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A novel nuclear 42-kDa casein kinase identified in *Chironomus tentans*

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We have purified and characterised an apparently novel nuclear 42-kDa casein kinase from epithelial cells of *Chironomus tentans* which comigrates with a phosphoprotein associated with transcriptionally active salivary gland genes. The protein kinase promotes phosphorylation of casein and phosvitin, using either ATP or GTP as phosphate donors, and undergoes autophosphorylation. The casein kinase activity of the 42-kDa protein is sensitive to heparin, 5,6-dichloro-1-β-D-ribofuranosylbezimidazole (DRB), spermine and spermidine indicating that it is a novel enzyme with similar but not identical properties to casein kinase II or nuclear protein kinase NII.

Protein kinase; Casein kinase; Protein phosphorylation; Enzyme purification; Inhibitor; Heparin; DRB

1. INTRODUCTION

DNA transcription is one of the central cellular processes which is subject to control by post-translational phosphorylation/dephosphorylation of basal as well as gene-specific components of the transcriptional machinery. RNA polymerase II, a multisubunit enzyme which catalyses the transcription of protein-coding genes, as well as numerous transcription factors has been shown to be regulated by phosphorylation [1]. Hence the identification and functional characterisation of protein kinases associated with the transcription process and the clarification of their mechanism of interaction are required to fully understand the transcription process.

We have, in previous reports, described a rapidly phosphorylated 42-kDa protein (pp42) from *Chironomus tentans* salivary gland cells, which possesses some characteristics related to the function of RNA polymerase II [2,3]. Previous in vivo studies of nuclear proteins and DNA transcription in salivary gland cells have shown that the salivary gland pp42 is preferentially associated with transcriptionally active chromatin and that the inhibition of its phosphorylation by DRB is coupled to a block in RNA polymerase II-promoted transcription initiation [2,4,5]. Furthermore, the salivary gland pp42, as well as pp42 from nuclei of a *Chironomus tentans* epithelial cell line, preferentially binds single-stranded promoter-containing gene probes [6]. In the light of these findings, it appears likely that pp42 plays an important role in DNA transcription and therefore the elucidation of its roll in the process is of considerable interest. The use of a *Chironomus tentans* epithelial cell culture enabled us to scale up the preparative work and to carry out enzymatic analyses. The pp42 protein purified from the nuclei of the epithelial cell line was shown to share the solubility-, phosphorylation- and DNA binding properties of its salivary gland counterpart [6,7]. From a partially purified pp42 protein extract a novel kinase (CK N42) was isolated whose characteristics suggest that it might be identical to the previously described pp42-phosphoprotein.

The purified 42-kDa protein kinase promotes phosphorylation of casein and phosvitin using either ATP or GTP as phosphate donors and is sensitive to heparin and the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Some properties of CK N42 are similar to those of casein kinase II (CK II) or nuclear protein kinase NII (NII) but CK N42 is not identical to any of known casein kinases.

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2. MATERIALS AND METHODS

2.1. *Isolation of nuclei and fractionation of nuclear proteins*

Cell nuclei from the *Chironomus tentans* embryonal epithelial cell line (L1), established by Wyss [8], were isolated as described by Widner et al. [9] with minor modifications. 10 g of frozen cells were rinsed in 20 ml buffer A1 (15 mM Tris-HCl, pH 7.0, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM DTT, 0.1 mM PMSF with 10 μg aprotinin per ml) and the cells pelleted in a swing-out rotor at 400 × *g* for 10 min. The cells were lysed for 10 min at 4°C in buffer A1 containing 0.5% Triton X-100 and subsequently homogenised in a teflon hand homogenizer (Thomas, size C). Nuclei were pelleted at 10,000 × *g* (Sorvall), washed twice in buffer A4 (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM DTT, 0.1 mM PMSF with 10 μg aprotinin per ml). The nuclear pellet was mixed with 20 ml extraction buffer (10 mM Tris-HCl, pH 8.0, 0.35 M NaCl and 0.1 mM DTT containing 10 μg aprotinin per ml solution) and the loosely bound nuclear proteins were extracted for 10 min at 4°C. The insoluble nuclear pellet was spun down at 100,000 × *g* for 16 h (Beckman L8-70 ultracentrifuge, rotor Ti50). To the 0.35

M NaCl-soluble nuclear proteins a saturated solution of $(NH_4)_2SO_4$ was added to obtain 50% saturation, the sample was centrifuged at 30,000 × g for 10 min (Sorvall) and the supernatant was removed.

2.2. *Casein-phosvitin-Sepharose 4B affinity chromatography*

Casein-phosvitin-Sepharose 4B was prepared as described by the manufacturer (Pharmacia, Uppsala, Sweden). 10 ml each of casein-Sepharose 4B and phosvitin-Sepharose 4B were mixed and transferred to a plastic syringe (5 ml) which was used as a column. The settled gel was first extensively washed with 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl, then with the coupling buffer (0.1 M $NaHCO_3$, pH 8.3 and 0.5 M NaCl) and finally equilibrated with the loading buffer (10 mM Tris-HCl, pH 7.5 and 0.15 M NaCl). The 50% $(NH_4)_2SO_4$ precipitate was solubilized in the loading buffer, applied and eluted at a flow rate of 15–20 ml/h using a peristaltic pump. After loading the sample, the column was washed with loading buffer and subsequently eluted with a linear 0.15–2 M NaCl gradient buffered with 10 mM Tris-HCl, pH 7.5. Fractions of about 1 ml were collected and the absorbance was monitored at 280 nm in a Beckman DUR 65 Spectrophotometer. The protein kinase was assayed with casein as substrate, and the fractions containing the highest activity were pooled and precipitated with 20% TCA (final concentration) at 20°C. The precipitate was dissolved in SDS sample buffer and assayed by 12% SDS-PAGE.

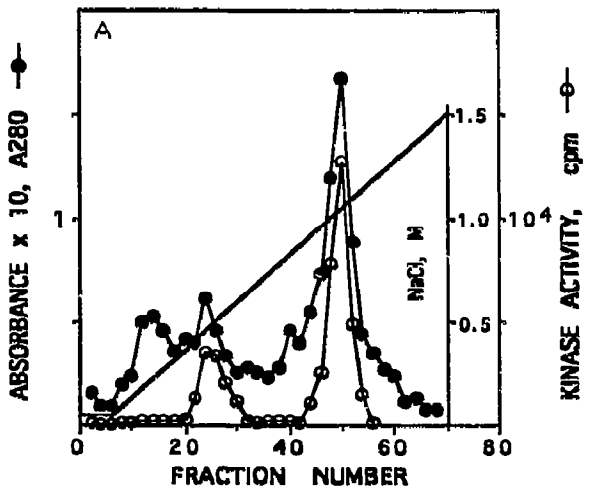
2.3. *Assays of protein kinase activity*

Reaction mixture consisted of 2.5 μl protein kinase extract and 1–3 μCi [γ-^{32}P]ATP (3,000 Ci/mmol) or [γ-^{32}P]GTP (5,000 Ci/mmol) (Amersham) in 10 μl final volume of the standard incubation buffer, 10 mM HEPES-KOH, pH 7.8, 10 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 80 mM KCl, 0.2 mM DTT [3]. As substrates, partially dephosphorylated casein (1 μg) and phosvitin (1 μg) were used. The enzyme activity was assayed in the absence and presence of heparin, DRB, spermine or spermidine. The reaction mixture was incubated for 15 min at 20°C and the reaction was stopped by addition of 10 μl SDS sample buffer. After incubation, the reaction mixture was subjected to 12% SDS-PAGE and the level of phosphorylation was estimated by scanning of the autoradiographs in a Shimadzu Dual-Wavelength Chromato-Scanner (model CS/930).

3. RESULTS

3.1. *Isolation and purification of a 42-kDa protein (CK N42) with casein kinase activity*

Differential $(NH_4)_2SO_4$ precipitation of the 0.35 M NaCl soluble protein extract from epithelial cell nuclei resulted in a considerable enrichment of pp42 in the 50% $(NH_4)_2SO_4$ precipitate. This material was used for preparation of the 42-kDa casein kinase by affinity chromatography on a casein-phosvitin-Sepharose 4B column. After sample application and subsequent elution of the proteins with a linear NaCl gradient, a major peak dominating the absorbance pattern eluted at about 1 M NaCl. The analysis of the protein kinase activity of the eluted material with casein as phosphate acceptor revealed that, although two smaller fractions eluted at lower salt concentrations, most of the casein kinase activity was associated with the main absorbance peak (Fig. 1A). An aliquot of the main peak material was then subjected to SDS-PAGE (Fig. 1B). The staining pattern exhibits the presence of one single band which, like pp42, comigrates with the actin marker indicating that this 42-kDa protein (CK N42) has the casein kinase



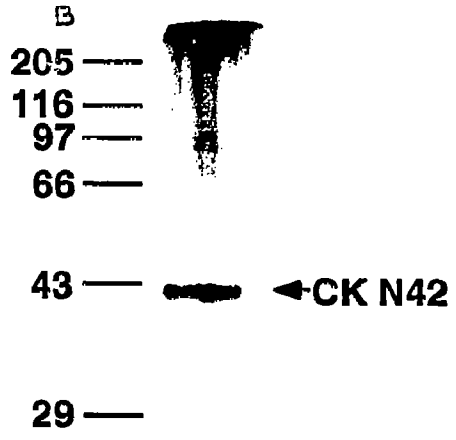


Fig. 1. Purification of CK N42 by casein-phosvitin-Sepharose chromatography (A) and the electrophoretic analysis of proteins in the main peak (pooled fractions 46–52) (B). The protein kinase was assayed with casein as substrate and [γ^{32}-P]ATP as phosphate donor. The protein bands were visualised by Coomassie blue staining.

activity. A significant portion of stained material does not enter the 12% SDS-PAGE. This material probably represents aggregated enzyme molecules which readily form in purified state. Autoradiographic analysis (Fig. 2) demonstrates CK N42 autophosphorylation as well as the phosphorylation of the casein and phosvitin. As in the case of the staining pattern of purified CK N42 (Fig. 1B), no other phosphorylated proteins can be seen apart from the kinase and in this case the two substrates.

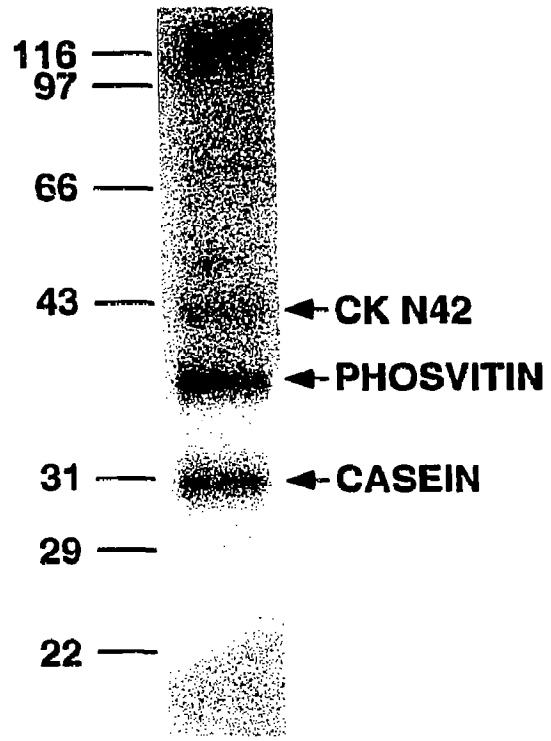


Fig. 2. Autoradiographic analysis after electrophoretic separation, of the affinity purified CK N42 after incubation with casein and phosvitin as substrates. The protein kinase was assayed with casein as substrate and [γ^{32}-P]ATP as phosphate donor.

3.2. *The effect of heparin, DRB, spermine and spermidine on activity of CK N42*

The results shown in Figs. 1 and 2 raise the question of whether the casein kinase identified and isolated here is of CK I/NI or CK II/NII type. This tissue was studied by assaying the protein kinase activity in the presence of known inhibitors of CK II or nuclear protein kinase NII as heparin [10–12] and DRB [13] and activators as spermine and spermidine [10–12,14] using [γ-^{32}P]ATP or [γ-^{32}P]GTP as phosphate donors. Due to the limited amount of purified kinase, the enzyme obtained from the 50% $(NH_4)_2SO_4$ precipitate was used in the inhibition studies. The dose-dependent inhibition of heparin on CK N42 phosphorylation of casein is shown in Fig. 3A. The autophosphorylation of CK N42 is blocked by heparin with an I_{50} value of about 7 μg/ml while phosphorylation of phosvitin and casein is inhibited by an I_{50} value of about 2.5 and 1 μg/ml, respectively.

The inhibitory activity of DRB on incorporation of ^{32}P into CK N42, phosvitin and casein is shown in Fig. 3B. As seen, the autophosphorylation of CK N42 is suppressed by about 80% at 65 μM DRB concentration while the phosphorylation of phosvitin and casein is lowered by 90% at the same concentration of inhibitor. Notably, at all concentrations used, the effect of DRB on autophosphorylation of CK N42 is somewhat lower than on phosphorylation of casein or phosvitin.

The effects of spermine on CK N42 activity is shown in Fig. 3C. Spermine blocks phosphorylation as well as the autophosphorylation of CK N42 by 90–100% at a 9 mM concentration. By analogy with the inhibitory properties of DRB, spermine has a slightly lower inhib-

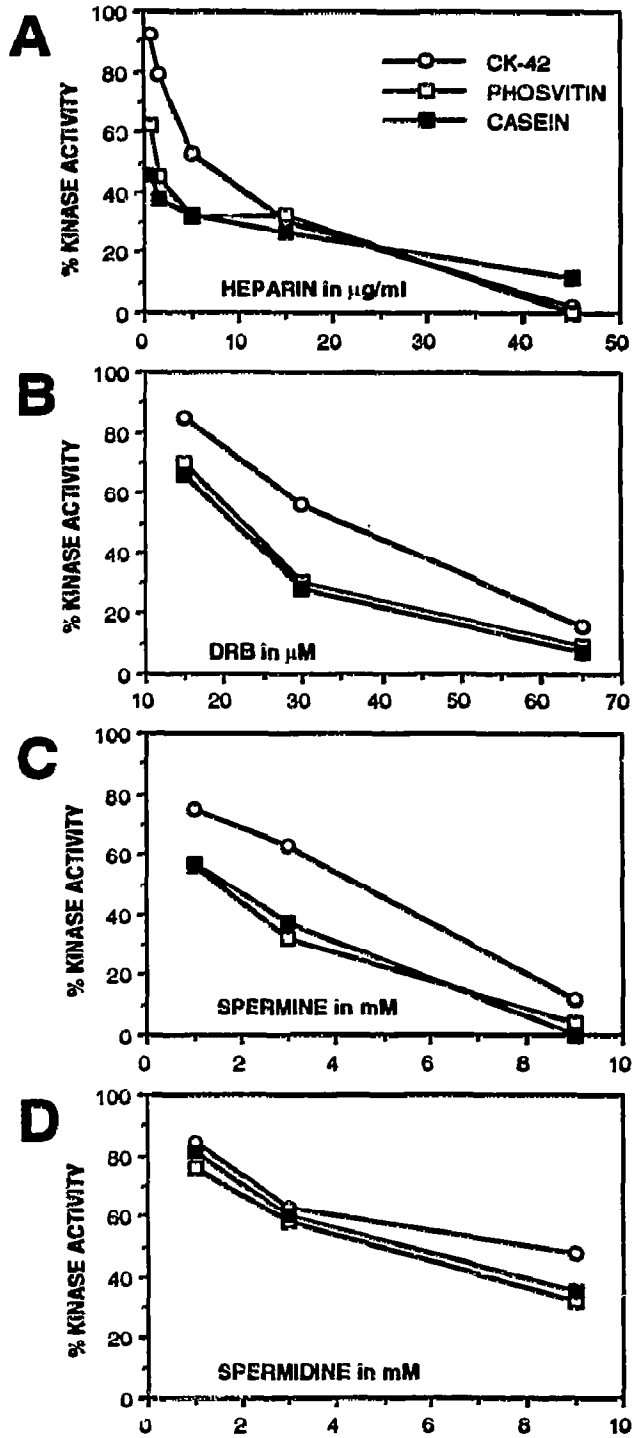


Fig. 3. The effect of heparin (A), DRB (B), spermine (C) and spermidine (D) on autophosphorylation of CK N42 (○) and phosphorylation of phosvitin (□) or casein (■). The protein kinase was assayed with [γ^{32}-P]ATP as phosphate donor.

itory action on autophosphorylation than on phosphorylation of the substrate proteins. The inhibition of CK N42 activity by spermidine, shown in Fig. 3D, is less efficient irrespective of whether casein or phosvitin is used as an acceptor protein. Thus, neither of the studied polyamines stimulates the activity of CK N42.

4. DISCUSSION

The present communication describes an apparently novel 42-kDa casein kinase, CK N42, isolated from the nuclei of *Chironomus tentans* epithelial cells. We have earlier observed that the pp42 nuclear protein, isolated from *Chironomus tentans*, could be phosphorylated by both ATP and GTP in a heparin and DRB sensitive manner [2,15] indicating that the enzyme that catalyses this phosphorylation might be nuclear protein kinase NII [13]. However, the experiments resulted in the isolation of a novel casein kinase. It is unlikely that CK N42 is of CK I/NI type since the latter enzyme is practically resistant to heparin [11] and uses only ATP as phosphate donor [16]. The enzyme also differs from CK II or nuclear protein kinase NII in a number of ways. (i) CK N42 is less sensitive to heparin which inhibits the activity at concentrations at least one order of magnitude higher than those required to inhibit the activity of the other two kinases [10–12]. (ii) The phosphorylation of casein by CK N42 is markedly inhibited by spermine and spermidine while similar concentrations of these polyamines cause a several-fold stimulation of the activity of CK II or NII [10–12, 14]. (iii) CK N42 elutes from the casein-phosvitin-Sepharose 4B column at about twice as high an NaCl concentration as nuclear protein kinase NII [10]. (iv) Polyclonal antibodies against Drosophila CK II [17], crossreacting with the α as well as the β subunits of *Chironomus* nuclear protein kinase NII, do not crossreact immunologically to CK N42 on Western blots (data not shown). (v) CK N42 appears to have a monodisperse appearance, although its native form and size has yet to be determined, while CK II and nuclear protein kinase NII are heterooligomers which contain two catalytic (35–45 kDa) subunits and two other smaller subunits (24–29 kDa) [11,18]. It is relevant to mention in this context that salivary gland pp42 forms a homooligomer in native state with an apparent molecular mass of 150–200 kDa [7].

The findings that CK N42 has the same apparent molecular weight as pp42, shares the same solubility characteristics during the initial purification steps, autophosphorylates in a manner identical to the previously characterised casein kinase-like phosphorylation of pp42 [2,6,7,15], strongly suggests that CK N42 and pp42 are identical.

The our knowledge, protein kinases with the ability to bind ss DNA with preference for promoter sequences have not been reported. If CK N42 is in fact identical to the ss DNA binding pp42 protein then it is logical to search for its target protein(s) among polypeptides, in the transcriptional machinery. Although all the characteristics of CK N42 do not seem to fully apply to any already characterised kinase, it is not unlikely that kinase activities or phosphoproteins, in the 40-kDa range, previously found by others are related to or identical with CK N42. It would for instance be most interesting to see whether CK N42 might be related to the 43-kDa subunit identified in a TFIIH-protein extract containing kinase activity [19]. Studies are now in progress in our laboratory to establish the relationship between the DNA-binding pp42 and CK N42 as well as to identify the intracellular substrate(s) of CK N42.

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REFERENCES

[1] Hunter, T. and Karin, M. (1992) Cell 70, 375–387.
[2] Egyházi, E., Pigon, A., Ossoinak, A., Holst, M. and Tayip, U. (1984) J. Cell Biol. 98, 954–962.
[3] Holst, M. and Egyházi, E. (1985) J. Cell Biochem. 29, 115–126.
[4] Egyházi, E. (1974) Proc. Natl. Acad. Sci USA 72, 947–950.
[5] Egyházi, E., Pigon, A. and Rydlander, L. (1982) Eur. J. Biochem. 122, 445–451.
[6] Stigare, J., Egyházi, E. (1991) Biochem. Biophys. Res. Commun. 176, 1565–1570.
[7] Egyházi, E., Stigare, J., Pretz, V., Holst, M. and Pigon, A. (1939) Biochem. Biophys. Res. Commun. 165, 895–901.
[8] Wyss, C. (1982) Exp. Cell Res. 139, 309–319.
[9] Widmer, R.M., Lucchini, R., Lezzi, M., Meyer, B., Sogo, J.M., Edström, J.-E. and Koller, Th. (1984) EMBO J. 3, 1635–1641.
[10] Delpech, M., Levy-Favatier, F., Moisand, F. and Kruh, J. (1986) Eur. J. Biochem. 160, 333–341.
[11] Rose, K.M., Bell, L.E., Siefken, D.A. and Jacob, S.T. (1981) J. Biol. Chem. 256, 7468–7477.
[12] Hara, T., Takahashi, K. and Endo, H. (1981) FEBS Lett. 128, 33–36.
[13] Zandomeni, R., Zandomeni, M.C., Shugar, D. and Weinmann, R. (1986) J. Biol. Chem. 261, 3414–3419.
[14] Hathaway, G.M. and Traugh, J.A. (1984) J. Biol. Chem. 259, 7011–7015.
[15] Egyházi, E. and Pigon, A. (1986) Chromosoma 94, 329–336.
[16] Dahmus, M.E. (1981) J. Biol. Chem. 256, 3319–3325.
[17] Padmanabha, R. and Glover, C.V.C. (1987) J. Biol. Chem. 262, 1829–1835.
[18] Saxena, A., Padmanabha, R. and Glover, C.V.C. (1987) Mol. Cell. Biol. 7, 3409–3417.
[19] Lu, H. Zawel, L., Fisher, L., Egly, J.-M. and Reinberg, D. (1992) Nature 358, 641–645.