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Abstract

In order to obtain a polyclonal antibody that recognizes various protein kinases, a peptide corresponding to an amino acid sequence of a highly conserved subdomain (subdomain VIB) of the protein kinase family was synthesized and used for immunization. When the synthetic peptide, CVVHRDLKPENLLLAS, was coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits, polyclonal antibodies that detected multiple protein kinases on a Western blot were generated. One of the antibodies obtained, KI98, detected a variety of purified Ser/Thr protein kinases, such as calmodulin-dependent protein kinase II (CaM-kinase II), calmodulin-dependent protein kinase IV (CaM-kinase IV), cAMP-dependent protein kinase, protein kinase C, and Erk2. The antibody detected as low as 0.2 ng of protein kinases blotted onto a nitrocellulose membrane by dot-immunobinding assay. When a rat brain extract was analyzed with this antibody, various protein kinases were simultaneously detected. The present anti-peptide antibody with a broad spectrum of cross-reactivity to multiple protein kinases may be a powerful tool for comprehensive analysis focused on protein kinases.

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1. Introduction

The eukaryotic protein kinases comprise large superfamilies of homologous proteins [1]. The molecular architecture of protein kinases includes regulatory regions with diverse amino acid sequences and a catalytic domain that composed of a highly conserved sequence. The 250-300 conserved amino acid residues can be divided into 12 subdomains, which may be essential sequences to provide structural features of the catalytic activity of the kinases [2].

There are hundreds of protein kinases in living cells and they are involved in numerous cellular signaling pathways. Since the expression of protein kinases varies depending on the cell type, cell cycle, developmental stage, or environmental conditions, it would be very useful if a variety of protein kinases expressed in the cells could be detected simultaneously with a simple procedure. In a previous study, we generated monoclonal antibodies to detect a variety of protein kinases by immunizing with synthetic peptides corresponding to amino acid sequences of a highly conserved subdomain (subdomain VIB) of the protein kinase family. These antibodies, designated M8C and M1C, proved to be useful not only for detecting various protein kinases but also for isolating cDNA clones of various known and novel protein kinases [3]. Therefore, such limited specificity antibodies, which possess broad cross-reactivity to multiple Ser/Thr protein kinases, may be useful tools for comprehensive analysis focused on protein kinases. For such a purpose, antibodies with a broader spectrum of cross-reactivity to protein kinases than the previous monoclonal antibodies would be highly desirable, because these monoclonal antibodies, for example, only weakly recognized protein kinase C, one of the typical multifunctional Ser/Thr protein kinases. The present study is an attempt to obtain an antibody with broader cross-reactivity using single antigen. Therefore, we generated polyclonal antibodies by immunizing rabbits with a synthetic peptide corresponding to the sequence of subdomain VIB of

calmodulin-dependent protein kinase II (CaM-kinase II), and examined the specificity and applicability of these antibodies for detection of various protein kinases in this study.

2. Materials and methods

2.1. Materials

The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described [4]. Protein kinase C was purified from rat brain by the method of Woodgett and Hunter [5] and this preparation was found to contain α, β, and γ isoforms of protein kinase C [3]. CaM-kinase II [6, 7] and calmodulin-dependent protein kinase IV (CaM-kinase IV) [8] were purified from rat brain as described previously. Recombinant mouse p42 MAP kinase (Erk2)-GST, recombinant human MAP kinase kinase 1 (MEK1), and anti-Erk1 antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Isozyme-specific antibodies to protein kinase C were obtained from Seikagaku Corporation (Tokyo, Japan). Antibodies against CaM-kinase II and CaM-kinase IV were obtained from BD Transduction (Lexington, KY, USA) and Life Technologies, Inc. (Maryland, USA), respectively. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, CA, USA). Bovine serum albumin was purchased from Sigma Chemicals (St.Louis, MO, USA). Goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase was obtained from ICN Pharmaceuticals (Aurora, OH, USA).

2.2. Preparation of rat brain extract and calmodulin-binding protein fraction

Rat brain extract was prepared essentially according to the method described previously [3, 9]. The calmodulin (CaM)-binding protein fraction was prepared essentially according to the method described previously [9]. Briefly, the extract was treated with ammonium

sulfate (50% saturation) for 30 min on ice and centrifuged. The precipitate thus obtained was dissolved in 40 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$, 50 mM NaCl, and 1 mM dithiothreitol, and applied onto a CaM-Sepharose column (1 ml). Proteins bound to CaM-Sepharose were eluted with 40 mM Tris-HCl (pH 7.5) containing 1 mM EGTA, 50 mM NaCl, and 1 mM dithiothreitol, and eluted protein fractions were pooled and used as the CaM-binding protein fraction.

2.3. Preparation of peptide conjugates

Peptides were synthesized using a Shimadzu PSSM-8 automated peptide synthesizer, and they were purified by reverse-phase HPLC on a C_{18} column. The identity and purity of the peptides were confirmed by fast atom bombardment-mass spectrometry. The purified peptides were coupled to KLH by a heterobifunctional reagent, N-(6-maleimidocaproyloxy)succinimide (Dojindo Laboratories, Kumamoto, Japan), through their amino-terminal cysteinyl residues as described previously [3, 10].

2.4. Production of antibody against multiple protein kinases

Eight-week old Japanese white rabbits (Japan SLC Inc., Hamamatsu, Japan) were used for antibody production. Approximately 200 μg of peptide-KLH conjugate (ca. 100 μg of peptide) emulsified with an equal volume of Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI. USA) was injected at multiple intradermal sites, followed 2 weeks later by injection with the same dosage of the peptide conjugate emulsified in Freund's incomplete adjuvant (DIFCO Laboratories, Detroit, MI, USA). Two intravenous boosters of 100 μg each of the conjugate (ca. 50 μg of peptide) in phosphate buffered saline (PBS) were given to rabbits in two-week intervals and the antisera were harvested 1 week after the final

injection. Thus obtained antisera were used as antibodies without further purification.

2.5. Analytical procedures

SDS-polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli [11] on slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. Western blotting and dot-immunobinding assay were carried out as described previously [3]. Proteins were determined by the method of Bensadoun and Weinstein [12] using bovine serum albumin as a standard.

3. Results

3.1. Generation of anti-peptide antibodies and their specificities

When primary sequences of a variety of protein kinases are compared, there are 12 common subdomains where amino acid sequences are conserved throughout the protein kinase family [1]. Among 12 subdomains, the subdomain VIB appears to be one of the most highly conserved regions in many protein kinases. Sequences of subdomain VIB of seven typical protein kinases are aligned and shown in Fig. 1. In order to generate a common polyclonal antibody directed against multiple protein kinases, we chose a peptide sequence of VVHRDLKPENLLLAS, which corresponds to the subdomain of CaM-kinase II α and β [13]. Cysteinyl residue was introduced on the amino terminal of the peptide in order to cross-link this peptide to carrier proteins. A peptide of CVVHRDLKPENLLLAS, which we designated as 16PEN, was conjugated to KLH and two rabbits were immunized with this antigen.

The specificity of antibodies thus obtained (designated KI98 and KI99, respectively) was examined by means of Western blotting. Purified preparations of CaM-kinase II,

CaM-kinase IV, cAMP-dependent protein kinase, protein kinase C, and Erk2-GST were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained with KI98 antibody and KI99 antibody. As shown in Fig. 2B, KI98 antibody detected all the protein kinases examined in this study including protein kinase C, which could be detected only marginally by the monoclonal antibodies, M8C and M1C [3]. On the other hand, KI99 antibody reacted with CaM-kinase II, CaM-kinase IV, cAMP-dependent protein kinase, and Erk2, but not with protein kinase C (Fig. 2C). Furthermore, both antibodies also detected MEK1 (data not shown). Since KI98 antibody exhibited broader specificity than KI99 antibody, KI98 antibody was used throughout the present study.

3.2. Sensitivity of detection of protein kinases

The sensitivity of the detection of protein kinases by the KI98 antibody was examined by dot-immunobinding assay. Purified preparations of CaM-kinase IV, cAMP-dependent protein kinase, and Erk2 were serially diluted and spotted onto nitrocellulose membrane, and detected by KI98 antibody. As shown in Fig. 3A, all three kinases, CaM-kinase IV, cAMP-dependent protein kinase, and Erk2, could readily be detected at the lowest amount (0.2 ng) used in this experiment. These spots greatly diminished when the antibody had been preincubated with the peptide antigen 16PEN, which was used for immunization (Fig. 3B). Thus, these reactive spots seem to be due to specific binding of the antibody to the proteins through the antigenic sequence.

3.3. Detection of protein kinases in rat brain extract and CaM-binding fraction

In order to examine the applicability of the KI98 antibody to detect protein kinases in

crude samples, rat brain extracts shown in Fig. 4A were analyzed by Western blotting using previously reported M8C monoclonal antibody and the present KI98 antibody. When M8C was used, various proteins were detected in the extracts from cerebral cortex and from cerebellum as reported in the previous paper [3] (Fig. 4B). However, a larger number of the protein bands could be detected by KI98 antibody than by M8C antibody as revealed by Fig. 4C. The bands less than 29 kDa might be proteolytic fragments of protein kinases recognized by the antibody. These reactive bands greatly diminished when the antibody had been preincubated with the peptide antigen 16PEN (Fig. 4D), suggesting that the antibody specifically recognized various proteins with this antigenic sequence.

In an attempt to identify the reactive bands recognized by KI98 antibody, the brain extract was fractionated by CaM-Sepharose column chromatography, and the CaM-binding proteins were analyzed by Western blotting with KI98 antibody (Fig. 5A). The major reactive bands detected at about 50-60 kDa in the crude extract were also observed in the CaM-binding fraction, suggesting that they were CaM-binding protein kinases. Of these, the band observed at about 50 kDa (Fig. 5A, band b) was identified as CaM-kinase II α by the specific antibody against the α-isoform of CaM-kinase II (Fig. 5B). The reactive protein band of about 60 kDa (Fig. 5A, band c/d) bound with KI98 may contain both CaM-kinase II β and CaM-kinase IV, because the 60-kDa protein band was detected either by CaM-kinase II or CaM-kinase IV antibody (Fig. 5C & D). Thus, the major reactive bands of about 50 and 60 kDa detected by the KI98 antibody in the crude extract may be CaM-kinase II α and CaM-kinase II β and/or CaM-kinase IV, respectively. On the other hand, the protein band around 80 kDa (Fig. 5A, band f) , which was observed in the rat brain extract but not in CaM-binding fractions, might be protein kinase C (Fig. 5F). The protein band of 44 kDa observed in the rat brain extract (Fig. 5A, band e, upper band) might be Erk1 as judged by its

mobility on SDS-polyacrylamide gel electrophoresis and cross-reactivity of the protein with the specific antibody (Fig. 5E).

4. Discussion

Antibodies to synthetic peptides have been often used as powerful tools for the molecular analysis of various proteins [14]. In most cases, unique sequences of the proteins of interest were chosen for preparation of peptide antigens in order to obtain highly specific antibodies. In the present study, on the contrary, a synthetic peptide corresponding to a highly conserved region of the protein kinase superfamily was employed for production of a "less specific " antibody that could detect a variety of protein kinases. From among several highly conserved subdomains of protein kinases, an amino acid sequence derived from subdomain VIB of CaM-kinase II was selected as a peptide antigen (Fig. 1).

Brown et al. produced anti-peptide antibodies against four highly conserved sequences in the kinase domain of human epidermal growth factor receptor kinase [15]. These antibodies were used for examination of topology and catalytic functions of these sequences in this receptor kinase. One of the antigens they used was a synthetic peptide with eight amino acids, HRDLAARN, which corresponds to the subdomain VIB of Tyr protein kinases [1]. Although similar amino acid sequences are found to be present in the same region of other Tyr protein kinases, such as platelet derived growth factor receptor kinase and insulin receptor kinase, cross-reactivity of the antibody, designated αHRD, to the other kinases has not been reported. They also reported that the antibody reactive with the entire epidermal growth factor receptor protein was produced only transiently as judged by immunoprecipitation [16].

In our previous report, we generated monoclonal antibodies, M8C and M1C, which

were raised against a synthetic peptide designated 16PEN corresponding to the highly conserved subdomain VIB of protein kinases, and showed that they detected a variety of known and novel Ser/Thr protein kinases [3]. In order to generate antibodies with a broader spectrum of cross-reactivity to Ser/Thr protein kinases, we employed the same synthetic peptide as an immunogen in rabbits in this study. Both rabbits used in the present study produced polyclonal antibodies, KI98 and KI99, that recognized various Ser/Thr protein kinases. In contrast to the αHRD antibody, KI98 and KI99 antibodies were stably produced as judged by dot-immunobinding assay using the entire CaM-kinase II protein as an antigen, with the titer of the antisera being substantially unchanged for at least two months (data not shown). Since we used a longer sequence for generation of KI98 and KI99 antibodies than that for the αHRD antibody, the N- and C- terminal extensions of the antigenic peptide for the former (VV and LLLAS, respectively) may be critical for stable production of the antibodies with broad cross-reactivity.

KI98 antibody reacted with all of the protein kinases examined, including protein kinase C (Fig. 2), whereas M8C and M1C detected protein kinase C only weakly. This is somewhat surprising, because the corresponding sequence in the subdomain VIB of protein kinase C is considerably different from that of the antigenic peptide: only seven amino acids in the former are matched to those of the latter (Fig. 1). This antibody could detect various proteins, which competed with the 16PEN peptide used as an antigen, in rat brain extract (Fig. 4C & D). Furthermore, a larger number of proteins could be detected by KI98 than by M8C in Western blotting analysis of rat brain extract (Fig. 4B & C). Thus, KI98 antibody had broader cross-reactivity to protein kinases than those thus far obtained. A BLAST homology search using the 16PEN peptide as a query showed more than 500 protein sequences corresponding to known and putative Ser/Thr protein kinases. Therefore, most

of the protein bands detected by KI98 antibody in the crude extract seem to be protein kinases. Western blotting analyses using specific antibodies to various known protein kinases supported this contention (Fig. 5B-F). Since less than 0.2 ng of protein kinases were detected by KI98 antibody (Fig. 3), and since various protein kinases in the crude extract could be analyzed simultaneously by this antibody (Fig. 4C & 5A), the antibody will be applicable to various comprehensive studies on protein kinases. Especially, this antibody will be useful for proteomic analysis focused on the protein kinase family. Alternatively, this antibody will be used for selection of positive clones in expression cloning of various protein kinases using lambda expression libraries.

Generation of KI98 and KI99 antibodies in rabbit further validate our claim proposed in our previous paper that the "multi-antibody", that is to say, "less specific" antibodies raised against highly conserved regions of protein kinases will be powerful tools for various protein kinase studies [3]. In addition, the present study made the "multi-antibody" strategy more convenient, because it is easier to prepare polyclonal antibodies than monoclonal ones. Mixed use of the "multi-antibodies" with different specificity thus obtained may also be useful, since we can design the "group specificity" by such blending. The "muti-antibody" strategy using multipurpose antibodies such as KI98, KI99, M8C, and M1C may provide a new approach for proteomic studies to clarify cellular functions of not only protein kinases but also other protein families.

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Legends for figures

Fig. 1. Alignment of subdomain VIB of typical protein kinases. Amino acid sequences of subdomain VIB regions of CaM-kinase II (CaMKII) , CaM-kinase IV (CaMKIV), Erk2, cAMP-dependent protein kinase (PKA), MEK1, protein kinase C (PKC), and epidermal growth factor receptor kinase (EGFR) were compared. Amino acids are represented by single-letter code. The identical amino acids are shown with shadows. Peptide 16PEN shown in the bottom line represents the peptide used as an antigen in this study.

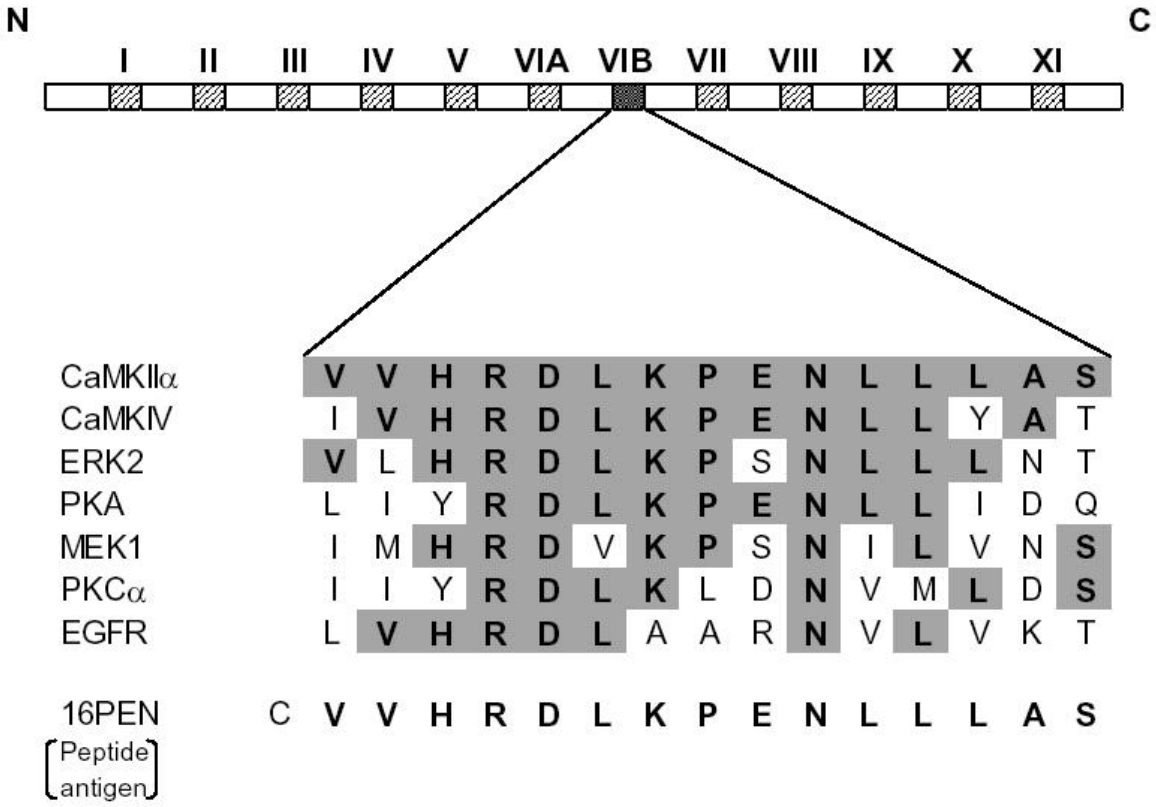
Fig. 2. Detection of purified Ser/Thr protein kinases by KI98 and KI99 antibodies. (A) Silver staining pattern of purified preparations of CaM-kinase II (100 ng, lane 1), CaM-kinase VI (50 ng, lane 2), cAMP-dependent protein kinase (50 ng, lane 3), protein kinase C (100 ng, lane 4), and Erk2-GST (20 ng, lane 5). M1 and M2 represent high molecular weight marker and low molecular weight marker proteins, respectively. (B)(C) Western blotting by anti-peptide antibodies. CaM-kinase II (40 ng, lane 1), CaM-kinase IV (20 ng, lane 2), cAMP-dependent protein kinase (10 ng, lane 3), protein kinase C (100 ng, lane 4), and Erk2-GST (20 ng, lane 5) were electrophoresed on SDS-polyacrylamide gels and detected by Western blotting using KI98 antibody (B) or KI99 antibody (C). The migration positions of standard marker proteins were shown on the left.

Fig. 3. Sensitivity of detection of protein kinases by KI98 antibody. Indicated amounts of CaM-kinase IV (CaMKIV), cAMP-dependent protein kinase (PKA), and Erk2-GST (Erk2) were spotted onto a nitrocellulose membrane and detected by dot-immunobinding assay using KI98 antibody (A) or the antibody that had been preincubated with 5% skim milk containing

1 mg/ml 16PEN at 0 °C for 2 hr (B).

Fig. 4. Comparison of reactivities of M8C antibody and KI98 antibody on Western blotting. (A) Approximately 20 μg of crude extracts from rat cerebral cortex (lane 1) and rat cerebellum (lane 2) were electrophoresed on SDS-polyacrylamide gel and stained with Coomassie brilliant blue. (B)(C)(D) SDS-polyacrylamide gel electrophoresis was carried out as in (A) and resolved proteins were blotted onto nitrocellulose membrane. Western blotting was performed using M8C antibody (B), KI98 antibody (C) or KI98 antibody that had been preincubated with 1 mg/ml 16PEN (D). The migration positions of the prestained marker proteins were indicated on the left.

Fig. 5. Analysis of a rat brain extract and a CaM-binding protein fraction by Western blotting with the antibodies directed to various protein kinases. Rat brain extract (20 μg, lane 1) and a CaM-binding fraction from rat brain (1 μg, lane 2) were electrophoresed on SDS-polyacrylamide gels and analyzed by Western blotting with KI98 antibody (A). Western blotting was also carried out using anti-CaM-kinase II α (B), anti-CaM-kinase II β (C), anti-CaM-kinase IV (D), anti-Erk1 (E), and anti-protein kinase C α (F). The positions corresponding to the proteins detected in panels B, C, D, E, and F are indicated in panel A by b, c, d, e, and f, respectively.



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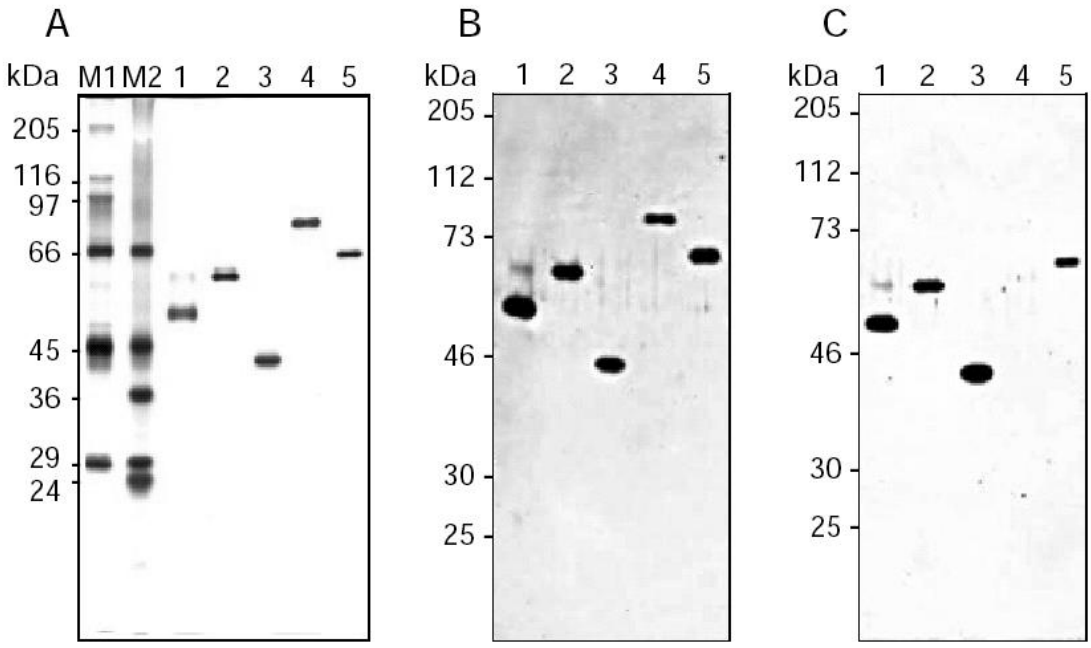
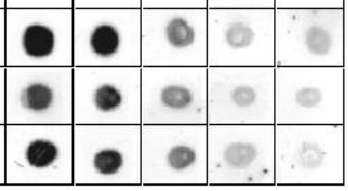


Fig. 2. Kameshita ***et al.***

A

	Protein Kinase (ng)				
	10	5	1	0.5	0.2
CaMKIV					
PKA					
ERK2					

B

	Protein Kinase (ng)				
	10	5	1	0.5	0.2
CaMKIV					
PKA					
ERK2					

Fig. 3. Kameshita ***et al.***

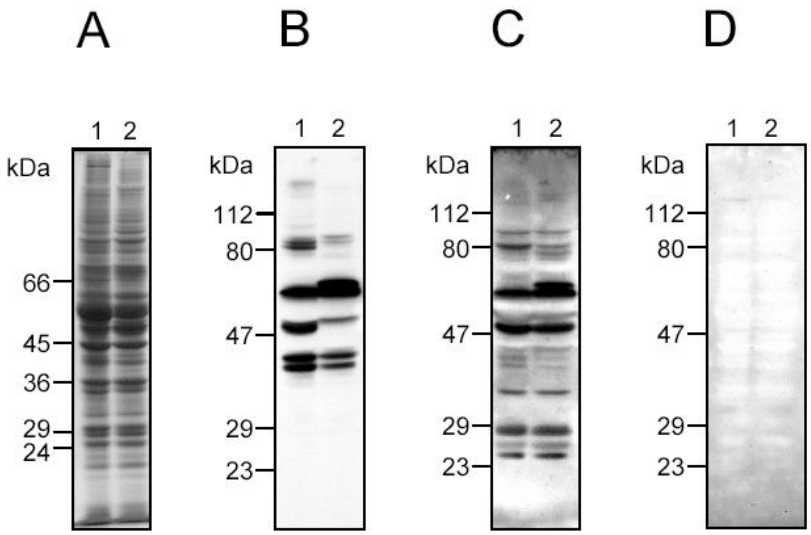


Fig.4. Kameshita ***et al.***

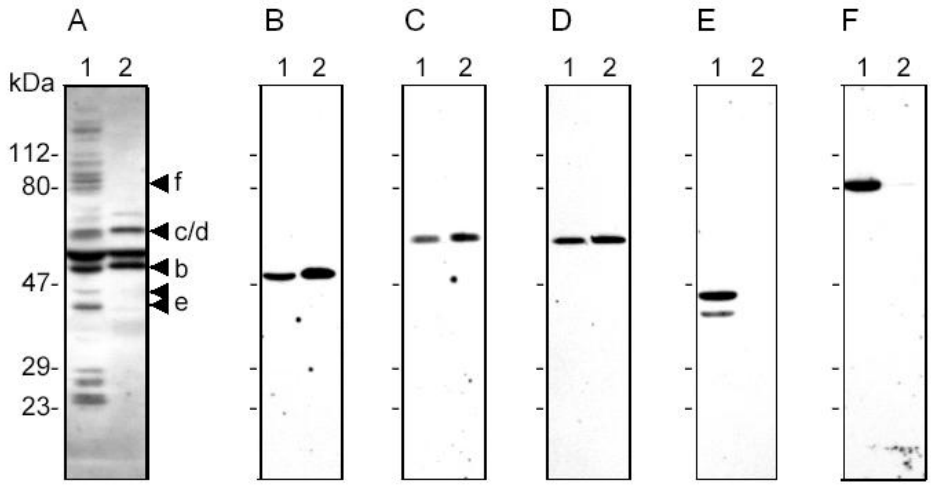


Fig.5. Kameshita ***et al.***