Purification of catalytic domain of rat spleen p72^{syk} kinase and its phosphorylation and activation by protein kinase C

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The catalytic domain of p72^{syk} kinase (CDp72^{syk}) was purified from a 30 000 g particulate fraction of rat spleen. The purification procedure employed sequential chromatography on columns of DEAE-Sephacel and Superdex-200, and elution from HA-Ultrogel by chloride. The analysis of the final CDp72^{syk} preparation by SDS/PAGE revealed a major silver-stained 40 kDa protein. The kinase was identified by covalent modification of its ATP-binding site with [¹⁴C]5'-fluorosulphonylbenzoyl-adenosine and by immunoblotting with a polyclonal antibody against the 'linker' region of p72^{syk}. By using poly(Glu₄, Tyr₁) as a substrate, the specific activity of the enzyme was determined as 18.5 nmol P_i/min per mg. Casein, histones H1 and H2B and myelin basic protein were efficiently phosphorylated by CDp72^{syk}. The kinase

exhibited a limited ability to phosphorylate random polymers containing tyrosine residues. CDp72^{syk} autophosphorylation activity was associated with an activation of the kinase towards exogenous substrates. The extent of activation was dependent on the substrates added. CDp72^{syk} was phosphorylated by protein kinase C (PKC) on serine and threonine residues. With a newly developed assay method, we demonstrated that the PKC-mediated phosphorylation had a strong activating effect on the tyrosine kinase activity of CDp72^{syk}. Studies extended to conventional PKC isoforms revealed an isoform-dependent manner ($\alpha > \beta_1 = \beta_{II} > \gamma$) of CDp72^{syk} phosphorylation. The different phosphorylation efficiencies of the PKC isoforms closely correlated with the ability to enhance the tyrosine kinase activity.

INTRODUCTION

A number of non-receptor tyrosine kinases with molecular masses between 30 and 50 kDa have been isolated from spleen and characterized [1–3]. Several lines of evidence suggest that some of the enzymes previously reported represent catalytic fragments of kinases generated by cellular processing or by proteolysis during the purification procedure. The conversion of the epidermal growth factor receptor (EGF-R) [4], murine PTK72 [5] and the *syk* or *abl* gene products [6,7] to such fragments *in vitro* have been shown. Thus there remain some possible sources of kinase fragments of similar size.

Here we report the purification of a tyrosine-specific protein kinase from the particulate fraction of rat spleen and its identification as the catalytic domain of p72^{syk} (CDp72^{syk}), by using an antibody recognizing the kinase's 'linker' region [8]. The subsequent determination of the biochemical properties of the kinase allows a comparative study with other similarly sized tyrosine kinases previously reported [1,5,9]. Using a newly developed assay, we demonstrate that the catalytic domain of p72^{syk} serves as a substrate for protein kinase C (PKC) *in vitro*, with PKC phosphorylation having an activating effect on CDp72^{syk}. These results suggest a new connection between two important intracellular signal transduction pathways.

MATERIALS AND METHODS

Materials

Affinity-purified PKC from rat brain was a gift from Dr. Hilz (University of Hamburg, Hamburg, Germany). PKC isoforms (α , β_1 , β_{II} and γ) were kindly provided by Dr. Stabel (Max-Delbrück-Laboratory, Köln, Germany). The polyclonal rabbit antiserum against the p72^{syk} 'linker' region [SYK(LR)] was purchased from Santa Cruz Biotechnology. DEAE-Sephacel and

Superdex-200 (High-Load) were purchased from Pharmacia. Catalytic subunit of the protein phosphatase 1 (γ isoform), protease inhibitors, histones and polyclonal rabbit anti-mouse antibody were obtained from Boehringer (Mannheim, Germany). Monoclonal anti-phosphotyrosine antibody, random polymers, hydroxyapatite (HA-Ultrogel) and all other chemicals were obtained from Sigma. [γ -³²P]ATP (3000 Ci/mmol) and [¹²⁵I]-protein A (91.1 μ Ci/ μ g) were obtained from Amersham; [methyl-¹⁴C]methylated protein marker kit, [³⁵S]adenosine 5'-[γ -thio]-triphosphate ([³⁵S]ATP[S]) (65 μ Ci/mmol), [³⁵S]adenosine 5'-[α,β -thio]triphosphate ([³⁵S]pp[S]pA) (12 μ Ci/mmol) and [¹⁴C]5'-fluorosulphonylbenzoyl-adenosine ([¹⁴C]FSBA) (47.6 mCi/mmol) were from Du Pont.

Purification of CDp72^{syk}

The solubilized particulate fraction from rat spleen was obtained and chromatographed on a DEAE-Sephacel column as described previously [3]. Fractions (numbers 4–6; each 7.5 ml) containing proteins not bound to the DEAE-Sephacel matrix were pooled, concentrated and chromatographed on the Superdex-200 column. The column was equilibrated and eluted with TGT buffer [20 mM Tris/HCl (pH 7.5)/10% (v/v) glycerol/0.05% (v/v) Triton X-100/1 mM EDTA/1 mM 2-mercaptoethanol] containing 200 mM KCl. The fraction (no. 17; 3.2 ml) showing the peak of kinase activity corresponding to proteins with a molecular mass of 40 kDa was concentrated; the KCl concentration was adjusted to 10 mM by dilution with TGT buffer. This material was applied to a 5 ml HA-Ultrogel column. The column was washed with 10 ml of TGT buffer, developed with a discontinuous MgCl₂ gradient (0–500 mM) and fractions (each 2 ml) were collected. The fraction with tyrosine kinase activity, which was eluted from the column at a salt concentration of

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; CDp72^{syk}, catalytic domain of p72^{syk}; FSBA, 5'-fluorosulphonylbenzoyl-adenosine; PKC, protein kinase C; EGF-R, epidermal growth factor receptor; PEG, poly(ethylene glycol).

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50–70 mM, was concentrated and the salt concentration adjusted to 10 mM by dilution with TGT buffer containing 25 % (v/v) glycerol. The column was reactivated with a continuous K_3PO_4 gradient (0–0.75 M).

Protein kinase assays

Tyrosine protein kinase activity of Cdp72^{syk} was determined as described previously [3], with the following modifications. The reaction mixture (20 μ l), containing 100 ng of kinase, was incubated at 37 °C for 5 min in the presence of 25 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$ /2 mM 2-mercaptoethanol/25 % (v/v) glycerol/0.1 mg/ml BSA/10 μ M [γ -³²P]ATP (0.5 μ Ci) containing 0.25 mg/ml of poly(Glu₄, Tyr₁) or protein substrates, as indicated in the figure legends. The reaction was terminated by the addition of 20 μ l of SDS sample buffer and boiling for 3 min. The samples were applied to SDS/PAGE [10 % (w/v) disc gel] as described by Laemmli [10]. After being stained with Coomassie Blue, the gels were dried and exposed to Kodak X-ray film at –70 °C. Subsequently, regions of the gel corresponding to phosphorylated proteins or polymers were excised and ³²P incorporation was measured by counting of Čerenkov radiation. Alternatively, the assays were performed with unlabelled ATP; after separation by SDS/PAGE, phosphoproteins were subjected to immunoblotting with anti-phosphotyrosine antibody.

The activity of PKC was assayed in a mixed-micelle assay as described previously [11]. One unit of activity was defined as the amount of enzyme that catalysed the transfer of 1 nmol of P_i per min from ATP to histone III-S.

Immunoblotting

After SDS/PAGE the proteins were electroblotted on nitrocellulose filter (BA 85, 0.45 μ m; Schleicher & Schüll). The filters were incubated for 1 h with 10 mg/ml BSA in 25 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.05 % (v/v) Tween 80 (TTBS buffer), and for 2 h with antisera [1:1000 in TTBS containing 10 % (v/v) glycerol and 3 mg/ml BSA]. The filters were washed again with 10 mg/ml BSA in TTBS, and antibody was detected with ¹²⁵I-protein A (0.5 μ Ci/ml) or with an anti-mouse antibody followed by ¹²⁵I-protein A. The nitrocellulose filters were dried and autoradiographed.

Covalent labelling with [¹⁴C]FSBA

The labelling of ATP-binding sites of the kinases with [¹⁴C]FSBA was performed by the method of Woodford and Pardee [12], with modifications. [¹⁴C]FSBA (20 μ l, 0.2 mg/ml; 0.4 μ Ci) was freeze-dried and dissolved in 2 μ l of DMSO. Aliquots of the fractions investigated were added and incubated for 2 h at 30 °C. Thereafter, SDS sample buffer was added, the probes were boiled and subjected to SDS/PAGE. In control experiments the [¹⁴C]FSBA binding was blocked with the non-hydrolysable ATP analogue ATP[S]. The samples were incubated with [¹⁴C]FSBA in the presence of ATP[S] added in a 5-fold excess.

Nucleotide binding assays

Kinase (500 ng) was incubated at 30 °C for 30 min with 0.5 nmol of [γ -³²P]ATP (0.5 μ Ci), [³⁵S]ATP[S] or [³⁵S]pp[S]pA in 50 μ l of reaction mixture containing 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 25 % (v/v) glycerol and 0.1 mg/ml BSA. Samples were subjected to non-denaturing PAGE by the method described previously by Davis [13], with the following modifications. The running gel (10 %, w/v) and stacking gel (5 %, w/v) additionally contained 10 % (v/v) glycerol

and 0.4 % (v/v) Triton X-100, and were run for 4 h at 4 °C and 20 mA. After electrophoresis, the gels were washed with TGT buffer until the radioactivity in the wash solution reached background levels. The gels were dried and autoradiographed.

Assay for determination of correlation between autophosphorylation of Cdp72^{syk} and substrate phosphorylation

The assay was performed in two steps: (i) autophosphorylation of Cdp72^{syk}, and (ii) determination of the kinase activity of the autophosphorylated Cdp72^{syk} towards exogenous substrate.

Autophosphorylation of the kinase was performed at 30 °C in 30 μ l of reaction buffer containing 25 mM Tris/HCl, pH 7.5, 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 25 % (v/v) glycerol, 3 mg/ml BSA, 0.5 % poly(ethylene glycol) (PEG), 0.05 % (v/v) Triton X-100 and 10 μ M [γ -³²P]ATP (0.5 μ Ci). The reaction proceeded for various periods. An aliquot of 10 μ l was removed and subjected to SDS/PAGE, and the level of autophosphorylation was determined. In step (ii) the kinase activity of the autophosphorylated Cdp72^{syk} was measured by phosphorylation of casein, histone H2B or poly(Glu₄, Tyr₁). The reaction was started by the addition of 5 μ l of reaction buffer containing the substrate to the remaining 20 μ l of reaction mixture. The final substrate concentration was 0.25 mg/ml. The substrate phosphorylation proceeded for 10 min at 30 °C and was terminated by the addition of 20 μ l of SDS sample buffer and boiling. The proteins were separated by SDS/PAGE and ³²P incorporation was determined by Čerenkov counting of excised phosphoproteins.

In control experiments the Cdp72^{syk} was incubated under the same conditions except that ATP [during step (i)] was omitted from the reaction mixture or was replaced by the non-hydrolysable ATP analogue ATP[S] (500 nM). In step (ii) of these control reactions the substrates were added together with [γ -³²P]ATP (0.5 μ Ci) to adjust its final concentration to 10 μ M.

Assay to determine the ability of PKC to phosphorylate and regulate Cdp72^{syk}

The assay was performed in two steps: (i) phosphorylation of Cdp72^{syk} by PKC, and (ii) measurement of tyrosine kinase activity of phosphorylated Cdp72^{syk} towards exogenous substrate.

For the first step, 250 ng of kinase was incubated under PKC phosphorylation conditions in 40 μ l of PKC reaction buffer. The buffer contained 12.5 μ g/ml PMA, 50 μ g/ml phosphatidylserine, 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 25 % (v/v) glycerol, 3 mg/ml BSA, 0.5 % PEG, 0.25 % (v/v) Triton X-100 and 10 μ M [γ -³²P]ATP (0.5 μ Ci). The reaction proceeded for 10 min at 30 °C.

During this incubation, 3.5 μ l of affinity-purified PKC preparation, corresponding to 0.5 m-unit, was added after 0, 2.5, 5 or 10 min. In some assays the catalytic subunit of protein phosphatase 1 (2 μ l, corresponding to 2 m-units) was added simultaneously with the PKC. Thereafter, 20 μ l of the reaction mixture was boiled with 20 μ l of SDS sample buffer and analysed by SDS/PAGE and autoradiography. To establish whether the increased phosphorylation of Cdp72^{syk} in the presence of PKC resulted from an amplified autophosphorylation or from phosphorylation by PKC, phosphoamino acid analysis of the 40 kDa phosphoprotein band corresponding to Cdp72^{syk} was performed.

In the step (ii) the remaining 20 μ l of the reaction mixture was assayed for tyrosine kinase activity. The assay was initiated by the addition of 5 μ l of PKC reaction buffer containing casein, histone H2B or poly(Glu₄, Tyr₁) to yield a final concentration of 0.25 mg/ml. The phosphorylation reaction was continued for a

further 10 min at 30 °C and terminated by the addition of 20 μ l of SDS sample buffer and boiling. The phosphoproteins were separated by SDS/PAGE and subjected to immunoblotting with anti-phosphotyrosine antibody. The ¹²⁵I-labelled proteins were excised and radioactivity was counted in a liquid-scintillation counter. These assays [steps (i) and (ii)] were performed with unlabelled ATP.

Other assays

Phosphoamino acid analysis was performed by the procedure described by Cooper et al. [14]. The amino acids were detected by ninhydrin staining, followed by autoradiography. Determination of the phosphatase activity and dephosphorylation assays were performed as recommended by the manufacturer (Boehringer-Mannheim). Protein concentration was measured by the method of Lowry et al. [15]. Silver staining of proteins in polyacrylamide gels was performed as described previously by Heukeshoven and Dernick [16].

RESULTS

Purification protocol

The 30000 *g* particulate fraction of rat spleen was solubilized with 1% (v/v) Triton and subjected to chromatography on DEAE-Sephacel. The antibody against the 'linker' region of p72^{syk} [SYK(LR)] recognized numerous polypeptides with molecular masses ranging from 35 to 80 kDa in the non-retained fractions (results not shown). With poly(Glu₄, Tyr₁) as a substrate, however, only a small amount of activity was detected in these fractions (Figure 1A). These fractions, subjected to size-exclusion chromatography on Superdex-200, showed two peaks of tyrosine kinase activity: a minor one containing proteins with

Figure 2 Detection of p72^{syk} derived and ATP-binding proteins in Superdex-200 fractions

(A) Immunoblot detection of the p72^{syk}-derived proteins was performed on aliquots of fractions 14 (lane 1) and 17 (lane 2) from Superdex-200. Aliquots of the fractions containing 150 μ g of protein were separated by SDS/PAGE, transferred to nitrocellulose and subjected to immunoblotting with SYK(LR) antibody. The blots were exposed for 14 h. (B) Aliquots of fractions 14 (lane 1) and 17 (lane 2) containing 300 μ g of protein were incubated with [¹⁴C]FSBA and separated by SDS/PAGE. The dried gel was exposed for 2 days. The migrations of molecular mass protein standards is indicated at the left (in kDa). Arrows indicate the position of the 40 kDa protein.

a molecular mass of 85–70 kDa and a major one of 60–35 kDa. The second peak contained two maxima of kinase activity corresponding to 55 kDa (fraction 14) and 40 kDa proteins (fraction 17) (Figure 1B). The antibody SYK(LR) bound to a protein with a molecular mass of 40 kDa in both fractions and additionally to a number of immunoreactive fragments (Figure 2A). To identify the catalytic kinase domain, we used covalent labelling of the ATP-binding sites with [¹⁴C]FSBA, with which fractions 14 and 17 were incubated, and the proteins separated by SDS/PAGE. The ¹⁴C-labelled proteins were detected by

Figure 1 Elution profiles of tyrosine kinase activity from consecutive chromatographic columns

Aliquots (10 μ l) of the tested fractions were assayed for tyrosine kinase activity with poly(Glu₄, Tyr₁) as described in the Materials and methods section. Elution profiles of the chromatographic steps: DEAE-Sephacel column chromatography of the 1% (v/v) Triton extract from particulate fraction of rat spleen (A); Superdex-200 column chromatography of the pooled DEAE peak (fractions 4–6) (B); hydroxyapatite (HA-Ultrogel) chromatography of the fraction 17 from the Superdex-200. The arrows indicate the start of the salt gradients (C). In (B) the molecular mass was estimated with ¹⁴C-labelled marker proteins: PB, phosphorylase *b* (97 kDa); BSA (966 kDa); OV, ovalbumin (45 kDa); CA, carbonic anhydrase (30 kDa).

Figure 3 Analysis of CDp72^{syk} from the last purification step on SDS/PAGE

(A) Aliquots (50 μ l) of fractions 9–12 from HA-Ultrogel (corresponding to lanes 1–4) were separated by SDS/PAGE; the protein pattern was revealed by silver staining. (B) Aliquots (50 μ l) of fractions 9–12 were subjected to autophosphorylation and separated by SDS/PAGE. The gel was dried and exposed for 10 h. (C) Aliquots (200 μ l) of fractions 9–12 were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with SYK(LR) antibody. The blot was exposed for 10 h. (D) Aliquots (500 μ l) of fractions 9–12 were incubated with [¹⁴C]FSBA, precipitated with 20% (w/v) trichloroacetic acid and subjected to SDS/PAGE. The gel was dried and exposed for 5 days. The protein concentrations in the fractions were: fraction 9, 15 μ g/ml; fraction 10, 110 μ g/ml; fraction 11, 75 μ g/ml; fraction 12, 150 μ g/ml. The molecular mass of the investigated protein was estimated by reference to protein standards indicated at the left (in kDa). Arrows indicate the position of the 40 kDa protein.

Table 1 Purification of the protein tyrosine kinase activity of CDp72^{syk} from rat spleen

The estimation of tyrosine kinase activity was performed under conditions described in the Materials and methods section, with poly(Glu₄, Tyr₁) as substrate. The kinase activity and recovery of CDp72^{syk} in the 240 000 g supernatant and in the DEAE-Sepharose fractions could not be estimated because of the presence of phosphatases and other kinases that also phosphorylated poly(Glu₄, Tyr₁). Consequently the purer preparation obtained after Superdex-200 was more accurate for the estimation of percentage yield and fold purification. Other kinases resolved by HA-Ultrogel have not been taken into account, so the purification factors shown in the Table are underestimated.

Step	Protein (mg)	Specific activity (pmol/min per mg)	Purification (fold)	Yield (%)
240 000 g supernatant	980	0.012		
SEAE-Sepharcel	85.5	170		
Superdex-200	5.5	735	1	100
HA-Ultrogel	0.162	7407	10.1	29.6

autoradiography. The autoradiographs revealed ¹⁴C-labelled proteins with molecular masses of 65, 58, 50, 46 and 33 kDa in fraction 14 and with molecular masses of 55, 46, 40 and 30 kDa in fraction 17 (Figure 2B). Fraction 17 was desalted and applied to an HA-Ultrogel column. The column was sequentially eluted with MgCl₂ and K₃PO₄ gradients. As shown in Figure 1(C), MgCl₂ eluted a single sharp peak of kinase activity (fraction 10). The same profiles of kinase activity were obtained when casein or histone H1 were used as substrates. Phosphoamino acid analysis revealed that only tyrosine residues were phosphorylated.

The analysis of HA-Ultrogel fractions 9–12 in silver-stained SDS/polyacrylamide gels revealed a major protein with a molecular mass of 40 kDa in fraction 10 (Figure 3A) constituting 40% of the total protein in fraction 10 as determined by densitometric analysis of the gel. Table 1 summarizes the purification protocol of the 40 kDa phosphoprotein-associated tyrosine kinase.

Evidence that the 40 kDa protein represents CDp72^{syk}

The fractions that were eluted with MgCl₂ and contained kinase activity were analysed by immunoblotting with antibody SYK(LR). As shown in Figure 3(C), the antibody reacted selectively with a 40 kDa protein. The following assays were

Figure 4 Co-migration of the [³⁵S]ATP[S]- and [³⁵S]pp[S]pA-binding capacity of CDp72^{syk} with ³²P-CDp72^{syk} under non-denaturing PAGE

The CDp72^{syk} samples were incubated with the nucleotides and subjected to non-denaturing PAGE. The gel was washed, dried and exposed for 12 h as described in the Materials and methods section. Lane 1, sample incubated with [³²P]ATP; lane 2, sample incubated with [³⁵S]ATP[S]; lane 3, sample incubated with [³⁵S]pp[S]pA. Only the section of the gel that includes labelled proteins was shown. The arrow indicates the position of CDp72^{syk}.

performed to demonstrate that the p72^{syk}-immunoreactive 40 kDa protein represented the catalytic domain of the kinase. Aliquots of HA-Ultrogel fractions 9–12 were subjected to autophosphorylation and analysed by SDS/PAGE and autoradiography. The resulting autoradiographs revealed only one ³²P-labelled band with a molecular mass of 40 kDa (Figure 3B). Some minor protein bands in the silver-stained gel were not phosphorylated. The phosphoamino acid analysis of the 40 kDa phosphoprotein showed that ³²P incorporation occurred exclusively on tyrosine residues (see also Figure 7B).

Aliquots of the investigated fractions were further probed for reaction with [¹⁴C]FSBA. Autoradiography of the SDS/PAGE-separated probes demonstrated a single ¹⁴C-labelled protein in fraction 10, migrating at 40 kDa (Figure 3D). The [¹⁴C]FSBA-binding was specific because it was completely blocked by co-incubation of the protein samples with ATP[S]. It is known that FSBA binds to proteins other than protein kinases [17]. Therefore the samples of the enzyme preparation were incubated with [³²P]ATP, [³⁵S]ATP[S] and [³⁵S]pp[S]pA under autophosphorylation conditions, and their migration was compared by non-denaturing PAGE. Autoradiography of the gel revealed identical migration patterns for the ³²P-labelled and ³⁵S-labelled proteins (Figure 4). The ³²P-labelled protein extracted from the native gel migrated in SDS/PAGE at 40 kDa (results not shown).

Figure 5 Phosphorylation of substrates by CDp72^{syk}

A CDp72^{syk} phosphorylation assay was performed as described in the Materials and methods section with proteins or polymers at 0.25 mg/ml as substrates. The reaction products were analysed by SDS/PAGE and autoradiography. The phosphoprotein bands shown corresponded to Coomassie Blue-stained proteins. Lane 1, autophosphorylation of CDp72^{syk}; lane 2, casein; lane 3, poly(Glu₄, Tyr₁); lane 4, poly(Glu₁, Ala₁, Tyr₁); lane 5, poly(Ala₆, Glu₂, Lys₅, Tyr₁); lane 6, poly(Glu₆, Lys₃, Tyr₁); lane 7, poly(Glu₁, Tyr₁); lane 8, poly(Glu₆, Ala₃, Tyr₁); lane 9, myelin basic protein; lane 10, histone H1; lane 11, histone H2B. The positions of molecular mass standards (in kDa) are indicated at the left.

These observations taken together demonstrated a co-eluting 40 kDa protein with tyrosine kinase activity, p72^{syk} immunoreactivity and an ATP-binding site, indicating that the CDp72^{syk} had been purified.

Substrates of CDp72^{syk}

CDp72^{syk} phosphorylated casein (K_m 18.9 μ M), myelin basic protein (K_m 6.5 μ M), histones H1 (K_m 8.9 μ M) and H2B (K_m 0.35 μ M) and random polymers poly(Glu₄, Tyr₁) (K_m 0.2 μ M) and poly(Ala₆, Glu₂, Lys₅, Tyr₁) (K_m 0.45 μ M) (Figure 5). Troponin, tropomyosin and acid-treated enolase were phos-

phorylated to a smaller extent (results not shown). Phosphoamino acid analysis of the phosphorylated proteins revealed exclusively phosphotyrosine (results not shown).

Correlation between autophosphorylation of CDp72^{syk} and its kinase activity

Incubation of CDp72^{syk} in the presence of [γ -³²P]ATP (10 μ M) resulted in an efficient autophosphorylation (Figure 6A, inset). The reaction was linear for 30 min and led to the incorporation of 0.65–0.70 mol of P_i per mol of kinase (Figure 6A). The influence of the autophosphorylation on the kinase activity was assayed in a two-step reaction. In the first step, aliquots of the kinase were prephosphorylated for various durations [corresponding to step (i) in the Materials and methods section]. In the second step, substrates [casein, histone H2B or poly(Glu₄, Tyr₁)] were added to the prephosphorylated CDp72^{syk} samples. Under these reaction conditions there was an enhancing effect of autophosphorylation on the kinase activity. The extent of this activating effect was dependent on the substrate investigated (Figure 6B). No changes in kinase activity were observed in control experiments performed with kinase incubated in step (i) without ATP. To eliminate the possibility that ATP stabilized or activated the kinase, aliquots of the enzyme were incubated in step (i) in the presence of 0.1 μ M non-hydrolysable ATP[S] for various durations. Thereafter, substrates (and [γ -³²P]ATP at 10 μ M) were added for a constant time of phosphorylation, corresponding to step (ii) of the assay. There were no differences in the kinase activity of probes incubated for different times with ATP[S] (results not shown).

Phosphorylation and regulation of CDp72^{syk} by PKC

Here we describe a newly developed method that allows the

Figure 6 Effect of autophosphorylation on the activity of CDp72^{syk} towards exogenous substrates

(A) Samples of CDp72^{syk} (250 ng) were incubated with [γ -³²P]ATP under autophosphorylation conditions. At the indicated time points, aliquots were removed and analysed by SDS/PAGE followed by autoradiography [inset; positions of molecular mass standards (in kDa) at the left]. The autophosphorylated bands were excised and ³²P incorporation was measured. (B) To the remaining part of the prephosphorylated kinase was added the substrate and the reaction was further continued as described in Materials and methods section. The phosphoproteins were separated by SDS/PAGE and excised from the gel, and the radioactivity incorporated was counted. The effect of the autophosphorylation on CDp72^{syk} activity is presented as a percentage of control (a sample incubated for 0 min is shown as 0%). Symbols: $\blacklozenge$, phosphorylated casein; $\bullet$, phosphorylated histone H2B; $\blacksquare$, phosphorylated poly(Glu₄, Tyr₁). The broken lines were drawn for better demonstration of the linear phase of the reaction. The results shown are representative of three independent experiments.

Figure 7 PKC phosphorylates and regulates Cdp72^{syk}

(A) Aliquots (250 ng) of Cdp72^{syk} were exposed to PKC in the presence of [γ -³²P]ATP for 0 min (lane 1), 2.5 min (lane 2), 5 min (lane 3) or 10 min (lane 4). The phosphoproteins were separated by SDS/PAGE and autoradiographed for 7 h. The positions of molecular mass markers (in kDa) are indicated at the left. (B) The ³²P-labelled Cdp72^{syk} band (in a) was extracted from the gel and subjected to acid hydrolysis followed by electrophoresis. Lane 1, Cdp72^{syk} exposed to PKC for 0 min (lane 1 in A); lane 2, Cdp72^{syk} exposed to PKC for 10 min (lane 4 in A). Abbreviations: P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine. (C) The phosphorylated Cdp72^{syk} was excised and P_i incorporation was determined by Čerenkov counting of the radioactivity. (D) Aliquots containing 250 ng of Cdp72^{syk} were phosphorylated by PKC similarly as in (A), but in the presence of unlabelled ATP. After the addition of PKC for 0, 2.5, 5 or 10 min, the substrate [casein, histone H2B or poly(Glu₄, Tyr₁)] was added and the reaction continued as described in the Materials and methods section. The proteins were separated by SDS/PAGE and transferred to nitrocellulose, and their relative phosphotyrosine content was estimated by immunoblotting with an anti-phosphotyrosine antibody. The ¹²⁵I-labelled proteins were excised and the radioactivity was counted in a liquid-scintillation counter. Symbols: ◆, phosphorylated casein; ●, phosphorylated histone H2B; ■, phosphorylated poly(Glu₄, Tyr₁). (E) Aliquots (250 ng) of Cdp72^{syk} were phosphorylated by PKC for 10 min as in (D), but in the presence or the absence of 2 m-units of the catalytic subunit of protein phosphatase 1 (PP1) inhibited or not with okadaic acid (O.a.) (50 nM). Substrate, poly(Glu₄, Tyr₁), was added and the reaction proceeded as described in the Materials and methods section. In (D) and (E) the effect of PKC on the Cdp72^{syk} activity is presented as a percentage of control (a sample incubated without PKC is shown as 0%). The results shown are representative of three experiments.

assessment of activity of a tyrosine kinase as a function of its phosphorylation state by a serine/threonine kinase. The method consists of two consecutive steps. In the first step the tyrosine kinase was phosphorylated by PKC; and in the second step the tyrosine kinase activity towards exogenous substrate was determined. In the first step, samples of Cdp72^{syk} were incubated with [γ -³²P]ATP for constant times. During the resulting autophosphorylation, affinity-purified rat brain PKC was added for various durations. Thus in the course of the incubation with ATP the Cdp72^{syk} was subject to two reactions: autophosphorylation and phosphorylation mediated by PKC. As shown in Figure 7(A), lengthening exposure to PKC was accompanied by an increase in the level of ³²P labelling of the Cdp72^{syk}. The velocity of phosphorylation was high: during the exposure of Cdp72^{syk} to PKC for 2.5 min the reaction approached its plateau. Extension of the exposure time to 5 min or the addition of further aliquots of PKC did not lead to a significant increase in the level of Cdp72^{syk} phosphorylation (Figures 7A and 7C). To differentiate between autophosphorylation and PKC-mediated phosphorylation, phosphoamino acid analysis of Cdp72^{syk} exposed to PKC was performed. As shown in Figure 7(B), the exposure

of the Cdp72^{syk} to PKC under phosphorylation conditions resulted in serine and threonine phosphorylation without change of phosphotyrosine content. Thus the increase in ³²P labelling of the 40 kDa phosphoprotein (shown in Figure 7C) resulted solely from PKC-mediated phosphorylation of serine and threonine residues.

The effect of PKC-mediated Cdp72^{syk} phosphorylation on its activity was investigated in step (ii) of the assay. For this assay the phosphorylation reactions in steps (i) and (ii) were performed with unlabelled ATP. The Cdp72^{syk} substrate was added to the PKC-phosphorylated Cdp72^{syk} sample. The phosphorylation of substrates was performed and after SDS/PAGE the products were analysed by immunoblotting with anti-phosphotyrosine antibody. We observed that increasing the time of incubation with PKC led to an increasing activation of Cdp72^{syk}. The degree of the activating effect was closely correlated with the kinetics of Cdp72^{syk} phosphorylation by PKC in step (i) of the assay (Figures 7C and 7D). Using proteins (casein, histone H2B) and poly(Glu₄, Tyr₁), we observed that the extent of Cdp72^{syk} activation varied with different substrates. As shown in Figure 7(D), the tyrosine phosphorylation of histone H2B was more

Figure 8 PKC isoform-specific phosphorylation and regulation of CDp72^{syk}

(A) Aliquots (250 ng) of CDp72^{syk} were phosphorylated by PKC isoforms in the presence of [γ -³²P]ATP for 0, 2.5, 5 or 10 min as described in the legend to Figure 7(A). The phosphoproteins were separated by SDS/PAGE, and ³²P incorporation into CDp72^{syk} was determined by Čerenkov counting of excised phosphoprotein bands. The symbols used reflect the CDp72^{syk} phosphorylation mediated by α -PKC (●), β_1 -PKC (▼), β_{II} -PKC (■) and γ -PKC (◆). The inset to (A) demonstrates the tyrosine phosphorylation of CDp72^{syk} phosphorylated by PKC isoforms for 0 or 10 min in the presence of unlabelled ATP, estimated by immunoblotting with anti-phosphotyrosine antibody. (B) Aliquots of CDp72^{syk} were phosphorylated by PKC isoforms as described in (A) in the presence of unlabelled ATP. After the phosphorylation, histone H2B was added, and the reaction continued as described in the Materials and methods section. Tyrosine phosphorylation of the substrate was determined by immunoblotting of transferred proteins with anti-phosphotyrosine antibody. The ¹²⁵I-labelling of histone H2B was counted in a liquid-scintillation counter. The effect of PKC on CDp72^{syk} activity is presented as a percentage of control (a sample incubated without PKC is shown as 0%). The symbols used reflect the activity of CDp72^{syk} phosphorylated by α -PKC (○), β_1 -PKC (▽), β_{II} -PKC (□) and γ -PKC (◇). The results shown are representative of three independent experiments.

efficiently increased than that of casein or poly(Glu, Tyr). After the addition of the substrates in step (ii), three reactions occurred at the level of CDp72^{syk}: its autophosphorylation, phosphorylation by PKC and phosphorylation of the substrates. As demonstrated above, the autophosphorylation of CDp72^{syk} was not influenced by PKC. In an additional experiment we tested the reversibility of the PKC effects. The catalytic subunit of protein phosphatase 1, a serine/threonine-specific protein phosphatase, was added simultaneously with PKC, during the reactions in step (i). The phosphatase completely abolished the PKC-mediated effects. Okadaic acid, an inhibitor of the phosphatase added to the reaction mixture, re-enabled these effects (Figure 7E). Therefore the differences in the tyrosine phosphorylation of substrates resulted solely from PKC-mediated serine and threonine phosphorylation of CDp72^{syk}.

Different regulatory effects of PKC isoforms on the kinase activity of CDp72^{syk}

Rat brain PKC consists of a mixture of isoforms that differ in their modes of activation, kinetic properties and regional distributions in mammalian tissues [18,19]. We therefore investigated the efficiency of PKC isoforms in phosphorylating and regulating CDp72^{syk}.

The specific activity of the α , β_1 , β_{II} and γ isoforms was adjusted with histone III-S as a substrate, and the same amounts of activity (0.5 m-unit) were used for the CDp72^{syk} phosphorylation assay. As shown in Figure 8(A), CDp72^{syk} was most efficiently phosphorylated by α -PKC, followed by β_1 -PKC and β_{II} -PKC with slightly lower efficiency. Surprisingly, CDp72^{syk} was a poor substrate for γ -PKC. Similarly to the assays with rat brain PKC, the α , β_1 , β_{II} and γ -isoform-mediated phosphorylation did not affect the autophosphorylation of CDp72^{syk} (Figure 8A, inset).

The activation of CDp72^{syk} by the PKC-isoform-mediated phosphorylation was assayed with histone H2B as a substrate in the two-step reaction described for rat brain PKC. As shown in Figure 8(B), PKC activated CDp72^{syk} in an isoform-dependent manner ($\alpha > \beta_1 = \beta_{II} > \gamma$) in parallel with phosphate incorporation into the tyrosine kinase during step (i).

DISCUSSION

In the present paper we describe the purification and characterization of a 40 kDa tyrosine kinase representing the catalytic domain of p72^{syk}. For its immunological identification we used an antibody against a polypeptide corresponding to amino acid residues 257–352 ('linker' region) of human p72^{syk} [termed SYK(LR)]. In addition to the holoenzyme and its N- and C-truncated forms, the antibody can detect series of partial proteolytic products of the enzyme that correspond to the catalytic or regulatory domain and result from cleavage in the 'linker' region of p72^{syk}. Because the molecular masses of these fragments are approximately equal [20], it was conceivable that the purified kinase might not be an intrinsic activity of a catalytic p72^{syk} fragment but might be derived from another co-purified kinase. To examine this possibility, the ATP-binding capability of the p72^{syk} immunoreactive fragments obtained at various stages of the purification was tested with FSBA, which alkylates conserved lysine residues within the ATP-binding sites of protein kinases [21]. Some immunoreactive proteins seen in fraction 14 (also a 40 kDa polypeptide) did not react with FSBA. Whether these polypeptides represent the regulatory domain of p72^{syk} remains to be elucidated. The final CDp72^{syk} preparation contained only one 40 kDa FSBA-binding protein, indicating that it was not contaminated with other kinases. This conclusion was supported by following observations: (1) autophosphorylated CDp72^{syk}

migrated in a native gel in the same way as the [35 S]ATP[S]- and [35 S]pp[S]pA-binding proteins, (2) the preparation contained no serine/threonine kinase activity as judged by using histones and casein as substrates, and (3) the kinase behaved homogeneously on chromatography on oligo(dA)-cellulose and heparin-agarose (results not shown).

The key step of our purification procedure was elution from HA-Ultrogel with chloride. The isoelectric point of CDP72^{syk} [pI 5.85 (SwissProt database)] permitted us to use an MgCl₂ gradient employed to elute proteins with an isoelectric point between 5 and 8. We exploited the ability of MgCl₂ to strengthen the binding of more acidic proteins to a hydroxyapatite column [22].

CDP72^{syk} (reported elsewhere as cytosolic 40 kDa tyrosine kinase [1]) is generated in the course of proteolytic cleavage of the membrane-associated holoprotein [20]. This proteolysis was postulated to be responsible for its translocation to the cytosolic compartment. Indeed, substantial amounts (0.2 mg per 100 g of tissue) of the 40 kDa kinase were purified from cytosol of pig spleen [1]. Comparable amounts of the kinase purified here (65 µg from 30 g of spleen) suggest that a certain part of the catalytic domain of p72^{syk} is membrane-associated. The mechanism responsible for the membrane attachment of CDP72^{syk} is not known. The kinase has neither potential myristoylation sites nor N-terminally located SH2 domains that mediate the binding to the sIgM-B-cell- or T-cell-receptor complex [20,23].

Our CDP72^{syk} differed from bovine thymus p40 kinase, proteolytically generated from a 72 kDa precursor, in its chromatographic behaviour and substrate specificity. CDP72^{syk} did not bind to anion-exchange chromatography columns and efficiently phosphorylated casein. Further, unlike the pig 40 kDa catalytic fragment of p72^{syk}, our CDP72^{syk} did not require Ca²⁺ for enzyme activity [24,25]. However, such a comparison permits only limited conclusions about the relationship of the tyrosine kinases because different substrates, reaction conditions or purification protocols were used.

CDP72^{syk} showed a limited ability to phosphorylate synthetic polymers containing tyrosine [26]. Only poly(Glu₄, Tyr₁) and poly(Ala₆, Glu₂, Lys₃, Tyr₁) were phosphorylated by CDP72^{syk}. Thus a set of polymers might be a useful approach for distinguishing between various kinases on the basis of substrate specificity.

The phosphorylation of both tyrosine residues (Tyr-518 and Tyr-519), putative p72^{syk} autophosphorylation sites, has been shown to activate the kinase [27]. Unlike the Src family kinases, it has no typical autophosphorylation YTAR motif in the catalytic domain of the kinase [23]. The corresponding region in p72^{syk} has the sequence YYKAQ. It is still not known whether the phosphorylation of the sites *in vivo* results from autophosphorylation or from phosphorylation by Src kinases [23], or whether an autophosphorylation or Src kinase-mediated phosphorylation of other tyrosine residues might be able to induce the phosphorylation of these sites in a co-ordinated process, as in the insulin receptor [28]. Our experiments indicate that CDP72^{syk} does not require a co-working tyrosine kinase for its autophosphorylation and activation.

It should be mentioned that phosphorylation of the YYTAR motif of the homologous T-cell-associated tyrosine kinase p70^{zap} also leads to a kinase activation. Interestingly, the tyrosine residues of p70^{zap} are not autophosphorylated: their phosphorylation is mediated by p56^{lck} and/or p59^{fyn} [29].

The extent of CDP72^{syk} activation by autophosphorylation was dependent to the nature of the substrate. This phenomenon was previously observed with tyrosine kinases from pig [30] and

from rat spleen [31]. The mechanism responsible remains unresolved, but it is probably dependent on individual superficial charge distributions of the substrates [32].

Using purified enzymes, we provide lines of evidence for CDP72^{syk} phosphorylation by PKC. The kinase is phosphorylated on serine and threonine residues by PKC, and the phosphorylation of these sites is associated with an increase in the CDP72^{syk} tyrosine kinase activity. The PKC-mediated phosphorylation of CDP72^{syk} is a time-dependent process closely correlated with activation. Dephosphorylation of serine/threonine residues by the catalytic domain of protein phosphatase I abolishes both PKC effects: CDP72^{syk} phosphorylation and its activation. Thus it is most likely that the CDP72^{syk} is activated via phosphorylation of serine/threonine residues and not as a result of a physical interaction of both kinases.

The level of CDP72^{syk} autophosphorylation was not altered in the course of PKC-mediated phosphorylation, indicating that serine/threonine phosphorylation and autophosphorylation are not co-ordinated processes. Distinct PKC isoforms differed in their kinetics and their intensity of CDP72^{syk} phosphorylation. Similar observations have previously been made. Ido et al. [33] observed that the α isoform of PKC phosphorylates the EGF-R more efficiently than the β or γ isoforms. However, the calibration procedure of the PKC isoforms with histone III-S as standard substrate led to variable amounts of the recombinant proteins per unit, as controlled in silver-stained SDS/gel. Therefore it is possible that the different efficacies of the CDP72^{syk} phosphorylation could reflect different amounts of enzymes employed. The expression pattern of PKC isoforms shows a heterogeneous distribution in mammalian tissues [18,19]. Thus it is conceivable that the distinct isoforms control different signal pathways within the cell.

The phosphorylation and regulation of tyrosine kinases by PKC is not without precedent. PKC phosphorylates receptor tyrosine kinases (e.g. EGF-R and insulin receptor [34,35]) and non-receptor kinases such as Src family kinases [36,37]. In receptor kinases the PKC-mediated serine/threonine phosphorylation inhibits their ligand-induced tyrosine kinase activity. The significance of serine/threonine phosphorylation of non-receptor kinases for their catalytic regulation is still unclear.

Although our results show that PKC phosphorylates and activates CDP72^{syk} *in vitro*, they do not allow us to conclude that it is a PKC function *in vivo*. However, it has been reported that a combination of a PKC inhibitor and a Ca²⁺ chelator inhibits the phosphorylation and activity of p72^{syk} in thrombin-stimulated platelets [38]. Further, μ -PKC, an atypical member of the PKC family, has been shown to phosphorylate the 'linker' region and, to a smaller extent, SH2 domains (but not the catalytic domain) of p72^{syk} and to inhibit its ability to phosphorylate phospholipase C γ 1 *in vitro* [39]. Results of kinase activity assays show very distinct modes of behaviour of PKC isoforms, owing to differences in their substrate preference and/or in their mode of activation [40]. Thus it is not clear whether the PKC isoforms tested here and μ -PKC used the same phosphate acceptors on the p72^{syk} molecule. However, some domains of p72^{syk} could influence the availability of the C terminus (CDP72^{syk}) as a substrate for protein kinases, as presumed for the catalytic domain of Ras GTPase-activating protein [41] or truncated lipocortin [32]. In the light of this result it is possible that the PKC-mediated activation of p72^{syk} or CDP72^{syk} results in the reinforcement of the signals mediated by p72^{syk} or its free catalytic domain.

Many tyrosine kinases have similar structural organization, with similarly sized catalytic domains (40–43 kDa) that are resistant to proteolysis [4,7,20]. It is tempting to suggest that proteolysis converts the holoproteins into constitutively active

protein kinases that could change their cell compartment and prolong the response of the activated holoenzyme.

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