Kinase activity associated with caldesmon is Ca^{2+} /calmodulin-dependent kinase II

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The relationship of the kinase which co-purifies with caldesmon to Ca^{2+} /calmodulin-dependent protein kinase II (CaM-kinase II) was investigated by studying the phosphorylation of bovine brain synapsin I, a well-characterized substrate of CaM-kinase II. Synapsin I is a very good substrate ($K_m = 90 \text{ nM}$) of the co-purifying kinase, which phosphorylates two sites in synapsin I, both of which are distinct from the single site phosphorylated by cyclic-AMP-dependent protein kinase. Phosphorylation of synapsin I is Ca^{2+} - and calmodulin-dependent: half-maximal activation occurs at $0.13 \mu\text{M-Ca}^{2+}$ and maximal activity at $0.4 \mu\text{M-Ca}^{2+}$. Phosphorylation of the co-purifying kinase slightly enhances the rate, but does not alter the stoichiometry, of subsequent synapsin I phosphorylation; it does, however, circumvent the requirement for Ca^{2+} and calmodulin. The properties of this kinase therefore closely resemble those of CaM-kinase II, and we conclude that it is probably a smooth-muscle isoenzyme of CaM-kinase II.

INTRODUCTION

We previously reported that smooth-muscle caldesmon is a substrate of a co-purifying Ca^{2+} /calmodulin-dependent protein kinase (Ngai & Walsh, 1984, 1985). Several attempts to separate the kinase from caldesmon were unsuccessful (Scott-Woo & Walsh, 1988a), which, taken with several indirect lines of evidence, suggested that this kinase activity resides within the caldesmon molecule itself (Scott-Woo & Walsh, 1988a). However, subsequent publication of the deduced amino acid sequence of chicken gizzard caldesmon did not reveal a consensus protein kinase sequence (Bryan *et al.*, 1989). There are five known Ca^{2+} /calmodulin-dependent protein kinases: Ca²⁺/calmodulin-dependent protein kinases (CaM-kinases) I, II and III, myosin light-chain kinase and glycogen phosphorylase kinase (Nairn *et al.*, 1985). The properties of the kinase co-purifying with caldesmon most closely resemble those of CaM-kinase II (Scott-Woo & Walsh, 1988b). One of the best characterized substrates of CaM-kinase II is synapsin I (Huttner *et al.*, 1981; Czernik *et al.*, 1987) and we have previously shown that synapsin I is phosphorylated by caldesmon preparations (Scott-Woo & Walsh, 1988a). We have therefore carried out a detailed study of the phosphorylation of synapsin I by caldesmon containing endogenous kinase activity, and conclude that this enzyme of smooth muscle is closely related to, and may be an isoenzyme of, CaM-kinase II.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Materials for SDS/PAGE were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). The *Staphylococcus aureus* V8 proteinase was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). General laboratory reagents were of analytical grade or better, and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

Protein purifications

The following proteins were purified by previously described methods: bovine brain CaM (Walsh *et al.*, 1984), chicken gizzard caldesmon containing endogenous kinase activity (Scott-Woo & Walsh, 1988a), bovine brain synapsin I (Ueda & Greengard, 1977), and the catalytic subunit of bovine cardiac type II cyclic-AMP-dependent protein kinase (A-kinase) (Demaillie *et al.*, 1977).

Enzymic assays

Protein phosphorylations were assayed at 30 °C under the following conditions: (i) 20 mM-Tris/HCl (pH 7.5), 5 mM-MgCl₂, CaM (42 µg/ml), caldesmon (20 µg/ml) containing endogenous kinase activity, synapsin I ($\pm 20 \mu\text{g/ml}$), 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (168 c.p.m./pmol); (ii) 20 mM-Tris/HCl (pH 7.5), 4 mM-MgCl₂, 1 mM-EGTA, A-kinase (catalytic subunit) (0.8 µg/ml), 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Total P_i incorporation into protein was quantified as previously described (Walsh *et al.*, 1983). Phosphorylation of individual proteins was quantified by Čerenkov counting of radioactivity in slices after SDS/PAGE of reaction mixtures, and by scanning laser densitometry of autoradiograms after SDS/PAGE. To determine the Ca^{2+} -dependence of synapsin I phosphorylation, a range of free Ca^{2+} concentrations was obtained by using Ca^{2+} -EGTA buffers (Fabiato, 1981).

'Cleveland' peptide mapping

Synapsin I (40 µg/ml) was phosphorylated under standard conditions by caldesmon containing endogenous kinase activity (to 1.8 mol of P_i/mol) or by A-kinase (to 1.0 mol of P_i/mol). Samples containing 2 µg of synapsin I were subjected to SDS/PAGE, gels were stained briefly and destained, and the synapsin I bands were cut out of the gel and digested with *S. aureus* V8 proteinase as previously described (Walsh *et al.*, 1984). Peptides were separated by SDS/PAGE and ³²P-labelled peptides detected by autoradiography.

Abbreviations used: A-kinase, cyclic-AMP-dependent protein kinase; CaM, calmodulin; CaM-kinase II, Ca^{2+} /calmodulin-dependent protein kinase II.

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Fig. 1. Phosphorylation of synapsin I by the kinase co-purifying with caldesmon and by A-kinase

Protein phosphorylations were carried out as described in the Materials and methods section. Key: ○, ■, P_i incorporation into synapsin I and caldesmon, respectively, when the two proteins were incubated together; □, P_i incorporation into synapsin I by A-kinase; ●, phosphorylation of caldesmon (no synapsin I present). The insets beneath the time-course data depict autoradiograms of SDS gels of the different reaction mixtures at corresponding times (reading from left to right within each of the three sets): K, kinase co-purifying with caldesmon; S, synapsin I; C, A-kinase catalytic subunit; CaD, caldesmon.

RESULTS

Caldesmon becomes phosphorylated when incubated with $MgATP^{2-}$ in the presence of Ca^{2+} and CaM (Fig. 1) as previously shown (Ngai & Walsh, 1984). In the presence of synapsin I, however, caldesmon phosphorylation is markedly inhibited, whereas the exogenous substrate is rapidly phosphorylated (Fig. 1). Consistent with this observation, the kinase has a very high affinity for synapsin I: the K_m for synapsin I was determined to be 90 nM (results not shown).

Phosphorylation of synapsin I by the catalytic subunit of A-kinase is shown in Fig. 1 for comparison. The Ca^{2+} /CaM-dependent kinase catalyses the incorporation of approximately twice as much [^{32}P] P_i (1.86 mol of P_i /mol) into synapsin I as does A-kinase (0.75 mol of P_i /mol). It is well known that A-kinase

Fig. 2. 'Cleveland' peptide mapping of phosphorylated forms of synapsin I

Phosphorylated forms of synapsin I (S) were purified by SDS/PAGE and subjected to 'Cleveland' peptide mapping as described in the Materials and methods section. This figure depicts the phosphopeptides on an autoradiogram after SDS/PAGE. Key to gel lanes: 1-4, synapsin I phosphorylated to 1.8 mol/mol by the kinase co-purifying with caldesmon, +0, 0.25, 1 and 2.5 μ g, respectively, of *S. aureus* V8 proteinase; 5-8, synapsin I phosphorylated to 1.0 mol/mol by A-kinase, +0, 0.25, 1 and 2.5 μ g,

respectively, of *S. aureus* V8 proteinase. The Coomassie-Blue-stained gel (not shown) indicated that identical peptides were generated from the two phosphorylated synapsin I samples.

Fig. 3. Loss of Ca²⁺/CaM-dependence of synapsin I phosphorylation

Caldesmon (0.2 mg/ml) containing endogenous kinase activity was incubated in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.1 mM-CaCl₂ containing CaM (42 µg/ml) and 0.2 mM-[γ-³²P]ATP (150 c.p.m./pmol) for 30 min at 30 °C. This preparation was then used to phosphorylate synapsin I under standard conditions (see the Materials and methods section) in the presence of 0.1 mM-CaCl₂ (○) or 1 mM-EGTA (●). The insets depict autoradiograms after SDS/PAGE: (a) + Ca²⁺; (b) + EGTA. Laser densitometry of the autoradiograms was used to determine the contribution of caldesmon phosphorylation to total [³²P]phosphate incorporation, and the numerical values have been corrected for caldesmon phosphorylation. Abbreviations: S, synapsin I; CaD, caldesmon.

phosphorylates a single site in synapsin I (Huttner *et al.*, 1981). Therefore, the Ca²⁺/CaM-dependent kinase probably phosphorylates two sites in synapsin I. The Ca²⁺- and CaM-dependence of synapsin I phosphorylation was verified in separate experiments (results not shown). In the presence of excess CaM, half-maximal activation occurred at 0.13 µM-Ca²⁺ and maximal activity at 0.4 µM-Ca²⁺. A small loss of activity was observed at higher [Ca²⁺].

The A-kinase phosphorylation site (site 1) and the two CaM-kinase II phosphorylation sites (sites 2 and 3) are located in distinct domains of synapsin I (Huttner *et al.*, 1981). Site 1 is located in a 10 kDa peptide generated by proteolysis with the *S. aureus* V8 proteinase. 'Cleveland' peptide mapping of synapsin I phosphorylated by the caldesmon preparation generated a 35 kDa phosphopeptide which retained both the phosphorylation sites (Fig. 2). There was no significant phosphorylation of the 10 kDa peptide. On the other hand, digestion of synapsin I after phosphorylation by A-kinase revealed no phosphorylation of the 35 kDa peptide. Rather, labelled peptides of 15 and 13 kDa were generated at low proteinase concentrations, and the 10 kDa phosphopeptide appeared at higher proteinase concentrations. We therefore conclude that the sites in synapsin I phosphorylated by the caldesmon preparation are distinct from the A-kinase site.

If the caldesmon preparation was incubated with MgATP²⁻ in the presence of Ca²⁺ and CaM for 30 min under standard conditions before addition of synapsin I, the stoichiometry of synapsin I phosphorylation was unaffected (2.0 mol of P_i/mol),

although the rate was enhanced from 119.1 to 310.3 pmol of P_i/min (results not shown). However, most interestingly, pre-phosphorylation of the caldesmon preparation was accompanied by loss of Ca²⁺/CaM-dependence of synapsin I phosphorylation (Fig. 3). We consistently find that the rate of phosphorylation in the absence of Ca²⁺ is lower than in the presence of Ca²⁺, but the maximum phosphate incorporation is the same.

DISCUSSION

Caldesmon is a major actin- and CaM-binding protein originally identified in smooth muscle (Sobue *et al.*, 1981), but now known to be widely distributed among muscle and non-muscle tissues (Clark *et al.*, 1986; Ngai & Walsh, 1985). Although its function is not clearly defined, it has been implicated in the regulation of actin-myosin interaction in smooth muscle (for review see Walsh, 1987). Isolated caldesmon can exist in phosphorylated and unphosphorylated forms (Ngai & Walsh, 1984, 1987); the unphosphorylated protein inhibits actin-activated myosin Mg²⁺-ATPase activity in a reconstituted smooth-muscle system *in vitro*, whereas the phosphorylated protein does not (Ngai & Walsh, 1987). Phosphorylation is catalysed by a Ca²⁺/CaM-dependent protein kinase. Caldesmon phosphorylation has also been demonstrated in intact vascular smooth muscle at rest and in response to several pharmacological agents (Adam *et al.*, 1989).

We suggested previously that the kinase activity which co-purifies with caldesmon actually resides in the caldesmon molecule itself (Scott-Woo & Walsh, 1988a). However, determination of the complete amino acid sequence of gizzard caldesmon did not reveal any protein kinase or ATP-binding-site consensus sequences (Bryan *et al.*, 1989), suggesting that the kinase activity found in caldesmon preparations is a contaminating enzyme rather than an intrinsic property of caldesmon. Comparison of the known properties of this kinase with those of well-characterized CaM-dependent kinases revealed that the kinase most closely resembles CaM-kinase II (Scott-Woo & Walsh, 1988b). A survey of potential exogenous substrates revealed only one protein which was phosphorylated rapidly and stoichiometrically, i.e. synapsin I (Scott-Woo & Walsh, 1988a). This protein is an excellent substrate of CaM-kinase II (Huttner *et al.*, 1981). Therefore, we characterized the phosphorylation of synapsin I by the caldesmon preparation in detail.

The following properties are shared with CaM-kinase II (cf. Colbran *et al.*, 1989). (1) The enzyme phosphorylates synapsin I in a Ca^{2+} /CaM-dependent manner. (2) There are two phosphorylation sites in synapsin I, both of which are distinct from the A-kinase site. (3) Autophosphorylation of the caldesmon preparation results in loss of the requirement of Ca^{2+} /CaM for synapsin I phosphorylation. (4) The autophosphorylated enzyme has lower activity in the absence than in the presence of Ca^{2+} /CaM. (5) Treatment of the caldesmon preparation with chymotrypsin generates a Ca^{2+} /CaM-independent form of the kinase (Scott-Woo & Walsh, 1988a). We conclude that the kinase activity associated with caldesmon preparations is most likely a smooth-muscle isoenzyme of CaM-kinase II. Several attempts to separate kinase activity from caldesmon have been unsuccessful (Scott-Woo & Walsh, 1988a), suggesting a tight association between the enzyme and its substrate.

After submission of this manuscript, a paper was published in which Abougou *et al.* (1989) also concluded, on the basis of a different experimental approach, that caldesmon preparations are contaminated with CaM-kinase II.

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REFERENCES

- Abougou, J.-C., Hagiwara, M., Hachiya, T., Terasawa, M., Hidaka, H. & Hartshorne, D. J. (1989) *FEBS Lett.* **257**, 408–410
- Adam, L. P., Haerberle, J. R. & Hathaway, D. R. (1989) *J. Biol. Chem.* **264**, 7698–7703
- Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G. & Lin, W.-G. (1989) *J. Biol. Chem.* **264**, 13873–13879
- Clark, T., Ngai, P. K., Sutherland, C., Gröschel-Stewart, U. & Walsh, M. P. (1986) *J. Biol. Chem.* **261**, 8028–8035
- Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K. & Soderling, T. R. (1989) *Biochem. J.* **258**, 313–325
- Czernik, A. J., Pang, D. T. & Greengard, P. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7518–7522
- Demaille, J. G., Peters, K. A. & Fischer, E. H. (1977) *Biochemistry* **16**, 3080–3086
- Fabiato, A. (1981) *J. Gen. Physiol.* **78**, 457–497
- Huttner, W. B., DeGennaro, L. J. & Greengard, P. (1981) *J. Biol. Chem.* **256**, 1482–1488
- Nairn, A. C., Hemmings, H. C., Jr. & Greengard, P. (1985) *Annu. Rev. Biochem.* **54**, 931–976
- Ngai, P. K. & Walsh, M. P. (1984) *J. Biol. Chem.* **259**, 13656–13659
- Ngai, P. K. & Walsh, M. P. (1985) *Biochem. Biophys. Res. Commun.* **127**, 533–539
- Ngai, P. K. & Walsh, M. P. (1987) *Biochem. J.* **244**, 417–425
- Scott-Woo, G. C. & Walsh, M. P. (1988a) *Biochem. J.* **252**, 463–472
- Scott-Woo, G. C. & Walsh, M. P. (1988b) *Biochem. J.* **255**, 817–824
- Sobue, K., Muramoto, Y., Fujita, M. & Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5652–5655
- Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 5155–5163
- Walsh, M. P. (1987) *Prog. Clin. Biol. Res.* **245**, 119–141
- Walsh, M. P., Hinkins, S., Dabrowska, R. & Hartshorne, D. J. (1983) *Methods Enzymol.* **99**, 279–288
- Walsh, M. P., Valentine, K. A., Ngai, P. K., Carruthers, C. A. & Hollenberg, M. D. (1984) *Biochem. J.* **224**, 117–127

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