Expression, purification and characterization of the ubiquitous protein kinase C-related kinase 1

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The recently described protein kinase C-related kinase (PRK) family is comprised of at least three members: PRK1, PRK2 and PRK3. Here the expression, purification and characterization of the ubiquitously expressed isoform, PRK1, is described. The enzyme was expressed in COS 7 cells and subsequently purified to apparent homogeneity by sequential column chromatography. The purified PRK1 protein migrates as a single 120 kDa polypeptide on SDS/PAGE. It displays a substrate specificity that in part resembles that of protein kinase C (PKC); however, unlike PKC, it is not activated by any combination of phorbol esters, diacylglycerol and Ca^{2+}. Nevertheless, it can be activated by limited proteolysis, indicating a negative regulatory role for the N-terminal domain(s). PRK1 is also activated by phospholipids. The physiological relevance of this activation is discussed.

INTRODUCTION

The protein kinase C-related kinase (PRK) gene family is comprised of at least three genes: *PRK1*, *PRK2* and *PRK3* [1]. As yet, few data exist to provide an insight into the regulation of the PRKs and no detailed purification and characterization of a member of this novel family of kinases has been described. The predicted structure suggests that the C-terminal region of the enzyme, which encodes a typical kinase catalytic domain, is subject to control by a large N-terminal regulatory region comprised of two readily defined domains, HR1 and HR2 [1]. This would be analogous to the various isotypes of the protein kinase C (PKC) family for which regulation of the C-terminal catalytic domain is exerted via the N-terminal regulatory portion of the molecule (recently discussed in ref. [2]). Although a similar structural arrangement can be seen for the PRKs, identity between the various PRK isotypes and the PKC family seems to be restricted to their highly similar kinase domains and not the putative regulatory domains [1,3]; the molecules that regulate PRKs have not yet been elucidated.

As a preliminary to understanding the mechanisms of PRK regulation and its capacity for activation, this study describes the purification and characterization of the PRK1 isotype. Purification was achieved by overexpressing PRK1 in COS 7 cells; the kinetic properties of the activity were then documented. PRK1 is shown to be activated by limited proteolysis and also by phospholipids. These properties are discussed with respect to the potential physiological regulation of the enzyme.

EXPERIMENTAL

Materials

Radioactive materials, enhanced chemiluminescence reagents, Hyperfilm, Hybond nylon membranes and first-strand cDNA-synthesis system were obtained from Amersham International. Poly(vinylidene difluoride) (PVDF) membrane was from Millipore. Trypsin was purchased from Worthington, and trypsin inhibitor from Sigma. Restriction enzymes came from NBL Biolabs.

DNA constructs

Full-length human PRK1 [1] was inserted into the expression vector pcDNA3 (Invitrogen). PRK1 cDNA was digested with *Bss*HI (a unique site before the start of translation) and *Xho*I (approx. 130 bp downstream of the translation stop); the 5′ *Bss*HI site was blunt-ended with Klenow polymerase (Boehringer). The purified fragment was inserted at the *Eco*RV (3′ to the cytomegalovirus promoter/enhancer region) and *Xho*I sites of pcDNA3; pcDNA3-PRK1 plasmids were then isolated and purified. All DNA-manipulation procedures were carried out using standard molecular-biology techniques [4].

COS 7 cell transfection

COS 7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (Gibco). They were then transfected by electroporation using a Gene Pulser (Bio-Rad) as follows. Actively growing cells were washed and resuspended in PBS at a density of 6.25×10^6/ml. The cell suspension (800 μl) was mixed with 40 μg of pcDNA3-PRK1 plasmid DNA in a 0.4 mm Gene Pulser cuvette (Bio-Rad) and placed on ice for 10 min. The Gene Pulser apparatus was set to 450 V and 250 μF. The cells were pulsed once and placed again on ice for 10 min. Cells were then seeded on to 150 mm-diameter dishes and incubated at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum.

Antisera and immunoblot analysis of PRK1

A C-terminal peptide corresponding to PRK1 amino acid residues AEQAAFLDFDFVAGGC was synthesized. The peptide was coupled to keyhole limpet haemocyanin with glutaraldehyde and used for the immunization of rabbits.

For immunoblot analysis, proteins were subjected to SDS/PAGE (8% gels) and transferred electrophoretically to PVDF membranes. The membranes were incubated for 1 h at room temperature in PBS containing 5% (w/v) skimmed milk and 0.1% (v/v) Tween 20. They were then incubated with rabbit anti-PRK1 serum for 1 h at room temperature. After being washed in PBS containing 1% (w/v) skimmed milk and 0.1%

Abbreviations used: PRK, protein kinase C-related kinase; PKC, protein kinase C; PVDF, poly(vinylidene difluoride); MBP, myelin basic protein.
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(v/v) Tween 20, the membranes were incubated with anti-rabbit IgG–horseradish peroxidase. Bands were visualized using enhanced chemiluminescence reagents.

Purification of PRK1

Buffers for chromatography contained: (A) 20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 2 mM EDTA, 0.3% (v/v) 2-mercaptoethanol, 0.05% (v/v) Triton X-100, 10 mM benzamidine; (B) 20 mM Tris/HCl, pH 7.5, 2 mM EDTA, 0.3% (v/v) 2-mercaptoethanol, 0.05% (v/v) Triton X-100, 10 mM benzamidine. For PRK1 purification, COS 7 cells were transfected with PRK1 (as detailed above); 10 × 15 cm plates were washed three times in ice-cold PBS and subsequently lysed in 500 μl of lysis buffer [60 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.3% (v/v) 2-mercaptoethanol, 100 μM Na_3VO_4, 50 mM NaF, 1% (v/v) Triton X-100, 50 μg/ml PMSF, 10 mM benzamidine, 125 μg/ml aprotinin, 250 μg/ml leupeptin] per plate. Lysates were clarified by centrifugation at 70000 *g* for 10 min at 4 °C. All subsequent procedures were carried out at 4 °C. The supernatant was loaded on to a 250 ml Sephacyl S-300 column (Pharmacia), equilibrated and developed in buffer A (see above). Eluted fractions (4 ml) were then assayed for PRK1 kinase activity (as detailed below) and peak fractions pooled. The S-300 pool was then diluted in buffer B (see above) to a final concentration of less then 0.1 M NaCl. This was chromatographed on a 5 ml HiTrapSP column (Pharmacia) and then eluted with a linear salt gradient from 0 to 2 M NaCl in buffer B. After being assayed for activity, peak fractions were again diluted to less than 0.1 M NaCl and subsequently loaded on to a 5 ml HiTrap Heparin column (Pharmacia). The column was again eluted with a linear salt gradient from 0 to 2 M NaCl in buffer B and fractions were assayed for PRK1 activity. Peak fractions were diluted to less than 0.1 M NaCl, loaded on to a 1 ml HiTrapQ column (Pharmacia) and eluted in a 0–1 M NaCl gradient in buffer B. Peak fractions of PRK1 activity were stored at −20 °C in 50% (v/v) ethanediol.

Assay of PRK1 activity

PRK1 activity was assayed routinely as follows. A 40 μl assay mixture typically contained 6.25 mM $MgCl_2$, 50 mM Hepes, pH 7.5, 0.25% (v/v) Triton X-100, 10 μg protamine sulphate (unless another substrate is specified) and 125 μM [γ-^{32}P]ATP. PRK1 was diluted into enzyme dilution buffer (20 mM Hepes, pH 7.5, 2 mM EGTA, 0.02% Triton X-100, 0.2 mM dithiothreitol) at 4 °C before addition to the assay. Reactions were carried out for 20 min, which was determined to be within the linear range for PRK1 (not shown), and stopped by spotting on to Whatman P81 paper and placing in 10% acetic acid. The filters were washed (3 × 10 min) in 30% acetic acid and Çerenkov radiation was determined. Where indicated, a sonicated dispersion of phosphatidylserine (1.25 mg/ml) or mixed brain phospholipids (1.25 mg/ml) was added.

Treatment of PRK1 with trypsin

Proteolytic activation of PRK1 was achieved by the following method. Purified PRK1 was cleaved in 10 mM Tris/HCl (pH 8.0)/10 mM 2-mercaptoethanol (in a final volume of 40 μl) by the addition of trypsin to the final concentrations specified in the Results section. Trypsin treatment was allowed to proceed for 4 min at 30 °C. The reactions were then stopped by the addition of 20 μl of 400 μg/ml trypsin inhibitor (Sigma).

Other procedures

SDS/PAGE was carried out by the method of Laemmli [5]. Protein was determined by the method of Bradford [6] with BSA as standard.

RESULTS

Purification of PRK1

Transfection of COS 7 cells with recombinant cDNA encoding human PRK1 (see the Experimental section) leads to a time-dependent increase in PRK1 protein levels that starts on day 1 and reaches a plateau between 2 and 3 days after transfection (results not shown). In order to purify PRK1, ten 15 cm-diameter plates of COS 7 cells were transfected with pcDNA3-PRK1 and harvested 2 days after transfection. The resulting cell extract was

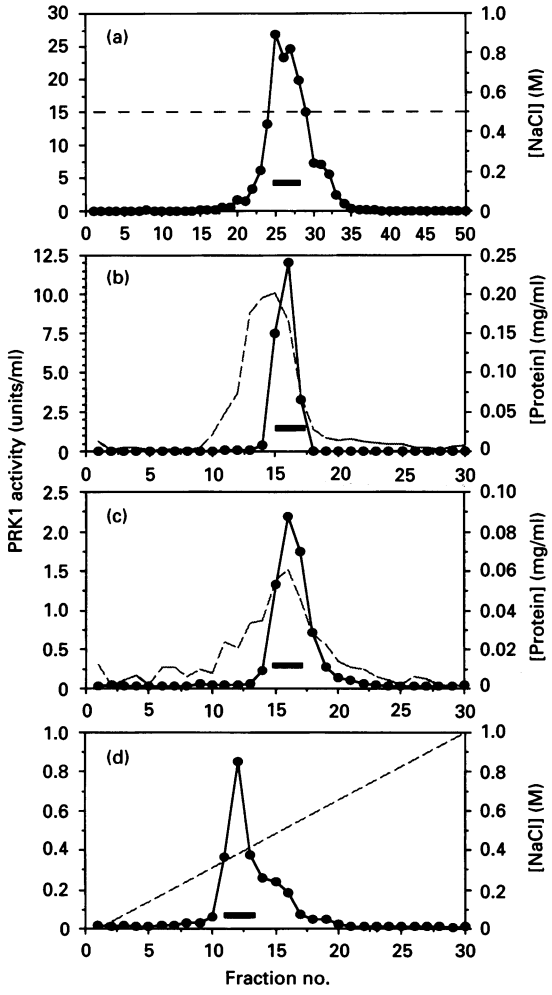

Figure 1 Sequential chromatography steps in the purification of PRK1

The activity of PRK1 (●) is shown for the four chromatography steps employed for purification. (**a**) Sephacryl S-300 column; (**b**) HiTrapSP column; (**c**) HiTrap Heparin column; (**d**) HiTrapQ column. PRK1 activity was assayed with protamine sulphate. One unit of PRK1 activity is defined as 1 nmol of ^{32}P incorporated into protamine sulphate/min. The bar indicates fractions taken for further purification. ----, NaCl gradient (**a** and **d**) or total protein elution profile (**b** and **c**).

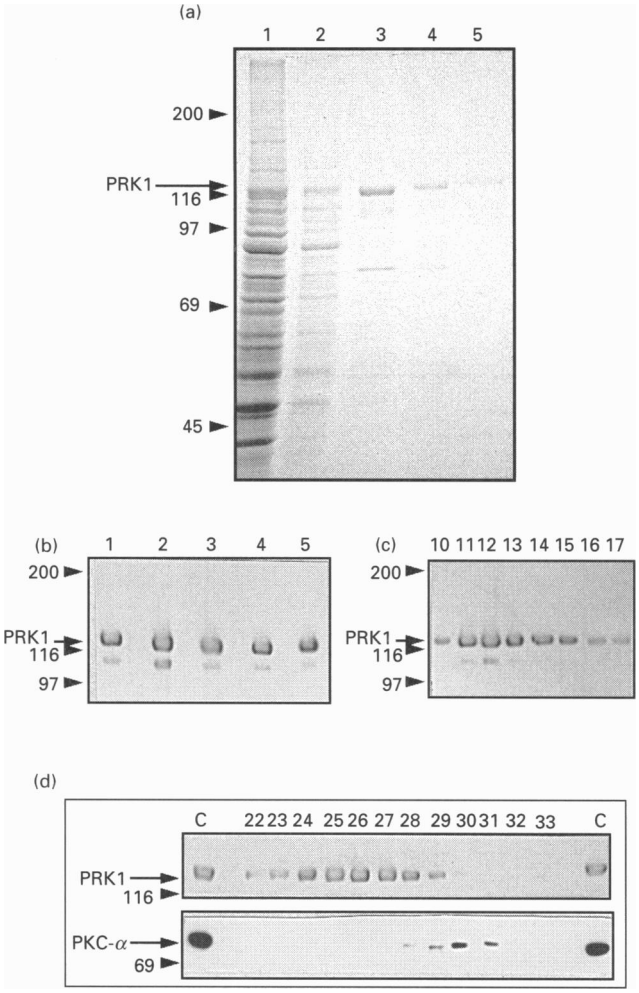

Figure 2 SDS/PAGE and Western-blot analysis of PRK1 purification

(**a**) Peak fractions at each step of the purification process were run on SDS/8% polyacrylamide gel and stained with Coomassie Blue. Lane 1, cell extract; lane 2, Sephacryl S-300 pool; lane 3, HiTrapSP pool; lane 4, HiTrap Heparin pool; lane 5, HiTrapQ pool. (**b**) The same peak fractions as in (**a**) were analysed for the presence of PRK1 protein by Western blot. PRK1 is indicated. (**c**) A 5 μl aliquot of the final HiTrapQ column fractions 10–17 was analysed by Western blot to demonstrate co-elution of PRK1 protein with activity. (**d**) The initial Sephacryl S-300 column profile fractions 22–33 were analysed for PRK1 (upper gel) and subsequently for the presence of PKC-α (lower gel). The Sephacryl S-300 column load (equivalent to the cleared cell extract) was included as control (C). Molecular-mass markers are indicated (kDa).

chromatographed on a Sephacryl S-300 column in buffer A (Figure 1a); if low-ionic-strength buffer was employed for gel filtration, PRK1 activity was not recovered (not shown). The activity peak, which was eluted at greater than 100 kDa, was pooled, diluted and loaded on to a 5 ml HiTrapSP column (Figure 1b) equilibrated in buffer B. Protein was then eluted in a linear NaCl gradient from 0 to 2 M and fractions were assayed for PRK1 activity. PRK1 was eluted at 0.8 M NaCl; the fractions containing the peak of activity were pooled and diluted to a final concentration of less than 0.1 M NaCl before being loaded on to a 5 ml HiTrap Heparin column (Figure 1c). This column was then developed in buffer B and protein was eluted on a 0–2 M linear NaCl gradient; peak fractions were then reloaded on to a 1 ml HiTrapQ column (Figure 1d). PRK1 was eluted at 0.8 M NaCl on a 0–2 M NaCl gradient and was apparently homogeneous as analysed by SDS/PAGE (Figure 2a). It was stored at -20 °C in 50% ethanediol and activity was stable for more than a month. The purification of PRK1 is summarized in Table 1. The low purification factor and yield achieved probably reflect the high level of functional PRK1 expression in this system; therefore, in the interests of producing a pure preparation of PRK1, only peak fractions were further processed. Western-blot analysis of the peak pools of activity confirmed the coincidence of PRK1 polypeptide with the peak of activity with protamine sulphate as substrate throughout the purification process (Figure 2b).

PKC-α, the major PKC isoform in COS 7 cells [7], is also capable of phosphorylating protamine sulphate in the assay used here. In order to confirm that it was not contaminating the PRK1 preparation, the peak pools were analysed for the presence of PKC-α (results not shown). It was present only in the original cell extract and not in any of the subsequent pools. Western-blot analysis using an antibody directed against the C-terminus of PRK1 throughout the S-300 column profile confirmed the presence of PRK1 in fractions 22–30, correlating with the peak of activity observed (Figure 2d, upper gel). Analysing the profile for the presence of PKC-α revealed it to be in fractions 28–31, coincident with the shoulder of activity observed in the S-300 activity profile (Figure 1a); the latter fractions were not included in the PRK1 pool (see the bar in Figure 1a). Three criteria were applied to confirm the functional purity of the PRK1 preparation: (1) autophosphorylation reactions produced only a single PRK1-labelled species; (2) similarly, reaction with fluorylsulphonylbenzoyladenosine (an ATP-site-reactive compound) produced only a single immunoreactive protein [8]; (3) depletion of the PRK1 preparation with an antiserum that recognizes the native protein [1] removed kinase activity (results not shown).

Properties of PRK1

Kinetic properties of PRK1 were determined using protamine sulphate as substrate (see the Experimental section). The apparent K_m values for ATP and Mg^{2+} were 11.8 ± 2.9 μM (Figure 3a) and 1.7 ± 0.2 mM (Figure 3b) respectively. There was no activation of PRK1 by Ca^{2+}; the only effect of Ca^{2+} is at high concentrations (> 1 mM) where it is inhibitory (results not shown).

Activation of PRK1

The regulatory potential of PRK1 was investigated by subjecting the enzyme to limited proteolysis. Trypsin treatment of the full-length purified PRK1 resulted in a dose-dependent increase in kinase activity towards the substrate myelin basic protein (MBP) (Figure 4). At the optimum concentration tested, PRK1 was activated 4-fold and the effect on MBP phosphorylation was essentially an effect on $V_{max.}$ with only a moderate effect on apparent K_m. The $V_{max.}$ values determined by the Enzyme Kinetics program (D. G. Gilbert, IN, U.S.A.) are 7.4 ± 0.2 and 31.6 ± 1.3 units/mg for intact and proteolysed PRK1 respectively; the apparent K_m values are 87 ± 7 and 60 ± 10 μg/ml for these preparations. Associated with the trypsin treatment was a dose-dependent cleavage of PRK1 into catalytic-domain fragments (detected with antiserum to the C-terminus; Figure 4b) of 67, 55 and 50 kDa. On the basis of the dose–response for activation of PRK1, it appears that all of these fragments express increased activity. The apparent increase in antigen (compare the first and last lanes in Figure 4b) is an artifact caused by the more efficient transfer of the lower-molecular-mass species (determined by protein-staining patterns). Interestingly, when protamine sulphate was employed as substrate, trypsin treatment did not lead to activation but a steady decline in activity (Figure 4a). In searching for potential activators of PRK1, it was found that

Table 1 Purification of PRK1 from COS 7 cells

COS 7 cells were transfected with pcDNA3-PRK1 as described in the Experimental section. Cells were harvested 2 days after transfection and the cell extract was chromatographed through the indicated steps. At each stage protein content and protamine sulphate kinase activity were measured. One unit of PRK1 activity is defined as 1 nmol of ^{32}P incorporated into protamine sulphate/min under the conditions described in the Experimental section. Data are shown for one of four similar purifications.

	Total protein (mg)	Volume (ml)	Total activity (units)	Recovery (%)	Specific activity (units/mg)	Purification factor
Cell extract	13.7	10	58.3	100	4.25	1
S-300 pool	1.55	6	22.4	38.4	14.4	3.4
HiTrapSP pool	0.30	2.7	12.2	20.9	40.9	9.6
HiTrap Heparin pool	0.27	2.7	4.47	7.7	36.4	8.6
HiTrapQ pool	0.063	1.5	1.60	2.7	25.29	6.0

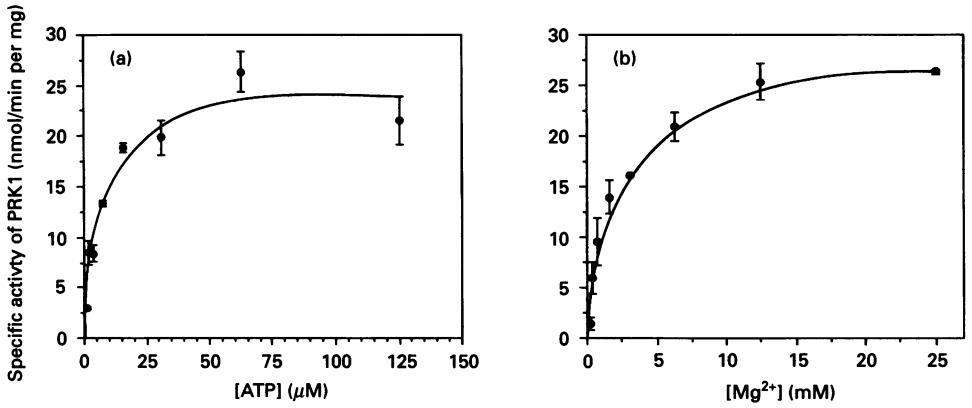

Figure 3 Effect of ATP and Mg^{2+} on PRK1 activity

PRK1 activity is plotted against ATP (**a**) and Mg^{2+} (**b**) concentration; 1.25 mg/ml protamine sulphate was used as substrate. Apparent K_m values for ATP and Mg^{2+} are 11.8 ± 2.9 μM and 1.7 ± 0.2 mM respectively. Values were determined using the program Enzfitter. All data points were determined in duplicate and determinations were carried out two or three times. Results are means and range for one set of determinations

sonicated dispersions of phosphatidylserine or mixed brain phospholipids cause activation (Figure 5). There was no difference in potency between these two lipid preparations suggesting that their interaction is non-specific. However, this behaviour of PRK1 is entirely consistent with the idea that it is an effector-dependent kinase.

Substrate specificity of PRK1

The activity of the purified PRK1 was examined using a range of potential substrates and the kinetic properties were determined (Table 2). Protamine sulphate and MBP were the only substrates tested that were phosphorylated significantly by PRK1. The K_m and $V_{max.}$ for MBP phosphorylation were determined after treatment of PRK1 with trypsin (see above); even under these conditions, the $V_{max.}$ value observed is no greater than that seen for protamine sulphate with intact PRK1.

DISCUSSION

The ubiquitously expressed member of the PRK family, PRK1, has been expressed in COS 7 cells, purified to apparent homogeneity and its properties characterized. In line with encoding a kinase domain closely related to PKC isotopes, PRK1 is shown to phosphorylate two typical PKC substrates, MBP and protamine sulphate, although a third, histone III, was not an efficient substrate for PRK1 (see Table 2). The purified PRK1 was found to be activated by limited proteolysis which is indicative of it being an effector-dependent kinase [9–11]. The finding that activation was observed with MBP as substrate but not protamine sulphate suggests that the latter can 'overcome' regulatory-domain constraints in acting as a substrate. This parallels results for PKC [12]; however, unlike PKC, PRK1 was found not to be activated by phorbol esters in a standard lipid/detergent mixed-micelle assay [13] based on the method of Hannun et al. [14]. Interestingly, phospholipid dispersions were found to activate PRK1.

The PRK family of cDNAs was originally identified in a PCR-based PKC gene screen [1]; PRK1 (PKN) was also identified by cross-hybridization to a PKC cDNA [3]. These previous descriptions of the PRKs have yielded little information on their kinase activity and regulation. It has been shown that immunoprecipitated PRK1 [3] and PRK2 [1] have an autophosphorylation capacity, and, further, that an ATP-binding-site point mutant of PRK1 displays no such activity [3]. Mukai and Ono [3] also expressed PRK1 in COS 7 cells but found the protein to be largely insoluble (as they also observed for PRK1 expressed in insect cells). The expression of PRK1 in COS 7 cells was here found to be efficient and yielded a soluble activity. The reason for the previous insolubility is not clear, although it is unlikely to be a function of high concentration, as, in the studies described

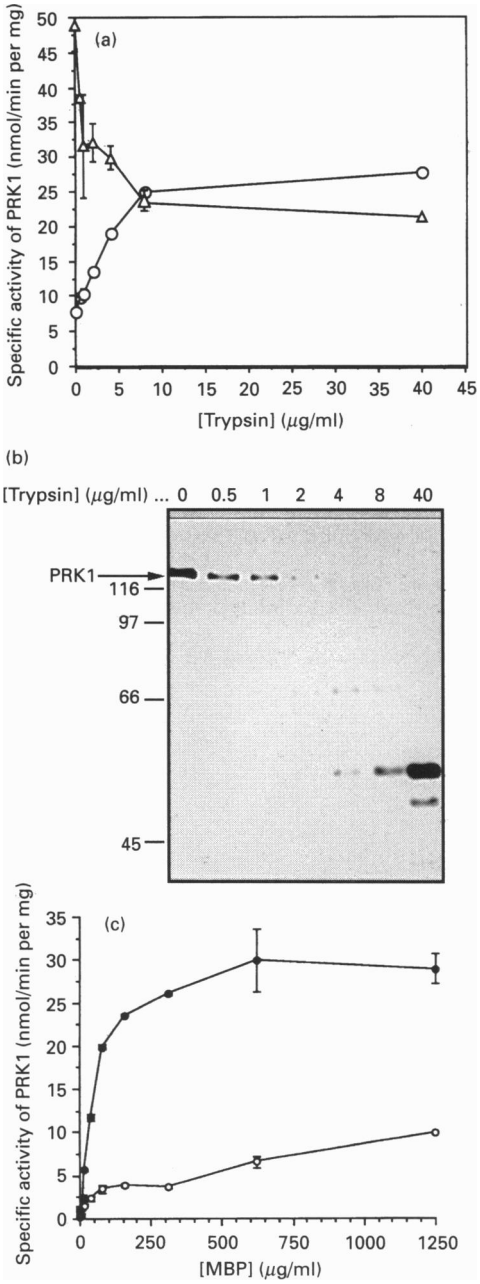

Figure 4 Effect of trypsin treatment on PRK1 activity

(**a**) PRK1 activity towards MBP (○) and protamine sulphate (△) is shown after treatment with an increasing concentration of trypsin as described in the Experimental section. (**b**) Western-blot analysis using a C-terminal epitope was carried out at increasing trypsin concentrations. (**c**) The kinetics of phosphorylation of MBP by intact (○) and proteolysed (●) PRK1 are compared.

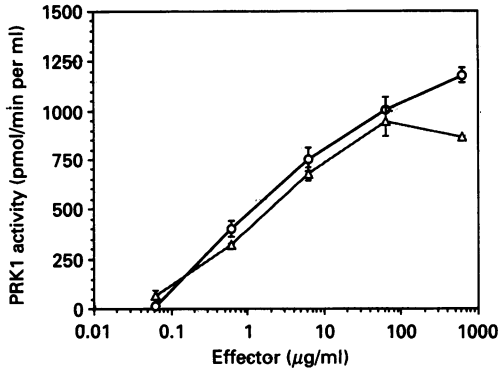

Figure 5 Lipid activation of PRK1 activity

PRK1 activity, measured with MBP as substrate, is plotted against increasing concentrations of phosphatidylserine (○) and mixed brain phospholipids (△). Lipid activation assays were carried out as described in the Experimental section with a final assay concentration of $MgCl_2$ of 1.6 mM.

Table 2 *In vitro* substrates of PRK1

The K_m and $V_{max.}$ values are indicted (means ± S.E.M. for four determinations). All values were calculated with the Enzyme Kinetics program. $V_{max.}$ for myelin basic protein was measured after treatment of PRK1 with trypsin (see the text and Figure 4). $V_{max.}$ for histone III, histone IIAS and casein were measured at substrate concentrations of 1.25 mg/ml.

Substrate	K_m (μg/ml)	$V_{max.}$ (nmol/min per mg)
Protamine sulphate	6.7 ± 1.7	30 ± 4.8
Myelin basic protein	60 ± 9.7	32 ± 1.3
Histone III		< 1.0
Histone IIAS		< 1.0
Casein		< 1.0

here, the expressed PRK1 protein represented approx. 10% of the extracted protein.

The high level of PRK1 expression in COS 7 cells readily permits purification of the protein. Although only a 10-fold enrichment factor is required for purification, it was essential to ensure removal of PKC (which displays some remarkably similar properties) and to achieve apparent homogeneity. Separation from PKC was obtained by an initial gel-filtration step; if omitted, PKC was found to be co-eluted with PRK1 on subsequent chromatographic steps. In order to achieve apparent homogeneity, a substantial cut in yield and loss of activity was encountered, particularly after chromatography on the HiTrap Heparin column. The reasons for this loss are not clear but do not seem to reflect any intrinsic instability of the purified protein which remained stable at −20 °C for over a month. That the purified PRK1 accounted for the observed kinase activity was suggested by the observations that (i) only this protein became radiolabelled in an autophosphorylation reaction, (ii) this was the only protein reactive with fluorylsulphonylbenzoyladenosine [8] and (iii) activity was depleted by an antibody that precipitates PRK1 (results not shown).

The purified protein displayed kinetic parameters for Mg^{2+} and ATP that are typical of this class of protein (e.g. the cPKC isotypes [13]). In contrast, the specific activity of the purified protein (20–30 units/mg) is some 10–50-fold lower than that of many other purified kinases. Although this low specific activity may be partly due to loss of activity during purification (see above), it is clear from the proteolysis studies that PRK1 is a latent kinase that can be activated on removal of the N-terminal regulatory domain. The true extent of proteolytic activation is underestimated as the catalytic domain is also gradually destroyed, as evidenced by the decline in protamine sulphate kinase activity. In the light of this decline, the optimum activation of PRK1 with MBP as substrate would be approx. 10-fold.

The tryptic activation observed for PRK1 was found to be due to an increase in $V_{max.}$ with little change in apparent K_m for the substrate MBP. This might imply the removal of some conformational constraints on the kinase domain as opposed to the removal of a competitive inhibitor of the substrate-binding site (i.e. pseudo-substrate site); visual inspection of the PRK1 sequence has not revealed any obvious pseudosubstrate site (discussed in ref. [1]). The fact that PRK1 shows latent activity implies that its physiological regulation may be driven by effector binding. The activation of PRK1 by phospatidylserine and mixed brain phospholipids is consistent with this notion. However, the similar potency of these two lipid preparations and indeed their nature suggest that neither of these are responsible for acute physiological regulation. This lipid-dependant behaviour of PRK1 is entirely consistent with a recent report on protease activated kinase 1, which is similar to if not identical with PKN/PRK1 [15].

To date comparison of purified PRK1 with purified PRK2 has not been possible because the latter cannot be expressed so efficiently in COS cells. However, the domain structure suggests that the properties of PRK2 will be at least similar to those described here for PRK1. The identification of the physiological effector(s) of the PRKs will be of paramount importance in placing these kinases in a signal-transduction pathway.

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