Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 34-38

IDENTIFICATION OF TESTIS SPECIFIC CALCINEURIN β SUBUNIT ISOFORM BY A MONOCLONAL ANTIBODY AND DETECTION OF A SPECIFIC SIX AMINO ACID SEQUENCE

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Received November 25, 1991

Summary: Two isoforms of calcineurin ß subunit(ß1 and ß2) were identified in rat testis by a monoclonal antibody Val. Both ß1 and ß2 were recovered in calmodulin binding protein fraction and showed calcium shift on SDS-polyacrylamide gel electrophoresis which is the specific character for EF-hand calcium binding protein. ß2 showed same apparent molecular weight on SDS-PAGE as that of brain calcineurin and was found in wide variety of tissues. ß1 was shown to have six amino acid polypepeptide sequence and it showed higher molecular weight than brain ß and was specific for testis.

Calcium and calmodulin-stimulated protein phosphatase(calcineurin or protein phosphatase 2B) has important roles in controlling sperm motility(1). We reported the presence of the enzyme in rat testis from which the sperms are produced(2). This enzyme is a heterodimer composed of a 61KDa calmodulin binding and catalytic subunit(calcineurin α) and 19KDa EF-hand Ca^{2+}binding and regulatory subunit(calcineurin ß (3, 4). While calcineurin α has multiple isoforms by different genes and by alternative splicing(5, 6) calcineurin ß has been thought to be a very conservative polypeptide with little species differences(7). In contrast to this view, we showed the presence of cDNA in rat testis coding for a polypeptide which was very similar to but distinctively different from brain calcineurin ß(8). One of the distinct differences was that testis ß had hydrophilic six amino acid polypeptide sequence at the c-terminal end. These data suggested the presence of calcineurin ß isoforms in rat testis.

In this report, we show and confirm the presence of two calcineurin ß isoforms(ß1 and ß2) in rat testis by specific monoclonal antibody Val and by calcium shift on SDS-PAGE. We also show that ß1 is specific for testis by a systematic tissue survey, and that ß1 has 6 amino acid polypeptide sequence which is specific for calcineurin ß in rat testis.

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0006-291X/92 $1.50

laterials and Methods

Materials: DEAE cellulose and Phenyl Sepharose CL-4B were obtained from Pharmacia Fine hemicals, Uppsala, Sweden. Keyhole limpet hemocyanin (KLH) was from Calbiochem, SanDiego, USA. -chloro-1-naphthol was from Wako pure chemical Industries, Osaka, Japan. Goat anti-rabbit IgG)rseradish peroxidase conjugate and molecular weight markers were from Bio-Rad Laboratories, ichmond, USA. All other chemicals were purchased from Sigma Chemical Company, Poole, UK. alcineurin and calmodulin was kindly provided from Dr.R.K.Sharma.

Gel electrophoresis and immunoblot analysis: SDS-polyacrylamide gel electrophoresis was performed / the method of Laemmli(9). Immunoblot analysis was performed by the method of Towbin et al(10) using)rseraddish peroxidase-linked second antibody and 4-chloro-1-naphthol as color substrate.

Preparation of synthetic peptides: Peptide(FVDHGQED) corresponding to carboxy-terminal of gulatory subunit of calcineurin cloned from rat testis cDNA library was synthesized manually by standard)lid phase technique(11). The peptide was purified by gel filtration chromatography and reversed-phase PLC column. Amino acid composition of the purified peptide was determined using a Beckman model 300 amino acid analyzer with ninhydrin determination.

Antiserum production: Purified peptide was coupled to keyhole limpet hemocyanin(KLH) through the /steine residue(12). Peptide-KLH conjugate was used to immunize the rabbits. Detection of titer of ntiserum was carried out using ELISA analysis. ELISAs were carried out according to the procedure of liles and Haber(13) using alkaline phosphatase-linked second antibody. Positive wells were detected using -nitrophenol as color substrate and preimmune serum as control.

Preparation of calmodulin binding proteins: Calmodulin binding proteins were prepared by the method f Sharma et al.(14) using DEAE cellulose and Calmodulin Sepharose 4B column chromatography. Affi-el Blue chromatography step was not performed.

esults

dentification of two isoforms of calcineurin ß in rat testis: Expression of regulatory subunit of alcineurin(phosphatase 2B) was examined in various rat tissues by a monoclonal antibody Val(Fig.1).)nly one immunoreactive band showing apparently same molecular weight as that of brain calcineurin ß on DS-PAGE was found in all tissues examined except testis, where two immunoreactive bands(ß1 and ß2) /ere found. ß2 showed apparently same molecular weight on SDS-PAGE as that of brain calcineurin ß. ß1 howed slightly higher molecular weight and was detected only in testis.

To characterize ß1 and ß2 in testis, we purified calmodulin binding proteins from rat testis as described n Materials and Methods. Immunoreactivity towards Val was retained in calmodulin binding protein raction but not detected in flow-through fraction of calmodulin affinity column chromatography. This howed that both ß1 and ß2 were recovered in calmodulin binding fraction. We also checked if ß1 and ß2 howed calcium shift on SDS-PAGE, which is the specific character of EF-hand calcium binding proteins.)oth ß1 and ß2 showed calcium shift on SDS-PAGE as was observed with brain ß subunit (Fig.2).

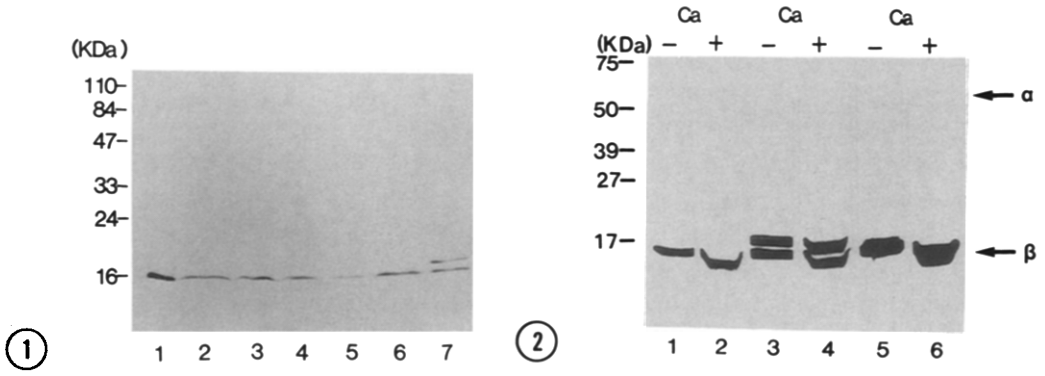

Figure 1. *Immunoblot analysis using various rat tissues by monoclonal antibody Val.*
After homogenization of tissues, samples were ultra-centrifuged at 100,000xg for 1hr. Supernatant fractions were applied to 12.5% SDS-PAGE,and immunoblot was performed as described in Materials and Methods. Lanes 1, 2, 3, 4, 5, 6 and 7 represent brain, spleen, lung, kidney, liver, skeletal muscle and testis respectively.

Figure 2. *Calcium shift of calcineurin ß.* Samples were prepared as described in Material and Methods, and applied to 12.5% SDS-PAGE. Immunoblot was performed using monoclonal antibody Val. Lanes 1, 2; calmodulin binding protein from rat brain. Lanes 3, 4; calmodulin binding protein from rat testis. Lanes 5, 6; purified calcineurin from bovine brain. Lanes 1, 3 and 5 contain 1mM EGTA. Lanes 2, 4 and 6 contain 1mM $CaCl_2$.

Identification of 6 amino acid polypeptide tail in isoforms: In our previous report, we isolated a rat testi cDNA for a calcium binding polypeptide homologous to brain calcineurin ß(8). Recently Mukai et al. als isolated a novel calcineurin ß-like protein from rat testis cDNA library(15). One of the striking differenc between the deduced amino acid sequence of the testis cDNA and brain calcineurin ß was the presence of th six amino acid polypeptide sequence in the testis cDNA at the c-terminal end.

To elucidate if ß1 and/or ß2 in rat testis contains 6 amino acid polypeptide sequence or not, a specifi antiserum against synthetic polypeptide (FVDHGQED) was prepared and was used for the immunoblot o ß1 and ß2(Fig.3). Strong immunoreactivity was detected in a band showing the same molecular weight a that of ß1. Some other positive bands were also seen. In order to exclude the non-specific immunoreactiv bands, the blocking experiment was performed. The synthesized polypeptide containing 6 amino aci sequence was preincubated with the antiserum solution to block the specific binding. Immunoblot analysi using antiserum containing synthesized peptide as first antibody showed that only one band correspondin to ß1 decreased in immunoreactivity, and that color intensity of other immunoreactive bands did not chang at all. Immunoreactivity of a band corresponding to ß1 was more absorbed by use of higher concentration o the synthesized peptide(data not shown). These results indicate that a band corresponding to ß1 contains a

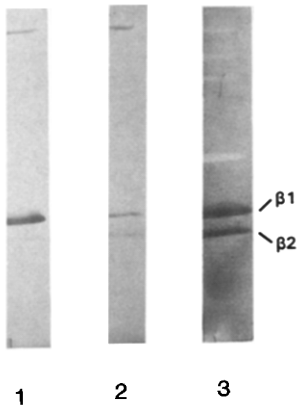

Figure 3. *Absorption of immunoreactivity by adding peptides containing 6 amino acid sequence to the antiserum.* Calmodulin binding proteins from rat testis were prepared as described in Materials and Methods. Samples were applied to 15% SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with different antibodies as described below. lane 1, antiserum; lane 2, antiserum + synthesized peptide (10µg/ml at final concentration); lane 3, Val.

mino acid polypeptide sequence and that other immunoreactive bands are due to nonspecific binding of the ntiserum.

)iscussion

In this study, we identified two polypeptides in rat testis by a monoclonal antibody Val. They were hown to have the epitope towards Val monoclonal antibody which is specific for calcineurin ß subunit, vere shown to have the characteristics of EF-hand calcium binding protein(16), and were recovered in :almodulin binding fraction. These results suggest that ß1 and ß2 are isoforms of calcineurin ß subunit.

We also show that ß1 posesses 6 amino acid polypeptide sequence and is specifically expressed in estis. In previous papers, Mukai et al.(15) and Sugimoto et al.(8) independently reported the cloning of :DNA coding for a testis specific calcineurin ß subunit having six amino acid polypeptide sequence. We suggest that ß1 is a translational product of testis specific mRNA.

ß2 did not posess the six amino acid polypeptide sequence and showed the same apparent molecular veight on SDS-PAGE as that of brain ß. A protein band recognized by ß specific monoclonal antibody Val, howing the same molecular weight as ß2, was seen in wide variety of tissues. Northernblot analysis by Mukai et al. showed that brain type ß subunit was also expressed in many tissues as ß2. These data suggest hat ß2 is actually a brain type calcineurin ß subunit.

Functional differences between ß1 and ß2 has not been elucidated yet. Interestingly ß1 is expresse only 3 weeks after birth, when spermatogenesis begins, and increases in amount depending on th maturation of spermatogenesis(17). The matured sperm is most likely to have only ß1(17). Thus the chan of calcineurin ß subunit isoform expression may have an important role in rat spermatogenesis and in th control of meiosis.

Acknowledgments

We thank Dr. Mikio Sugimoto and Dr. Ikumasa Takenaka for preparing calomodulin binding protein: We also thank Dr. Heung Chin Cheng and Dr. Jerry H. Wang for helping us to synthesize polypeptide. W are grateful to Dr. Najma Janjua for reviewing the English of this manuscript. This work was supported b Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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