Short sequence-paper

Molecular cloning and expression profile of *Xenopus* calcineurin A subunit[1]

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Abstract

We have cloned a cDNA encoding a catalytic subunit of calcineurin (CnA) expressed in *Xenopus* oocytes. The deduced amino acid sequence indicates 96.3% and 96.8% identities with the mouse and human CnAα isoforms, respectively. *Xenopus* CnA (XCnA) RNA and protein are expressed as maternal and throughout development. Recombinant XCnA protein interacted with calmodulin in the presence of Ca^{2+}. Deletion of calmodulin binding domain and auto-inhibitory domain revealed calcium independent phosphatase activity, thereby showing that XCnA is likely to be modulated by both calmodulin and calcium. © 2000 Elsevier Science B.V. All rights reserved.

In early embryogenesis, the inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} signal transduction pathway is involved in many biological processes including fertilization, cell cleavage and dorso–ventral axis formation [1]. Although the importance of IP_3-Ca^{2+} signaling has been appreciated, the immediate target of free Ca^{2+} has not been documented so far.

Calcineurin (Cn), a calcium/calmodulin (CaM) dependent phosphatase 2B, is most abundant in brain, but it is also detected in a wide variety of non-neuronal tissues, and plays an important role in many biological processes including T-cell signal transduction [2–8]. Cn has been implicated in the early events of T-cell activation. In lymphoid cells, stimulation of the T-cell receptor leads to the nuclear translocation of the nuclear factor of activated T-cell (NF-AT) family via activation of Cn, which in turn activate immune response genes such as interleukin-2. This process confers resistance to the immunosuppressants, FK506 and cyclosporin A (CsA). It was shown that the immunophilins, cyclophilin and FK506 binding protein (FKBP), which function as specific receptors for CsA and FK506, interact with Cn and inhibit its activity, which in turn inhibits nuclear translocation of NF-AT [9]. Recently, the Cn-NF-AT pathway is also reported to be involved in non-lymphoid cells and tissues [10–14]. This suggests that

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A

B

Fig. 1. (A) Comparison of the predicted amino acid sequence of the XCnA with that of mouse (GenBank accession number J05479; mCnA) and human (L14778; hCnA). Amino acid residues identical to those of the *Xenopus* homologue are indicated by black boxes. Conservatively substituted amino acid residues are indicated by shaded boxes. Catalytic domain is boxed with a line. CnB BD is underlined with a solid line. CaM BD is underlined with a broken line. AID is underlined with asterisks. The nucleotide sequence of XCnA is deposited in DDBJ/DMBL/GenBank DNA database under the accession number AB037146. (B) Comparison of splicing sites of CnA isoforms. Splicing sites were compared among CnAα (human and murine [6], *Xenopus* [16]), CnAβ [17,25], CnAγ isoforms (human [18] and mouse [26]) and *D. melanogaster* CnA [8]. Amino acid residues are numbered on the right.

the Cn pathway has a variety of roles in both the immune and non-immune systems.

In order to characterize the role of Cn during early development, we cloned *Xenopus* CnA subunit (XCnA) cDNA expressed in *Xenopus* oocytes, and studied its expression pattern in *Xenopus* early embryonic development.

We isolated the XCnA cDNA by using an EST

clone similar to mouse CnA (Intergrated Molecular Analysis of Genome Expression, I.M.A.G.E., clone ID, my38b01; GenBank database accession no. AA239356) as a probe to screen a *Xenopus* oocyte cDNA library [15]. The nucleotide sequence of XCnA contains a presumptive ATG start codon preceded by 373 bp of 5′-flanking sequence, an open reading frame encoding 508 amino acids, and 906 bp of 3′-untranslated sequence. The predicted molecular weight of the full length peptide is 57.4 kDa, in reasonable agreement with the value of ∼60 kDa estimated by Western blot analysis (Fig. 3). The cloned cDNA is 93.6% and 93.8% identical at the amino acid level to murine CnAα [6] and human CnAα [5], respectively. XCnA consists of four putative functional domains, which are 'catalytic (residues 70–333) domain', 'regulatory domain' that contains the CnB binding domain (CnB BD) (residues 348–368), 'CaM binding domain (CaM BD)' (residues 391–414) and 'auto-inhibitory' domain (residues 457–480). The catalytic domain shows 96.3%, while the other domains (CnB BD, CaM BD and auto-inhibitory domain (AID)) show 100% amino acid identities with those of both human and murine CnA. Based on the high degree of similarity, we concluded that this molecule was the *Xenopus* homologue of CnA (XCnA). Recently, a splicing variant form of XCnA expressed in *Xenopus* brain was reported [16]. The XCnA cDNA expressed in *Xenopus* brain has an insertion of 10 amino acids (amino acids 447–456, accession no. AF019569), which is absent in our present XCnA cDNA clone. These insertions/deletions are found in exactly the same position in mammalian CnAα [6], CnAβ [17] and CnAγ isoforms [18]. In *Drosophila melanogaster*, only the 10 amino acids CnA spliced-out form has been reported [8] (Fig. 1B). It seems likely that the ancestral gene for CnA obtained the 10 amino acid insertion during the course of evolution into vertebrate. This site is conserved among α-, β-, and γ-isoforms in mammalians. CnA with the 10 amino acids has not been found in adult and embryonic fly [8,19]. Therefore, the spliced-in amino acids may have a role in higher biological activities such as neural or immune system that was gained during evolution. This is supported by our result that the major expression site of the CnA spliced-in form is in brain (Fig. 2).

Based on this discrepancy, we designed a primer pair to specifically distinguish between these two splicing variants by reverse transcription (RT)-PCR
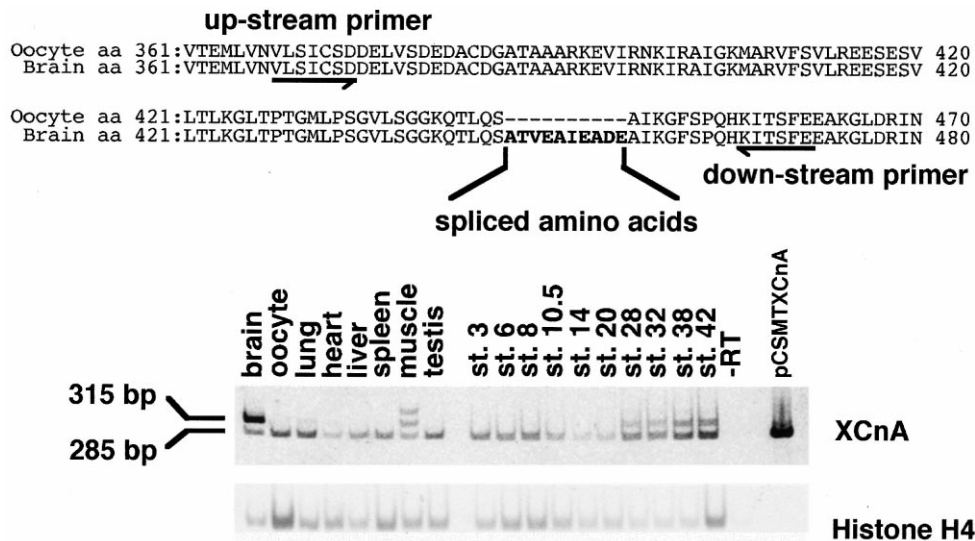
Fig. 2. RNA profile of XCnA in embryos. Schematic representation of the primer pair for detection of oocyte subtype (spliced-out form) and brain subtype of XCnA transcripts (upper panel). Ten amino acid insert in brain type is indicated by boldface type. Amino acid (aa) number is shown at both sides of the sequence. RT-PCR analysis of expression of XCnA in adult tissues and embryonic stages (lower panel). The 315 bp PCR product corresponds to the brain subtype and the 285 bp band corresponds to the oocyte subtype. pCS2+MTXCnA is used as a control template (lane, pCSMTXCnA). Histone H4 is used as a loading control. −RT, stage 8 embryo RNA without reverse transcription. Staging was according to Nieuwkoop and Faber [27].

amplification, and examined tissue distribution and developmental expression of these two subtypes of transcripts by performing a semi-quantitative RT-PCR [20]. The primers used are: up-stream primer, 5′-CTCCTCGAAGCTAGTGATCTTA-3′; down-stream primer, 5′-GTCCTTAGTATCTGCTCA-GATG-3′. XCnA transcripts corresponding to the present oocyte subtype ('spliced-out' form) or brain subtype show sizes of 285 bp or 315 bp, respectively (Fig. 2). Histone H4 was used as a loading control (up-stream primer: 5′-CGGGATAACATTCAGGG-TA-3′, down-stream primer: 5′-TCCATGGCGG-TAACTGTC-3′). These semi-quantitative PCRs were performed at 94°C (30 s), 58°C (10 s), 72°C (30 s) for 20–23 cycles using KOD′ polymerase (Toyobo). For each set of primers, serial dilutions of cDNA and cycle numbers required for the linear range in the RT-PCR were determined empirically. One fourth of the RT-PCR products were loaded onto 5% non-denaturing polyacrylamide gel electro-phoresis (PAGE) and the gels were overlaid with DNA sensitive dye, SYBR Green I (Molecular Probes), and analyzed using FluoroImager (Molecu-lar Dynamics). A single band of 285 bp was ampli-fied from XCnA oocyte cDNA (Fig. 2, lane: oocyte). Two bands (315 bp and 285 bp) were amplified from adult brain cDNA (Fig. 2, lane: brain). The spliced-out form of XCnA (oocyte subtype) is expressed in unfertilized eggs, and decreased gradually during early cleavage stages. After gastrulation, the zygotic XCnA (oocyte subtype) expression level increased and reached a substantial level by tailbud stage of stage 28 (Fig. 2). The 'brain subtype' first appeared in tailbud stage, and increased gradually thereafter. Skeletal muscle expressed a third subtype of RNA (Fig. 2, lane: muscle). This muscle subtype was not expressed during early stages of embryogenesis. Tak-en together, the present XCnA is an alternative spliced form of XCnA [16], which is expressed both in early embryonic developmental stages and many adult tissues.

In order to determine the size of the protein en-coded by XCnA RNA and study XCnA protein ex-pression profile during embryonic stages, Western blot analysis was performed using crude lysate of *Xenopus* embryos overexpressing full length XCnA, or myc-tagged XCnA (MTXCnA) RNA. The coding region of XCnA was amplified by PCR. Primers used

are as follows: 5′ up-stream primer including a *Stu*I restriction enzyme site, is 5′-AGGCCTTCTGAG-CACAAGGCCAATGAAGC-3′; 3′ down-stream primer including a *Xba*I site, is 5′-TCTAGAT-CACTGAATATTGCTGCCGTTG-3′. The ampli-fied fragment was subcloned into *Stu*I and *Xba*I sites of pCS2+ or pCS2+MT to generate pCS2+XCnA or pCS2+MTXCnA. Crude lysates of *Xenopus* embryos
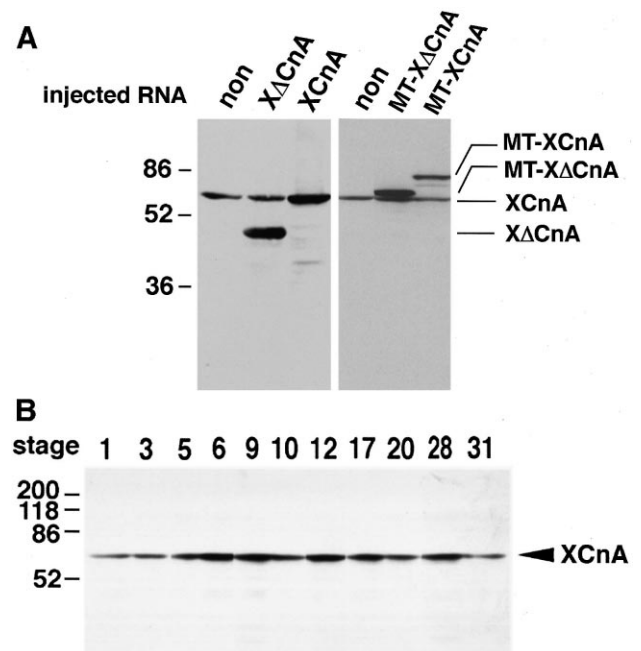
Fig. 3. Expression of XCnA protein in embryos. (A) Anti-hu-man CnA recognizes endogenous XCnA or exogenously intro-duced XCnA or XΔCnA. RNA transcribed from pCS2+XΔCnA, pCS2+MTXΔCnA, pCS2+XCnA or pCS2+MTXCnA was in-jected into four blastomers of *Xenopus* four-cell stage embryos at 4 ng per embryo. Ten µg of crude lysates from stage 8 em-bryos was loaded onto a 10% SDS–PAGE, and blotted with an anti-human CnA antibody (Transduction Laboratories, C26920). Anti-human CnA antibody recognized endogenous XCnA in uninjected embryo (lanes, non). In embryos overex-pressed with XCnA, a dense band of the same size was detected (lane, XCnA). In embryos injected with XΔCnA (lane, XΔCnA), myc-tagged XCnA (lane, MT-XCnA) or myc-tagged XΔCnA (lane, MT-XΔCnA), additional bands corresponding to the exogenously introduced RNA were detected. The bands cor-responding to each exogenously introduced RNA are denoted at the right. (B) Protein profile of XCnA was analyzed by Western blot analysis. Twenty µg of crude lysates prepared from indicated stages of embryos was loaded onto a 10% SDS–PAGE, and blotted with an anti-human CnA antibody. Staging was according to Nieuwkoop and Faber [27].

at indicated stages were prepared as follows. *Xenopus* embryos were homogenized in lysis buffer, containing 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Nonidet P-40, 4 mM phenylmethylsulfonyl fluoride. The homogenate was briefly centrifuged at $1000 \times g$ for 5 min and supernatants were mixed with $2 \times$ SDS–PAGE sample buffer and boiled for 5 min, and were loaded onto 10% SDS–PAGE. A monoclonal antibody raised against human CnA (Transduction Laboratories, C26920) recognized both endogenously and exogenously expressed XCnA (Fig. 3A). The XCnA protein profile during developmental stages was analyzed by Western blot analysis using the same anti-human CnA antibody (Fig. 3B). Interestingly, XCnA protein was kept at a constant level compared to the biphasic expression of XCnA RNA. The differences of expression profiles between XCnA RNA and protein may be due to difference in stabilities between RNA and protein, or post-transcriptional regulations of XCnA.

The primary structure of XCnA indicates that XCnA consists of a putative CaM BD, a CnB BD and an AID (Fig. 1B). To confirm that XCnA was a functional molecule, we performed CaM overlay and phosphatase assays, and compared the activities between full length XCnA and XΔCnA. We first prepared bacterially expressed glutathione-S-transferase (GST) fusion proteins with XCnA and a putative constitutive active form, XΔCnA (Fig. 4A). XΔCnA (amino acids 1–398 of XCnA) was generated by PCR amplification. The 5′ up-stream primer used was the same as that used to generate full length XCnA. The 3′ down-stream primer used was 5′-TCTAGAT-CAGTTTCTGATAACTTCCTTTCGGG-3′ which included a *Xba*I site. For GST fusion protein production, *Stu*I and *Not*I fragments of pCS2+MTXCnA or pCS2+MTXΔCnA were subcloned into pGEX4T-3 (Pharmacia) to generate pGEX-XCnAFL or pGEX-XΔCnA, respectively. pGEX-XCnAFL or pGEX-XΔCnA were transformed into *Escherichia coli* BL21 (DE3). Recombinant proteins were purified following the manufacturer's instructions (Pharmacia). GST-XCnAFL and XΔCnA were resolved on 8% SDS–PAGE and then electrophoretically transferred onto a PVDF membrane (Millipore Corp.). The membrane was blocked with blocking buffer (0.1 M NaCl, 50 mM Tris–HCl
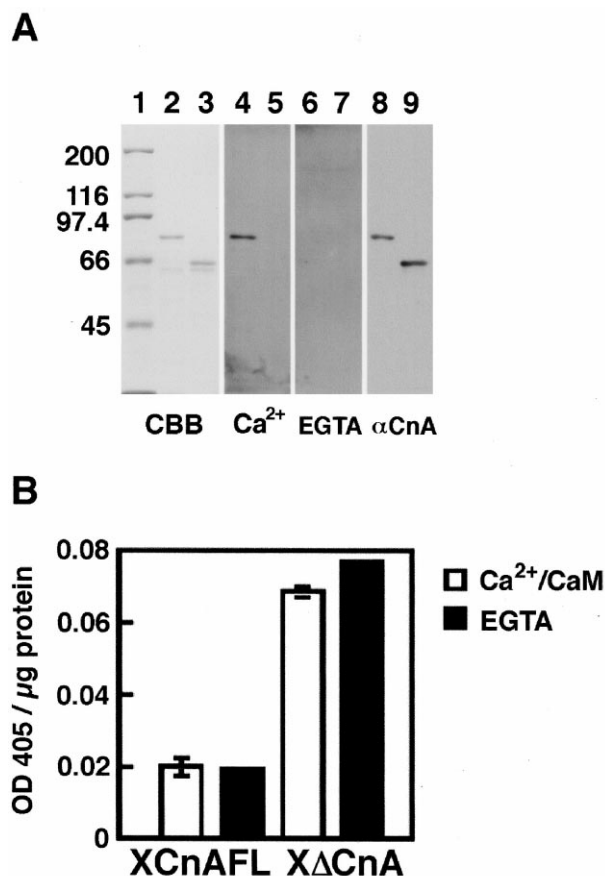

Fig. 4. CaM binding activities and phosphatase activities of recombinant XCnA. (A) Purified GST fusion XCnAs were separated by 8.0% SDS–PAGE and stained with Coomassie brilliant blue R250 (lanes 1–3), or blotted onto a PVDF membrane (lanes 4–9). The blots were reacted with a biotinylated CaM in the presence of either 1 mM $CaCl_2$ (lanes 4 and 5) or 1 mM EGTA (lanes 6 and 7), or probed with an anti-human CnA antibody (αCnA) (lanes 8 and 9). Molecular weight standard was lane 1, GST-XCnAFL was lanes 2, 4, 6 and 8, GST-XΔCnA was lanes 3, 5, 7 and 9. (B) Protein phosphatase activities were measured using pNPP as a substrate at 30°C for 30 min. Solid bars indicate the phosphatase activities in the presence of EGTA, white bars indicate in the presence of $CaCl_2$. Values are mean ± S.E.M. of triplicate experiments, each done in duplicate.

(pH 7.5), 0.1% bovine serum albumin, 0.05% Tween 20) in the presence of 1 mM $CaCl_2$ or 2 mM EGTA for 30 min at room temperature. Biotinylated CaM (Calbiochem) was added at a final concentration of 0.5 μg/ml in the blocking buffer to the blotted membrane and incubated for 30 min at room temperature. After washing with the blocking buffer, the membranes were incubated with 1:5000 diluted avi-

din-conjugated horseradish peroxidase (Prozyme) in blocking buffer. The membranes were washed well with blocking buffer, and developed with ECL (Amersham). Wild type XCnA protein (GST-XCnA) bound to biotinylated CaM in the presence of Ca^{2+} (lane 4), but not in the presence of EGTA (lane 6). Whereas XΔCnA, which was deleted with CaM BD, did not bind to CaM (lanes 5 and 6). The GST-tagged recombinant proteins apparently showed larger molecular weights compared to their intact molecules which was confirmed by Western blot analysis using anti-human CnA (Fig. 4A, lanes 8 and 9). The enzymatic activity of XCnA was further confirmed by a phosphatase assay (Fig. 4B). CaM dependent phosphatase activity was measured using *p*-nitro-phenyl-phosphate (pNPP) as a substrate at 30°C in 50 mM MOPS (pH 7.0), 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.03 μM CaM, 1 mg/ml bovine serum albumin, 0.5 mM dithiothreitol and 1.0 μM okadaic acid. CaM independent activity was done in the above buffer, in which 1 mM EGTA was added instead of 0.5 mM $CaCl_2$. After incubation for 5 min at 30°C, reactions were initiated by the addition of pNPP at a final concentration of 20 mM. After incubation for 30 min at 30°C, the reactions were stopped by the addition of 0.5 M Na_2CO_3. GST-XCnAFL did not show any phosphatase activity, probably due to the absence of B-subunit (Fig. 4B). XΔCnA, which was deleted of CaM BD and AID, did not bind to CaM (Fig. 4A), however showed phosphatase activity both in the absence and the presence of Ca^{2+}. These results thereby suggest that XCnA shows Ca^{2+} dependent association with CaM, and that XΔCnA functions as a constitutive active mutant in that its activity is independent of CaM and Ca^{2+}, as was previously proposed with those of the mammalian counterparts [2,21].

It is reported that CnA acts corporately with FKBP to dephosphorylate the IP3R, which in turn regulates the activity of IP3R [22,23]. We have previously reported that functional inhibition of XIP3R in the ventral side of the embryo led to conversion of ventral mesoderm to adopt a dorsal fate [20]. Nishinakamura et al. reported that injection of *Xenopus* FKBP into the ventral side of early stage embryo induced an ectopic axis [24]. Taken together, these lines of evidence suggest that FKBP-Cn may be involved in dorso–ventral axis formation, possibly through modification or as a result of the activation of IP3R. However, the immunosuppressants, FK506 and CsA, did not show any effect on the early embryogenesis in *Xenopus* embryos (T.S. and S.K., unpublished data). Since yolk protein is abundant in the embryo, these drugs might be non-specifically absorbed by yolk protein. Alternatively, the concentration of FKBP or cyclophilin in the embryos was insufficient to inhibit endogenous XCnA.

In conclusion, we have cloned a splicing variant of XCnA expressed in early embryonic development, characterized its Ca^{2+} dependent activities and analyzed its RNA and protein profile. Further analysis is needed to dissolve various aspects of CnA function in early development including dorso–ventral axis formation or neural development.

References

[1] S. Kume, Cell. Mol. Life Sci. 56 (1999) 296–304.
[2] S.J. O'Keefe, J. Tamura, R.L. Kincaid, M.J. Tocci, E.A. O'Neill, Nature 357 (1992) 692–694.
[3] C.B. Klee, H. Ren, X. Wang, J. Biol. Chem. 273 (1998) 13367–13370.
[4] N.A. Clipstone, G.R. Crabtree, Nature 357 (1992) 695–697.
[5] T. Muramatsu, R.L. Kincaid, Biochim. Biophys. Acta 1178 (1993) 117–120.
[6] R.L. Kincaid, P.R. Giri, S. Higuchi, J. Tamura, S.C. Dixon, C.A. Marietta, D.A. Amorese, B.M. Martin, J. Biol. Chem. 265 (1990) 11312–11319.
[7] M.S. Cyert, R. Kunisawa, D. Kaim, J. Thorner, Proc. Natl. Acad. Sci. USA 88 (1991) 7376–7380.
[8] L. Brown, M.X. Chen, P.T. Cohen, FEBS Lett. 339 (1994) 125–128.
[9] A. Rao, C. Luo, P.G. Hogan, Annu. Rev. Immunol. 15 (1997) 707–747.
[10] J.D. Molkentin, J.R. Lu, C.L. Antos, B. Markham, J. Richardson, J. Robbins, S.R. Grant, E.N. Olson, Cell 93 (1998) 215–228.
[11] A. Musaro, K.J. McCullagh, F.J. Naya, E.N. Olson, N. Rosenthal, Nature 400 (1999) 581–585.
[12] E.R. Chin, E.N. Olson, J.A. Richardson, Q. Yang, C.

Humphries, J.M. Shelton, H. Wu, W. Zhu, R. Bassel-Duby, R.S. Williams, Genes Dev. 12 (1998) 2499–2509.

[13] T. Hoey, Y.L. Sun, K. Williamson, X. Xu, Immunity 2 (1995) 461–472.

[14] K.L. Abbott, B.B. Friday, D. Thaloor, T.J. Murphy, G.K. Pavlath, Mol. Biol. Cell 9 (1998) 2905–2916.

[15] M.R. Rebagliati, D.L. Weeks, R.P. Harvey, D.A. Melton, Cell 42 (1985) 769–777.

[16] N.J. Lautermilch, N.C. Spitzer, J. Neurosci. 20 (2000) 315–325.

[17] A.E. McPartlin, H.M. Barker, P.T. Cohen, Biochim. Biophys. Acta 1088 (1991) 308–310.

[18] T. Muramatsu, R.L. Kincaid, Biochem. Biophys. Res. Commun. 188 (1992) 265–271.

[19] D. Guerini, C. Montell, C.B. Klee, J. Biol. Chem. 267 (1992) 22542–22549.

[20] S. Kume, A. Muto, T. Inoue, K. Suga, H. Okano, K. Mikoshiba, Science 278 (1997) 1940–1943.

[21] A. Tsuboi, E.S. Masuda, Y. Naito, H. Tokumitsu, K. Arai, N. Arai, Mol. Biol. Cell 5 (1994) 119–128.

[22] A.M. Cameron, J.P. Steiner, A.J. Roskams, S.M. Ali, G.V. Ronnett, S.H. Snyder, Cell 83 (1995) 463–472.

[23] A.M. Cameron, J.P. Steiner, D.M. Sabatini, A.I. Kaplin, L.D. Walensky, S.H. Snyder, Proc. Natl. Acad. Sci. USA 92 (1995) 1784–1788.

[24] R. Nishinakamura, Y. Matsumoto, U. Ta, M. Asashima, T. Yokota, Biochem. Biophys. Res. Commun. 239 (1997) 585–591.

[25] D. Guerini, C. Klee, Proc. Natl. Acad. Sci. USA 86 (1989) 9183–9187.

[26] T. Muramatsu, P.R. Giri, S. Higuchi, R.L. Kincaid, Proc. Natl. Acad. Sci. USA 89 (1992) 529–533.

[27] Nieuwkoop and Faber, Normal Table of *Xenopus laevis* (Daudin), North-Holland Pub. Co., Amsterdam, 1967.