ELSEVIER

FEBS Letters 337 (1994) 128–130

FEBS 13508

FEBS LETTERS

The role of the autoinhibitory domain in differential metal ion activation of calmodulin-stimulated phosphatase

Noriko Yokoyama[a,*], Jerry H. Wang[b]

[a]*Department of Physiology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271, Japan*
[b]*Department of Biochemistry, MRC Group in Signal Transduction, The University of Calgary, Calgary, Alba, T2N 4N1, Canada*

Received 1 November 1993; revised version received 23 November 1993

Abstract

Metal ion activators, Ni^{2+} and Mn^{2+}, have been suggested to induce different conformations of calmodulin (CaM)-stimulated phosphatase. In the present study, an autoinhibitory domain previously implicated in the conformation transition of CaM stimulation of the phosphatase is shown to participate in defining the differential metal ion activation. A proteolytic derivative of the phosphatase deleted from the autoinhibitory domain displayed CaM-independent Mn^{2+}-stimulated activity which was about 4-times that of the CaM-stimulated activity of the native enzyme. The Ni^{2+}-stimulated activity of the derivative, on the other hand, retained slight CaM-dependence, and the CaM-stimulated activity was 90% of that of the native enzyme. A synthetic peptide corresponding to the autoinhibitory domain could inhibit the Mn^{2+}-stimulated activity of the phosphatase derivative by 80%, but had little effect on the Ni^{2+}-stimulated activity.

Key words: Autoinhibitory domain; Synthetic autoinhibitory peptide; CaM-stimulated phosphatase; Metal ion activation

1. Introduction

CaM-stimulated phosphatase (also known as calcineurin or protein phosphatase 2B) possesses complex regulatory properties [1,2]. In addition to regulation by CaM, the purified enzyme shows a dependence on specific metal ion activators for activity. The various metal ions, Ni^{2+}, Mn^{2+} and Mg^{2+} plus Ca^{2+}, activate the enzyme to different levels of phosphatase activity with different degrees of CaM dependence [3–5]. The enzyme activations by Ni^{2+} and Mn^{2+} are both time-dependent, suggestive of conformational changes of the enzyme. Kinetic analysis of the timecourse of activation and immunological evidence suggest that the Ni^{2+}- and Mn^{2+}-induced forms of the phosphatase are distinct [4,5,7,8]. The nature of the differential metal ion activations, however, is not defined in terms of the enzyme structure.

CaM-stimulated phosphatase is composed of two subunits, α and β, of molecular weights of approximately 61,000 and 20,000 Da, respectively [9–14]. Structure–function analysis of the enzyme using controlled proteolysis has revealed several functional domains. The α subunit is the catalytic subunit, but it also contains regulatory domains such as the CaM-binding domain and an autoinhibitory domain, as well as binding sites for Mn^{2+} and Ni^{2+} [2,6,9,12,15]. The β subunit is a Ca^{2+}-binding unit with four EF hand structures [9,14,16]. Proteolytic derivatives of the phosphatase with specific domains deleted have been obtained [17]. A synthetic peptide derived from the inhibitory domain has been shown to inhibit CaM-stimulated phosphatase activity using ^{32}P-labelled myosin light chain as a substrate [18]. In this study, we use the controlled proteolysis and synthetic peptide approaches to demonstrate that the inhibitory domain plays an important role in the differential metal ion activation of the enzyme.

2. Materials and methods

2.1. Materials

Clostripain and *N-p*-tosyl-L-lysine chloromethylketone was purchased from Sigma. Synthetic autoinhibitory peptide, ITSFEEAKGLDRINERMPPRRDAMP, was from Takara Biomedicals (Japan).

2.2. Proteins

The major isoform of bovine brain CaM-stimulated phosphatase, designated as BPI, was purified as described previously [19] and used in this study.

2.3. Digestion of CaM-stimulated phosphatase with clostripain

Digestion was carried out according to the procedure of Hubbard et. al. [17]. Digestion was carried out in 115 μl of reaction mixture containing 40 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 8 mM dithiothreitol and CaM-stimulated phosphatase (20 μg) with or without CaM (20 μg) at 30°C. Proteolysis was started by the addition of 0.17 U of clostripain, and stopped by the addition of *N-p*-tosyl-L-lysine chloromethylketone (24 μg). Digestion with CaM was carried out for 2.5 min when more than 95% of CaM-stimulated phosphatase α subunit had been converted to a 57 kDa derivative. For some experi-

*Corresponding author. Fax: (81) (473) 646 295.

SSDI 0014-5793(93)E1425-L

ments, CaM was removed from the phosphatase derivative by chromatography on a FPLC-Superose 12 column and then the 57 kDa derivative was used. Digestion in the absence of CaM was carried out for 15 min when all CaM-stimulated phosphatase α subunit was converted to a 43 kDa and 40 kDa doublet.

2.4. Assay for CaM-stimulated phosphatase activity

CaM-stimulated phosphatase was assayed using pNPP as described previously [5]. The reaction mixture contained 50 mM Tris-HCl (pH 7.0), 3.4 mM pNPP, 0.1 mg/ml bovine serum albumin with or without 10–30 μg/ml CaM, and metal ion activators such as Ni^{2+}, Mn^{2+}, Mg^{2+} or Ca^{2+} as indicated. To analyze the effect of the autoinhibitory domain peptide, CaM-stimulated phosphatase was preincubated with metal ion as indicated and the synthetic peptide with or without CaM for 15 min and the assay was started by addition of pNPP. Specific activity of CaM-stimulated phosphatases which were used in this study was 1.5–3.2 μmol pNPP hydrolyzed/min/mg protein in the presence of Ni^{2+} and CaM.

3. Results and discussion

3.1. Effect of proteolysis on metal ion activation of CaM-stimulated phosphatase

Hubbard and Klee [17] showed that controlled digestion of CaM-stimulated phosphatase by clostripain produced well-defined active phosphatase derivatives with the carboxy-terminal region of the α subunit truncated, one with a 57 kDa α subunit derivative and another with a 43 kDa α subunit derivative. While the native phosphatase is strongly dependent on CaM for activity, the amino-terminus truncated derivatives were shown to display CaM-independent activity [17]. These results have led to the suggestion that CaM-stimulated phosphatase contains, in the carboxy-terminal region of the α subunit, an autoinhibitory domain which interacts with the catalytic domain to suppress the enzyme activity. CaM-stimulation of the enzyme is due to the disruption of the interaction between the autoinhibitory and the catalytic domains, whereas proteolytic activation of the enzyme results from the removal of the autoinhibitory domain from the enzyme protein.

Purified bovine brain CaM-stimulated phosphatase depends on various added metal ions for activity. The different metal ion activation conditions have been suggested to induce different active forms of the enzyme. To explore the possibility that the differential metal ion activation involves the participation of the carboxy-terminal region of the α subunit of the enzyme, the effects of clostripain digestion on the enzyme activity under the various metal ion activation conditions were examined.

In the original study [17], the enzyme activity was examined only by using Mg^{2+} plus Ca^{2+} as the activator. Table 1 shows that the effects of clostripain digestion on the phosphatase activity are distinct under different activation conditions. When the enzyme was assayed using Mg^{2+} plus Ca^{2+} or Ni^{2+} as metal activators, the conversion of the native phosphatase to the 57 kDa derivative was accompanied by a marked increase in CaM-independent phosphatase activity or an activity slightly lower than the CaM-stimulated activity of the native enzyme.

Table 1
Effect of proteolysis on metal ion activation of CaM-stimulated phosphatase activity

	CaM	Phosphatase activity (%)		
		Native	57 kDa	43 kDa
Ni^{2+}	+	100	90.41 ± 7.41	33.24 ± 4.31
	−	18.34 ± 4.26	45.47 ± 5.01	–
Mn^{2+}	+	16.58 ± 2.58	60.05 ± 10.26	38.08 ± 6.62
	−	5.23 ± 1.81	60.02 ± 5.49	–
Ca^{2+}, Mg^{2+}	+	9.72 ± 4.35	6.11 ± 2.45	2.82 ± 1.55
	−	1.50 ± 0.77	5.23 + 2.89	–

The concentration of activators used were: 1 mM Ni^{2+}, 1 mM Mn^{2+}, 0.1 mM Ca^{2+}, 3 mM Mg^{2+} and CaM 10–30 μg/ml. Reactions were carried out at pH 7.0, n = 6–24. Phosphatase activity was expressed as the percentage of Ni^{2+}/CaM-stimulated activity as 100%.

Under the Mn^{2+} activation conditions, a more drastic increase in CaM-independent activity resulted from the proteolytic digestion, so that the CaM-independent activity reached almost 4-times that of the CaM-stimulated activity on the native enzyme. One possible explanation for the drastic increase in Mn^{2+}-stimulated phosphatase activity upon clostripain treatment is that the carboxy-terminal region exerts inhibitory activity on the enzyme even when CaM is bound.

The 57 kDa derivative maintains the CaM-binding activity, but it is not activated by CaM under the Mn^{2+} or Mg^{2+} plus Ca^{2+} activation conditions. However, it could be activated, about 2-fold by CaM when Ni^{2+} was used as the metal activator (Table 1). The 43 kDa α subunit derivative displayed a generally lower enzyme activity than the 57 kDa derivative. It showed no CaM-activation. This is to be expected since the CaM-binding domain of this enzyme is absent.

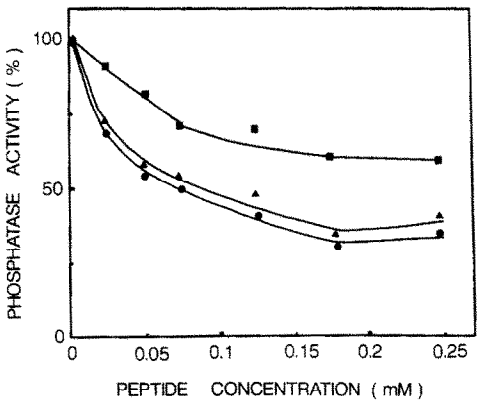

Fig. 1. Effect of autoinhibitory peptide on CaM-stimulated phosphatase under various metal ion activation conditions. The pNPP phosphatase activity with CaM was expressed as the percentage of the enzyme activity in absence of the peptide. (▲), (●) and (▲) were activities assayed by using 1 mM Ni^{2+}, 1 mM Mn^{2+} and 3 mM Mg^{2+} plus 0.1 mM Ca^{2+} as metal ion activator, respectively. The reaction were carried out at pH 7.0.

Table 2
Effects of an autoinhibitory peptide on CaM-stimulated phosphatase and proteolyzed phosphatase activity

	CaM	Phosphatase activity (%)		
		Ni^{2+}	Mn^{2+}	Ca^{2+}, Mg^{2+}
Native	+	75.8 ± 8.4	48.8 ± 9.8	55.3 ± 5.3
57 kDa	+	96.3 ± 6.4	20.1 ± 4.2	48.7 ± 4.9
	−	95.3 ± 10.2	–	–
43 kDa	+	89.4 ± 11.2	18.1 ± 0.8	50.5 ± 5.6

Phosphatase activity without the autoinhibitory peptide was taken as 100%. The autoinhibitory domain peptide concentration was 0.125 mM. The concentration of metal ions were the same as in Table 1. Reactions were carried out at pH 7.0, n = 3–9.

3.2. *Effect of synthetic autoinhibitory peptide on differential metal ion activation of CaM-stimulated phosphatase*

Hashimoto et al. [18] identified a peptide sequence within the 4 kDa carboxy-terminal fragment, ITSFEEAKGLDRINERMPPRRDAMP, which showed specific and relatively potent inhibition of the phosphatase. This peptide has been suggested to represent the autoinhibitory domain, and may be used to further study the involvement of the autoinhibitory domain in the differential metal ion activation of CaM-stimulated phosphatase. Fig. 1 shows that the CaM-stimulated phosphatase activity was inhibited by the autoinhibitory peptide under all activation conditions with a similar dose dependency. However, the extents of inhibition differed. With Mg^{2+} plus Ca^{2+} or Mn^{2+} as metal activators, the CaM-stimulated phosphatase activity was inhibited maximally 65%, whereas the Ni^{2+}/CaM-stimulated activity was inhibited about 40% (Fig. 1).

The differential effects of autoinhibitory peptide on the phosphatase activity of the enzyme under different metal ion activation conditions can be more clearly demonstrated using the proteolytic derivatives of het phosphatase. Table 2 shows that the inhibition of both the 57 and 43 kDa enzyme species by the autoinhibitory peptide showed strong dependence on metal ion activation conditions. In particular, while the enzyme derivatives were only marginally inhibited by the peptide (about 10%) when Ni^{2+}-activated activity of the enzyme was measured, the Mn^{2+}-activated activity was drastically inhibited (about 80%). The result further supports the involvement of the carboxy-terminal region of the α subunit in the induction of different metal ion activation forms of the enzyme. Under the Mn^{2+}-activation conditions, the autoinhibitory domain appears to maintain a strong interaction with the catalytic domain to suppress the enzyme activity even in the presence of CaM. On the other hand, in the Ni^{2+}-activated form, the autoinhibitory domain does not exert strong action towards the enzyme activity.

Acknowledgements: This study was partially supported by a Nihon University Grant in 1992.

References

[1] Pallen, C.J., Sharma, R.K., Matsui, H. and Wang, J.H. (1985) in: Advances in protein phosphatases 1 (W. Merlevede and J. Di Salvo, eds.) pp. 147–161, Leven University Press, Belgium.
[2] Klee, C.B. and Cohen, P. (1988) in: Molecular Aspects of Cellular Regulation, vol. 5 (Cohen, P. and Klee, C.B. eds.) pp. 225–245, Elsevier, Amsterdam.
[3] King, M.M. and Huang, C.Y. (1983) Biochem. Biophys. Res. Commun. 144, 955–961.
[4] King, M.M. and Huang, C.Y. (1984) J. Biol. Chem. 259, 8847–8856.
[5] Pallen, C.J. and Wang, J.H. (1983) J. Biol. Chem. 258, 8550–85536.
[6] Pallen, C.J. and Wang, J.H. (1986) J. Biol. Chem. 261, 16115–16120.
[7] Matusi, H., Pallen, C.J., Adachi, A.-M., Wang, J.H. and Lam, H.-Y. (1985) J. Biol. Chem. 260, 4174–4179.
[8] Yokoyama, N., Ali, Z. and Wang, J.H. (1993) Arch. Biochem. Biophys. 300, 615–621.
[9] Klee, C.B., Crouch, T.H. and Krinks, M.H. (1979) Proc. Natl. Acad. Sci. USA 76, 6270–6273.
[10] Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B. and Cohen, P. (1982) FEBS Lett. 137, 80–84.
[11] Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120–126.
[12] Sharma, R.K., Desai, R., Waisman, D.M. and Wang, J.H. (1979) J. Biol. Chem. 254, 4276–4282.
[13] Wallace, R.W., Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1979) J. Biol. Chem. 254, 377–382.
[14] Aitken, A., Klee, C.B. and Cohen, P. (1984) Eur. J. Biochem. 139, 663–671: Li, H.-C. (1984) J. Biol. Chem. 259, 8801–8807.
[15] Kincaid, R.L., Nightingale, M.S. and Martin, B.M. (1988) Proc. Natl. Acad. Sci. USA 85, 8983–8987.
[16] Kretsinger, R.H. (1980) CRC, Crit. Rev. Biochem. 8, 119–174.
[17] Hubbard, M.J. and Klee, C.B. (1989) Biochemistry 28, 1868–1874.
[18] Hashimoto, Y., Perrino, B.A. and Soderling, T.R. (1980) J. Biol. Chem. 265, 1924–1927.
[19] Yokoyama, N. and Wang, J.H. (1991) J. Biol. Chem. 266, 14822–14829.