Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 449-454

PHOSPHORYLATION OF TROPONIN T BY CASEIN KINASE TS

Lorenzo A.Pinna, Flavio Meggio and Marina M.Dediukina*

Istituto di Chimica Biologica dell'Università di Padova, Via Marzolo 3, 35100 Padova (Italy).

Received April 3,1981

SUMMARY

Casein kinase TS (Ck-TS), a protein kinase ubiquitous in mammalian tissues, whose natural substrates are still unknown, can phosphorylate in vitro Troponin T -even more efficiently than casein fractions. The phosphorylation site corresponds to the acetylated N terminal seryl residue which is also phosphorylated in native Troponin T and which actually fulfils the structural requirements of Ck-TS as evidenced with model substrates. It is conceivable therefore that Troponin T might be comprised among the physiological targets of Ck-TS.

INTRODUCTION

"Casein kinase TS" (Ck-TS) is the main cAMP-independent protein kinase of rat liver cytosol active on "acidic" phosphoproteins (caseins and phosvitin) rather than on histones (1,2). It is also present in several other tissues like brain, heart and kidney and in organisms other than mammalians, like chicken, frog and possibly yeast (3). Its physiological targets are still unknown. On the other hand its site specificity, evidenced with model substrates, is fairly well known: apparently this protein kinase recognizes the sequences Ser(Thr)-X-Glu (Asp) (4,5), which are also recognized by the mammary gland casein kinase(s) (reviewed in ref.6); the efficiency of Ck-TS however is especially high whenever two or more acidic residues - rather than only one - are clustered on the C terminal side of the amino acid undergoing phosphorylation.

---

* Permanent address: Perm Polytechnic, U.S.S.R.

Abbreviations: Ck-TS, Casein kinase TS; SDS, Sodium dodecyl sulphate; PMSF, Phenylmethyl sulfonyl fluoride; AE, aminoethylated.

0006-291X/81/090449-06$01.00/0

Interestingly phosphorylated Serines N terminal to very acidic clusters are actually present in some proteins undergoing physiologically meaningful phosphorylations, like Nucleolar Nonhistone phosphoproteins (7) and Troponin T (8). This finding suggested that protein kinases belonging to the Ck-TS type might be responsible for the phosphorylation of these proteins in vivo.

In order to obtain additional information about this hypothesis the phosphorylation of Troponin T by Ck-TS purified from rat liver and heart has been studied. The results obtained provide the clearcut demonstration that in vitro troponin T behaves as a very suitable target for Ck-TS, undergoing phosphorylation at the same N terminal seryl residue which is also phosphorylated in vivo.

EXPERIMENTAL

Casein kinase TS (Ck-TS) was purified from rat liver cytosol according to ref.1: however the Sepharose 6B and Phosphocellulose steps were reversed. 50 μM PMSF was also present throughout the whole preparation. Purified Ck-TS (molecular weight 130,000 according to Sephacryl S200 gel filtration) constantly displayed only two protein bands of Mr 38,000 and 36,000 and a faint band of Mr 24,000 when submitted to Polyacrylamide gel electrophoresis in 0.1% SDS (unpublished data). Quite similar behaviour was observed for rat heart Ck-TS, purified by the same procedure.

Troponin T, isolated from rabbit skeletal muscle and enzymatically dephosphorylated (9) was kindly provided by Dr.B.Gusev.

Protein kinase activity was assayed by incubating for 15 min at 37° Ck-TS with the protein substrates (final concentration 2 mg/ml) in 0.1 ml of a reaction mixture also containing: 60 μM [$^{32}\gamma$P] ATP (specific radioactivity: 25 μCi/μmole), 100 mM Tris-HCl buffer pH 7.5 and 12 mM $MgCl_2$. The reaction was stopped by addition of trichloroacetic acid to 10% (w:v) final concentration. The precipitated protein was washed three times with 3 ml of 10% trichloroacetic acid and dissolved in the minimum volume of 0.1 M NH_4HCO_3. An aliquot was counted in a liquid scintillator. The remaining sample was sometimes employed for gel electrophoresis and/or enzymatic digestion. One unit of Ck-TS was defined as one pmole ^{32}P transferred to whole casein in one min under the conditions described above.

10% Polyacrylamide gel electrophoresis of ^{32}P labeled Troponin T was run in the presence of 0.1% SDS on vertical plates prepared essentially according to ref.10. A single radioactive protein band co-migrating with authentic unlabeled Troponin T and accounting for more than 90% of the radioactivity incorporated by either liver or heart Ck-TS was constantly detected.

Table I: Phosphorylation of Troponin T by Ck-TS.

General conditions are described in the experimental section. 50 units of Ck-TS were added to the reaction mixture.

	^{32}P incorporated into protein (cpm/min)
Troponin T plus Ck-TS (liver)	4,619
Troponin T plus Ck-TS (heart)	3,824
Troponin T, enzyme omitted	107
Ck-TS (liver), Troponin T omitted	45

Sephadex G50 gel filtration of Troponin T digestion products was performed through a 1.8x70 cm column equilibrated with 15% formic acid and operated at a flow rate of 12 ml/hr. 3.4 ml fractions were collected. The column was calibrated with synthetic hexa and dodecapeptides, glucagon, Salmine A1 and ACTH. High voltage paper electrophoresis was run at pH 1.9 (formic acid-acetic acid-H_2O, 2.5:7,8:89.7) for 1 hr (200 V/cm).

Trypsin and pronase digestions of ^{32}P-Troponin T were accomplished according to ref.9.

The isolation and detection of labeled phosphoaminoacids after 6M HCl hydrolysis at 105° were performed according to ref.5.

Materials: α_{s1} and β-caseins were prepared according to ref.11. Pepsin was from Worthington and it was reduced and aminoethylated according to ref.12 before being used as a substrate for Ck-TS. Trypsin and Pronase were from Serva. [$^{32}\gamma$P] ATP was purchased from The Radiochemical Center, Amersham, U.K. All the other chemicals were either from Sigma or from Merck.

RESULTS

The phosphorylation of Troponin T by Ck-TS purified from either liver or heart is shown in Table I. The whole radioactivity incorporated actually co-migrates with the Troponin T protein band upon Polyacrylamide gel electrophoresis in 0.1% SDS. After prolonged incubations with nearly saturating ATP concentrations the amount of ^{32}P incorporated into Troponin T - fully accounted for by Ser-^{32}P - was found to approximate 0.8 mole/mole protein.

In Table II the kinetic constants obtained with Troponin T are compared with those of typical model substrates of Ck-TS. It can be

Table II: Kinetic constants of some protein substrates of Ck-TS.

Ck-TS was purified from liver. Apparent Km and Vmax were calculated by the double reciprocal Lineweaver-Burk plot from 10 min. incubation experiments under the conditions described in the experimental section.

SUBSTRATE	Km, app. μM	Vmax (pmole ^{32}P/min)	Vmax/Km
Troponin T	39	839	21.5
α_{s1}-casein	57	49	0.8
β-casein	60	363	9.3
AE-pepsin	253	180	0.7

seen that the phosphorylation efficiency of Troponin T, and hence its fitness as a phosphate acceptor, is greater than those of α_{s1} and β-casein and of pepsin.

Fig.1 describes the procedure followed for the identification of the phosphorylation site of Troponin T affected by Ck-TS. Upon tryptic digestion of ^{32}P labeled Troponin T all the radioactivity was recovered in the largest predicted fragment, corresponding to the N terminal sequence of 40 aminoacids, which can be readily isolated by Sephadex G50 gel chromatography from all the other digestion products. Such a fragment includes two seryl residues, at its 1st and 18th positions respectively. Upon pronase subdigestion however the whole radioactivity was accounted for by a single hexapeptide, not retained by Dowex 50, furtherly purified by Sephadex G50 gel filtration and displaying an unusual anodic mobility upon paper electrophoresis at pH 1.9 (see Fig.1). Such properties and the aminoacid analysis fully support the identification of such a radioactive hexapeptide with the acetylated N terminal fragment of Troponin T displaying the sequence:

AcSer(P)-Asp-Glu-Glu-Val-Glu (9),

and rule out any involvement of Ser_{18} in the protein kinase reaction.

CONCLUSIONS

The above results allow the following conclusions:

1) Troponin T in vitro behaves as a substrate of Ck-TS more efficient than are the casein fractions routinely employed for assaying this kind of protein kinases.

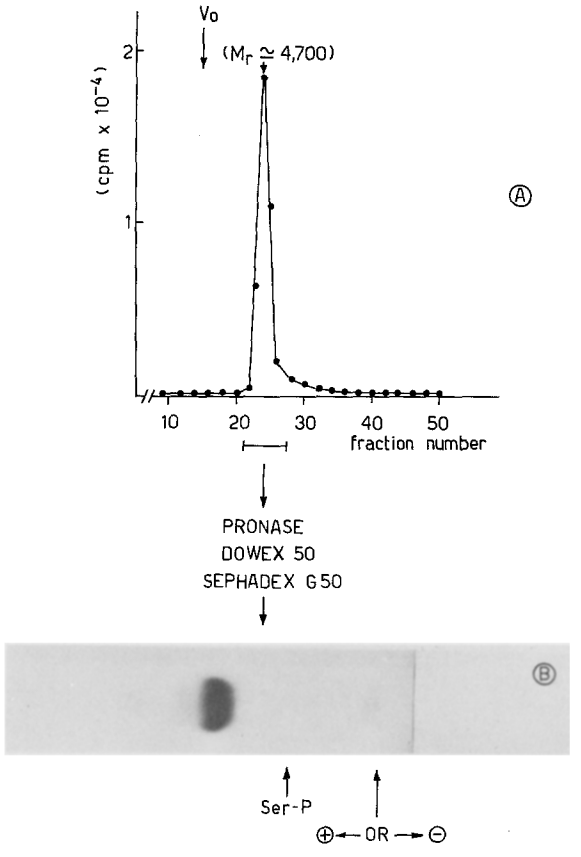

Figure 1: Isolation and identification of the radioactive fragments obtained by Trypsin and Pronase digestion of Troponin T phosphorylated by Ck-TS.
The procedure followed was similar to that of ref.9. 200 µg of trypsin digested ^{32}P-labeled troponin T were firstly submitted to Sephadex G50 gel filtration (A). The radioactive fraction collected as indicated in A was liophylised and furtherly digested with pronase. The pronase digestion products were applied to a Dowex 50 (x4, H^+ form) 0.5x8 cm column. The whole radioactivity was not adsorbed and was eluted by water: once resubmitted to Sephadex G50 gel filtration it gave rise to a single radioactive peak with molecular weight expectable for an hexapeptide (not shown) which, upon high voltage paper electrophoresis at pH 1.9 gives a single anodic radioactive spot devoid of fluorescamine reactivity (autoradiography in B). Before the Dowex 50 treatment the same radioactive spot could be evidenced together with several cathodic fluorescamine positive spots which disappeared after Dowex 50. Further details are given in the experimental section.

2) The residue of Troponin T affected by Ck-TS is the acetylated N terminal Ser_1 exhibiting the structural features actually required for the optimal activity of this enzyme. This finding also supports the

view that the site recognition by Ck-TS is not dependent on residues laying on the N terminal side of the phosphate acceptor aminoacid.

Considering that the N terminal Ser_1 of Troponin T is also phosphorylated in vivo (8) and that Casein kinase TS is present in several tissues, including heart (3) and muscle (A.Donella Deana, personal communication) it is conceivable that Ck-TS might behave as a multifunctional protein kinase exhibiting in some tissues also Troponin T kinase activity.

In the light of these results the possible relationship between Ck-TS and the "Troponin T kinase" reported to affect the same phosphorylation site (9) should be carefully investigated.

ACKNOWLEDGMENTS

We are very grateful to Dr. N.B. Gusev, Moscow State University, for donating Troponin T. The skillful technical assistance of Mr.G. Tasinato and the secretarial aid of Ms.M. Cuccia are gratefully acknowledged.

REFERENCES

1. Meggio,F., Donella Deana,A., Pinna,L.A. and Moret,V. (1977) FEBS Lett. 75, 192-196.
2. Clari,G., Pinna,L.A. and Moret,V. (1976) Biochim.Biophys. Acta 451, 484-490.
3. Meggio,F., Donella Deana,A. and Pinna,L.A. (1981) Anal.Biochem. 110, in press.
4. Pinna,L.A., Donella Deana,A. and Meggio,F. (1979) Biochem. Biophys.Res.Comm. 87, 114-120.
5. Donella Deana,A., Meggio,F. and Pinna,L.A. (1979) Biochem. J. 179, 693-696.
6. Mercier,J-C. (1981) Biochimie 63, 1-17.
7. Mamrack,M.D., Olson,O.J. and Busch, H. (1979) Biochem. 18, 3381-3386.
8. Pearlstone,J.R., Carpenter,M.R., Johnson,P. and Smillie,L.B.(1976) Proc.Natl.Acad.Sci.U.S.A. 73, 1902-1906.
9. Gusev,N.B., Dobrovolskii,A.B. and Severin,S.E. (1980) Biochem. J. 189, 219-226.
10. Laemmli,U.K. (1970) Nature 227, 680-685.
11. Mercier,J-C., Maubois,J.L., Pornanski,S. and Ribadeau-Dumas,B. (1968) Bull.Soc.Chim.Biol. 50, 521-530.
12. Chen,K.C.S., Tao,N. and Tang,J.(1975) J.Biol.Chem. 250, 5068-5075.