S100α, CAPL, and CACY: Molecular Cloning and Expression Analysis of Three Calcium-Binding Proteins from Human Heart[†,‡,§]

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ABSTRACT: Elevated levels of intracellular calcium are a major cause of myocardial dysfunction. To find possible mediators of the deregulated calcium we searched for EF-hand calcium-binding proteins of the S100 family. By PCR technology we identified three members of the S100 protein family (S100α, CACY, and CAPL) in the human heart. We cloned the corresponding cDNAs and examined their expression levels in various human tissues by Northern blot analysis. All three proteins are expressed at high levels in the human heart. Whereas CACY and CAPL mRNAs are expressed ubiquitously, S100α mRNA is restricted to heart, skeletal muscle, and brain. Interestingly, the expression pattern of S100α, CACY, and CAPL in human tissues differs significantly from that in rodent tissues.

Mechanisms leading to cardiac dysfunction are not well understood. Altered calcium regulation may contribute to myocardial diseases [for reviews, see Barry (1991) and Morgan (1991)]. For example, in hypertrophic or ischemic myocardium the intracellular calcium levels are elevated (Morgan et al., 1990; Kihara et al., 1989). During ischemia the responsiveness of the troponin C–myofilament system to calcium is impaired and the calcium activated force is decreased (Korestune et al., 1990). In prolonged ischemia the elevation of the intracellular calcium level even leads to cell injury. Increased calcium may activate calcium-dependent proteases, nucleases, phospholipase A_2, or kinases, resulting in a breakdown of membrane components (Chien et al., 1985; Nicotera et al., 1989) and cytoskeletal components (Steenbergen et al., 1986), degradation of nuclear DNA (Cantoni et al., 1989), and phosphorylation of proteins (Klee, 1991).

Good candidates to mediate the intracellular calcium response are Ca^{2+}-binding proteins of the EF-hand type (e.g., calmodulin, troponin C, and S100 proteins). Whereas calmodulin is ubiquitously distributed in vertebrate tissues, and thus probably possesses a universal function, S100 proteins are expressed in a tissue- and cell-specific manner [for review, see Hilt et al. (1991)]. This suggests that S100 proteins may have distinct cellular functions depending on the phenotype of the cells in which they are expressed. It is thought that S100 proteins regulate phosphorylation of proteins, influence cellular differentiation, cell cycle progression, and signal transduction. S100 proteins (11 members of ≈10 kDa are presently known) contain two distinct EF-hand calcium-binding loops of 14 and 12 amino acids, respectively. The 14 amino acid loop is specific for S100 proteins, and both calcium-binding loops are of relatively low affinity. Like calmodulin, S100 proteins change their conformation upon calcium binding, exposing hydrophobic regions that interact with target proteins. In the human heart, S100 proteins are highly enriched; S100α, for example, represents approximately 0.2% of soluble heart proteins (Kato et al., 1986).

Some members of this family are associated with human diseases. S100α is increased in serum after myocardial infarction (Usui et al., 1990). S100β may be associated with Down's syndrome (Allore et al., 1988; Kato et al., 1990), Alzheimer's disease (Griffin et al., 1989), and chronic lung disease (Kahn et al., 1991). Also, the calgranulins CAGA and CAGB are present at elevated levels in patients with cystic fibrosis (Wilkinson et al., 1988; Dorin et al., 1987) and rheumatoid arthritis (Odink et al., 1987).

To study the role of S100 proteins in cardiac function, we first examined which of the 11 known members are expressed in the human heart. Using the polymerase chain reaction (PCR), we identified three members of the S100 protein family from which we isolated the corresponding cDNAs. Thus, we report here new human sequences of S100α and CAPL. Furthermore, we examined their expression levels in human tissue by Northern blot hybridization.

MATERIALS AND METHODS

Isolation of RNA. Tissue samples, obtained from autopsy (10–20 h after death), were immediately frozen on dry ice and stored at −70 °C. Total cytoplasmic RNA was isolated according to Chirgwin et al. (1979). In short, 1 g of tissue was quick-frozen in liquid N_2, crushed, and homogenized in 3 mL of GTC buffer (5 M guanidinium thioisocyanate, 60 mM Tris-HCl, pH 7.4, 15 mM EDTA, 1% 2-mercaptoethanol, 0.5% lauroylsarcosine) using a polytron homogenizer. Insoluble material was removed by centrifugation (10 min, 4000*g*). The supernatant was layered onto a 1.5-mL cushion of 5.7 M CsCl and 0.1 M EDTA (pH 7.5) in a Sorvall TST 60.1 tube and centrifuged (36 000 rpm, 16 h, 20 °C). The pellet was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and the RNA precipitated at −20 °C in the presence of 0.1 vol of 3 M sodium acetate (pH 5.2). The RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated H_2O and stored at −70 °C.

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‡ The cDNA sequences described in this report are listed in the GenBank/EMBL database under the Accession Nos. X58079 (human S100α) and M80563 (human CAPL).

§ As several different names have been given to S100 proteins, we will use the nomenclature of the committee of the Tenth International Workshop on Human Gene Mapping (Bruns et al., 1989). All other names are listed in Figure 1.

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Oligonucleotide Primers. Oligonucleotides were synthesized (Pharmacia Gene Assembler), removed from the cartridge, and purified by a single ethanol precipitation. Finally, they were dissolved in H_2O and stored at −20 °C. The degenerated oligonucleotide primers, ConsI, ConsII, and ConsIII, were designed on the basis of an alignment of all available cDNA sequences of the S100 protein family. Primers ConsI and ConsII were constructed against the first calcium-binding loop and primer ConsIII against the second calcium-binding loop. Degeneracy varied from 13 824- to 576-fold. All primers include 17–19 bases of the coding region. For subcloning they were extended at their 5′-end with recognition sequences of restriction endonucleases.

ConsI: 5′-AGAATTCCA(CT)(ACG)(AC)(ACG)(CT)A(CT)TC(ACGT)GG-3′

ConsII: 5′-CTGCTCGAGC(AG)(CT)(ACGT)TT(CT)CAC(ACG)(ACG)(ACG)(CT)A(CT)(GT)C-3′

ConsIII: 5′-GTACTCGAGGAAGT(CT)(AC)(ACT)(CG)(CGT)T(CG)(ACGT)(CT)(CGT)(AG)(AT)C-3′

First Strand cDNA Synthesis. Three micrograms of total cytoplasmic RNA and 0.1 nmol of oligo$(dT)_{15}$ were heated (10 min at 65 °C) and then quickly chilled on ice and diluted into a total volume of 20 μL with a solution containing 100 units of RNasin (Promega), 2 mM each of all four deoxynucleotides (Boehringer), 50 mM Tris (pH 8.3), 75 mM KCl, and 10 mM DTT. After short preincubation at 37 °C, 200 units of reverse transcriptase (Superscript, BRL) was added. The reaction was stopped after 30 min at 37 °C by heating (10 min at 65 °C).

Polymerase Chain Reaction. The polymerase chain reaction (PCR) was performed according to a modified procedure originally described by Saiki et al. (1988). PCR was performed in 100 μL of reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 5 mM KCl, pH 7.5) containing 200 nmol each of the degenerated primers (ConsI and ConsIII or ConsII and ConsIII, respectively), 20 nmol each of all four dNTPs (Boehringer), 1 μL of the first strand cDNA mixture, and 2.5 units of Taq DNA polymers (Boehringer). The reaction mixture was overlayed with 75 μL of mineral oil and placed into a thermal cycler (Perkin Elmer). The cycle steps were as follows: 5 cycles at 37 °C annealing temperature (1 min at 95 °C, 1 min at 37 °C, heating to 65 °C in 2 min 30 s, 1 min at 65 °C) followed by 30 cycles at 42 °C annealing temperature (1 min at 95 °C, 1 min at 42 °C, heating to 65 °C in 1 min, 1 min at 65 °C). Following amplification, the PCR products were extended for an additional 8 min at 65 °C.

Subcloning of the PCR Products. Following a phenol/chloroform extraction, the PCR product was ethanol precipitated, dissolved in the appropriate restriction enzyme buffer, and digested for 1 h at 37 °C with either 25 units of *Eco*RI and *Xho*I (if primer ConsI was used in the PCR) or *Spe*I and *Xho*I (if primer ConsII was used). The PCR product of 200 bp length was gel purified and subcloned into the Bluescript SK^+ vector (Stratagene). The subcloned fragments were sequenced by the method of Sanger et al. (1977), using a T7 sequencing kit (Pharmacia).

Screening a cDNA Library with Antibodies against S100α. Nitrocellulose filters (Schleicher & Schuell) were soaked with 10 mM IPTG and dried at room temperature. 100 000 phages of a human heart expression cDNA library were plated on four 140-mm dishes. After 3.5 h preincubation at 42 °C, dishes were overlayed with the pretreated nitrocellulose filters and incubated for an additional 3.5 h at 37 °C. The removed filters were screened using a monoclonal antibody directed against bovine S100α (JIMRO, Japan) (Noda et al., 1988) and the Proto Blot kit (Promega). After blocking with 1% FCS (fetal calf serum) and 3% BSA (bovine serum albumin) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM Ca_2Cl, 0.05% Tween 20) for 15 min at 37 °C, the filters were incubated with monoclonal antibodies (1:100 dilution in TBST) for 1 h at room temperature. Filters were then washed two times with TBST, incubated with an alkaline phosphatase coupled secondary antibody (1:7500 dilution in TBST), washed again two times with TBST, and finally developed using NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

Screening a cDNA Library Using Radiolabeled Probes. Gel-purified DNA fragments of the partial S100α cDNA clone (obtained by the antibody screening procedure) and of the partial CAPL clone (one of the subcloned PCR products) were labeled with [^{32}P]dCTP by the method of Feinberg and Vogelstein (1983), using the random prime labeling kit of Boehringer. Specific activities of 3×10^9 and 1×10^9 cpm/μg were obtained. 200 000 phages were screened using standard procedures (Sambrook et al., 1989). Washing conditions: twice at 65 °C with 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) and once with 0.2× SSC for 20 min each.

Excision and Sequencing of the cDNA Clones. cDNAs that showed a positive signal after the second round of hybridization were clonally purified. The in vivo excision of the cDNA containing Bluescript vector from the lambda Zap phages followed the protocol of Stratagene. Every isolated clone was sequenced using the T7 sequencing kit (Pharmacia) and double-stranded DNA as template. In order to obtain unambiguous sequences of the full length cDNA clones of S100α and CAPL, we used single-stranded DNA as template. Both clones were sequenced in both directions in an overlapping manner.

Northern Blot Hybridization. This was performed using standard procedures (Sambrook et al., 1989). Total cytoplasmic RNA (10 μg) from different tissues was separated on a formaldehyde-containing agarose gel, blotted onto Nytran N membranes (Schleicher & Schuell), and cross-linked to the membrane by UV irradiation (Stratalinker). Gel-purified cDNA inserts of S100α, CACY, CAPL (this report), and β-actin (Ponte et al., 1984) were ^{32}P labeled using the random prime labeling kit (Boehringer) (Feinberg & Vogelstein, 1983). Specific activities of $2–4 \times 10^9$ cpm/μg of DNA were obtained. Hybridization was performed in an Hybaid hybridization oven at 42 °C for 15 h in 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.1% SDS, 2× Denhardts' (1× Denhardts' = 1% ficoll, 1% BSA, 1% polyvinylpyrrolidone), 100 μg/mL denatured DNA from salmon sperm, and 2×10^6 cpm/mL of the radiolabeled probe. After hybridization, the filters were washed two times at 65 °C for 30 min with 2× SSC/0.1% SDS and one time for 30 min with 0.5× SSC/0.1% SDS. Filters were exposed for 24 h (S100α), 2 days (β-actin), or 4 days (CAPL, CACY) at −70 °C using intensifying screens.

RESULTS

Identification of S100 Proteins That Are Expressed in the Human Heart. Eleven members of the S100 family of calcium-binding proteins are presently known that are expressed in different vertebrate tissues. To search for S100 proteins in the human heart, we adapted the PCR technique. PCR using degenerated oligonucleotides as primers is a powerful method for the identification of proteins with a high degree of homology

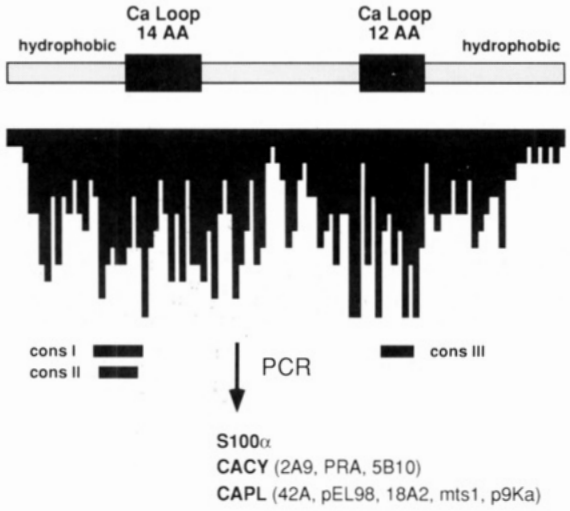

FIGURE 1: Homology profile of S100 proteins. All presently known S100 proteins were aligned to maximum homology. The length of the columns reflects the number of identical amino acids at this particular position. The general structure of S100 proteins with two calcium loops and hydrophobic regions is indicated at the top. The location of the degenerated oligonucleotides used for PCR is indicated by bars. The following sequences were used for the alignment: S100α, human (this report); S100β, human (Allore et al., 1990); S100L, human (Lee et al., 1992) [sequence comparison strongly indicates that the cDNA clone CaN19 isolated by Lee et al. (1992) is the human homologue of bovine S100L identified by Glenney et al. (1989)]; S100C, porcine (Ohta, et al., 1991) (synonym: calgizzarin); S100P, human (Emoto et al., 1992); CACY, human (Calabretta et al., 1986) (synonyms are calcyclin, 2A9, PRA, and 5B10); CAPL, human (this report) (synonyms are p9Ka, 18A2, pEL98, 42A, mts1, and calvasculin); Ca[1], human (Kube et al., 1991) (synonyms are p10, p11, 42C, and calpactin light chain); CaBP9K, bovine (Fullmer et al., 1980) (synonyms are IaBP and calbindin-D_{9K}); CAGA, human (Odink et al., 1987) (synonyms are CFAg, MIF, MRP8, p8, L1 Ag 1c, MAC387 Ag α chain, and 60B8Ag); CAGB, human (Odink et al., 1987) (synonyms are CFAg, MIF, MRP14, p14, L1 Ag 1c, MAC387 Ag β chain, 60B8Ag, and CFP).

(Kamb et al., 1989; Compton, 1989). To construct the proper PCR primers, the cDNA sequences of all S100 proteins were aligned for a maximum of homology (Figure 1). PCR primers ConsI and ConsII were designed to regions in the first calcium-binding loop that show the highest degree of homology; PCR primer ConsIII was designed to a region of the second calcium-binding loop. We used two different primers aligned to the first calcium-binding loop to allow amplification of all members of this family. Every primer was extended at the 5′-end with recognition sequences for restriction endonucleases.

As template for the PCR, total cytoplasmic RNA was isolated from human heart and the first strand cDNA synthesized by reverse transcription using avian reverse transcriptase and oligo(dT) as a primer. We used low annealing temperatures to allow for mismatches to occur during the PCR. Two different amplifications were performed, using either oligonucleotides ConsI and ConsIII or ConsII and ConsIII as primers. Agarose gel electrophoresis revealed a single DNA band of 200 bp in both cases. The products were then subcloned and 35 clones were sequenced. Twelve clones showed a high degree of homology to the published sequence of bovine (Kuwano et al., 1986) and rat (Zimmer et al., 1991) S100α, eight clones were highly homologous to the murine (Jackson-Grusby et al., 1987) and rat (Masiakowski et al., 1988) sequences of CAPL, and eight clones had a sequence identical to the published human CACY cDNA clone (Calabretta et al., 1986). Six could not be identified as members of the S100 protein family.

The high degree of homology of the short sequences to the published vertebrate S100α and CAPL cDNAs suggested that we cloned fragments of the corresponding human cDNAs. To confirm this hypothesis for the entire coding sequence, we isolated the corresponding full length cDNAs.

Isolation of Full Length Human cDNA Clones of S100α and CAPL. To date, a human cDNA clone has been described only for CACY (Calabretta et al., 1986). Therefore, we decided to screen for full length cDNA probes of human S100α and CAPL. A partial cDNA clone of S100α was obtained by screening fusion proteins of a human heart cDNA library (Stratagene) with a monoclonal antibody directed against bovine S100α (JIMRO, Japan) (Noda et al., 1988). This incomplete clone was used to rescreen the same library. Out of 400 000 screened phages, 15 showed a positive signal after the second round of screening. They were identified by partial sequencing as cDNA clones coding for human S100α. The longest cDNA clone was chosen for complete sequencing. This cDNA of 607 bp contains a 13 bp poly(A) tail, the complete open reading frame, and 113 bp of the 5′ untranslated region (Figure 2a). Of the entire human nucleotide sequence, 78% was identical to the bovine sequence (Kuwano et al., 1986). Two short cytidine-rich insertions in the 5′ (11 bp) and 3′ (32 bp) untranslated regions were present in the human but not in the bovine sequence. The homology in the translated region is much higher (93%). In the deduced amino acid sequence, only one difference in the human protein is found when compared to the bovine sequence (position 51: alanine in bovine, valine in human; see Figure 3). We therefore concluded that we have isolated the true human homologue of S100α. The incomplete rat S100α cDNA published recently (Zimmer et al., 1991) (the 3′ end is missing) shows an overall identity of 80% to the human sequence; seven amino acids are different. The short insertion in the 5′ untranslated region of the human sequence is also not present in the rat sequence.

We used the same human heart cDNA library as described above, for the cloning of the S100α cDNA, to isolate a human cDNA coding for the second set of PCR clones, homologous to the rodent CAPL sequences. As a probe, the radiolabeled subcloned PCR fragment was used. Out of 400 000 phages screened, four showed a positive signal after the second round of screening and were identified by partial sequencing to encode the PCR product. The longest clone was chosen for complete sequencing. The cDNA contains a poly(A) tail of 20 nucleotides, an open reading frame of 100 amino acids, and 135 bp of the 5′ untranslated part (Figure 2b). The overall sequence homology is 79% and 78%, compared to the mouse (Jackson-Grusby et al., 1987) and rat (Masiakowski et al., 1988) CAPL sequences, respectively. The translated part of the cDNA clone shows a higher homology of 89%. Only seven or nine amino acids are different in our human sequence when compared to the rat or mouse sequences, respectively (see Figure 3), hence we conclude to have isolated the human homologue of CAPL.

In both cDNA clones, the first methionine codon of the longest open reading frame was chosen as translation start site. Only the CAPL cDNA contains a stop codon preceding the start codon. For S100α, no stop codon preceding the methionine codon was observed. We defined the translation start by comparison to the protein sequence of bovine S100α (Isobe et al., 1981). The context surrounding the AUG codon defined by Kozak (1991) $GCC^{A}/_{G}CC\underline{AUGG}$ fits well with the translation start we have chosen. The purine in position −3 and the G in position +4, which have the strongest effect

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GGACTGTTGAAGACAGGTCTCCACACACAGCTCCAGCAGCCACATTTGCAACCTTGGCCATCTGTCCAGAACCTGCTCCCACCTCAGGCCCAGGCCAACC  100

GTGCACTGCTGCAATGGGCTCTGAGCTGGAGACGGCGATGGAGACCCTCATCAACGTGTTCCACGCCCACTCGGGCAAAGAGGGGGACAAGTACAAGCTG  200
            M  G  S  E  L  E  T  A  M  E  T  L  I  N  V  F  H  A  H  S  G  K  E  G  D  K  Y  K  L

AGCAAGAAGGAGCTGAAAGAGCTGCTGCAGACGGAGCTCTCTGGCTTCCTGGATGCCCAGAAGGATGTGGATGCTGTGGACAAGGTGATGAAGGAGCTAG  300
 S  K  K  E  L  K  E  L  L  Q  T  E  L  S  G  F  L  D  A  Q  K  D  V  D  A  V  D  K  V  M  K  E  L

ACGAGAATGGAGACGGGGAGGTGGACTTCCAGGAGTATGTGGTGCTTGTGGCTGCTCTCACAGTGGCCTGTAACAATTTCTTCTGGGAGAACAGTTGAGC  400
D  E  N  G  D  G  E  V  D  F  Q  E  Y  V  V  L  V  A  A  L  T  V  A  C  N  N  F  F  W  E  N  S

AGACAGCCACATTGGGCAGCGCCCTTCCTCTCCACCCTCCCAGACCTGCCTCTTCCCCCTGCTTCCACCTCACCCCACTTATCCCTCTCCATAACCCCAC  500

CCTTGCCCACCCCACCCCCACCCCCACCAAGGGCGCAAGAGTAGCGGTCCAAGCCTGCAACTCATCTTTCATTAAAGGCTTCTCTCTCACCAGC(A)13  594


GGCAGTTGAGGCAGGAGACATCAAGAGAGTATTTGTGCCCTCCTCGGGTTTTACCTTCCAGCCGAGATTCTTCCCCTCTCTACAACCCTCTCTCCTCAGC  100

GCTTCTTCTTTCTTGGTTTGATCCTGACTGCTGTCATGGCGTGCCCTCTGGAGAAGGCCCTGGATGTGATGGTGTCCACCTTCCACAAGTACTCGGGCAA  200
                              M  A  C  P  L  E  K  A  L  D  V  M  V  S  T  F  H  K  Y  S  G  K

AGAGGGTGACAAGTTCAAGCTCAACAAGTCAGAACTAAAGGAGCTGCTGACCCGGGAGCTGCCCAGCTTCTTGGGGAAAAGGACAGATGAAGCTGCTTTC  300
 E  G  D  K  F  K  L  N  K  S  E  L  K  E  L  L  T  R  E  L  P  S  F  L  G  K  R  T  D  E  A  A  F

CAGAAGCTGATGAGCAACTTGGACAGCAACAGGGACAACGAGGTGGACTTCCAAGAGTACTGTGTCTTCCTGTCCTGCATCGCCATGATGTGTAACGAAT  400
 Q  K  L  M  S  N  L  D  S  N  R  D  N  E  V  D  F  Q  E  Y  C  V  F  L  S  C  I  A  M  M  C  N  E

TCTTTGAAGGCTTCCCAGATAAGCAGCCCAGGAAGAAATGAAAACTCCTCTGATGTGGTTGGGGGGTCTGCCAGCTGGGGCCCTCCCTGTCGCCAGTGGG  500
F  F  E  G  F  P  D  K  Q  P  R  K  K

CACTTTTTTTTTTCCACCCTGGCTCCTTCAGACACGTGCTTGATGCTGAGCAAGTTCAATAAAGATTCTTGGAAGTTT(A)20                  578
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FIGURE 2: Nucleotide and deduced amino acid sequence of human S100α (a, top) and human CAPL (b, bottom). The start codon and the polyadenylation signal are underlined.

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EF-Hand I

S100α - human    1 G S E L E T A M E T L I N V F H A H S G K E G D K Y K L S K K E L K E L L Q T E L S G F L D A Q K D  50
S100α - bovine   1                                                                                                  50
S100α - rat      1 ? ? ? ? ? ? ? ? ? ?     H                                   D           S     V                   50

CAPL - human     1 A C P L E K A L D V M V S T F H K Y S G K E G D K F K L N K S E L K E L L T R E L P S F L G K R T D  50
CAPL - mouse     1   R     E       I                                 T                                               50
CAPL - rat       1   R     E       I               N                 T                           R                   50


EF-Hand II

S100α - human   51 V D A V D K V M K E L D E N G D G E V D F Q E Y V V L V A A L T V A C N N F F W E N S  93
S100α - bovine  51 A                                                                                  93
S100α - rat     51 A         I                          F                                             93

CAPL - human    51 E A A F Q K L M S N L D S N R D N E V D F Q E Y C V F L S C I A M M C N E F F E G F P D K Q P R K K 100
CAPL - mouse    51           V                                                                 C     E               100
CAPL - rat      51              N                                                              C     E               100
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FIGURE 3: Amino acid sequence comparisons of S100α (human, bovine, and rat) and CAPL (human, mouse, and rat). In bovine, rat, and mouse sequences, only the amino acids differing from the human sequence are shown. The N-terminus of rat S100α is not known (as indicated by ?). Positions of the two EF-hands are indicated by shaded bars.

in modulating the translation efficiency, are present in both clones.

In summary, we have cloned the first human cDNAs of S100α and CAPL. Both clones show a high degree of homology to the published bovine and rodent sequences. The deduced translation products have the same size, and most amino acid exchanges are conservative. The predicted secondary structures [according to the algorithm of Garnier et al. (1987)] of the corresponding human, bovine, and rodent sequences are very similar (data not shown).

Northern Blot Analysis. As PCR is a very sensitive method, the number of the corresponding S100α, CAPL, and CACY clones we obtained does not reflect the appropriate expression levels. To examine if the three proteins identified by PCR are expressed in the human heart at high levels, we performed Northern blot analysis. Ten micrograms of total cytoplasmic

RNA was size fractionated by gel electrophoresis, blotted, and hybridized to ^{32}P-labeled cDNA probes. Our CACY cDNA clone corresponds to nucleotides 39–398 of the cDNA isolated by Calabretta et al. (1986). As a control, we hybridized a human β-actin clone (Ponte et al., 1984), which recognizes all actin isoforms including the muscle specific actins (lower bands). Exposure times were 24 h (S100α), 2 days (β-actin), and 4 days (CACY, CAPL). The results are illustrated in Figure 4. S100α and CAPL hybridized to mRNA of about 650 bp, indicating that we cloned nearly full length cDNAs. CACY hybridized to a shorter mRNA of about 550 bp in length. Small amounts of a S100α mRNA species of about 750 bp were coexpressed in all tissues that express the 650 bp mRNA. This could be due to different S100α mRNA species, originated by alternative splicing or polyadenylation, or to cross-hybridization to a homologous unknown S100 species. The mRNAs of S100α, CACY, and CAPL are expressed at high levels in the human heart, but the strongest signal was observed for S100α. Interestingly, S100α is expressed at higher levels in the left heart ventricle, whereas CAPL and CACY are expressed predominantly in the right heart ventricle. All three mRNAs are also expressed at high levels in skeletal muscle. Examining various human tissues, the expression pattern of S100α is mainly restricted to heart and skeletal muscle and, to a lesser extent, to brain. Low levels of S100α are found in kidney, visible only at longer exposure times (not shown). CAPL and CACY mRNAs are distributed in a ubiquitous manner. CACY is expressed at low levels in brain, stomach, skeletal muscle, and the left heart ventricle. Higher levels are found in lung, kidney, thymus, and the right heart ventricle. CAPL is expressed at low levels in skeletal muscle, stomach, and kidney and at higher levels in heart, lung, and thymus. In brain, CAPL is not detectable. S100α, CACY, and CAPL are not expressed in the human liver.

We conclude that three S100 mRNAs identified by PCR, S100α, CACY, and CAPL are expressed at high levels in the human heart. S100α shows the most restricted and interesting tissue distribution as well as the highest expression level.

DISCUSSION

To search for members of the S100 protein family in the human heart, we constructed degenerated oligonucleotides directed against two conserved regions and used them as primers in a polymerase chain reaction with reverse-transcribed human heart RNA as template. We analyzed the PCR products by subcloning and sequencing and identified three S100 proteins: S100α, CACY, and CAPL.

S100α was originally isolated from bovine brain as a low molecular weight calcium-binding protein (Moore, 1965). It is predominantly expressed in neurons (Isobe et al., 1984), slow twitch muscle fibers (Haimoto et al., 1986; Zimmer, 1991), and heart muscle (Kato et al., 1986). It is suggested that S100α stimulates the Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmatic reticulum (Fanò et al., 1989). CACY (synonyms used by other laboratories are: 2A9, PRA, and 5B10) was isolated first by Calabretta et al. (1986) as a mRNA that is inducible by growth factor addition to G_0 phase arrested fibroblasts. It has been proposed that CACY may be involved in cell cycle progression (Calabretta et al., 1986). CAPL (synonyms are 42A, pEL98, 18A2, and mst1) was isolated by Jackson-Grusby et al. (1987) as a mRNA species that is induced by serum stimulation of growth-arrested fibroblasts and by Masiakowski and Shooter (1988) as a mRNA whose level is increased in PC12 cells upon treatment with NGF.

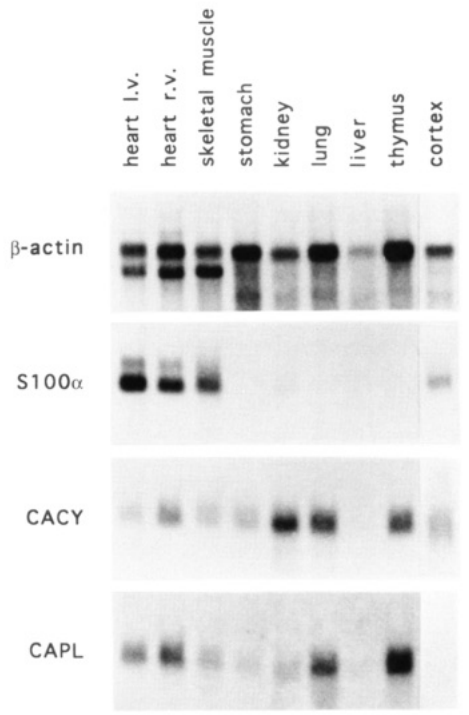

FIGURE 4: Northern blot analysis. Each lane corresponds to 10 μg of total cytoplasmic RNA isolated from various human tissues (l.v., left ventricle; r.v., right ventricle), size fractionated by formaldehyde gel electrophoresis, and blotted onto nylon membranes. The same blot was hybridized successively with ^{32}P-labeled human cDNAs of S100α, CAPL, CACY, and β-actin and after washing exposed for 1 day (S100α), 2 days (β-actin), and 4 days (CACY, CAPL).

Increasing amounts of CAPL are expressed during the S phase of the cell cycle (Jackson-Grusby et al., 1987). An important function in the regulation of the metastatic behavior of tumor cells has been proposed (Ebralitze et al., 1989).

To demonstrate that three different S100 proteins, S100α, CACY, and CAPL, are present in the human heart, we purified these proteins from human cardiac muscle (data not shown). According to their biochemical properties (Ca^{2+}-dependent binding to phenyl-*Sepharose*, hydrophobicity on reverse-phase HPLC, ^{45}Ca binding by the gel overlay method, and low molecular weight in SDS–PAGE), we were able to distinguish three S100 proteins. By immunochemical methods, we identified two of them as S100α and CACY. To identify S100α, we used the same monoclonal antibody as has been used to isolate the S100α cDNA. To identify CACY, we used a polyclonal antibody directed against mouse CACY (Kuznicki et al., 1989). Antibodies directed against CAPL were not available. As protein purification and PCR revealed similar results, we conclude that at least three members of the S100 protein family are expressed at high levels in the human heart.

We isolated the corresponding cDNA clones and obtained for the first time the human sequences of S100α and CAPL. Their primary structures were found to be highly conserved when compared to published sequences of other vertebrates. S100α shows only one conservative amino acid exchange between the human and bovine proteins. The differences between rodent and human sequences are much higher. We identified seven or nine amino acid exchanges when comparing the human and the corresponding rat and mouse sequences (Figure 3). In the mRNA of S100α, two short sequences are inserted in the human 5′ and 3′ untranslated regions. These insertions are not present in the bovine and rat sequences.

The transcription levels of S100α, CACY, and CAPL are high in the human heart as demonstrated by Northern blot

hybridization. Both S100α and β-actin are expressed at similarly high levels. This high content of S100α is in agreement with the results of Kato et al. (1986). As demonstrated by immunoprecipitation, they showed that in the human heart S100α represents 1.8 μg/mg of total soluble proteins. The water solubility and the high concentration in the human heart are reasons why S100α has been suggested as a marker for cell damage (e.g., after infarction) in the human heart (Usui et al., 1990).

CACY and CAPL mRNA levels in the human heart are lower than those of S100α. Densitometric scanning of the hybridization signal yielded mRNA ratios of about 2.0 to 1.4 for S100α/CACY and 10 to 5 for S100α/CAPL. Whether the mRNA ratio reflects the real protein ratio will be further tested by Western blot analysis. From histochemical experiments (Kuznicki et al., 1989; Zimmer, 1991), we expect that S100α and CACY are expressed in different muscle cell types. This might also be the case in the human heart. The differences in the expression levels of S100α versus CACY/CAPL in left and right ventricles may be a reflection of a different cell-specific expression.

Two mRNA species hybridized to the S100α probe in all tissues examined. These mRNAs are 650 and 750 bp long (Figure 4). In every case, the 750 bp species is present in lower abundance. The ratio of the hybridization signals is similar in all RNA samples. It is possible that the two S100α mRNAs result from the usage of different polyadenylation signals, as can be observed, e.g., for parvalbumin (Epstein et al., 1986). However, we only found one cDNA species in our screening. The analysis of the genomic structure might give an answer by showing a second polyadenylation signal downstream. The published partial genomic sequence of S100α contains no sequences preceding the 3'-end (Morii et al., 1991).

For the first time, the transcription levels of S100α, CACY, and CAPL were examined in human tissues. But looking at the corresponding transcription levels in rodents [S100α, Kuwano et al. (1987) and Zimmer et al. (1991); CAPL, Ebralitze et al. (1989) and Jackson-Grusby et al. (1987); CACY, Guo et al. (1990) and Murphy et al. (1988); and our own results], we observed a different tissue distribution of S100α, CAPL, and CACY. The most striking differences between the expression patterns in rodent and human tissues are the following: (a) S100α is expressed at low levels in all mouse tissues (including heart and skeletal muscle); (b) CAPL shows a very low transcription level and is not expressed in mouse heart and skeletal muscle; and (c) S100α and CACY are expressed in the mouse liver at low but detectable levels. This raises the following question: Do these proteins have different functions in humans and rodents or is the dissimilar expression pattern due to differences between human and rodent organs? Histochemical stainings of human and rodent tissue sections using specific antibodies should give more insights.

The mRNA levels of S100 proteins are regulated during development. This has been shown for S100α in rat leg muscle (Zimmer, 1991) and for S100β (Landry et al., 1989; De León et al., 1991), CAPL, and Ca[1] (De León et al., 1991) in the central and peripheral nerve system. We therefore examined the transcription level of S100α and CACY in the developing mouse heart (not shown). Levels of both mRNAs increase about 5–10-fold from postnatal day 1 to day 40, indicating a developmental regulation of S100α and CACY in the murine heart.

Our aim was to characterize the members of the S100 protein family that are expressed in the human heart. By PCR using degenerated oligonucleotides, we identified three members, i.e., S100α, CACY, and CAPL, expressed in the human heart. In future experiments we will concentrate on the function of S100α in the human heart because of its specific expression pattern and because of its high level in this tissue. In addition, we started to search for different expression levels of S100α in normal and diseased heart (autopsies from patients with infarction or hypertrophy). Transfection of cultured heart cells with S100α combined with a calcium overload of the cells may allow the correlation of morphological and cytoskeletal alterations as has been observed in the diseased heart.

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