Biochemical and Biophysical Research Communications 475 (2016) 277–282

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

High-performance CaMKI: A highly active and stable form of CaMKIδ produced by high-level soluble expression in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 8 May 2016
Accepted 11 May 2016
Available online 17 May 2016

Keywords:
Autonomous
Catalytic fragment
One-step purification
Overexpression
Thermal stability
Truncation

ABSTRACT

We describe here the expression and characterization of a constitutively active fragment of zebrafish Ca^{2+}/calmodulin-dependent protein kinase (CaMK) Iδ designated zCaMKIδ(1–299) that lacks an autoinhibitory domain. We used a simple one-step purification method to isolate the recombinant enzyme at high yield (220 mg/l of the culture medium) from the soluble fraction of lysates prepared from *Escherichia coli*. Unlike the corresponding fragment of CaMKIα (CaMKIα(1–294)), the kinase activity of zCaMKIδ(1–299), without activation procedures, was comparable to that of wild-type zCaMKIδ activated by CaMK kinase. zCaMKIδ(1–299) exhibited broad substrate specificity highly similar to that of wild-type zCaMKIδ, and complementary to that of the cAMP-dependent protein kinase catalytic subunit (PKAc). The protein kinase activity of zCaMKIδ(1–299) was higher compared with that of PKAc as well as CX-30K-CaMKII that comprises a constitutively active fragment of CaMKII fused to the N-terminal region of *Xenopus* CaMKI. Furthermore, kinase activity was highly stable against thermal inactivation and repeated freezing-thawing. Thus, zCaMKIδ(1–299) represents a readily available alternative that can be used as a "High-performance phosphorylating reagent" alone or in combination with PKAc in diverse experiments on protein phosphorylation and dephosphorylation.

1. Introduction

Multifunctional protein kinases such as cyclic AMP (cAMP)-dependent protein kinase (PKA) and Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKs) phosphorylate diverse protein substrates and play important roles in cellular signal transduction. PKA, which comprises two regulatory subunits with a cAMP-binding site and two catalytic subunits, is extensively studied as a prototypical Ser/Thr protein kinase. The well-characterized catalytic subunit of PKA (PKAc) phosphorylates various proteins in a cAMP-independent manner [1]. Therefore, PKAc is widely used as a commercially available "phosphorylating reagent" for a variety of experiments concerning protein phosphorylation and dephosphorylation [2].

CaMKI, CaMKII, and CaMKIV, which are collectively called as CaMKs, are known as another type of multifunctional protein kinases [3]. They are composed of an N-terminal catalytic domain and a central regulatory domain called the autoinhibitory domain including CaM-binding domain. Binding of Ca^{2+}/CaM to the autoinhibitory domain of CaMK leads to activation of the kinase. CaMK-kinase (CaMKK), which resides functionally "upstream" in the CaMK signaling cascade, activates CaMKI and CaMKIV through Ca^{2+}/CaM-dependent phosphorylation of a Thr residue in the activation loop of the latter kinases. Meanwhile, Ca^{2+}/CaM-dependent autophosphorylation of CaMKII induces dramatic

Abbreviations: CaM, calmodulin; CaMKs, multifunctional Ca^{2+}/CaM-dependent protein kinases; CaMKK, CaMK-kinase; 30K-CaMKII, constitutively active 30-kDa fragment of CaMKII; CREB, cAMP-responsive element-binding protein; MBP, myelin basic protein; MLC, myosin light chain; PKAc, catalytic subunit of cAMP-dependent protein kinase.

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http://dx.doi.org/10.1016/j.bbrc.2016.05.060
0006-291X/

changes in enzymatic properties such as generation of Ca^{2+}/CaM-independent activity [3].

Chymotryptic digestion of autophosphorylated CaMKII produces a constitutively active (autonomous) 30-kDa fragment (30K-CaMKII) [4]. The Ca^{2+}/CaM-independent 30K-CaMKII fragment is therefore used as a convenient "phosphorylating reagent" instead of PKAc [5–7]. However, its preparation from rat brain requires sacrificing numerous rats, the protocol is extremely time-consuming and laborious, and its kinase activity is highly labile [8]. To circumvent these drawbacks, we generated a recombinant 30K-CaMKII fused with the N-terminal region of *Xenopus* CaMKI (CX-30K-CaMKII) that is expressed at high levels in *Escherichia coli* [9]. Nevertheless, the improved procedure requires tedious solubilization, denaturation, and renaturation steps.

CaMKI and CaMKII play pivotal roles in neuronal functions [3]. The kinase activity of a constitutively active fragment of rat CaMKIα (rCaMKIα(1–294)) [10,11] produced in *E. coli* is much lower compared with that of wild-type rCaMKIα (rCaMKIα(WT)) when fully activated by CaMKK. Because the activity of rCaMKIα(1–294) prepared from cultured cells is equivalent to that of activated rCaMKIα [12], the low activity of rCaMKIα(1–294) produced in *E. coli* is likely due to lack of activation by CaMKK.

We previously isolated cDNA clones encoding zebrafish CaMKIδ (zCaMKIδ) to investigate its biological significance during embryogenesis [13,14]. In the present study, we prepared a constitutively active fragment of zCaMKIδ (zCaMKIδ(1–299)) corresponding to rCaMKIα(1–294) and were surprised to discover that the former, which was produced in *E. coli* without an activating procedure, exhibited activity comparable to that of fully activated zCaMKIδ(WT). Moreover, we used a simple one-step purification method to produce zCaMKIδ(1–299) at high yield from the soluble fraction of *E. coli* lysates. Therefore, zCaMKIδ(1–299) represents a readily available alternative to PKAc as a "High-performance phosphorylating reagent" in experiments aimed to define biochemical and physiological functions of protein phosphorylation and dephosphorylation.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (111 TBq/mmol) was purchased from PerkinElmer. A HiTrap Chelating HP column was purchased from GE Healthcare Bio-Sciences. Mixed histones from calf thymus were from Sigma-Aldrich. Myosin light chain (MLC) was obtained as described previously [15]. Recombinant rat CaM [16], mouse CaMKKα [17], zebrafish CaMKIδ (CaMKIδ-LL) [13], mouse CaMKIδ [14], rat CaMKIα [18], CX-30K-CaMKII [9], mouse myelin basic protein (MBP) [19], and cAMP-responsive element-binding protein (CREB) [20] were expressed in *E. coli* and purified as described.

2.2. Construction of plasmids

pETzCaMKIδ(1–299), pETmCaMKIδ(1–297), and pETrCaMKIα(1–294) were generated using the inverse polymerase chain reaction (PCR) [21]. Sense primer: 5′-CTC GAG CAC CAC CAC CAC CA-3′ (common); antisense primers: 5′-CAT CTG TCG ACT GAC GGA CTC ATG-3′ (pETzCaMKIδ(1–299)), 5′-GAT CTG GGC ACT GAC AGA TTC GTG-3′(pETmCaMKIδ(1–297)), 5′-GAT CTG CTC ACT CAC TGA CTG GTG G-3′ (pETrCaMKIα(1–294)), and templates pETz-CaMKIδ-LL [13], pETmCaMKIδ [14], pETrCaMKIα [18] were used for PCR, respectively. The 5′-ends of PCR products were phosphorylated using T4 polynucleotide kinase (Nippon Gene) and self-ligated using T4 DNA ligase (Nippon Gene). To generate the plasmid pETrPKAc, the primers used for PCR to amplify a rat brain

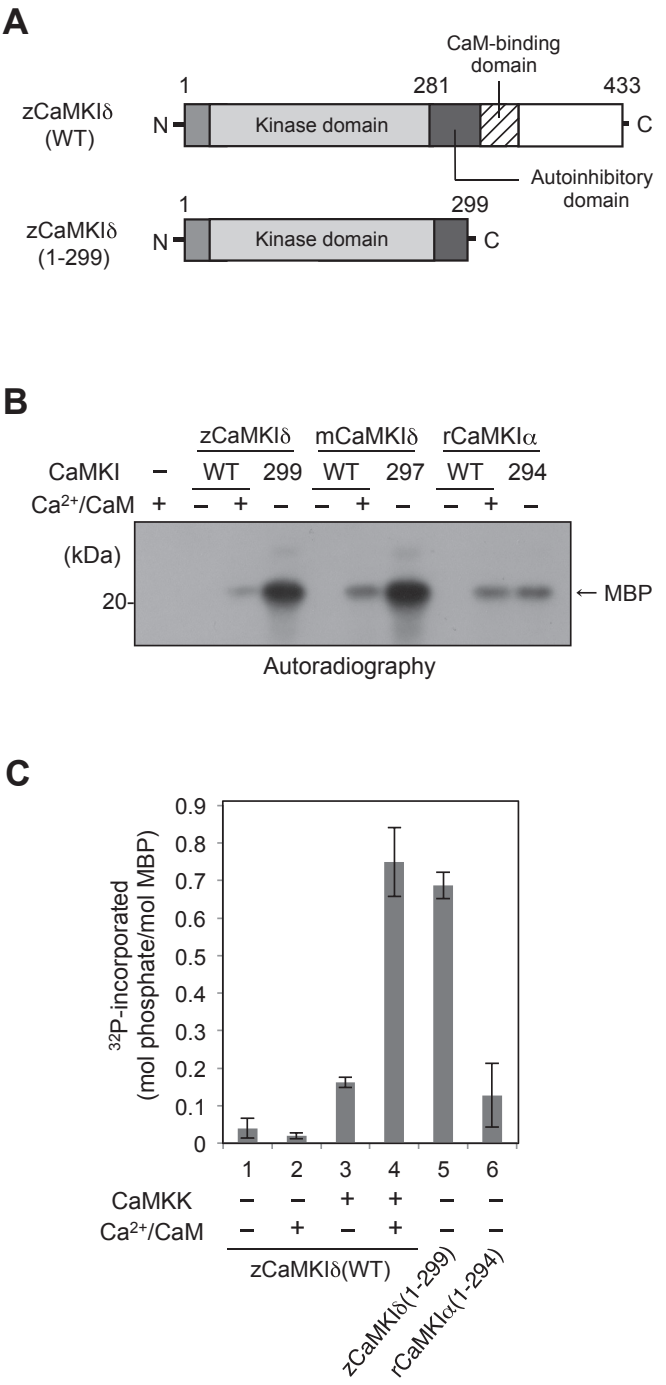

Fig. 1. Construction and characterization of zCaMKIδ(1–299). (A) Primary structures of zCaMKIδ(WT) and zCaMKIδ(1–299). (B) Comparison of the kinase activity of each CaMKI(WT) with constitutively active CaMKIs in the presence or absence of Ca^{2+}/CaM. The kinase activity of CaMKI (100 ng) was measured using an *in vitro* kinase assay with MBP (400 ng) as a substrate in the standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P]ATP in the presence (+) or absence (−) of Ca^{2+}/CaM. Phosphorylation of MBP was analyzed by SDS-PAGE, followed by autoradiography. (C) Comparison of the kinase activities of CaMKIs(WT) with constitutively active CaMKIs in the presence or absence of Ca^{2+}/CaM and CaMKK. Each CaMKI (2.5 μg) was incubated in the standard phosphorylation mixture (20 μl) containing 100 μM ATP in the presence (+) or absence (−) of CaMKK (125 ng). Subsequently, the kinase activity of CaMKI (20 ng) was measured using an *in vitro* kinase assay with MBP (100 ng) as a substrate in the standard phosphorylation mixture (10 μl) containing 100 μM [γ-^{32}P] ATP in the presence (+) or absence (−) of Ca^{2+}/CaM. Phosphate incorporation into MBP was determined as described in Materials and methods. Three independent experiments were performed, and the data are presented as the means ± standard deviation (SD).