protein kinases using rabbit anti-GTPEYLAPE serum. Kinases were analyzed by SDS-PAGE, electroblotted onto nitrocellulose and immunoblotted as described in section 2. Each lane was probed with a 1:500 dilution of rabbit preimmune and immune serum. The first channel of each lane (indicated by the marks at the top of each lane) represents preimmune serum and the second and third immune serum. Lane 1 represents casein kinase I (0.2 $\mu$g); lane 2, phosphorylase kinase (20 $\mu$g); lane 3, cAMP dependent protein kinase catalytic subunit (3 $\mu$g); lane 4, cGMP dependent protein kinase (5 $\mu$g); lane 5, myosin light chain kinase (9 $\mu$g); lane 6, $Ca^{2+}$/calmodulin dependent protein kinase II (3.5 $\mu$g) and lane 7, protein kinase C (3 $\mu$g).

### 2.4. *Western immunoblotting*

Purified protein kinases were analyzed by SDS-PAGE and electroblotted at 4°C onto nitrocellulose paper using a Bio-Rad mini-protean II apparatus [10,11]. Unreacted sites on nitrocellulose were blocked with 5% nonfat dry-milk and 1% normal goat serum in 20 mM Tris, pH 7.4, with 154 mM NaCl during a 2 h incubation at room temperature. Nitrocellulose was then washed once with 20 mM Tris, pH 7.4, 154 mM NaCl and 0.1% Tween-20 (10 min each) followed by two washes in the same buffer without detergent. For the studies represented in Fig. 1 nitrocellulose was inserted into an Immunetics Miniblotter template (Cambridge, MA) using lanes with prestained molecular weight markers as guides to position the nitrocellulose paper. This allowed one lane to be probed with both preimmune and immune serum. In other studies strips of nitrocellulose paper containing antigen were incubated with antibodies as described in the figure legends. Rabbit preimmune serum or affinity-purified antibodies were incubated with nitrocellulose bound proteins for 2 h, washed with 20 mM Tris-HCl, pH 7.4, 154 mM NaCl and 0.1% Tween-20 followed by two washing steps in the same buffer without detergent and finally incubated for 1 h with goat anti-rabbit IgG conjugated to alkaline phosphatase. Immunoblots were washed as described above and developed with NBT and BCIP following the manufacturer's (Promega) instructions. All procedures after electroblotting were conducted at room temperature.

## 3. RESULTS AND DISCUSSION

Three out of a total of 8 purified serine-threonine protein kinases tested were recognized by anti-GTPEYLAPE polyclonal antibodies during Western

Table I

Correlation between sequence of enzyme homologous region and ability of anti-peptide antibody to bind Western blotted enzyme

| Enzyme | Sequence[a] | Immunoblot[b] |
|---|---|---|
| cAMP dependent kinase (catalytic subunit) | GTPEYLAPE | + |
| Phosphorylase kinase ($\gamma$ subunit) | GTPSYLAPE | + |
| $Ca^{2+}$/calmodulin kinase II | GTPGYLSPE | + |
| cGMP dependent kinase | GTPEYVAPE | – |
| Protein kinase C | GTPDYIAPE | – |
| Myosin light chain kinase (skeletal muscle) | GTPEELSPE | – |
| Casein kinase II | ASRYFKGPE | – |
| Casein kinase I[c] | | – |

[a] Single-letter amino acid code for the enzyme region homologous with the GTPEYLAPE peptide antigen. Residues differing from the GTPEYLAPE sequence are underlined

[b] A positive signal following immunoblotting with anti-GTPEYLAPE serum is represented by (+) and no signal by (–)

[c] Sequence of homologous region of casein kinase I has not been reported

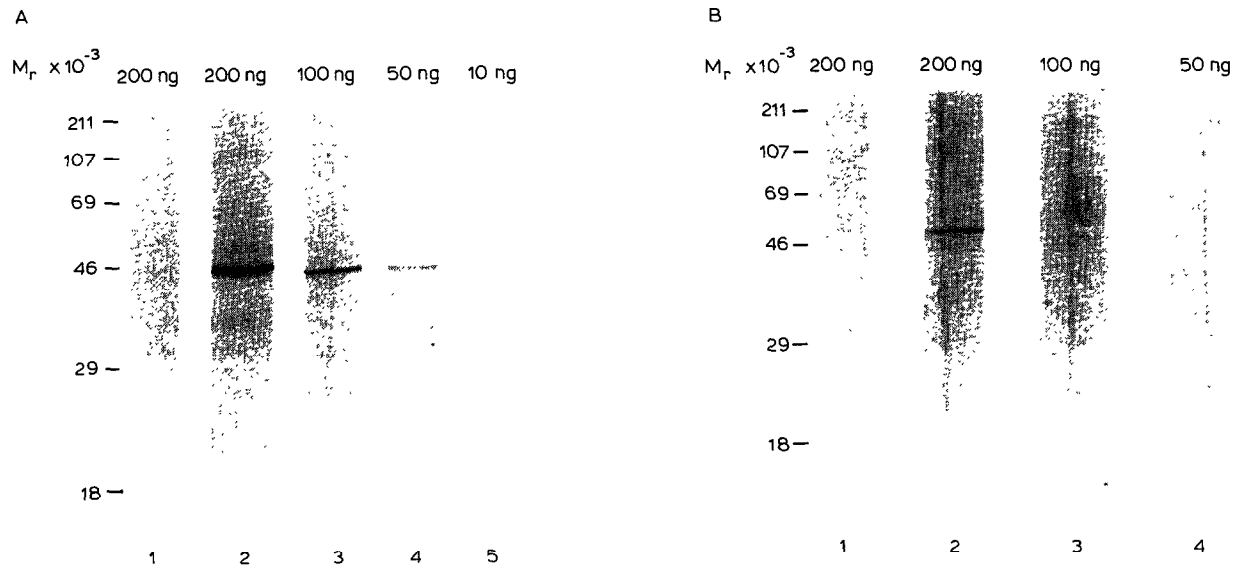


Fig. 2. Sensitivity of immunoblot detection of purified cAMP dependent protein kinase catalytic subunit and $Ca^{2+}$/calmodulin dependent protein kinase II. SDS-PAGE, Western blotting and immunoblotting were conducted as in Fig. 1 except that affinity-purified antibodies were used. (A) represents the immunoblot in which the quantities of cAMP dependent protein kinase catalytic subunit indicated above each lane were applied in the SDS-PAGE step prior to electroblotting. Prestained molecular mass standards appeared to be completely transferred from the gel to nitrocellulose under the conditions described in section 2. Lane 1 represents preimmune serum and lanes 2–5 affinity-purified antibody diluted 1:3000. (B) represents the immunoblot of $Ca^{2+}$/calmodulin dependent protein kinase II performed in the same manner as (A). The quantities of enzyme applied in the SDS-PAGE step are indicated above each lane.

immunoblotting. cAMP dependent protein kinase, calcium calmodulin dependent protein kinase II and the $\gamma$ subunit of phosphorylase kinase all consistently gave clearly positive signals with immune serum but not with preimmune (Fig. 1). On the other hand, protein kinase C, casein kinase I and II, myosin light chain kinase and cGMP dependent protein kinase were not recognized by anti-GTPEYLAPE serum under the immunoblotting conditions used. These results demonstrate that it is possible to develop polyclonal antibodies against relatively short conserved sequences, in this case 9 amino acids, present in protein kinases and that they can be used to detect identical or highly homologous regions in different enzymes. Positive results were obtained with an enzyme with the same sequence, cAMP dependent protein kinase, and two other enzymes that differed at one or two out of 9 residues (Table I). However, antiserum did not react with cGMP dependent protein kinase, protein kinase C, or myosin light chain kinase which differ from the oligopeptide antigen by one, two and two amino acids, respectively. These results indicate that simply the number of amino acids that are different between the peptide antigen and the homologous region in native enzyme does not explain the inability of anti-peptide antibodies to react with Western blotted enzyme. Moreover, the negative result with protein kinase C indicates that substitution of residues in the GTPEYLAPE sequence with amino acids having the same R group charge does not necessarily permit recognition of the enzyme homologous sequence by the anti-peptide antibody.

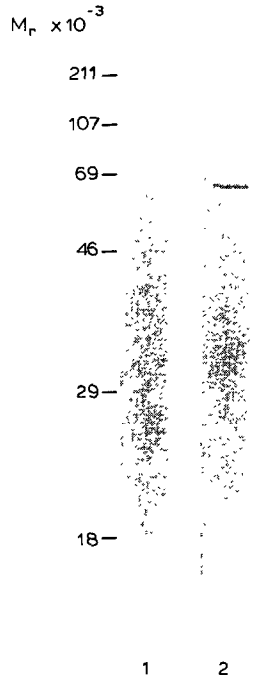


Fig. 3. Immunoblotting of A431 cell plasma membrane vesicles with affinity-purified anti-GTPEYLAPE antibodies. Samples of membrane vesicles (50 $\mu$g) were analyzed by SDS-PAGE, transferred to nitrocellulose and immunoblotted as in Fig. 2. Lane 1 represents an immunoblot with affinity-purified anti-mRNA cap binding protein (eIF-4E) polyclonal antibody (control) and lane 2 represents an immunoblot with affinity-purified anti-GTPEYLAPE antibodies.

The limits of sensitivity of affinity-purified anti-peptide antibody in detecting protein kinases by Western immunoblotting methods were tested by immunoblotting known quantities of purified enzymes that contain a homologous sequence. Immunoblots of cAMP dependent protein kinase catalytic subunit demonstrated that as little as 50 ng of enzyme can be detected with affinity-purified anti-peptide antibody and the ELISA method described (Fig. 2, panel A). However, with $Ca^{2+}$/calmodulin dependent protein kinase II the limits of detection were 200 ng of enzyme (Fig. 2, panel B).

To determine if affinity-purified anti-GTPEYLAPE antibody would react with a tyrosine protein kinase we immunoblotted A431 cell plasma membrane vesicles which are enriched with a 170 kDa form of the EGF receptor [5]. Although no 170 kDa protein was detected in immunoblots, a protein of approximately 55 kDa consistently reacted in immunoblots. This result suggests that a protein kinase that has not been previously characterized is present in A431 cell membrane vesicles.

We conclude that it is possible to raise polyclonal antibodies against a 9 residue homologous sequence present in many protein kinases. These antibodies can be used to identify, by Western immunoblotting methods, intact kinases that contain regions differing by as many as two amino acids from the original peptide antigen. As demonstrated by our analysis of A431 cell membranes such antibodies can be used to indicate the presence of previously uncharacterized protein kinases in tissues or cell fractions. We are presently exploring the possibility of using monospecific polyclonal or monoclonal antibodies against conserved kinase regions as an affinity method to purify nonabundant or rapidly inactivated protein kinases.

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