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δ-Ca^{2+}/Calmodulin-Dependent Protein Kinase II Expression Pattern in Adult Mouse Heart and Cardiogenic Differentiation of Embryonic Stem Cells

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Abstract δ-isoforms of the Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) are considered to substantially influence cardiac functions. However, no data exist on the expression of these isoforms in the mouse heart. We analyzed the transcript pattern of non-neuronally expressed δ-isoforms in heart and skeletal muscle of adult mice by RT-PCR. For members of the δ-CaMKII subclass with both variable domains (subclass II), weak transcriptional expression of isoforms δ_2 and δ_3 was found in the heart. In skeletal muscle no δ_3-specific transcript was detectable. In cardiac tissue, stronger signals result from amplifications of δ_9 and from members of the subclass I lacking the second variable domain. Western blotting was performed using a subclass II-specific antibody. In murine cardiac and skeletal muscle tissue a δ-CaMKII protein pattern was obtained similar to that described for rat. To gain insight into the expression of δ-CaMKII during the earliest steps of cardiogenic differentiation, we analyzed the transcript pattern of murine embryonic stem cell-derived cardiomyocytes in various differentiation stages. Reproducible RT-PCR signals could be obtained for δ_6 and δ_{10}, both belonging to the δ-CaMKII subclass I. Transcripts for δ_6 were ubiquitously expressed, whereas transcripts for δ_{10} were detectable in increasing amounts after 7–10 days of the onset of cardiogenic differentiation. Our results point to a differentiation-dependent expression of the two δ-CaMKII subclasses, and also to differences in the expression of individual members of subclass I during the early stages of cardiogenic differentiation. J. Cell. Biochem. 79:293–300, 2000.

Key words: multifunctional Ca^{2+}/calmodulin-dependent protein kinase type II; murine cardiac δ-isoforms; embryonic stem cell differentiation

INTRODUCTION

The multifunctional CaMKII is a central mediator of Ca^{2+} regulated processes in the myocardium and it plays a critical role in heart function. In isolated cardiomyocytes, CaMKII affects the activity of the sarcolemmal L-type Ca^{2+} channel and the ryanodine receptor [Anderson et al., 1994; Li et al., 1997]. The controversial role of CaMKII in cardiac relaxation has been discussed [Odermatt et al., 1996; Reddy et al., 1996]. However, there is increasing evidence that CaMKII may increase the v_{max} of the sarcoplasmic reticulum Ca^{2+} pump [Mattiazzi et al., 1994; Li et al., 1997; Xu and Narayanan, 1999]. Implications of CaMK and especially CaMKII on mouse cardiomyocyte function was recently shown [Okazaki et al., 1994; Li et al., 1998].

The CaMKII holoenzyme is an oligomere consisting of a varying number of either identical or differing subunits [Kanaseki et al., 1991; Shen et al., 1998]. Alternative splicing generates subunit variants of primary transcripts encoded by four genes (α, β, γ, δ) [Tobimatsu and Fujisawa, 1989; Brocke et al., 1995]. The phosphorylation state [Heist et al., 1998; Strack and Colbran, 1998], isoform composition [Srinivasan et al., 1994; Shen et al., 1998], and anchoring proteins [Bayer et al., 1998] determine the targeting of the holoenzyme to different cellular compartments. Thus, specific functions in gene expression are achieved [Nghiem et al., 1994; Ramirez et al., 1997], such as cell cycle control [Planas-Silva and Means, 1992] and differentiation [Wang and

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Simonson, 1996; Masse and Kelly, 1997]. Autophosphorylation of the enzyme results in an autonomous activity characterized by an increased affinity for calmodulin and independence from Ca^{2+}, thus enabling CaMKII in vitro to decode the frequency of Ca^{2+} spikes [De Koninck and Schulman, 1998].

The CaMKII δ-class is characterized by the existence of two subclasses defined by the presence (subclass II) or absence (subclass I) of a C-terminal extension that forms the second variable domain. The subclass I members δ_6, δ_7, δ_8, and δ_{10} , and the subclass II isoforms δ_2, δ_3, δ_4, and δ_9 are expressed in non-neuronal tissues in rat [Schworer et al., 1993; Mayer et al., 1995]. A growing number of studies describe cardiac expression patterns of distinct δ-CaMKII isoforms in rat [Schworer et al., 1993; Edman and Schulman, 1994; Hoch et al., 1998; Hagemann et al., 1999] and human [Hoch et al., 1999]. Members of the CaMKII δ-subclass II represent the cardiac-typic and abundant isoforms of the CaMKII family [Edman and Schulman, 1994; Baltas et al., 1995]. In human dilated cardiomyopathy and chronic atrial fibrillation, changes in the protein content of the δ-subclass II can be demonstrated [Hoch et al., 1999; Tessier et al., 1999].

Accessibility to transgenic and gene targeting approaches, expanding knowledge of the genome, a short gestation period, and relatively low breeding costs made the murine system an attractive model to investigate cardiovascular function and pathology [Doevendans et al., 1998]. However, no information is available on the expression of individual δ-CaMKII isoforms in mouse heart. Mouse embryonic stem (ES) cells can be successfully manipulated with high reproducibility and have the potency to differentiate into a cardiomyocyte-like phenotype representing the earliest steps of cardiogenic differentiation [Wobus et al., 1997; Wobus and Guan, 1998]. Thus, ES cells are an attractive model to gain insights into the expression pattern of CaMKII isoforms during the early steps of cardiac development.

In this study, we investigated the expression of non-neuronal δ-CaMKII isoforms in cardiac muscle from adult mouse, and analyzed the transcript pattern of these isoforms in various stages of cardiogenic differentiation of ES cells.

METHODS

Materials

RT-PCR reagents were obtained from Eurogentec (Seraing, Belgium; GoldStar red DNA polymerase and buffers), United States Biochemical (Cleveland, OH; dNTP), Boehringer (Mannheim, Germany; 10× hexanucleotide mix), Pharmacia (Freiburg, Germany; RNase-free DNaseI, RNAguard) and Gibco BRL (Eggenstein, Germany; Superscript™ II RnaseH⁻ Reverse transcriptase). All other chemicals were of analytical grade and were obtained from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany). Kits for extraction of nucleic acids were from Qiagen (Hilden, Germany; RNeasy system, Qiaquick gel extraction kit). Size marker for agarose gel electrophoresis was the ready-load 100 bp DNA standard (Gibco BRL, Eggenstein, Germany). Antirabbit IgG conjugated with peroxidase and the enhanced chemoluminescence kit were obtained from Sigma (Deisenhofen, Germany) and Amersham (Braunschweig, Germany), respectively. Materials for cell culturing are given in the corresponding methods section.

Tissue Samples

Cardiac and thigh skeletal muscle tissue samples were obtained from adult mice of the inbred strains AB and XVII (both obtained from the Animal Facility of the Max Delbrück Center). Strains were described by Staats [1976] and von Magdon [1962]. Immediately after killing the animals, tissue samples were frozen in liquid nitrogen and stored at −80°C. Experiments were performed in accordance with the Helsinki Declaration and the Guiding Principles in the Care and Use of Animals.

Cell Cultures

The ES cell line D3 [Doetschman et al., 1985], cultivated in the undifferentiated state on the feeder layer of primary cultures of mouse embryonic fibroblasts, was used [Wobus et al., 1984; Wobus et al., 1991]. Cells were cultivated on gelatine (0.1%)-coated petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany) in DMEM (Gibco BRL, Eggenstein, Germany), supplemented with 15% fetal calf serum (FCS; selected batches, Gibco), 2 mM L-glutamine (Gibco), β-mercaptoethanol (final concentration 5×10^{-5} M; Serva, Heidelberg, Germany), and nonessential amino acids (stock solution diluted 1:100;

TABLE I. Primers Used for Polymerase Chain Reaction†

Primer	Sequence 5′ → 3′	Product length (bp) with P5/P33	Ref.*
Forward primer for δ_2/δ_6	CCGGATGGGGTAAAGGAGTCAACTGAGAGCT	531/381	L13406
Forward primer for δ_3/δ_7	AAAAGGAAGTCCAGTTCGAGTGTTCAGATGAT	549/399	L13407
Forward primer for δ_4/δ_8	CTACCCCGGCGCTGGAGTCAAC	530/380	L13408
Forward primer for δ_9/δ_{10}	GTAAAGGAGCCCCAAACTACTGTAA	564/414	Mayer et al., 1995
P5 (reverse primer for δ_2, δ_3, δ_4, δ_9)	TCAGATGTTTTGCCACAAAGAGGTGCCTCCT		J05072
P33 (reverse primer for δ_6, δ_7, δ_8, δ_{10})	TTCTGACTGCATTGTCTTTGGCAT		J05072
Forward primer for GAPDH	CAGTCCATGCCATCACTGCC	584 bp	M32599
Reverse primer for GAPDH	GGGTCTGGGATGGAATTGTG		M17701

†All primers were purified by high-performance liquid chromatography (HPLC) and purchased from BioTez (Berlin, Germany).
*Cited are references or GenBank accession numbers.

Gibco) as described [Wobus et al., 1991]. For cardiac differentiation, Iscove's modification of DMEM (Gibco) containing 20% FCS (supplemented as described above) was used, with the exception that β-mercaptoethanol was replaced by 450 μM monothioglycerol (Serva). Cells (n = 600) were cultivated as embryoid bodies (EBs) in hanging drops for three days, followed by suspension culture in 6 cm bacteriological petri dishes (Greiner, Nuertingen, Germany). Undifferentiated ES cells ($1–2 \times 10^6$), as well as EBs (n = 25) in various developmental stages were collected and frozen in 400 μl lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 1% β-mercaptoethanol).

Reverse Transcription Coupled Polymerase Chain Reaction (RT-PCR)

Total RNA from muscle tissue was extracted according to methods described by Kingston et al. [1994], and from cell pellets with the RNeasy system according to the manufacturer. After isolation, remaining DNA contaminations were digested and RNA was re-extracted [Hoch et al., 1998]. RT-PCR and gel electrophoresis was performed as described [Hoch et al., 1998] with the following minor modifications. After reverse transcription, an aliquot of the reaction equivalent to 250 ng total RNA was used for PCR. The volume of this aliquot was increased to 50 μl and to a final amount of 5 μl 10xPCR buffer, 4 μl 25 mmol/L $MgCl_2$, 2 μl dNTP (2.5 mmol/L each nucleotide), 50 pmol of each specific primer (see Table 1), and 0.1 μl thermostable DNA polymerase. For sequencing, amplification products were extracted from agarose gels and standard cycle sequencing reactions were performed commercially by InViTek (Berlin-Buch, Germany) to confirm specificity of the single amplification products.

Preparation of Total Homogenates and Western Blotting

Tissue homogenization and Western blotting were performed according to Hoch et al. [1998]. For immunodetection, a δ-subclass-specific antibody that recognizes δ-CaMKII isoforms of subclass II was used in a dilution of 1 μg/ml. Specificity of the antibody was demonstrated previously [Hoch et al., 1998].

RESULTS

δ-CaMKII Variants in Cardiac and Skeletal Muscle Tissue of Adult Mouse

Primers obtained from corresponding sequences of rat were used for specific amplification of mouse δ-CaMKII isoforms (Table I). In preparations from adult mouse heart, transcripts of all investigated δ-CaMKII subclass I isoforms were detected (Fig. 1a, upper right). The strongest RT-PCR signal of an isoform of

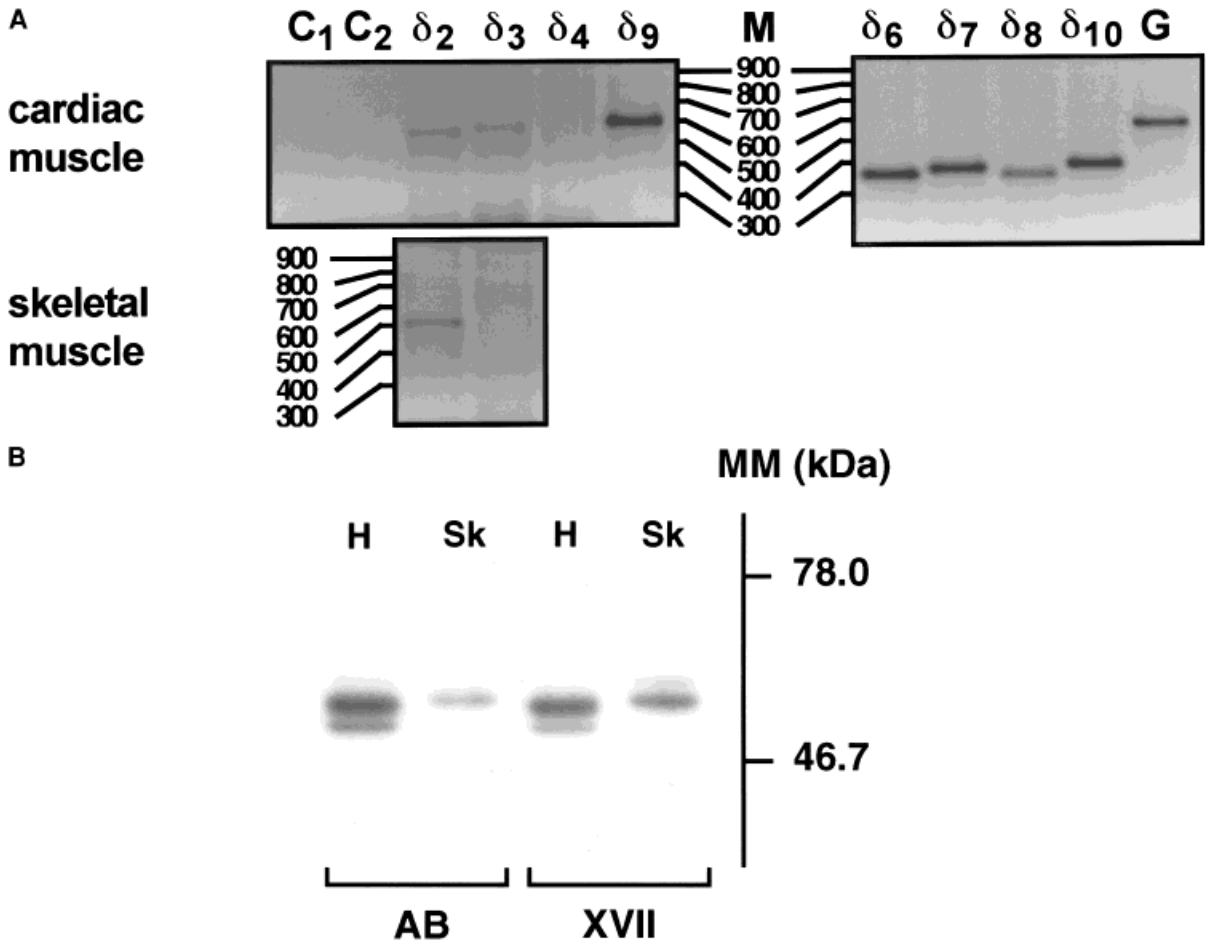

Fig. 1. δ-CaMKII isoform pattern in cardiac and skeletal muscle tissue of mouse. **a:** RT-PCR signals derived from mouse cardiac (upper) and skeletal muscle (lower) tissues obtained with various primer combinations specific for δ-CaMKII isoforms and GAPDH (G) (see Table I). **Lanes C1** and **C2** contain buffer control (PCR without nucleic acid) and RT-PCR in the absence of reverse transcriptase, respectively. Marker positions (M) are given as numbers of base pairs. Similar results were obtained with both investigated mouse strains, AB and XVII. **b:** δ-CaMKII protein in cardiac (H) and skeletal muscle (Sk) tissue of mouse strains AB and XVII. δ-isoforms containing the second variable domain were detected on immunoblots of tissue homogenates from cardiac and skeletal muscle by using a δ-CaMKII subclass-specific antibody. Amount of homogenate protein was 10 μg per lane.

subclass II was detected for δ_9. Much weaker signals were obtained for δ_2 and δ_3 (Fig. 1a, upper left). To specify tissue typic presence of the rat cardiac isoform δ_3 in mouse myocardium, we compared the transcriptional expression of δ_2 and δ_3 in the heart with that in skeletal muscle tissue. Whereas RT-PCR signals for δ_2 were detectable in skeletal muscle, no δ_3-specific amplification products could be obtained (Fig. 1a, lower left).

On the protein level, the C-terminal second variable domains from rat [Tobimatsu and Fujisawa, 1989] and mouse (GenBank accession number AF059029) are identical. Therefore, we were able to analyze murine δ-CaMKII subclass II protein in cardiac and skeletal muscle tissue by using an antibody specific for the second variable domain of rat [Hoch et al., 1998] (Fig. 1b). In both tissues, the major immunoreactive protein band was detected at about 58 kDa. In cardiac preparations there was a second protein band at 56 kDa that was not seen in skeletal muscle. However, in skeletal muscle an additional faint band at about 59 kDa appeared above the dominant immunoreaction. In mouse strain AB skeletal muscle, the amount of δ-CaMKII protein relative to total homogenate protein appeared to be lower than in strain XVII. Therefore, in this strain the weak 59 kDa signal became visible only

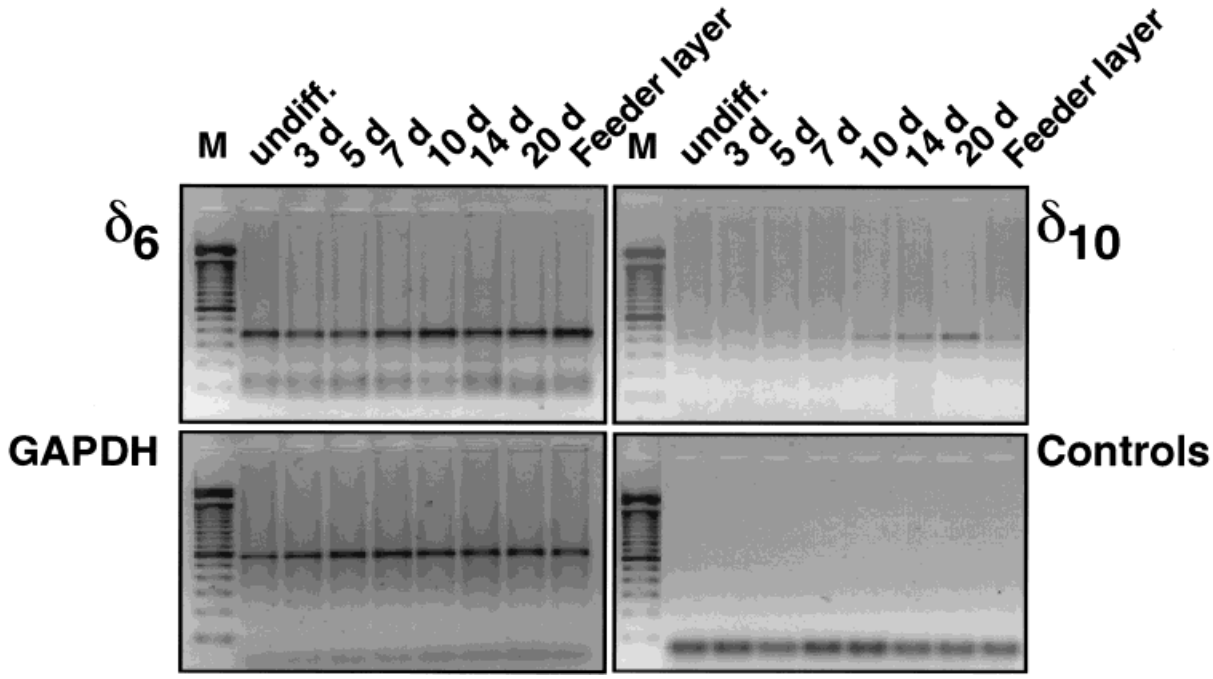

Fig. 2. δ-CaMKII isoform pattern in mouse embryonic stem cell-derived embryoid bodies (EBs) at various times of cardiogenic differentiation. Representative RT-PCR results for isoforms δ_6 and δ_{10} in undifferentiated ES cells (undiff.), and in EBs 3, 5, 7, 10, 14, and 20 days (d) after the onset of differentiation and in feeder layer cells. For comparison, GAPDH-specific amplification was done in parallel. Control lanes contain RT-PCR of the various samples in the absence of reverse transcriptase. Marker lanes are denoted as M.

after prolonged exposure of immunoblots to autoradiography (data not shown).

Expression of Transcripts of δ-CaMKII Isoforms During Cardiogenic Differentiation of ES Cells

ES cells from mouse were differentiated into cardiomyocyte-like cells up to 20 days. The δ-CaMKII transcript pattern was analyzed at various time points after the onset of cardiogenic differentiation. In two independent experiments reproducible RT-PCR signals for two isoforms could be detected, both belonging to δ-CaMKII subclass I (Fig. 2). Transcripts for δ_6 were found in similar amounts at all investigated stages as well as in control samples (undifferentiated ES cells and feeder layer cells). RT-PCR signals for δ_{10}, however, appeared only after 7–10 days of differentiation with increasing amounts up to 20 days of cardiogenic differentiation. Corresponding signals were also obtained from control samples but in much lower amounts. RT-PCR of the other investigated isoforms did not generate amplification products (δ_3, δ_4), or occasionally revealed a weak expression that was not reproducible (δ_2, δ_7, δ_8, δ_9) (data not shown).

DISCUSSION

This is the first report on the δ-CaMKII expression pattern in the murine heart. We describe tissue and differentiation-dependent changes in the expression of individual isoforms of both δ-subclasses in the mouse myocardium or cardiogenic differentiated ES cells.

Isoform δ_3 is typically expressed in cardiac muscle and aorta of the rat [Schworer et al., 1993; Edman and Schulman, 1994; Hagemann et al., 1999] and in the human heart [Hoch et al., 1999]. In agreement with these observations, we were able to demonstrate transcripts for δ_3 only in cardiac, but not in skeletal muscle tissue of the adult mouse. Due to the similar sizes of proteins for δ_3 [57.7 kDa in rat (Edman and Schulman, 1994)] and δ_9 [differing in rat only by three amino acids (Mayer et al., 1995)] and due to the specificity of our antibody, we were unable to separate the protein signals for δ_3 and δ_9. To what extent these two isoforms contribute to the major signal observed in cardiac tissue remains to be elucidated with an isoform-specific antibody that is not yet available. In skeletal muscle tissue, where no δ_3-

specific transcript was detected (Fig. 1a, lower left), the major signal therefore most likely represents δ_9.

On immunoblots of mouse skeletal muscle tissue, a weak signal of the expected size of δ_4 [Mayer et al., 1995; Hagemann et al., 1999] was detected. In adult rat, δ_4 was described as a specific isoform of skeletal muscle tissue [Schworer et al., 1993; Hagemann et al., 1999]. In cardiac tissue, the lack of a δ_4-specific amplification product and corresponding protein signal points to the skeletal muscle-specific expression of this isoform also in the adult mouse.

In cardiac homogenates, the minor signal below the major immunoreaction corresponds in size to the molecular mass of δ_2 [56.4 kDa in rat (Edman and Schulman, 1994)]. In mouse skeletal muscle tissue we identified transcripts for isoform δ_2 (Fig. 1a, lower left). However, there was no evidence for the presence of this isoform in skeletal muscle on the protein level (Fig. 1b). Hagemann et al. [1999] found a similar discrepancy for δ_2 between the transcript and protein levels in developing rat heart. Collectively, these results suggest a posttranscriptional regulation of the expression of δ_2 in muscle tissues.

Despite considerable differences in physiologically relevant cardiac parameters between mouse strains [Doevendans et al., 1998], we obtained similar cardiac δ-CaMKII expression patterns in two inbred mouse strains, suggesting the observed isoform pattern to be typic for the adult mouse.

We investigated the expression of both δ-subclasses in murine ES cell-derived cardiomyocytes representing early stages of cardiogenic differentiation. We were unable to demonstrate expression of members of the δ-subclass II in the cardiogenic differentiated ES cells. In one experiment, we detected a δ_9-specific signal 20 days after the onset of cardiogenic differentiation and a weak δ_2-specific signal after five days, but were not able to reproduce these results. However, reproducible RT-PCR signals were obtained for δ-isoforms belonging to subclass I. Significant amounts of transcripts for isoform δ_6 were detected at all investigated time points in differentiated and undifferentiated ES cells, as well as in feeder layer cells and the adult mouse heart. Thus, isoform δ_6 seems to be expressed ubiquitously and independently from the differentiation state. Also reproducible was the increase of the amount of transcripts for δ_{10} from days 7–10 of cardiogenic differentiation in ES cells. This may indicate a specific function of this isoform during cardiogenesis.

Bayer et al. [1999] analyzed transcripts for the CaMKII classes α, β, γ, and δ in the developing nervous and cardiac systems of five-day-old and adult mice (γ, δ). This study did not discriminate between individual δ-isoforms. However, it identified the early onset of δ-CaMKII expression in the brain, and significant transcript levels of the δ-class in five-day-old animals. Hagemann et al. [1999] investigated the expression pattern of cardiac δ-subclass II isoforms in developing rat heart, and classified δ_4 to be the typical isoform of the embryonic and neonatal rat heart. As we were unable to demonstrate expression of this isoform in cardiogenic differentiated ES cells, these cells may represent an earlier differentiation state than the earliest (embryonic day 14) studied by Hagemann et al. [1999].

Substrate specificity of the CaMKII holoenzyme is thought to be achieved by strictly controlling its localization, e.g. by isoform composition [Srinivasan et al., 1994; Shen et al., 1998]. Essential for holoenzyme formation seems to be the interaction of isoform monomers via their C-terminal association domains [Shen et al., 1998]. The presence or absence of a unique C-terminal extension is what differentiates the two δ-CaMKII subclasses. The differentiation-dependent expression of individual members of subclass I in ES cell-derived cardiomyocytes suggest a specific role of various members of the δ-CaMKII family in cardiac differentiation. This may substantially influence the formation of the holoenzyme and thus its intracellular localization.

These changes may lead to alterations in the accessibility for substrates in the differentiating cardiomyocyte, and are therefore of potential physiological relevance for cardiogenesis. Such processes may affect the phosphorylation and regulation of substrates involved in the developing excitation/contraction mechanism of the beating cardiomyocyte [Anderson et al., 1994; Li et al., 1997; Xu and Narayanan, 1999], as well as changes of gene expression when CaMKII isoforms containing nuclear localization signals as isoforms δ_3 and δ_7 are expressed [Ramirez et al., 1997]. To address these issues, future investigations may involve the overexpression of individual δ-CaMKII isoforms in ES

cells in order to study their influence on the cardiogenic differentiation of these cells.

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