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Developmental Regulation of 14-3-3 ϵ Isoform in Rat Heart

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Abstract Human heart cDNA sequencing yielded a cDNA clone that is similar in DNA and amino acid sequences to that of mouse 14-3-3 ϵ isoform. The 6xHis-tagged H1433ϵ recombinant protein was expressed in *Escherichia coli* and its size was approximately 30 kDa. From Northern blot results with human multiple tissues, human skeletal muscle was found to have the highest level of h1433ϵ mRNA expression, whereas Northern blots of human cancer cell lines detected the highest mRNA level of h1433ϵ in colorectal adenocarcinoma SW480. The protein expression level of h1433ϵ and Raf-1 is found to be regulated coordinately during rat heart development, and their protein expression was highest from 14.5 to 16.5 days postcoitum. J. Cell. Biochem. 68:195–199, 1998.

Key words: 14-3-3 protein; developmental regulation; heart development; Raf-1

14-3-3 proteins belong to a family of acidic proteins named after their migration positions on two-dimensional DEAE-cellulose chromatography and starch-gel electrophoresis [Aitken, 1995]. Seven mammalian brain isoforms of 14-3-3 proteins have been discovered, named α to η after their respective elution positions on high-performance liquid chromatography (HPLC) [Robinson et al., 1994]. The 14-3-3 proteins may exist as dimers with subunit molecular mass of 29–30 kDa [Suen et al., 1995]. Comparison of the amino acid sequence of the ϵ isoform to the sequence of the plant and vertebrate 14-3-3 proteins indicated that the ϵ isoform is the isoform closest to the plant counterpart [Roseboom et al., 1994]. Besides having the highest sequence homology, the ϵ isoform also has a similar length as the plant isoform. This suggests that the ϵ isoform is the least diverged mammalian isoform of the 14-3-3 proteins. 14-3-3 proteins are expressed in most tissues, and their protein sequences are conserved across a range of mammalian species [Aitken et al., 1992]. Along with the discovery of 14-3-3 protein homologues in plants [Lu et al., 1992] and yeasts [Ford et al., 1994], it has been suggested that these proteins might have important and diverse physiological functions.

A number of functions have been proposed for 14-3-3 proteins, including inhibition of protein kinase C (PKC) [Robinson et al., 1994], activation of Raf-1 protein kinase [Li et al., 1995], regulation of tyrosine/tryptophan hydroxylases [Ichimura et al., 1988] and N-acetyltransferases [Aitken et al., 1992], and determination of the timing of mitosis [Ford et al., 1994]. The association of the 14-3-3 proteins with all these enzymes further supports an important role for the 14-3-3 proteins in the signal transduction pathway. Since signal transduction pathway involves a vast number of protein kinases, protein phosphatases, and various transducing proteins, successful cloning and expression of the 14-3-3 proteins should help unravel the signal transduction pathways in cell proliferation and development through in vivo and in vitro studies.

Abbreviations: DEAE, diethylaminoethyl; DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminoprophyl) carbodiimide; h1433ϵ, human 14-3-3 ϵ isoform; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; PKC, protein kinase C; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris(hydroxymethyl)aminomethane-buffered saline-Tween 20.

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Previous studies on 14-3-3 proteins have been done in the brain [Robinson et al., 1994]; however, their mode of expression in other tissues such as the heart is unclear. In the present study, h1433ϵ cDNA was cloned from a human fetal heart cDNA library and was characterized using Northern hybridization and Western hybridization. H1433ϵ isoform was also expressed as a histidine-tagged recombinant protein in *Escherichia coli*.

MATERIALS AND METHODS

Cloning and Protein Expression

PCR of h1433ϵ was done by using a pair of h1433ϵ cloning primers (forward: 5′-GCGCGGATCCGATGACGATGACAAAGATGATCGAGAGGATCTGGTG-3′; reverse: 5′-TAGGGCGTCGACTGGTTTCTCTTGGCTTATGTCTCACTGTTG-3′)and a human fetal heart cDNA library [Hwang et al., 1994] as the template. The procedures were performed as described previously [Hwang et al., 1995]. A *Bam*H1 site and a *Sal*I site, shown as the underlined bases, were designed in the h1433ϵ forward and reverse cloning primers, respectively. End-clamps that consist of GCGC in the forward primer and TAGGGC in the reverse were added to facilitate cleavage by the restriction enzymes. After cutting with restriction enzymes, the polymerase chain reaction (PCR) product was subcloned into the T5 expression vector, pQE-30. The recombinant plasmid pQE-30-h1433ϵ was transformed into the *E. coli* strain M15(pREP4). The synthesis of the 6xHis-tagged h1433ϵ protein in *E. coli* was induced by the addition of IPTG at a concentration of 0.4 mM. The bacterial crude extract was electrophoresed in a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). H1433ϵ protein was purified using Ni-NTA affinity chromatography by QIAexpress purification system (Qiagen).

Northern Hybridization

The human multiple tissue Northern (MTN) blot, and human cancer cell line multiple tissue Northern (MTLN) blot were purchased from Clontech. The integrity of the poly A^+ RNAs of the Northern hybridization was examined by denaturing gel electrophoresis and probing with a radioactively labeled human β-actin cDNA control probe (unpublished data, quality control sheet supplied from Clontech, Palo Alto, CA). A pair of primers flanking the coding region of the h1433ϵ were designed to give a PCR product of 814 base pairs (bp). A radioactive random-primed probe was made using the purified PCR product as the template. Northern hybridization was done by hybridizing the nylon membrane-bound RNA with the [^{32}P]-labeled probe at 42°C in the presence of 50% formamide for 24 h. The membrane was washed with 0.1× SSC/0.1% SDS to remove the nonspecific signals. Autoradiography was performed at −70°C for 4 days.

Protein Preparation

Various developmental stages of rat fetal heart were isolated and rinsed with cold PBS. The heart tissues were immediately homogenized and washed three times with pre-chilled either. They were then freeze-dried overnight and kept at −70°C until use. The dried homogenized tissues were lysed in a minimal volume of buffer, consisting of 8M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl, 10 mM DTT at pH 8.0. Supernatants of lysate were collected and the total cardiac protein (100 μg/lane) were normalized using BioRad protein assay kit before loading onto a 12% SDS polyacrylamide gel.

Immunological Procedures

Anti-h1433ϵ antibodies were raised against an acetylated h1433ϵ peptide, residues 30–48 (Ac-VAGMDVELTVEERNLLSV); 0.1 mg of the h1433ϵ peptides were mixed with KLH solution (5 mg/ml) for 30 min. EDAC (10 mg/ml) was added to the mixture and stirred for 1 h. The peptide conjugate was purified with a G-50 Sephadex desalting column (Pharmacia, Piscataway, NJ), and injected subcutaneously with complete Freund's adjuvant for the first injection and incomplete one for the subsequent ones.

Anti-Raf-1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western Hybridization

Proteins were run on a 12% SDS polyacrylamide gels and were visualized with Coomassie blue staining. SDS–PAGE gel used in Western hybridization was immersed for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) and transferred with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) onto a PVDF membrane (Millipore, Bedford, MA). The PVDF membrane was blocked with 5% nonfat milk powder in

TBST (20 mM Tris, 137 mM sodium chloride, 1 M hydrochloric acid (HCl), 0.05% Tween-20, pH 7.6) for 2 h and probed with h1433ϵ-specific antibodies (1 : 300) or with Raf-1 polyclonal antibodies (1 : 300) in TBST overnight. HRP-labeled goat antirabbit antibodies were used as the secondary antibodies (1 : 3,000). Binding was detected by enhanced chemiluminescence (ECL) detection reagents (Amersham, Life Science, Arlington Heights, IL), and exposed on BioMax MR films (Kodak, Rochester, NY).

RESULTS

Cloning and DNA Sequencing of h1433ϵ

H1433ϵ cDNA has an open reading frame of 771 bp that codes for 257 amino acids. The DNA sequence of h1433ϵ from the human fetal heart cDNA library is in good agreement with the one cloned from the HeLa cell two-hybrid library [Conklin et al., 1995]. The isoelectric point as determined by the software PROSIS is 4.48.

Expression of h1433ϵ in *E. coli*

H1433ϵ cDNA was expressed in *E. coli* M15(pREP4). The purified 6xHis-tagged h1433ϵ protein was analyzed in 15% SDS–PAGE. The size of the expressed protein is consistent with the calculated molecular mass of the 6xHis-tagged h1433ϵ protein (data not shown). Western hybridization result also confirmed the identity of the 6xHis-tagged h1433ϵ protein (data not shown).

Northern/Western Hybridization

Analysis of the tissue distribution of the h1433ϵ mRNA shows that the mRNA level of h1433ϵ is highest in skeletal muscle (Fig. 1, lane 6). H1433ϵ mRNA level was lower in heart, brain, placenta, kidney and pancreas and lowest in lung and liver (Fig. 1). However, previous Northern hybridization of 14-3-3 β, η, and γ isoforms showed a different patterns of tissue distribution from that of h1433ϵ [Watanabe et al., 1993]. It has been shown that the mRNA expression of the three isoforms β, η, and γ, was relatively high in lymphoid tissues, spleen, and thyroid, and lower in heart and kidney. From the human cancer cell line multiple tissue Northern blot, colorectal adenocarcinoma SW480 showed the highest h1433ϵ mRNA level (Fig. 2, lane 6). HeLa cell S3, chronic myelogenous leukemia K-562, Burkitt's lymphoma Raji, lung carcinoma A549, and melanoma G361 were lower whereas promyelocytic leukemia HL-60, and lymphoblastic leukemia MOLT-4, showed the lowest h1433ϵ mRNA level (Fig. 2).

From the Western hybridization result, our anti-h1433ϵ antibody is shown to be able to detect a single 30-kDa band, which corresponds to the h1433ϵ intact protein without any obvious degradation product. From our developmental time course study, the expression of the h1433ϵ protein was found to be highest from 14.5 to 16.5 days postcoitum and return to its previous level thereafter (Fig. 3a). The Western hybridization signals were quantitated by comparing with the bacterial expressed h1433ϵ protein, and we found that the amount of h1433ϵ protein is approximately 0.1% of the total rat cardiac protein at its peak level of expression. This pattern of changes in the h1433ϵ protein expression is reproducible upon repeated analysis of additional sets of the developing rat cardiac proteins. This difference in the h1433ϵ protein expression level is not due to an increase in protein degradation of the total cellular proteins, since Coomassie blue staining of the total protein pattern remains unchanged

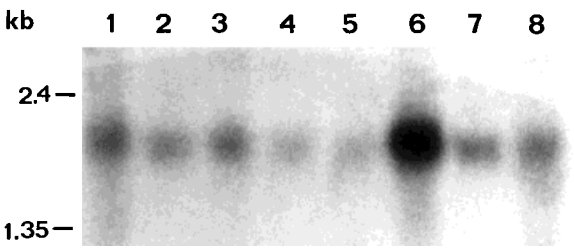

Fig. 1. Tissue distribution of h1433ϵ mRNAs of the various human tissues. Northern hybridization used h1433ϵ as a probe. **Lane 1,** heart; **lane 2,** brain; **lane 3,** placenta; **lane 4,** lung; **lane 5,** liver; **lane 6,** skeletal muscle; **lane 7,** kidney; **lane 8,** pancreas.

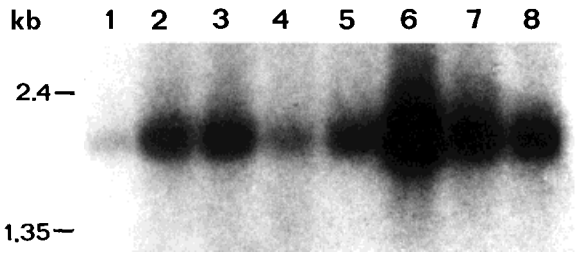

Fig. 2. H1433ϵ protein distribution in the various cancer cell lines. Northern hybridization used h1433ϵ as a probe. **Lane 1,** promyelocytic leukemia HL-60; **lane 2,** HeLa cell S3; **lane 3,** chronic myelogenous leukemia K-562; **lane 4,** lymphoblastic leukemia MOLT-4; **lane 5,** Burkitt's lymphoma Raji; **lane 6,** colorectal adenocarcinoma SW480; **lane 7,** lung carcinoma A549; **lane 8,** melanoma G361.

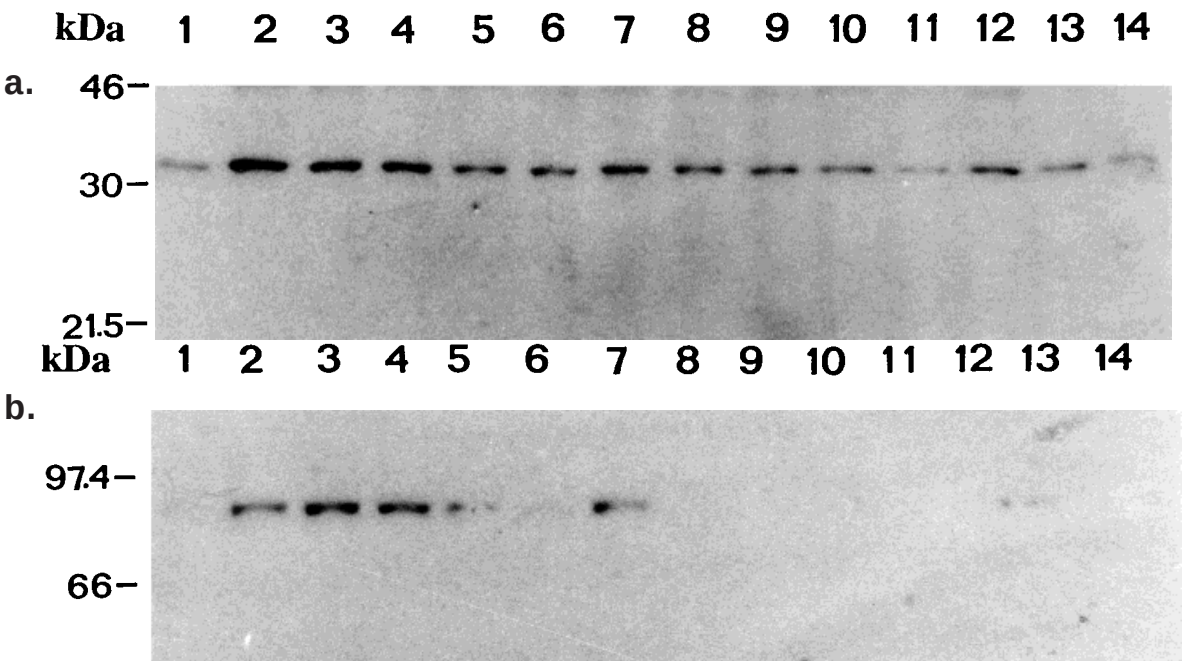

Fig. 3. **a:** Detection of the h1433ϵ isoforms at the various stages of rat heart development during gestation by Western hybridization. **lane 1,** 13.5 dpc; **lane 2,** 14.5 dpc; **lane 3,** 15.5 dpc; **lane 4,** 16.5 dpc; **lane 5,** 17.5 dpc; **lane 6,** 18.5 dpc; **lane 7,** 19.5 dpc; **lane 8,** 20.5 dpc; **lane 9,** 21.5 dpc; **lane 10,** newborn; **lane 11,** day 1; **lane 12,** day 2; **lane 13,** day 3; **lane 14,** day 4. The h1433ϵ protein was detected using anti-h1433ϵ antibody. **b:** The Western blot from a was regenerated for the detection of the protein expression of Raf-1 isoforms at the various stages of rat heart development. Raf-1 protein was detected using anti-Raf-1 polyclonal antibody.

(data not shown). Furthermore, in a parallel study, the protein expression pattern of Raf-1 was found to be similar, but not identical, to that of h1433ϵ (Fig. 3b).

DISCUSSION

It has been found that 14-3-3 proteins associate with a number of proto-oncogenes and oncogenes, such as Raf-1, polyomavirus middle T antigen, Bcr, Bcr/Abl, and cdc25 [Aitken, 1995]. All these proteins are membrane-bound proteins involved in signal transduction. Common features amongst these proteins are the presence of both cysteine- and serine-rich regions. From our Northern hybridization experiment with the cancer cell line, colorectal adenocarcinoma SW480 showed the highest h1433ϵ mRNA level (Fig. 2, lane 6); however, the phosphorylation level of Raf-1 was found to be very low [Eggstein et al., 1996]. In promyelocytic leukemia HL-60, the mRNA level of h1433ϵ was low (Fig. 2, lane 1), but Raf-1 was found to be highly phosphorylated [Okuda et al., 1994]. These may support the observation that the phosphorylation state of the 14-3-3 interacting protein is important for association with 14-3-3 protein; this may be the general mechanism used by the 14-3-3 proteins to interact with others [Michaud et al., 1995].

From our Western hybridization results, the expression level of 1433ϵ protein in rat heart was highest from 14.5 to 16.5 days postcoitum and returns to its previous level thereafter (Fig. 3a). Our results indicate that the expression of h1433ϵ is developmentally regulated in the rat heart. Previous results also demonstrated that the expression pattern of the rat 1433ϵ protein in the pineal gland decreases as it goes from embryonic day 19 to day 60; this expression pattern was suggested to correlate with the development of the photoreceptor in the pineal gland [Roseboom et al., 1994]. These observations indicated that the expression of the h1433ϵ protein is controlled developmentally in a similar fashion in both heart and pineal gland. It has been suggested that 14-3-3 isoforms may function independently or that they may form heterodimers with other 14-3-3 isoforms [Roseboom et al., 1994]. It would be interesting to see whether the formation of either homodimers or

heterodimers, or both, would contribute to the difference in their functions and protein expression in the different tissues. Further examination of the h1433ϵ isoform is needed to find out whether this represents a general trend of h1433ϵ protein expression in all tissues.

A parallel study on the protein expression pattern of Raf-1 in the developing rat heart was performed. From the Western hybridization of the developing rat heart, Raf-1 was shown to have a protein expression pattern similar to that of h1433ϵ (Fig. 3b). Along with the Northern hybridization result of the cancer cell line, our Western blot results suggest a close relationship between h1433ϵ and Raf-1. From previous results, 14-3-3 proteins are found to associate with both the active and inactive forms of Raf-1 [Freed et al., 1994]. In order to account for the inconsistency, it had been suggested that different 14-3-3 isoforms might be associated with a different level of phosphorylated Raf [Aitken, 1995]. These protein complexes can, in turn, govern the downstream phosphorylation events in the signal transduction pathway, leading to cell proliferation or cell differentiation. Nevertheless, it had been suggested that the role of the 14-3-3 proteins might act as a chaperone protein, stabilizing or facilitating multimeric complexes formed between Raf-1 and other signaling proteins, rather than being involved in the regulation of catalytic activities [Suen et al., 1995]. Further characterization of the h1433ϵ protein is needed to resolve the correlation among h1433ϵ, Raf-1 and other protein kinases.

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