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Purification and Kinase Assay of PKN

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Abstract:

PKN is a serine/threonine protein kinase, which has a catalytic domain highly homologous to that of protein kinase C (PKC) in the carboxyl-terminal region and three repeats of the antiparallel coiled coil (ACC) domain in the amino-terminal region. Mammalian PKN has three isoforms each derived from different genes, PKN1 [= PKNα / PRK1 / PAK1], PKN2 [= PRK2 / PAK2 / PKNγ], and PKN3 [= PKNβ]. PKN isoforms show different enzymatic properties and tissue distributions, and have been implicated in various distinct cellular processes (reviewed in (Mukai, 2003)). This article discusses methods to prepare purified enzymes and to assay substrate phosphorylation activities.

Introduction

PKN cDNA was first identified in 1994 by a low stringency hybridization using the catalytic domain of PKC βII as a probe (Mukai and Ono, 1994), and PKN protein was later purified to homogeneity from rat testes using a specific antibody raised against recombinant PKN as a guide (Kitagawa et al., 1995). This enzyme was first described as a fatty acid- or phospholipid- activated protein kinase and also as a protease-activated protein kinase (Kitagawa et al., 1995;Mukai et al., 1994;Peng et al., 1996). On the other hand, PKN was elucidated as a target molecule of the small GTPase RhoA using GTP-RhoA affinity chromatography (Amano et al., 1996) and a gel overlay method (Watanabe et al., 1996). Subsequent molecular cloning and protein analysis revealed that PKN is a family consisting of multiple isoforms, and hence the original PKN was redesignated as "PKN1". Since these studies have been carried out independently in several laboratories, different nomenclatures are proposed for the PKN isoforms derived from different genes. The nomenclature adopted herein uses PKN1 [instead of PKNα (Mukai and Ono, 1994) / PRK1 (Palmer et al., 1995b) / PAK1 (Peng et al., 1996)], PKN2 [instead of PRK2 (Palmer et al., 1995b) / PAK2 (Yu et al., 1997) / PKNγ], and PKN3 [instead of PKNβ (Oishi et al., 1999)] according to the Human Kinome (Manning et al., 2002). Sequence comparison reveals that PKN isoforms have conserved domains: ACC domains and the C2-like domain in the amino-terminal region, and the catalytic serine/threonine kinase domain in the carboxyl-terminal region. However, PKN isoforms show different enzymatic properties and tissue distributions, and have been implicated in various distinct cellular processes (reviewed in (Mukai, 2003)).

This article describes methods to purify endogenous PKN from mammalian tissues and recombinant enzyme from insect cells, and to assay for kinase activity. Modifiers of PKN kinase activity are also summarized.

Purification of PKN

This section describes the purification procedures of PKN from various sources. The first two methods enable purification of the endogenous PKN1 to homogeneity from mammalian tissues as determined by silver staining after SDS-polyacrylamide gel electrophoresis (PAGE), and the third method is to purify recombinant PKN isoforms from insect cells. (The apparent molecular weights of PKN1, PKN2, and PKN3 are different from one another, and these enzymes are easily distinguished on SDS-PAGE.)

PKN1 is expressed in every tissue and is especially enriched in the testes and spleen. In so far as cell lines, leukemic cell lines such as Jurkat and U937 cells are also abundant in PKN1. It should be noted at this point that PKN1 actually consists of at least two types of alternatively spliced isoforms with almost the same molecular weight (manuscript in preparation) and the relative amounts of these isoforms differ among tissues. PKN2 is also widely expressed in adult tissues, with the highest levels being in the liver and lung (Vincent and Settleman, 1997). PKN3 is almost undetectable in normal adult tissues. However, culture cells such as HeLa, chronic myelogenous leukemia K-562, and colorectal adenocarcinoma SW480 cells contain significant amounts of this enzyme (Oishi et al., 1999). Subcellular fractionation experiments revealed that about half of all

endogenous PKN1 resides in the soluble cytosolic fraction (Kitagawa et al., 1995) of brain tissue. It was recently suggested that a part of PKN1 exists as the integral membrane form and has modifications different from the cytosolic/peripheral membrane form of the enzyme (Zhu et al., 2004). The source of the endogenous enzyme should be determined in view of the above information.

Purification of PKN1 from the soluble cytosolic fraction of rat testes

The purification protocol for endogenous PKN1 from rat testes was published in detail (Kitagawa et al., 1995). An outline of the method and previously undescribed information will be provided herein. The flow chart of the procedure is depicted in Fig 1A. All procedures are carried out at 0-4°C. Rat testes (60 g fresh weight) are added to 4 volumes of the buffer Te-A [50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.5 mM dithiothreitol, 10 μg/ml leupeptin, 0.02 % NaN_3, and 1 mM phenylmethanesulfonyl fluoride], and homogenized with a Polytron homogenizer (KINEMATICA), or a Teflon homogenizer. The resulting crude homogenate is then centrifuged at 24000 *g* for 1 hr and the supernatant is loaded onto a 180 ml DE52 (Whatman) column equilibrated in buffer Te-B [25 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 100 ng/ml leupeptin, and 0.02 % NaN_3]. After washing, PKN1 is eluted with buffer Te-B containing 200 mM NaCl and immediately subjected to a 40% ammonium sulfate (AS) fractionation. The precipitate is resuspended in buffer Te-B containing 20 % AS and loaded onto a 20 ml Butyl-Sepharose column (Amersham) equilibrated with the same buffer. The eluate from Butyl-Sepharose with buffer Te-B containing

10% AS is dialyzed against buffer Te-B and is loaded onto a 5ml HiTrap Heparin Sepharose column (Amersham) equilibrated with buffer Te-B, which is connected to a Pharmacia FPLC system. PKN1 is eluted from the column by application of a linear concentration gradient of NaCl (0-0.6 M) in buffer Te-B. The major peak of PKN1 detected by anti-PKN1 antibody is dialyzed against buffer Te-B, and then loaded onto a Mono Q 5/5HR column (Amersham) equilibrated with the same buffer using an FPLC system. PKN1 is eluted from the column by application of a 40 ml linear concentration gradient of NaCl (0-0.4 M) in buffer Te-B, and the major peak of PKN1 is loaded onto a 4ml protamine-CH-Sepharose column. (This column is prepared by coupling protamine sulfate to the activated CH-Sepharoase 4B according to the methods of Amersham.) Purified PKN1 tightly bound to the column is eluted by 2M NaCl (Fig. 2). Approximately 12 μg of PKN1 is purified about 8000-fold to an apparent homogeneity from 60 g of starting material. Purified enzyme should be dialyzed against Tris/dithiothreitol buffer for enzyme assays because the salt at high concentrations inhibits the activity of PKN1 (refer to the "Modifiers of PKN" section). The purified enzyme is stable for more than 6 months in 50% glycerol at -80ºC. (Glycerol does not inhibit and rather mildly increases the kinase activity of PKN1) Even in the absence of glycerol, PKN1 can be preserved for about 1 month at -80ºC without a significant loss of enzyme activity, although freeze-thaw cycles should be avoided.

A Thr-Sepharose column with a salt gradient is useful for partial purification of PKN1, although we did not include this step in the above protocol. We previously loaded the sample from the MonoQ 5/5HR in Fig.1A onto the Thr-Sepharose column, and applied the eluate to a TSK G3000SW gel filtration column in the presence of 0.3M NaCl. PKN1 was separated in roughly two peaks with high (>200kDa) and low (= monomer)

molecular weight fractions, suggesting the presence of PKN1 which has oligomerized or has interacted with other proteins under these conditions.

Rapid purification of PKN1 from the membrane fraction of bovine brain

PKN1 binds to the small GTPase RhoA in a GTP dependent manner. So the GTPγS-loaded glutathione S-transferase (GST)-RhoA affinity column is applicable for the purification of PKN1 (Amano et al., 1996). The GTPγS-GST-RhoA affinity column is prepared as described previously (Amano et al., 2000). The flow chart of the purification procedure is depicted in Fig 1B. All procedures are carried out at 0-4°C. Bovine brain is cut into small pieces. A 30 g aliquot is homogenized with 90 ml of buffer Br-A [25 mM Tris-HCl at pH 7.5, 10% sucrose, 5 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 0.1 μg/ml leupeptin] and 0.1 mM phenylmethanesulfonyl fluoride in a Teflon-glass homogenizer and filtered through three layers of gauze. The crude homogenate from 200 g of brain in total is put together and is centrifuged at 18,000 *g* for 30 min. The precipitate is resuspended in 400 ml of buffer Br-A, and the protein from the membrane fraction is extracted by mixing with 400 ml of buffer Br-A containing 4 M NaCl, being stirred for 1 hr. After the solution is centrifuged at 18,000 *g* for 30 min, the supernatant is filtered through three layers of gauze, and is dialyzed against buffer Br-B [20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 5 mM $MgCl_2$]. After centrifugation at 18,000 *g* for 30 min, solid ammonium sulfate is added to the supernatant to a final concentration of 40% saturation. The precipitate is dissolved in 16 ml of buffer Br-B, dialyzed against buffer

Br-B, and centrifuged at 100,000 *g* for 1 hr followed by filtrations through 0.80μm and 0.45 μm filters sequentially. After being passed over a 1-ml glutathione-Sepharose column, the flowthrough fraction is loaded onto a 1-ml glutatione-Sepharose column carrying GTP-γS-GST-RhoA. The column is washed extensively (more than 20 volumes) of buffer Br-B, and proteins are eluted by addition of buffer Br-B containing 0.2 M NaCl. One-ml fractions are collected, and the amount of PKN1 purified is examined by silver staining and/or immunoblotting with anti-PKN1 antibody. (Typically, fractions #3 - #10 contain relatively rich amounts of PKN1.) Fractions containing PKN1 are pooled together and dialyzed against buffer Br-B. The dialyzed sample is subjected to a 0.3 ml DE52 column, and the column is washed with buffer Br-B containing 50 mM NaCl. PKN1 is eluted by loading buffer Br-B containing 75 mM NaCl, and 0.3 ml fractions are collected. (Typically, fractions #2-#4 contain purified PKN1.)

Expression and Purification of recombinant PKNs from insect cells

It is difficult to obtain active PKN1 from *Escherichia coli* expression systems. PKN2 can be expressed as an active enzyme in *E. coli*, but its specific activity is low (Yoshinaga et al., 1999) and it is difficult to obtain rich amounts of the full-length enzyme from the bacteria. The eukaryotic insect cell expression system is useful to obtain isotype-pure and sufficient amount of functional PKNs. The GST/glutathione Sepharose or His_6 tag/Ni-NTA one-step purification system is useful for preparing active enzymes quickly. Further purification of the enzyme can be carried out by combining these two affinity chromatographies sequentially or by adding some of the purification steps described in the previous section. The GST and His_6 tag may

potentially induce steric hindrance or conformational changes of the enzyme itself and affect substrate recognition due to dimer formation of GST (Kaplan et al., 1997). However, the wild type PKN1 expressed as a fusion protein with the GST tag or His_6 tag in Sf9 cells has almost the same properties as endogenous PKN1 purified from the soluble cytosolic fraction of rat testes with regard to the substrate specificity and response of effectors (Yoshinaga et al., 1999). The insect cell expression system is useful for obtaining each isoform as described above, although we should keep in mind that the purified PKN still might be composed of heterogeneous molecules with different modifications in insect cells. Construction of a baculovirus for expression of PKN1 and the purification protocols have been described elsewhere in detail (Mukai and Ono, 2003), so the outline of the procedure with previously undescribed information will be commented herein.

pBlueBacHisGST (pR538) vector, a transfer vector for the expression of His_6-GST-tagged fusion proteins, is made by subcloning the coding region for GST and a thrombin recognition site in frame to the multiple cloning site of pBlueBacHis-B (Invitrogen). The cDNA for PKN is subcloned into the multicloning site of this vector in frame. For the protein product, the His_6 tag can be cleaved by enterokinase if so desired, yielding a GST fusion protein. Both the His_6 tag and the GST tag can be cleaved by thrombin. The pAcGHLT vector for expression of GST-His_6- tagged fusion proteins is commercially available (Pharmingen). However, it should be kept in mind that the PKA phosphorylation site is included in the product enzyme without cleavage tags. PKN efficiently phosphorylates this PKA phosphorylation site. Information about construction of the catalytically active fragment or introduction of the kinase-negative mutation has been described (Yoshinaga et al., 1999). Sf9 cells are seeded at 1×10^7 cells on a 10 cm culture dish. About 2 - 20

dishes are used for purification of each enzyme depending on the expression levels of the recombinant proteins. Cells are incubated at 28°C for 1 hr so that they attach to the dish. After removing the medium, recombinant baculovirus is added at an appropriate multiplicity (usually in a range of 5 – 10 pfu/cell) to the cells. After incubating the virus/cells for 1 hr at room temperature, 10 ml of medium is added to the dish and incubated at 28°C for 2- 3 days until a cytopathic effect is observed. The cells are harvested by scraping them from the dish with a rubber policeman. The cell pellets are collected by centrifugation at 1000 *g* for 5 min at 4°C, and stored at -80°C. It is basically easier to get rich amounts of PKN1 in the soluble fraction than PKN2 and PKN3, although the amounts of the soluble recombinant enzymes vary among the deleted and mutated constructs. To increase the recovery of PKN2 and PKN3, higher multiplicities of infection and longer incubation times after infection with virus are necessary, although too high of a concentration of virus and long incubation times will sometimes result in a decrease of recovery of the enzyme in the supernatant fraction.

The sequential affinity purification of His_6- and GST- tagged PKN is as follows. All procedures are carried out at 0-4°C. The cell pellet is resuspended in 4 ml of buffer Sf-A [50 mM Tris-HCl at pH 8.0, 1 μg/ml of leupeptin, 1 mM $MgCl_2$, 0.3 M NaCl, and 10 mM β-ME] containing 1 mM phenylmethanesulfonyl fluoride and 10 mM imidazole, and is homogenized with a Dounce homogenizer with 30 strokes. Triton X-100 is added at 1% and incubated for 10 min. After centrifuging at 100,000 *g* for 30 min, the supernatant is added to 400 μl of 50% Ni-NTA agarose equilibrated with buffer Sf-A and is rotated for 1 hr. The lysate-resin mixture is loaded into an empty column, and washed with 30 column volumes or more of buffer Sf-A containing 10 mM imidazole. The recombinant protein is eluted with 800 μl of buffer Sf-A containing 250 mM imidazole. 200

μl of 50% glutathione Sepharose (Amersham) equilibrated with buffer Sf-B [50 mM Tris-HCl at pH 7.5, 1μg/ml leupeptin, 1 mM EGTA, 1 mM EDTA, 3 mM $MgCl_2$, and 1 mM dithiothreitol] is added to the eluate, and rotated for 1 hr. The sample is loaded onto an empty column, and washed with 30 column volumes or more of buffer Sf-B. The recombinant protein is eluted 4 times with 100 μl buffer Sf-C [50 mM Tris-HCl at pH 8.0, 1 μg/ml leupeptin, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM glutathione] in each tube. The advantage of this sequential chromatography method is to provide highly pure enzyme quickly and to remove imidazole, which is inhibitory to PKN, during the glutathione Sepharose column step.

Kinase assay

To assess the protein kinase activity of PKN isoforms, purified enzyme (~10 ng) is incubated for 5 min at 30°C in a reaction mixture (final volume 25 μl) containing 20 mM Tris-HCl at pH 7.5, 4 mM $MgCl_2$, 40 μM ATP, 18.5 kBq of [γ-^{32}P]ATP, 40 μM oligopeptide phosphate acceptor as described below, 0.1 mg/ml BSA or recombinant GST as a stabilizer, and in the presence or absence of modifiers such as 40 μM arachidonic acid (Table I). (PKN exhibits a characteristic Mg^{2+} dependence for a protein kinase, with an optimal Mg^{2+} ion concentration in the range of 2-5 mM (Morrice et al., 1994)). Reactions are terminated by spotting the mixture onto P81 phosphocellulose paper (Whatman) and submersing them in 75 mM phosphate, and then washing them three times for 10 min. The incorporation of ^{32}P phosphate into the oligopeptide phosphate acceptor is assessed by the Cherenkov counting.

As speculated from the structural resemblance among catalytic domains of PKNs and PKCs, the

consensus phosphorylation motif sites for PKNs are very similar to each other and also to those of PKCs (manuscript in preparation). This is also supported by the report that PKN1 phosphorylates the same sites of MARCKS (Palmer et al., 1996) and vimentin (Matsuzawa et al., 1997) as PKCs *in vitro*. Oligopeptide substrates synthesized based on the pseudosubstrate sites of PKCs can be used as good phosphate acceptors for PKN (Mukai et al., 1994). For example, AMFPTMNRRGSIKQAKI is an efficient substrate for PKN and corresponds to amino acids 137 – 153 of PKCδ, substituting Ser for Ala_{147}. Thus PKN seems to phosphorylate PKC substrates unscrupulously. However, relatively optimized peptide substrates for either PKN or PKC are available for kinase assays (manuscript in preparation). Protein substrates such as myelin basic protein (MBP), protamine sulfate, and histone H1 can be used instead of oligopeptide phosphate acceptors. In this case, the radiolabeled bands can be visualized by autoradiography after resolving the reacted mixture by SDS-PAGE. In the absence of modifiers such as arachidonic acid, these protein substrates are efficient substrates. However, this test does not reflect well the conformational change of PKN by the modifier (Kitagawa et al., 1995). PKN does not phosphorylate GST alone, so GST fused to an oligopeptide optimized for the PKN assay can be used as a good stoichiometric protein substrate instead of those mentioned above. (It is noteworthy that PKN weakly phosphorylates the Ser residue coded by the *Bam*HI [$GGA\text{-}_{Gly}$:$TTC\text{-}_{Ser}$] site of some pGEX vectors especially when Met follows this Ser residue.) This method is more sensitive than using the P81 phosphocellulose paper method as described above.

Lipid activators are freshly prepared as follows. The required amounts of lipids in organic solvent are dried in a nitrogen stream and then sonicated on ice in distilled water for 5 sec to make sonicated micelles.

Palmer et al. (Palmer et al., 1995a) employed the detergent mixed micellar assay, first developed by Hannun et al. for the study of PKC activity (Hannun et al., 1985), to the kinase assay for PKN1. In the case of PKC, phosphatidylserine (PtdSer) is a potent activator when presented alone. However, in detergent mixed miscelles, activation of PKC becomes dependent upon diacylglycerol or phorbol esters (Hannun et al., 1985), suggesting that detergent mixed micelles, while not physiological, support an *in vitro* behavior of PKC consistent with that observed *in vivo*. It was reported that PKN1 is activated by both phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3) with similar potency either as pure sonicated lipids or in detergent mixed micelles. However, sonicated phosphatidylinositol (PtdIns) and PtdSer are less effective in detergent mixed micelles than in pure sonicated ones. The detergent mixed micellar assay might be useful especially for the specific modulation of PKN by lipids under defined conditions.

The kinase assay can be performed conventionally using immunoprecipitation of PKN from cell extracts. However, especially in this case, the following points should be taken into account: i) Various detergents such as non-ionic Triton X-100 and NP-40 affect the kinase activity of PKN in a biphasic manner (Kitagawa et al., 1995). PKN is also sensitive to various ions. For example, $CaCl_2$, $MnCl_2$, and NaCl inhibit PKN activity in a dose dependent manner with IC_{50} values of ~600 μM, 40 μM, and 160 mM, respectively for the basal kinase activity (Table II). Thus the final precipitate should be washed before application to the enzyme assay to remove these reagents commonly used for the immunoprecipitation. ii) The Kd value for PKN1 with GTPγS-RhoA is ~ 0.4 μM (Amano et al., 1996) and the *in vivo* binding between these proteins is not

strong enough to be kept in the extensive washing step. When the recombinant PKN is immunoprecipitated and subjected to the kinase assay, the following additional points should be noted. The carboxyl-terminally tagged catalytic domain of PKN1 bound to anti-tag (such as FLAG and HA) antibody does not show a significant kinase activity. The non-tagged catalytic domain of PKN1 also does not have kinase activity when bound to the anti-carboxyl-terminal region antibody such as αC6 (Mukai et al., 1996). The kinase activity can be restored once the enzyme is dissociated from the antibody by low pH treatment after immunoprecipitation (Takahashi et al., 1998). Therefore, the antibody bound to the carboxyl-terminal region of the catalytic domain of PKN probably induces steric hindrance or a conformational change in the enzyme. This is not the case for the full length PKN1. Leenders et al. (Leenders et al., 2004) reported that the catalytic region fragment has just slight or no kinase activity compared to the full length fragment of PKN3. This controversial result might be obtained with the immunoprecipitation kinase assay when using an antibody against the carboxyl-terminal tag or a part of PKN.

Since PKN is sensitive to detergents and salts *in vitro* and may change its activity drastically within a few minutes after stimulation *in vivo*, a quick and accurate assay method of PKN in the crude extract is longed for studying the physiological significance of this enzyme. However, the development of this type of assay has been hampered by the lack of a highly specific and efficient substrate. From another point of view, the previous substrates which are supposed to be specific for PKC are not always specific for PKC since the finding of the related kinase, PKN. Although some commercial peptide substrates have been available for the *in vitro* assay of PKC in crude extracts, these same peptides also serve as substrates for PKN and might partly reflect

PKN activation.

PKN1 is phosphorylated at several sites *in vivo* and also by autophosphorylation *in vitro*. Phosphorylation dependent activation is suggested (Peng et al., 1996;Yoshinaga et al., 1999), and the activation of PKN seems to be correlated with the appearance of hyperphosphorylated bands migrating more slowly (Mellor et al., 1998). Therefore, it may be helpful for the assessment of PKN kinase activity to measure the electrophoretic mobility shift of the kinase. However, it should be noted that the activation loop phosphorylation corresponding to the Thr 774 site of human PKN1, which is thought to be critical for activity, is not directly responsible for the apparent band-shift of PKN1 (manuscript in preparation), and that the extent of autophosphorylation is diminished especially in the presence of good substrate peptides *in vitro*. Anti-phospho-Thr (in the activation loop) antibody is now commercially available, and several groups have suggested the *in vivo* signal-induced activation of PKN by detecting the phosphorylation of this site (Dong et al., 2000;Leenders et al., 2004;Torbett et al., 2003;Yau et al., 2003). However, care should be taken about the interpretation of activation loop phosphorylation. At least for PKN2, activation loop phosphorylation may not be a critical point of acute regulation of the enzyme (discussed in detail in (Mukai, 2003)).

Modifiers of kinase activity

The effects of various activators on the kinase activity of PKN1 are summarized in Table I. The effective doses of modifiers of PKN1 are listed in Table II. Some of the well-known potent inhibitors for PKC such as

bisindolylmaleimide I and H-7, and for Rho kinase/ROCK such as Y27632, also inhibit PKN1 kinase activity with a similar efficiency. Despite the many similarities among the isoforms of PKN, responsiveness to some modifiers is different for each isoform (Oishi et al., 1999;Yu et al., 1997). For example, responsiveness to unsaturated fatty acid, one of the potent activators of PKN1, is clearly different among isoforms, with the order of PKN1>PKN2>PKN3 when a PKCδ peptide is used as a substrate.

Comment

As discussed in the "kinase assay" section, there is no standard appropriate measure to monitor PKN activity in vivo thus far. PKN-specific *in vivo* substrates have not been identified yet, and the correlation of phosphorylation of PKN itself with its substrate phoshorylation activity has not been established. Biochemical assays of PKN activation have limited predictive value for the understanding of structure-activity relationships because of extensive modulation of PKN behavior by the cellular environment, which includes contributions from lipids, ions, and cellular binding proteins including Rho family members. A number of studies have shown that fluorescence resonance energy transfer (FRET) reporters are useful tools with which to probe kinase-mediated phosphorylation in living cells. Much information can be obtained by these kinds of reporters, provided that such reporters do not significantly perturb cellular function (for example, by the buffering of cell signals resulting from reporter overexpression) and provided that reporter specificity is maintained in cells. Some of these reporters consist of portions of a kinase substrate fused to a fluorescent protein in an arrangement such that a conformational change occurs upon phosphorylation, resulting in a change in FRET signal (Kunkel

et al., 2005;Nagai et al., 2000;Sato et al., 2002;Ting et al., 2001;Violin et al., 2003). Among these includes a C kinase activity reporter (CKAR), which reports phosphorylation driven by PKC (Violin et al., 2003). With the fact that PKN1 phosphorylates the original CKAR *in vitro*, this reporter might also be affected by PKN activity. Further studies are expected to develop an efficient PKN-specific reporter, "NKAR".

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Figure legends

Fig. 1. Outline of the PKN1 purification procedure. sup, supernatant; ppt, precipitate

Fig. 2. PKN1 obtained from rat testes after protamine-CH-Sepharose chromatography. **A**. Silver stained SDS/polyacrylamide gel **B**. Western blotting using polyclonal antiserum against PKN1. The arrows indicate the position of PKN1.

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Table I Effects of various activators on PKN1 activity

modifier	activity (%)	enzyme	substrate	reference
10 μg/ml cardiolipin	380	purified PKN1 from rat liver	S6-peptide (AKRRRLSSLRA)	(Morrice et al., 1994a)
40 μM arachidonic acid	850	purified PKN1 from rat testes	PKC δ peptide (AMFPTMNRRGSIKQAKI)	(Kitagawa et al., 1995)
40 μM linoleic acid	900			
8μg/ml lyso-PA	430			
60 μg/ml PtdIns-4,5-P_2, 60 μg/ml PtdIns-3,4,5-P_3	robust activation	recombinant human PKN1 purified from COS7 cells	MBP	(Palmer et al., 1995)
0.04% DOC	730	purified PKN1 from rat testes	PKC δ peptide (AMFPTMNRRGSIKQAKI)	(Kitagawa et al., 1995)
0.004% SDS	690			

Phosphate incorporation in the absence of modifiers was taken as 100% activity. Each value of "activity (%)" represents the mean of the observations.

Table II $A_{0.5}$ and IC_{50} values for the various agents on PKN1 activity

modifier	IC_{50}	enzyme	substrate	reference
sodium glycerophosphate	54 mM	PKN1 purified from rat liver, activated by trypsin	S6-peptide (AKRRRLSSLRA)	(Morrice et al., 1994b)
NaF	30 mM			
staurosporine	0.03 μM			
bisindolylmaleimide I (Gö6850)	0.2 μM	recombinant full length GST-PKN1 from Sf9 cells, activated by 40 μM arachidonic acid,	PKC δ peptide (AMFPTMNRRGSIKQAKI)	
H-7	1 μM			
HA-1077	1.7 μM	GST-catalytic domain of PKN1 from Sf9 cells,	PKC substrate peptide (RFARKGSLRQKNVHEVK)	(Amano et al., 1999)
Y-32885	0.4 μM			
Y-27632	0.5 μM	GST-catalytic domain of PKN1 from Sf9 cells,	PKC substrate peptide (RFARKGSLRQKNVHEVK)	(Amano et al., 2000)
NaCl	160 mM	purified PKN1 from rat testes	PKC δ peptide (AMFPTMNRRGSIKQAKI)	(Kitagawa et al., 1995)
$MnCl_2$	40 μM			
$CaCl_2$	610 μM			
His_6-I alpha fragment (human PKN1 455-511 peptide)	0.05 μM	GST-catalytic domain of PKN1	PKC δ peptide (AMFPTMNRRGSIKQAKI)	(Yoshinaga et al., 1999)

modifier	$A_{0.5}$	enzyme	substrate	reference
arachidonic acid	7 μM	purified PKN1 from rat testes	PKC δ peptide (AMFPTMNRRGSIKQAKI)	(Kitagawa et al., 1995)
lyso-PA	5 μg/ml			
DOC	0.005%			
SDS	0.0004%			
cardiolipin	1.7 μM	purified PKN1 from rat liver	S6-peptide (AKRRRLSSLRA)	(Morrice et al., 1994a)

For agents that inhibit kinase actiivty, the concentration of the agent which causes 50% maximal inhibition of kinase activity (IC_{50}) is listed. For agents that activate kinase activity, the concentration of the agent which causes 50% maximal activation of kinase activity ($A_{0.5}$) is listed. Each value represents the mean of the observations.

Reference List for Tables

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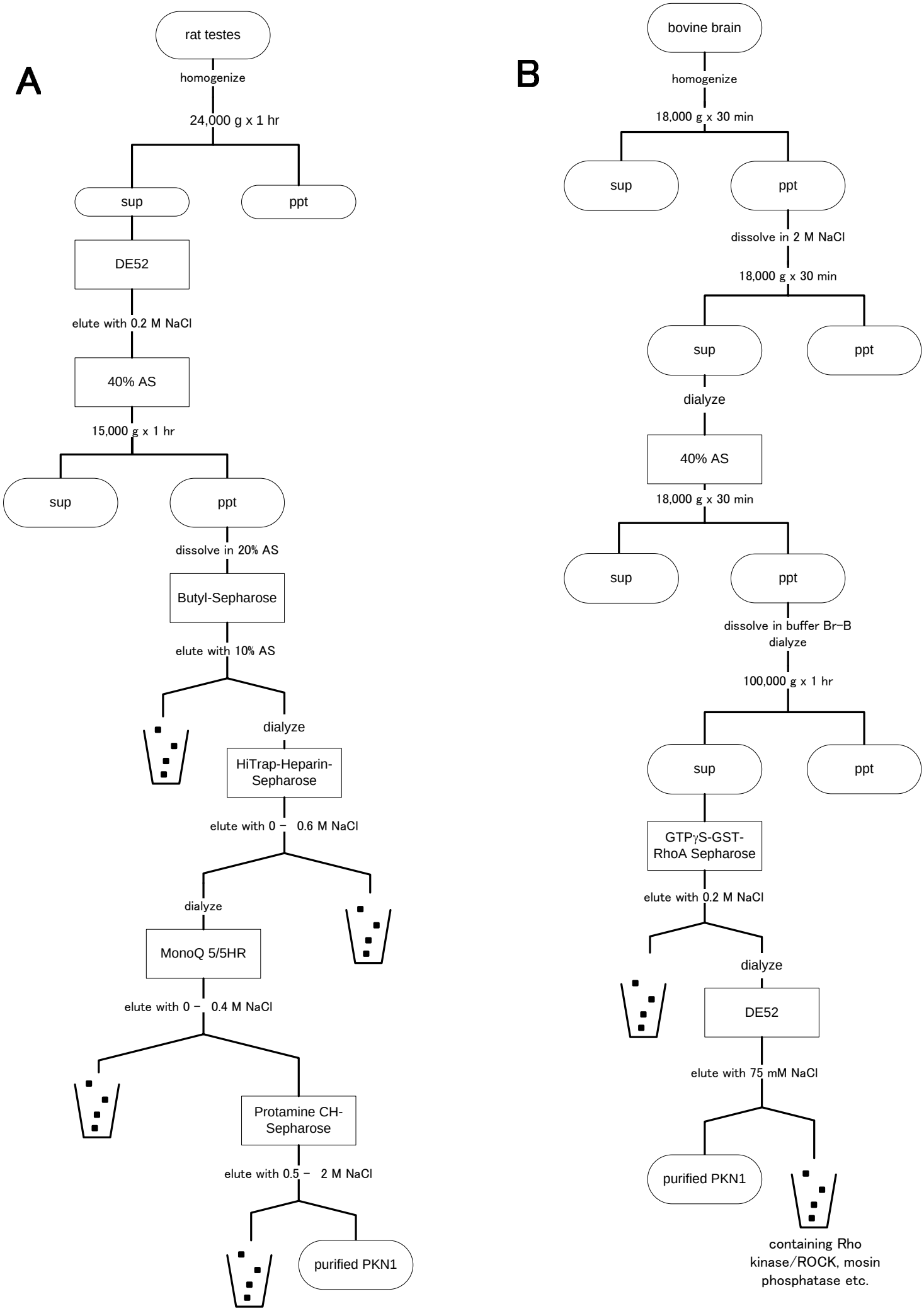
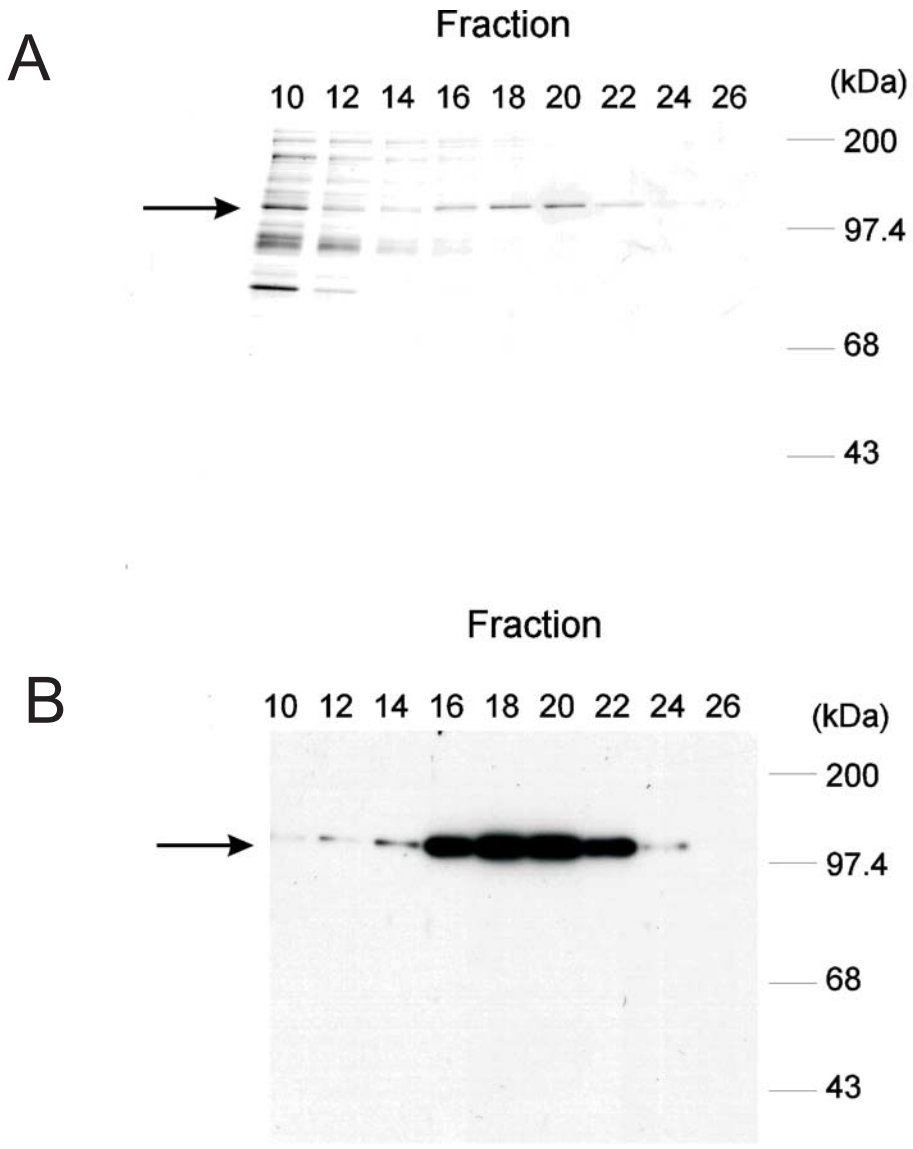


Fig. 1 Mukai and Ono ↑



Mukai and Ono Fig. 2