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BREAKTHROUGHS AND VIEWS

Senescence Marker Protein-30 (SMP30): Structure and Biological Function

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Senescence marker protein-30 (SMP30), which we previously identified, is notable for its androgen-independent decrease in the livers of aging rats. Hepatocytes and renal tubular epithelia express large amounts of SMP30 in their cytosol throughout the tissue-maturing process and adulthood, but its level decreases thereafter. Upon cloning cDNAs that encode SMP30 in rats, mice, and humans, we found that the amino acid sequence of SMP30 is well conserved with remarkable homology among these species. However, this gene, which is so strongly conserved in these higher animals, does not appear in yeast. We also determined the genome organization and 5′ flanking region of SMP30 in mouse genome. In the meantime, SMP30 turned out be identical to a Ca^{2+}-binding protein called regucalcin (RC). To learn how this protein functions, we transfected Hep G2 cells with human SMP30 cDNA so that these cells stably express large amounts of SMP30. The results suggest that SMP30 regulates Ca^{2+} homeostasis by enhancing Ca^{2+}-pumping activity in the plasma membranes. Thus, SMP30 seems to play a critical role in the highly differentiated functions of the liver and kidney and to exert a major impact on Ca^{2+} homeostasis. If so, down-regulation of SMP30 with aging would attribute greatly to the related deterioration of these organs, as indicated in this brief overview of the structure, expression, and function of SMP30.

Key Words: **aging; Ca^{2+}-binding protein; Ca^{2+} homeostasis; calbindin-D 28 kDa; cDNA; kidney; hepatocyte; liver; plasma membrane Ca^{2+}-pumping activity; renal tubular epithelium; regucalcin; SMP30; senescence marker protein-30.**

Abbreviations used: CaBP, calbindin-D 28 kDa; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; nt, nucleotide(s), RC, regucalcin; SMP30, senescence marker protein-30.

1. SMP30 DECREASES ANDROGEN-INDEPENDENTLY WITH AGING IN THE RAT LIVER

The liver is a key player in nutritional metabolism, production of essential proteins and factors as well as detoxification of harmful substances. The liver also has a potency for cell proliferation; classified as direct cell proliferation induced by chemicals, and compensatory cell proliferation, observed in partial hepatectomy (1). Certain chemicals cause undue proliferation of hepatic cells, leading to hyperplasia. Additionally, the age-dependent deterioration of hepatic proteins may cause liver dysfunction.

To understand these age-associated alterations at the gene level, we investigated the transcription factors c-jun, c-fos, and others with the ability to alter the expression of their target genes. Clearly, the expression of c-jun and c-fos was upregulated in aged rat livers compared with that of adult rats (2).

Next, to assess the age-associated changes in hepatic proteins, we surveyed soluble proteins in the livers of adult as well as aged rats by two-dimensional electrophoresis. The outcome was detection of a novel hepatic protein (SMP30), the amounts of which were down-regulated in aged rats compared with their younger counterparts (3). Since senescent males and females were affected equally, the effect was androgen-independent. Because of its relationship to aging and its molecular mass of 30 kDa, this peptide was designated senescence marker protein-30 (SMP30). That hepatic expression of the rat androgen receptor gene declines markedly with aging has been established (4). Accordingly, proteins responsive to androgen also decrease with age. Indeed, we formerly detected a protein whose quantity drastically diminished in the livers of aged male rats, but was barely detectable in the females at any age (3). However, we focused on SMP30, because its effects are not limited by sex.

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To characterize SMP30 biochemically and histochemically, we purified SMP30 from rat livers and prepared a specific antibody. This anti-SMP30 serum was also used for quantitative rocket immunoelectrophoresis. These methods enabled us to show a 60 to 70% decrease of SMP30 in the livers of aged males and females compared to the concentrations in younger adults (3). In addition, this quantitative analysis revealed that the amounts of SMP30 constituted 2% of the total soluble protein fraction in the livers of adult rats (3).

2. PRIMARY STRUCTURE OF SMP30

A cDNA clone encoding rat SMP30 was then isolated and characterized (5). The full cDNA segment, 1,600 bp in length, has an open reading frame of 897 bp, which encodes 299 amino acids. The estimated molecular weight of the deduced polypeptide is 33,387 and estimated pI is 5.101. The 3′ untranslated region contains two polyadenylation signals. 'PEST'n regions, which are thought to mediate rapid degradation of proteins, have also been found in the amino acid sequence of SMP30. Since, no significant homology was found to known nucleic acid or amino acid sequences, SMP30 was considered to be a novel protein. Independently, in later experiments, a cDNA encoding a Ca^{2+}-binding protein called regucalcin (RC) was proved to be identical to SMP30 (6). Since the primary structure of SMP30 lacks known Ca^{2+}-binding motifs, such as EF-hand motif, suggesting a novel class of Ca^{2+}-binding protein.

Genomic Southern hybridization analysis showed that SMP30 is evolutionarily conserved only in higher animals; this gene is not found in yeast (5). To characterize the gene's molecular evolution, we then cloned human, rat, and mouse cDNA encoding SMP30 (7, 8). All these cDNA clones have a single open reading frame consisting of 299 amino acids. Alignment of their amino acid sequences reveals that rat SMP30 closely resembles human SMP30 (88.9% identity in amino acid sequence) and also mouse SMP30 (94.3% identity). In fact, the entire primary structure of SMP30 is conserved among humans and rodents, suggesting that this complete structure is required for the physiological function of SMP30. These data indicate the biological importance of SMP30 in multiple animal species.

Finally, the SMP30 gene has been assigned to the p11.3-q11.2 segment of X chromosome (7). This gene could, thus, be a candidate as the cause of X-linked diseases mapped to that region.

3. GENOMIC ORGANIZATION AND 5′ FLANKING SEQUENCE OF THE SMP30 GENE

After isolating the genomic locus encompassing the murine SMP30 gene, we characterized the putative promoter and cis-regulatory sequences in its 5′ flanking region (8). SMP30 is organized into 7 exons and 6 introns, spanning approximately 17.5 kb. Primer extension analysis revealed two major transcription initiation sites located 101 and 102 bp upstream from the ATG translation initiation codon. For identification of the potential cis-regulatory elements of SMP30, the promoter region was sequenced and searched for the motifs of transcription factor binding sites. In the proximal promoter region, a TATA-like sequence, a CAAT box and Sp1 sites could reasonably be located at nt −29, −72, and −169, respectively. In addition to these conventional transcription factor binding sites, we found two clustered Sp1 boxes with AP-2 at nt −900 and −1376 in the distal promoter region. Interestingly, Sp1 DNA binding efficiency declines greatly and specifically in nuclear extracts from tissues of aged rats, including their livers and brains. Conceivably, then, this decrease of Sp1 binding activity may influence downregulation of the androgen-receptor gene or expression of the heavy chain ferritin gene with aging (4, 9). Since, SMP30 gene also contains three Sp1 sites, the loss of Sp1-binding activity may account for the downregulation of SMP30 gene expression with age in rat livers. Our experiments also yielded binding sites for two classes of C/EBP transcription factors that are highly expressed in the liver in addition to AP-2, AP-1, GATA-1, and AP-1/GRE. Overall, these results provide important clues about the regulatory mechanism(s) of SMP30 and its relationship to aging.

4. TISSUE DISTRIBUTION

Immunohistochemical and Northern blot analyses of multiple rat tissues pinpointed specific and strong SMP30 reactivity in the liver and kidney (5). In paraffin-embedded sections of liver, the response was strongly positive in the centrilobular to midlobular areas of hepatocytes. However, the immunoreactivity was localized mainly in proximal tubular epithelia and less intensely in distal and collecting tubular epithelia in the kidney. By immunoelectron microscopic examination, the immunoreactivities for SMP30 antibody was strong and restricted to the cytosol of hepatocytes and to the cytosol and brush border of the renal proximal tubular epithelia. Unlike rats, mice showed an abundant expression of SMP30 only in the liver (8). In humans, SMP30 was more widely distributed but was also strongly reactive in the liver (7). Clearly, SMP30 is highly expressed in the livers of all these mammalian species. Again, the importance of SMP30 in liver function is suggested by this protein's evolutional conservation in higher animals and the remarkable homology of its entire sequence in human and rodents.

5. COORDINATED UP-REGULATION OF SMP30 WITH TISSUE MATURATION AND ITS DOWN-REGULATION WITH AGING

When we charted the expression of SMP30 in embryonic, neonatal, young, adult and senescent rats, no SMP30 mRNA was detectable in either the livers or kidneys of 18-day-old embryos (10). However, in the liver, SMP30 protein was started to increase at neonatal day 7 and rapidly reached a plateau at day 10. Thereafter, substantial amounts of this protein and its transcripts were maintained in adult livers until the animals reached 3 to 6.5 months of age. In the kidney, SMP30 started its sharp increase in 21-day-old rats and reached a maximal level at day 35. Those high levels of transcript and protein were maintained in the kidneys of young and adult rats. Later, as aging progressed to senescent stages, amounts of SMP30 significantly decreased both in the liver and the kidney. The heightened expression of SMP30 during tissue-maturing stages and adulthood witnessed in this study suggests that SMP30 is required for the maintenance of differentiated hepatocytes and renal tubular epithelia.

6. SMP30 RESCUES CELL DEATH BY ENHANCING PLASMA MEMBRANE Ca^{2+}-PUMPING ACTIVITY

Ca^{2+} is essential for various cellular functions, although prolonged high cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) lead to cell death. A low $[Ca^{2+}]_i$ ensures necessary cellular activities and survival, so cells tightly regulate their $[Ca^{2+}]_i$ levels through numerous binding and specialized extrusion proteins. For this purpose, Ca^{2+} pumps transport Ca^{2+} to the extracellular space or to intraorganellar spaces, such as the endoplasmic reticulum. SMP30 presumably participates in this and possibly other physiological functions; although, related studies using hepatocytes have been inconclusive. Therefore, to elaborate on the biological significance of SMP30, we have generated Hep G2 cell lines that stably express large amounts of SMP30 after transfection with human SMP30 cDNA (11). With these cell lines, after adding extracellular ATP, we investigated $[Ca^{2+}]_i$ and Na^+-independent Ca^{2+} efflux (11). Although stimulation of cells with ATP causes a transient increase of $[Ca^{2+}]_i$ in both SMP30- and mock-transfectants, after reaching a peak, $[Ca^{2+}]_i$ decreases at a 2-fold enhanced rate in SMP30-transfected cells. Correspondingly, Na^+-independent Ca^{2+} efflux is 2-fold greater in SMP30 transfectants compared with mock transfectants. This Ca^{2+} efflux presumably results from the augmentation of plasma membrane Ca^{2+}-pumping activity. In addition, after Ca^{2+} ionophore treatment to induce cell death, more SMP30 transfectants survive than mock transfectants. Because the typical DNA ladder pattern was detected in this experiment, the cell death observed has been attributed to apoptosis. Apparently, SMP30 rescues Hep G2 cells from apoptosis by enhancing plasma membrane Ca^{2+}-pumping activity. However, since we measured Na^+-independent Ca^{2+} efflux into the medium as plasma membrane Ca^{2+}-pumping activity, the possibility remains that SMP30 enhances sarco/endoplasmic reticulum Ca^{2+}-pumping activity as well as plasma membrane Ca^{2+}-pumping activity. If so, the net degree of increased Ca^{2+} efflux could mask sequestration of Ca^{2+} into internal Ca^{2+} stores.

7. DISCUSSION AND PERSPECTIVES

Ca^{2+} is a second messenger for the common signal transduction element in cells. Therefore, Ca^{2+} is involved in such critical cellular functions as proliferation, differentiation, adhesion and apoptosis. Presumably, then impaired Ca^{2+} signaling affects some or all of these activities. SMP30 evidently plays an important role in $[Ca^{2+}]_i$ homeostasis and in the modulation of effector molecules including some enzymes required for Ca^{2+} to perform its actions. Like SMP30, the tissue-specific Ca^{2+}-binding protein, calbindin-D 28 kDa (CaBP), also decreases with aging in the cerebella of rats and humans (12, 13). These two molecules share other similarities, e.g., (i) the expression of both is developmentally regulated in a tissue-specific manner, and (ii) the overexpression of CaBP rescues cells from apoptosis caused by Ca^{2+} ionophore (14). The remarkable similarities between SMP30 and CaBP may contribute to our understanding of Ca^{2+} homeostasis in aging. That is, the dual age-associated decrease in SMP30 and CaBP suggests a possible scheme for the dysregulation of Ca^{2+} homeostasis, which may alter the signaling system in aged tissues. Without proper Ca^{2+} homeostasis, the logical outcome would be a failure in the response to harmful stimuli as well as in an age-associated deterioration of cellular functions in tissues and organs. Our results suggest that the down-regulation of SMP30 in liver and kidney may increase the tissue susceptibility for harmful stimuli in aged tissues and may cause the deterioration of particularly hepatic and renal functions.

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