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Plasma membrane Ca^{2+}-ATPase mRNA expression in murine hepatocarcinoma and regenerating liver cells

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Abstract

The plasma membrane calcium ATPase (PMCA) is an ubiquitous enzyme that extrudes calcium from the cytoplasm to the extracellular space. Four PMCA genes through alternative splicing produce a large diversity of isoforms of this enzyme. We reported previously that the PMCA contained in AS-30D hepatocarcinoma cells showed significant differences in activity in comparison to normal and regenerating liver. In the present study we investigate if the difference in PMCA activity could be related to differential expression of mRNAs encoding different isoforms of PMCA. Using RT-PCR we found that variants 1b, 1x, and 4b are expressed in all liver samples. The hepatoma AS-30 and liver at 2 days of regeneration express low amounts of isoforms 2w, 4b and 4x, and do not express isoforms 4a, 4d and 4z. Fetal and neonatal liver do not express variants 4a and 4d, but they do express variants 4x and 4z. Immunoblot analysis showed a higher ratio ATPase/total protein in the hepatoma AS-30D in comparison to normal liver. Our results suggest that the Ca^{2+}-ATPase kinetic pattern previously observed by us in the AS-30D cells, could be at least partially explained by changes in the mRNA expression of several of the PMCA isoforms expressed in the liver. (Mol Cell Biochem **247**: 177–184, 2003)

Key words: plasma membrane calcium ATPase, calcium pump, mRNA expression, regenerating liver, hepatocarcinoma AS-30D

Introduction

The plasma membrane calcium pump (PMCA) is one of the main systems involved in the delicate balance of controlling localized intracellular calcium concentrations [1]. This enzyme belongs to the family of P-type ATPases characterized by the formation of a phosphorylated intermediate and calmodulin modulation [2]. A large diversity of enzyme isoforms is produced by four PMCA genes, which present four potential splicing sites named A through D [3, 4]. The housekeeping variants correspond to those from mRNAs of genes 1 and 4 [5]. Alternative splicing occurs mainly at two sites, site A and site C. Site A includes exons that encode residues in the vicinity of the phospholipid responsive domain [6]. Interestingly, all genes except that of PMCA1 have shown alternative splicing at site A [5]. Splicing in this region has been demonstrated for rat [7–9] and human isoforms: PMCA2, PMCA3, and PMCA4 [5, 10]. Alternative splicing at site A involves 3 exons in the PMCA2 gene [7], and a single exon in PMCA3 and PMCA4 genes [8, 9, 11].

Site C includes exons encoding the 3′-end of PMCA mRNAs, which contain alternative calmodulin-binding domains at the C-terminus. Alternative splicing at site C of PMCAs has been demonstrated in primary transcripts of the

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four known PMCA genes in human [5] and rat [8]. Splicing at site C for PMCA1 involves a single exon of 154 bp, which can be included (PMCA1a) or excluded (PMCA1b). Variants 1c, d, and e, are generated by the use of internal donor splice sites at nucleotide positions 87, 114, and 152 respectively [12]. Alternative splicing of PMCA4 at site C generates two variants, PMCA4a that includes a 175 bp exon and PMCA4b that excludes it [12]. Recently, we described in heart muscle a third splice variant for PMCA4 at site C named PMCA4d. This splice variant uses an internal donor splice site at position 108 of the 175 bp exon [13].

In liver, a novel PMCA that is not modulated by calmodulin, nor inhibited by vanadate, shows an apparent higher molecular weight than the average for the family of Ca^{2+}-ATPases [14]. Since we have been interested with the kinetic behavior of this special liver calcium ATPase during different development stages [15], the purpose of the present study has been to analyze the isoforms expressed in the murine hepatocarcinoma AS-30D cells, in comparison to the adult normal and regenerating rat liver. According to previous studies, the most abundant isoforms are those from genes 1 and 4 in adult rat liver cells [5, 16], as well as during different fetal stages in the mouse [17]. In contrast with the transformed cell systems, the regeneration process represents an interesting model of fast growth in a controlled fashion.

Since liver PMCA cannot be purified by affinity columns coupled to calmodulin and represents a low percentage of the proteins embedded in the membrane [18], we first screened a cDNA library constructed from AS-30D cells. Furthermore, we analyzed by RT-PCR the mRNA expression pattern of two of the main PMCA genes (PMCA1 and PMCA4) during different developmental stages of the liver. Although under our conditions we were not able to find a cDNA encoding for a new isoform in the hepatoma AS-30D through library screening, using RT-PCR we found a qualitative low expression of PMCA4 isoforms. We also found expression of the 4d isoform previously reported to be expressed in human heart [13]. Noteworthy, we found an apparent increased expression of PMCA2w isoform in regenerating, neonatal and adult normal liver compared to hepatoma cells.

Materials and methods

Materials

Reagents employed were of the highest quality available. Restriction enzymes, synthetic DNA oligonucleotides and the dsDNA Cycle Sequencing System were provided by GIBCO BRL (Grand Island, NY, USA). The oligolabeling kit was obtained from Pharmacia LKB (Uppsala, Sweden). Radionucleotides were supplied by DuPont, NEN (Boston, MA, USA). The custom AS-30D cDNA library was constructed by Clontech Laboratories Inc. (Palo Alto, CA, USA). Taq polymerase, murine reverse transcriptase and the 9600 thermocycler were from Perkin-Elmer Cetus. The monoclonal antibody anti-PMCA (clone 5F10) that recognizes a conserved region located between transmembrane domains M4 and M5, was obtained from Affinity Bioreagents, Inc. (Golden, CO, USA), the secondary antibody goat antimouse peroxidase-conjugated was from Alpha Diagnostic International (San Antonio, TX, USA). The ECL detection system used was from Amersham (Buckinghamshire, UK).

AS-30D cell line

In order to expand the hepatocarcinoma cell line, we used male Wistar rats with an average weight of 200 g. They were inoculated in the peritoneal cavity with one ml of a concentrated stock of AS-30D cells (3×10^6 cells/ml). The cell stocks were stored in liquid nitrogen in Dulbecco's Modified Eagle Medium (DMEM) plus 10% dimethyl sulfoxide until needed. Seven days after the inoculation, the ascites fluid was collected, centrifuged and the cells washed under sterile conditions with 150 mM NaCl, 5 mM KCl, and 20 mM Tris-HCl, (pH 7.4). Finally, cells were collected in sterile PBS and quickly frozen in liquid nitrogen until needed.

cDNA library screenings

The cDNA library was constructed in λgt10 from total mRNA from AS-30D cells using an EcoRI linker. Briefly, the *E. coli* strain C600 was infected, the clones presented inserts with an average size in the range of 2 kb, with 1×10^6 pfu, representing the whole number of independent clones in the library. The screenings were done by hybridization of nitrocellulose replica filters as previously described [19]. Briefly, filters were prehybridized overnight at 42ºC in 5X SSC (20X SSC: 3 M NaCl, 300 mM sodium citrate-NaOH, pH 7.0), 5X Denhardts, 50% formamide, 0.1% SDS, and 100 µg/ml of salmon sperm DNA. Filters were hybridized overnight at 42ºC using the same prehybridizing solution plus a probe containing a full length cDNA encoding the rat PMCA2 isoform [3], labeled by random primer extension using the Klenow fragment and α^{32}P-dCTP. Washing was performed at high stringency with 0.2X SSC. The positive clones were directly sequenced in λgt10 or subcloned in pTZ19R. The DNA sequences were analyzed using the nucleotide analysis programs BLAST (NCBI) and MacVector 6.5.3 (Oxford Molecular Group PLC).

RT-PCR analysis

Total RNA from fetal, and adult rat liver or AS-30D hepatocarcinoma cells was isolated by the guanidinium thiocy-

anate method [20]. For the regenerating animal model, we partially hepatectomized rats according to Higgins [21] and total RNA was isolated at the indicated times after hepatectomy. Briefly, 5 µg of total RNA were incubated at 65°C with 50 pmol of oligo dT, and 50 pmol of random hexamers for 5 min and then cool down to 25°C. First strand cDNA synthesis was performed with a Gene Amp RNA PCR kit (Perkin-Elmer Cetus), following the manufacturer's recommendations. One fifth of the RT reaction was used for the polymerase chain reaction (PCR) in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 unit of Taq polymerase using a hot start procedure. Samples were heated at 82ºC, and 10 pmol of specific primer pairs (Table 1) were added in a final volume of 50 µl. The reaction was followed by 35 cycles: 94ºC/45 sec, 55ºC/45 sec, 72ºC/2 min. A final extension step was included at 72ºC for 10 min. The PCR products were analyzed in 2% agarose gels stained with ethidium bromide and photographed. Gels were denatured, neutralized and transferred overnight to nylon membranes (Hybond, Amersham) with 20X SSC [19]. The DNA was fixed with an ultraviolet crosslinker (Stratagene). PCR products from site A were hybridized with cDNA probes corresponding to rPMCA2 [13]. PCR products corresponding to PMCA1 site C were hybridized with specific complementary oligonucleotides of rat whose sequences were: 5′-GCTCTAAGGCGACAACCCTCC-3′ (3661–3681), and 5′-CTTCGTTGGCTTCTAAGTCGC-3′ (3796–3817) for PMCA4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as an internal standard [5] (Table 1). The ssDNA synthetic oligonucleotides were labeled using T4 polynucleotide kinase and γ^{32}P-ATP at 37ºC. Hybridization with cDNA probes was performed using a random primer labeling kit (Pharmacia LKB) as previously described [19]. Labeled blots were exposed to X-OMAT film (Kodak).

Immunoblot analysis

Plasma membrane fractions from normal adult liver, regenerating liver and AS-30D cells were isolated as indicated [15]. The membranes were solubilized for 20 min at 4°C in 25 mM Na-HEPES, 10 mM MgCl pH 7.4 containing 0.5% of Triton X-100 as a final concentration and centrifuged at 100,000 × g for 30 min. The supernatant was collected and 20 µg of the indicated samples separated by SDS gel electrophoresis and blotted into nitrocellulose membranes (Bio-Rad). Pure fractions of PMCA isolated from human erythrocyte ghosts were used as positive control. The non specific binding was blocked for 1 h with fat free milk (2.5%) in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20 (milk-TBST). Membranes were then incubated for one hour with the antibody mAb5F10 (1:1000 dilution) in milk-TBST, washed for one hour with the same buffer without milk. Finally, the membranes were incubated with the secondary antibody for 1 h and washed for 1 h with TBST and once more with TBS (buffer without Tween 20). Blots were developed with the ECL system and exposed to X-OMAT film. All incubations were done at room temperature.

Results

Cloning the PMCA1 isoform from AS-30D hepatocarcinoma

We analyzed a cDNA library constructed from the hepatocarcinoma AS-30D by making an exhaustive screening of its clones (1×10^6 pfu) with cDNA inserts showing an average size of 2 kb. Identity of the positive clones was confirmed by Southern blot analysis using a cDNA probe for rPMCA2

Table 1. Primers employed for RT-PCR amplification of PMCAs

Primer sequence	Position		Splice site
5′-CAGGTACTCATGTAAGGGAAGGCTC-3′	785–809	(F)	1A*
5′-CACTCTTCAATGGCTGCATTTCC-3′	1015–993	(R)	
5′-GAGATCCCTGAGGAGGAATTGG-3′	3502–3523	(F)	1C
5′-TGTGCGGCTCTGAATCTTCTATC-3′	3893–3871	(R)	
5′-TGGTCAGCTTATACAACTCCCAGTG-3′	752–776	(F)	4A
5′-GGTCCCTTGCTTGCTTTCTTCTC-3′	1201–1179	(R)	
5′-AGAGATCGACCATGCCGAGATG-3′	3398–3419	(F)	4B/C
5′-AACAGTTTCAGCATCCGACAGG-3′	3852–3831	(R)	
5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	71–95	(F)	GAPDH**
5′-CATGTGGGCCATGAGGTCCACCAC-3′	1030–1053	(R)	

*Sequence reported in [13]. **Sequence reported in [5], glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for sites 1A, 1C, 4A and 4B/C, were designed according to references 23, 9, 12, and 7, using the MacVector 6.5.3 analysis software.

(data not shown). Eleven positive clones with a size range of 1.6–2 kb were sequenced. All these clones overlapped and covered the full length of rPMCA1 (Fig. 1).

RT-PCR analysis of PMCAs at splicing sites A and C

When we used primers corresponding to the splice site A of the human PMCA1 (Table 1), we observed the expression of the expected 231 bp band corresponding to the 1x variant (Fig. 2A). In addition, we also amplified a second product of 312 bp, which was further cloned, sequenced and identified as the variant 2w [7] (Figs 2A and 3). Basically, the 1x levels were quite constant, while the isoform 2w showed apparently a lower expression than the variant 1x. The lowest level for the expression of 2w was observed during earlier fetal stages and was gradually increased to reach a maximum in the neonatal stage. A low expression of 2w was evident in the hepatoma AS-30D compared to adult normal liver (Fig. 2A). As shown successfully by others [5], glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard in order to validate our RT-PCR analysis (Fig. 2C).

Using a primer pair designed for the splice site A of PMCA4 (Table 1), we mainly observed in all samples of regenerating liver the amplification of a 450 bp product corresponding to the isoform 4x and a lower expression of a 420 bp product corresponding to the isoform 4z (Fig. 4A). The 4x isoform was shown to increase through the regeneration process. All samples, but the fetal stage of 13 days and the hepatoma expressed the 4z isoform.

For site C, we found that the housekeeping isoform 4b was

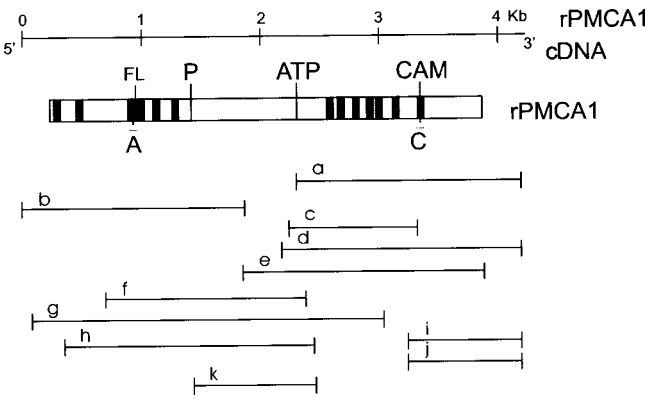

Fig. 1. Schematic representation for the isolated AS-30D PMCA1 clones. The cDNA coding sequence for rPMCA1 is shown in the upper bar, the transmembrane regions are indicated as bold bars, and the splice sites A and C are indicated in the binding domains for phospholipids and calmodulin, respectively. The length and location of the cDNA clones isolated are indicated by solid lines (named 'a' through 'k'). All clones localized at splice site A correspond to the isoform 1x. At splice site C, clones correspond to the isoform 1b.

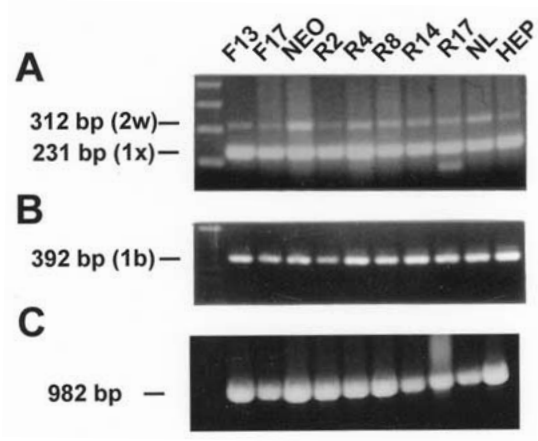

Fig. 2. Ethidium bromide stained agarose gel of PCR products corresponding to the PMCA1 spliced at site A (panel A) and site C (panel B). (C) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal standard. F13 – fetal liver at 13 days; F17 – fetal liver at 17 days; Neo – neonatal liver; R2 – regenerating liver at 2 days; R4 – regenerating liver at 4 days; R8 – regenerating liver at 8 days; R14 – regenerating liver at 14 days; R17 – regenerating liver at 17 days; NL – adult normal liver; HEP – hepatoma AS-30D cells.

expressed in all tissues; however, the hepatoma AS-30D and liver samples at early regeneration times showed an apparent lower expression (Fig. 4B). The 4a and 4d variants presented a similar expression but were not expressed in fetal, neonatal stages, or hepatoma cells. Nevertheless, qualitatively their highest level of expression was seen in late regeneration stages of the liver (17 days) (Fig. 4B). The 4d variant has been previously reported by our group to be expressed in adult and fetal human heart [13], and now found in adult normal and regenerating liver, but not in fetal liver or in hepatoma cells. In regenerating liver, the 4b isoform presents a very low level of expression at 2 days posthepatectomy; nevertheless, it appears to be consistently expressed during development as well as in adult normal liver. Interestingly, the hepatoma cells showed a low expression level of the 4b isoform and did not express the 4a and 4d isoforms (Fig. 4B).

Based on different PCR reactions under the same conditions, we observed that the level for the 2w isoform seems not to be negligible in all samples throughout the developmental stages of rat liver, since it is known that in human liver less than 1% is estimated to correspond to the 2x and 2w isoforms [5]. The main isoform spliced at site A was consistently the 1x isoform in the hepatoma AS-30D, followed by the 4x and 4z isoforms.

Immunoblot analysis

Soluble fractions from liver plasma membranes were analyzed employing polyacrylamide gel electrophoresis (PAGE)

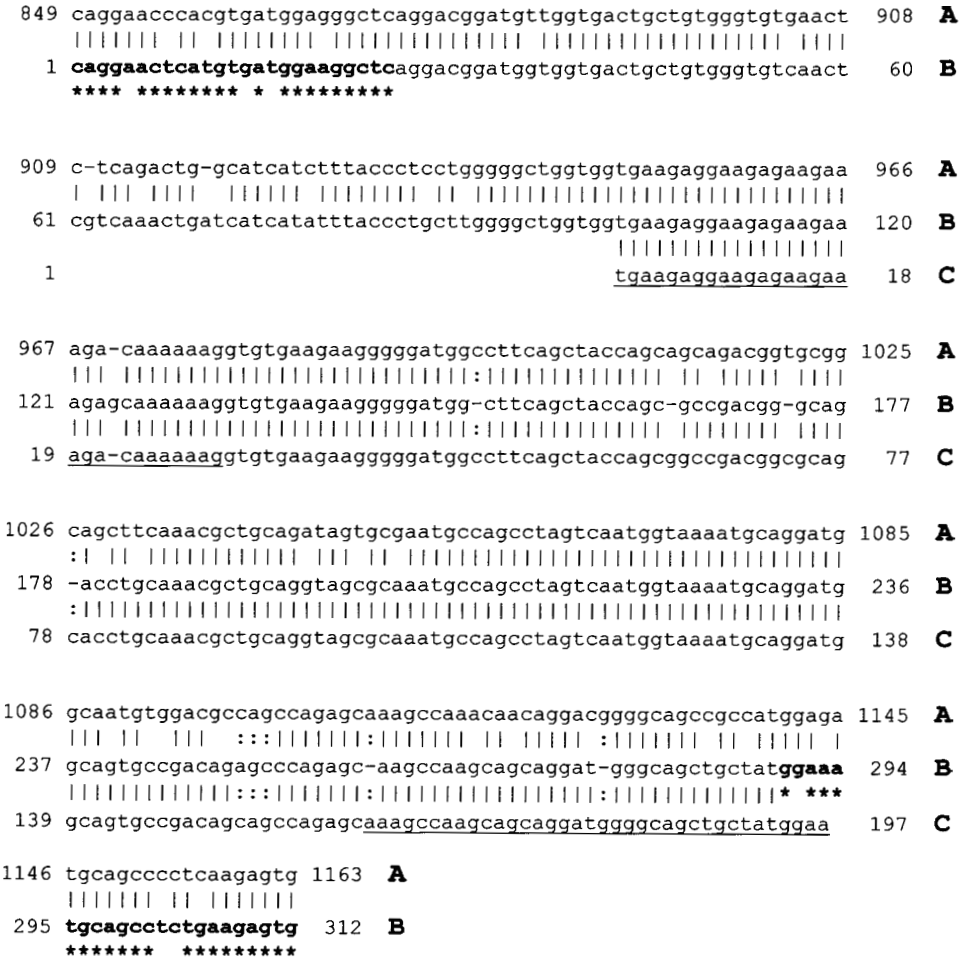

Fig. 3. Nucleotide sequence alignment of a rat liver PMCA2w fragment. The 312 bp PCR amplification product obtained using primers designed for human PMCA1 at site A (see Table 1), was sequenced (lane B). The fragment sequence revealed high homology with the published human PMCA2w sequence (lane A) [22] and a partial sequence for the rat PMCA2w (lane C) [7]. The nucleotide sequence of the two primers used to amplify the rat PMCA2w isoform at site A is highlighted in bold letters, and identical nucleotides of the PMCA1 primers sequence are indicated by asterisks under the rat PMCA2 sequence. The sequences of the PMCA2 primers used by Adamo [7] to amplify the PMCA2 splice site A region, are underlined.

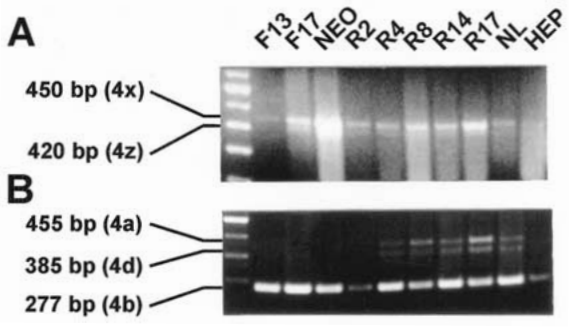

Fig. 4. Ethidium bromide stained agarose gel of PCR products corresponding to PMCA4. (A) PCR products generated by alternative splicing at site A. (B) PCR products produced by alternative splicing at site C. In a similar fashion as seen in Fig. 2, GAPDH levels did not significantly change between conditions. Abbreviations used as in Fig. 2.

and immunodetected using the monoclonal antibody mAb5F10 raised against the purified PMCA from human erythrocytes. Although the use of this antibody does not permit us to distinguish different levels of ATPase directly related to a specific isoform, we can study differences in total ATPase levels associated to the different tissues investigated. Figure 5A shows the main band of 138 kD corresponding to the erythrocyte calcium ATPase. This band is found in adult normal liver, as well as in the hepatoma AS-30D. Interestingly, membranes from normal hepatic tissue showed a lower amount of PMCA in comparison to the hepatoma AS-30D. Figure 5B shows the Western blot analysis of the regenerating liver tissue in comparison to our erythrocyte positive control. An increase in Ca^{2+}-ATPase protein content was evident in the shortest time post-hepatectomy (R2), followed by a progressive reduction in later regeneration stages. Slight changes in

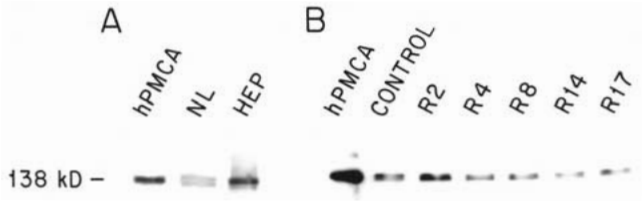

Fig. 5. Immunoblots showing the expression of PMCA in different liver samples. Plasma membrane soluble fractions from the indicated samples were separated by SDS-gel electrophoresis and transferred to nitrocellulose membranes. The PMCA was detected using the monoclonal antibody 5F10 (Affinity Bioreagents). (A) Immunoblot showing the expression of PMCA in normal liver (NL) and the hepatoma AS-30D (HEP). (B) Immunoblot showing the expression of PMCA in liver at the indicated times of regeneration. As a control, a solubilized sample obtained from a sham-operated rat was used. In both panels, hPMCA corresponds to the human erythrocyte PMCA used as a positive control. Abbreviations used as in Fig. 2.

the mobility of the Ca^{2+}-ATPase could be related to a PMCA lacking the subdomain B included in the calmodulin-binding domain [23, 24].

Discussion

The liver plasma membrane calcium ATPase is not as well understood as those from other tissues. Although in general it belongs to the P-type ATPases, the regulation of the enzyme does not share all the canonical features of most plasma membrane Ca^{2+}-ATPases. It seems to be regulated in a different way and therefore may involve molecules that never have been related to the regulation of other calcium pumps before; for instance regucalcin [25, 26] and G proteins [27].

The role that calcium plays in tumor tissues has been studied by many groups [28, 29]. Studying the hepatocarcinoma AS-30D, a high plasma membrane and mitochondrial calcium uptake activity has been reported [30]. We have shown that AS-30D cells transport calcium with an efficiency similar to the one found in normal cells, but the ATPase activity in the AS-30D cells is much lower than in normal liver cells. This phenomenon has been explained through an increased ATP $\leftrightarrow$ Pi exchange mechanism that would support calcium transport representing a more efficient mechanism for regulating the movement of calcium through an optimum expenditure of energy [15].

In this study we were puzzled by the differential expression of some of the PMCA isoforms present in the AS-30D hepatoma cells in comparison to fetal and adult normal liver, as well as throughout the regeneration process of the liver. In accordance to other groups [31], we consider that the production of a large diversity of variants might be an interesting mechanism by which cells are provided with a delicate control of intracellular calcium. In this respect, several reports support the idea of a fine regulation in the Ca^{2+} signaling machinery at the isoform level during the regeneration process [32, 33]. The cytosolic calcium elevation produced during liver regeneration involves the mitochondria as well as the endoplasmic reticulum associated to the IP_3 and ryanodine receptors embedded in these membranes.

In the case of the plasma membrane calcium pump, the housekeeping genes correspond to PMCA1 and 4, whereas PMCA2 and 3 genes show a tissue specific location, mainly in the central nervous system [5]. In accordance to previous reports using PCR techniques as well as Northern and Western analysis to estimate the relative abundance of PMCA isoforms in rat liver [5, 16], our exhaustive screening of a cDNA library from the hepatoma AS-30D revealed that apparently, the most abundant housekeeping isoform for this system corresponds to PMCA1 (Fig. 1). Since PMCA1 has been shown to present a unique exon involved in splicing at site A [5], in our screenings we found the complete exon included in some of the clones studied (Fig. 1). This finding correlates well with previous reports showing it in every PMCA1 transcript studied [11]. Among the different PMCA genes, the alternative splice possibilities at site A considered highly variable, have been thoroughly characterized in rat and human [5, 7–10].

Previous studies showed that PMCA1 and PMCA4 were abundantly expressed in human liver, unlike PMCA2, which is expressed at very low levels [5]. PMCA1 has also been shown to be more abundant than PMCA2 in adult normal rat liver [16]. In developing rat brain it has been described that the stage of transcription induction varies among the isoforms [34].

Using RT-PCR analysis to study the alternative splicing of PMCA mRNAs at sites A and C in regenerating liver, normal liver at different developmental stages and the hepatocarcinoma AS-30D, we found that splice variants 1b and 1x are expressed in all tissue samples in a similar fashion. The hepatoma AS-30D and liver at two days of regeneration express very low levels of variants 2w, 4b and 4x, and did not express the 4a, 4d, and 4z variants. Fetal and neonatal liver did not express variants 4a and 4d, but expressed variants 4x and 4z. Our results indicate that PMCA1 is constantly expressed as indicated by apparent constant levels of splice variants at sites A and C of this gene.

With respect to the expression of variants of PMCA4, previous work using mixed primers discounted the expression of PMCA4 in rat liver [16], we show that several differently spliced isoforms from this gene are expressed in normal liver during development as well as in regenerating liver, and the hepatoma AS-30D.

Studies using quantitative PCR have shown that most of the expression from the isoforms spliced at site A in human normal liver are due to the 1x variant (70%) followed by the variant 4x (28%), and less than 1% is represented by the variants 2x and 2w [5]. The isoforms spliced at site C ex-

pressed in human liver at the highest level are 1b and 4b, with 70 and 28% respectively [5]. Qualitatively, our data suggest that in the different rat liver samples tested, the PMCA 1x seems to be the main isoform spliced at site A. Accordingly, we could suggest that in fetal, neonatal liver, liver at two days posthepatectomy, and the hepatoma AS-30D, variants 1b and 4b represent the main isoforms edited at site C. However, in adult rat liver and in late stages of regenerating liver, the splice variants 4a and 4d may have some contribution.

The hepatocarcinoma AS-30D, rat liver at early stages of regeneration (day two), and fetal liver mainly express variants 1b and 4b, considered as the housekeeping isoforms. In contrast, adult rat liver and liver during the regeneration process showed a more complex pattern of PMCA splice variants, including variants 4a and 4d. Previously, we have shown that human fetal heart at early stages of development also express the housekeeping isoforms [13]. The differential expression of several isoforms in the hepatocarcinoma AS-30D suggests that the neoplastic system suppresses the expression of several of the PMCA isoforms. Whether this has an impact on the calcium homeostasis of the neoplastic cell line remains to be determined.

We observed an apparent decrease in the expression of the constitutive variant 4b, especially at earlier times posthepatectomy (2 days) and in the tumor tissue. Interestingly, the variant 4b has been shown to present a mechanism of down regulation mediated by calcineurin, recently described in rat neurons exposed to a sustained increase of cytosolic Ca^{2+} [35]. In general, a common factor in these systems is the elevated cytosolic calcium concentration observed at the beginning of the regeneration process [32], and in tumor cells [30, 36].

Another interesting mechanism for regulation consists in the apparent subexpression simultaneously with an overexpression of several systems that mobilize calcium. This type of regulation has been observed in stable transfected Chinese ovary cells [37] and in rat aortic endothelial cells [38]. For instance, the overexpression of PMCA4b from 15–20 times has been shown to induce a down regulation of the endogenous sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2b) [37]. The opposite is observed when an overexpression of sarcoplasmic ATPase (SERCA3) is induced [39].

At the protein expression level, our immunoblot analysis using a monoclonal antibody raised against a conserved region of the ATPase of the human erythrocyte PMCA, showed that the highest amount of total enzyme per mg of membrane protein in comparison to normal liver is present in the hepatoma AS-30D (Fig. 5A). There is also an apparent increase in the total ATPase in the shortest regeneration time (R2). This increase in enzyme is higher than that found in sham operated rats (Fig. 5B). These observations indicate the relevant function played by this Ca^{2+}-ATPase in liver during stress conditions suggesting a translation and/or post-translation regulation for this ATPase. It is noticeable that the ATPase contained in the hepatoma cell line corresponds to the same molecular weight as the one found for the erythrocyte ATPase. In order to find out a direct relationship between RT-PCR products and expressed isoform levels, real time PCR techniques will also allow us to study changes in mRNA levels at different developmental stages.

Also the implementation of suitable techniques to isolate the different isoforms known to exist, will help us to directly correlate each one of them to their specific enzyme activity.

Our study represents the first report that investigates the mRNA expression of several liver PMCAs isoforms in comparison to the expression of hepatoma cells with that of normal liver during development, adulthood as well as during posthepatectomy regeneration. The differential expression of PMCA isoforms most probably defines a very fine regulation of important cellular processes directly related to the homeostasis of calcium.

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References

1. Carafoli E, Guerini D: The plasma membrane calcium pump. In: J.R. Sotelo, J.C. Benech (eds). Calcium and Cellular Metabolism. Plenum Press, New York, 1997, pp 73–84
2. Pedersen PL, Carafoli E: Ion motive ATPases I: Ubiquity, properties, and significance to cell function. Trends Biochem Sci 12: 146–150, 1987
3. Shull GE, Greeb J: Molecular cloning of two isoforms of the plasma membrane Ca^{2+}-transporting ATPase from rat brain. J Biol Chem 263: 8646–8657, 1988
4. Shull GE, Greeb J: Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skeletal muscle. J Biol Chem 264: 18569–18576, 1989
5. Stauffer TP, Hilfiker H, Carafoli E, Strehler E: Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skeletal muscle. J Biol Chem 268: 25993–26003, 1993
6. Zvaritch E, James P, Vorherr T, Falchetto R, Modyanov N, Carafoli E: Mapping of functional domains in the plasma membrane Ca^{2+} using trypsin proteolysis. Biochemistry 29: 8070–8076, 1990
7. Adamo HP, Penniston JT: New Ca^{2+} pump isoforms generated by alternative splicing of rPMCA2 mRNA. Biochem J 283: 355–359, 1992
8. Burk SE, Shull GE: Structure of the rat plasma membrane $Ca(^{2+})$-ATPase isoform 3 gene and characterization of alternative splicing and transcription products. Skeletal muscle-specific splicing results in a plasma membrane $Ca(^{2+})$-ATPase with a novel calmodulin-binding domain. J Biol Chem 267: 19683–19690, 1992

9. Keeton TP, Shull GE: Primary structure of rat plasma membrane Ca^{2+} ATPase isoform 4 and analysis of alternative splicing patterns at splice site A. Biochem J 306: 779–785, 1995
10. Heim R, Hug M, Iwata T, Strehler EE, Carafoli E: Microdiversity of human-plasma-membrane calcium-pump isoform 2 generated by alternative RNA splicing in the N-terminal coding region. Eur J Biochem 205: 333–340, 1992
11. Strehler EE, Zacharias DA: Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol Rev 81: 21–50, 2001
12. Keeton TP, Burk SE, Shull GE: Alternative splicing of exons encoding the calmodulin-binding domains and C termini of plasma membrane Ca^{2+} ATPase isoforms 1, 2, 3, and 4. Alternative splicing of exons encoding the calmodulin-binding domains and C termini of plasma membrane Ca^{2+} ATPase isoforms 1, 2, 3, and 4. J Biol Chem 268: 2740–2748, 1993
13. Santiago-García J, Mas-Oliva J, Saavedra D, Zarain-Herzberg A: Analysis of mRNA expression and cloning of a novel plasma membrane Ca^{2+} ATPase splice variant in human heart. Mol Cell Biochem 155: 173–182, 1996
14. Kessler F, Bennardini F, Bachs O, Serratosa J, James P, Caride AJ, Gazzoti P, Penniston JT, Carafoli E: Partial purification and characterization of the Ca^{2+}-pumping ATPase of the liver plasma membrane. J Biol Chem 265: 16012–16019, 1990
15. Mas-Oliva J, Pérez-Monfort R, Cárdenas-García M, Rivas-Duro M: Altered coupling states between calcium transport and (Ca^{2+},Mg^{2+})-ATPase in the AS-30D ascites hepatocarcinoma plasma membrane. Mol Cell Biochem 100: 39–50, 1991
16. Howard A, Barley NF, Legon S, Walters JRF: Plasma-membrane calcium-pump isoforms in human and rat liver. Biochem J 303: 275–279, 1994
17. Zacharias DA, Kappen C: Developmental expression of the four plasma membrane calcium ATPase (Pmca) genes in the mouse. Biochim Biophys Acta 1428: 397–405, 1999
18. Knauf PA, Proverbio F, Hoffman JF: Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and N-K-ATPase in human red cell ghosts. J Gen Physiol 63: 324–336, 1974
19. Sambrook J, Maniatis T, Fritsch EF: Molecular Cloning. A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York, 1989
20. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159, 1987
21. Higgins GM, Anderson RM: Experimental pathology of the liver. Arch Pathol 12: 186–202, 1931
22. Latif F, Duh FM, Gnarra J, Tory K, Kuzmin I, Yao M, Stackhouse T, Modi W, Geil L, Schmidt L, Li H, Orcutt M, Maher E, Richards FO Jr, Ferguson-Smith MA, Le Paslier D, Linehan M, Zbar B, Lerman MI: Von Hippel-Lindau syndrome. Cloning and identification of the plasma membrane Ca(++)-transporting ATPase isoform 2 gene resides in the von Hippel-Lindau gene region. Cancer Res 53: 861–867, 1993
23. Verma AK, Filoteo AG, Stanford DR, Wieben ED, Penniston JT, Strehler EE, Fischer R, Heim R, Vogel G, Mathews G, Strehler-Page MA, James P, Vorherr T, Krebs J, Carafoli E: Complete primary structure of a human plasma membrane Ca^{2+} pump. J Biol Chem 263: 14152–14159, 1988
24. Carafoli E: The Ca^{2+} pump of the plasma membrane. J Biol Chem 267: 2115–2118, 1992
25. Takahashi H, Yamaguchi M: Regulatory effect of regucalcin on (Ca^{2+}-Mg^{2+})- ATPase in rat liver plasma membranes: Comparison with the activation by Mn^{2+} and Co^{2+}. Mol Cell Biochem 124: 169–174, 1993
26. Takahashi H, Yamaguchi M: Activating effect of regucalcin on (Ca^{2+}-Mg^{2+})-ATPase in rat liver plasma membranes: Relation to sulfhydril group. Mol Cell Biochem 136: 71–76, 1994
27. Jouneaux C, Audigier Y, Goldsmith P, Pecker F, Lotersztajn S: Gs mediates hormonal inhibition of the calcium pump in liver plasma membranes. J Biol Chem 268: 2368–2372, 1993
28. Hickie R, Kalant H: Calcium and magnesium content of rat liver in Morris hepatoma 5123tc. Can Res 27: 1053–1057, 1967
29. Swierenga SH, Whitfield JF, Karasaki S: Loss of proliferative calcium dependence: Simple *in vitro* indicator of tumorigenicity. Proc Natl Acad Sci 75: 6069–6072, 1978
30. Fiskum G: Intracellular levels and distribution of Ca^{2+} in digitonin-permeabilized cells. Cell Calcium 6: 25–37, 1985
31. Enyedi A, Verma AK, Heim R, Adamo H, Filoteo AG, Strehler E, Penniston JT: The Ca^{2+} affinity of the plasma membrane Ca^{2+} pump is controlled by alternative splicing. J Biol Chem 269: 41–43, 1994
32. Díaz-Muñoz M, Canedo-Merino R, Gutiérrez-Salinas J, Hernández-Muñoz R: Modifications of intracellular calcium release channels and calcium mobilization following 70% hepatectomy. Arch Biochem Biophys 349: 105–112, 1998
33. Magnino F, St-Pierre M, Lüthi M, Hilly M: Expression of intracellular calcium channels and pumps after partial hepatectomy in rat. Mol Cell Biol Res Commun 3: 374–379, 2000
34. Brandt P, Neve RL: Expression of plasma membrane calcium-pumping ATPase mRNAs in developing rat brain and adult brain subregions: Evidence for stage-specific expression. J Neurochem 59: 1566–1569, 1992
35. Guerini, D, Wang X, Li L, Genazzani A, Carafoli E: Calcineurin controls the expression of isoform 4CII of the plasma membrane Ca($^{2+}$) pump in neurons. J Biol Chem 275: 3706–3712, 2000
36. Cittadini A, Dani AM, Wolf F, Bossi D, Calviello G: Calcium permeability of Ehrlich ascites tumor cell plasma membrane *in vivo*. Biochim Biophys Acta 686: 27–35, 1982
37. Guerini D, Schröder S, Foletti D, Carafoli E: Isolation and characterization of a stable Chinese hamster ovary cell line overexpressing the plasma membrane Ca^{2+} ATPase. J Biol Chem 270: 14643–14650, 1995
38. Liu BF, Xu X, Fridman R, Muallem S, Huo TH: Consequences of functional expression of the plasma membrane calcium pump isoform 1a. J Biol Chem 271: 5536–5544, 1996
39. Kuo TH, Liu BF, Yu Y, Wuytack F, Raeymaekers L, Tsang W: Coordinated regulation of the plasma membrane calcium pump and the sarco(endo)plasmic reticular calcium pump gene expression by Ca^{2+}. Cell Calcium 21: 399–408, 1997