

Senescence marker protein-30 (SMP30) and its gerontological significance

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We have isolated and characterized a novel protein designated as senescence marker protein-30 (SMP30). The amount of SMP30 decreases in an androgen-independent manner with increasing age in the rat liver. SMP30 turned out to be identical to a calcium-binding protein called regucalcin. In this review, we give an overview of the structure, expression and possible physiological role(s) of SMP30. We also discuss the hypothetical role of SMP30 in aging and calcium homeostasis.

THE liver plays important roles in nutritional metabolism, detoxification of various harmful substances, and the production of essential proteins and factors. Hence, age-dependent changes of hepatic proteins may influence the hepatic functions in the elderly people. With respect to age-associated alterations of gene/protein expression in the liver, several reports have described such changes in aged rats¹⁻³. We investigated the expression of transcription factors and found that the expression of *c-jun* and *c-fos* was up-regulated in aged rat livers as compared to that of adult rats⁴. Transcription factors, such as *c-jun*, *c-fos*, or other factors may alter the expression of their target genes. In order to assess the age-associated changes in hepatic proteins, we surveyed soluble proteins in adult rat livers as well as aged rat livers by two-dimensional electrophoresis. We detected and purified a novel hepatic protein, which is down-regulated androgen-independently in aged rat liver⁵. We designated this protein as senescence marker protein-30 (SMP30) because of its molecular mass of 30 kDa. Down-regulation of SMP30 was sex-independent and its amount decreased in aged females as well. To elucidate physiological function(s) of SMP30 and its expressional regulation in aged rat liver, we prepared specific antibody for the detection of SMP30 and characterized cDNA clones encoding rat, human and mouse SMP30 (refs 5-8). In addition, we isolated mouse genomic locus encompassing SMP30 gene and characterized the putative promoter and *cis*-regulatory sequences in its 5' flanking region⁸. An immunohistochemical analysis showed that SMP30 was specifically localized in

hepatocytes and renal tubular epithelia⁶. The primary structure of SMP30 was well conserved in higher animals with remarkable sequence homology^{6,7}. These results suggested the important role(s) of SMP30 in the liver and kidney of higher animals. SMP30 was recently found to be a novel type of calcium-binding protein, implying that the regulation of intracellular calcium ions in hepatocytes and renal tubular epithelia may be altered in aged animals.

Detection of SMP30 and its androgen-independent decrease with age in the rat liver

To identify age-associated changes in the soluble proteins of the rat liver, we used two-dimensional cellulose acetate membrane electrophoresis (2D-CAME) and microcomputer-assisted two-dimensional densitometry^{9,10}. This method has the advantage of defining different profiles of water-soluble proteins from those detected by the ordinary two-dimensional polyacrylamide gel electrophoresis of O'fferrell¹¹. By using this method, we analysed undenatured liver proteins from young as well as aged rats. The results showed the profiles of these water-soluble proteins from the livers of 6 and 24-month-old rats (Figure 1). Among 10 well-resolved proteins, 5 are indicated in Figure 1. Next, we quantified these spots on the filter by a microcomputer-assisted densitometry. The amounts of electrophoresed proteins were measured as integrated optical density (IOD)¹⁰. Quantitative analysis showed that the amounts of two proteins, p/4.9 and p/7.3, were significantly decreased in the liver of aged male rat (24 months) in comparison to those of adult male rat liver (6 months). These decreases were 37% and 93%, respectively (Figure 2). In contrast, other well-resolved proteins at spots 2, 8, and 9 (Figure 1) failed to show significant age-associated changes (Figure 2). The decrease of p/7.3 protein was drastic, while this protein was hardly detected in the liver of female rats of all ages. On the other hand, the amount of p/4.9 protein was specifically down-regulated in aged female rat liver as well as aged male rat liver. In order to characterize the expressional regulations of these molecules, we investigated the effect of castration and postoperative administration of testosterone on the ex-

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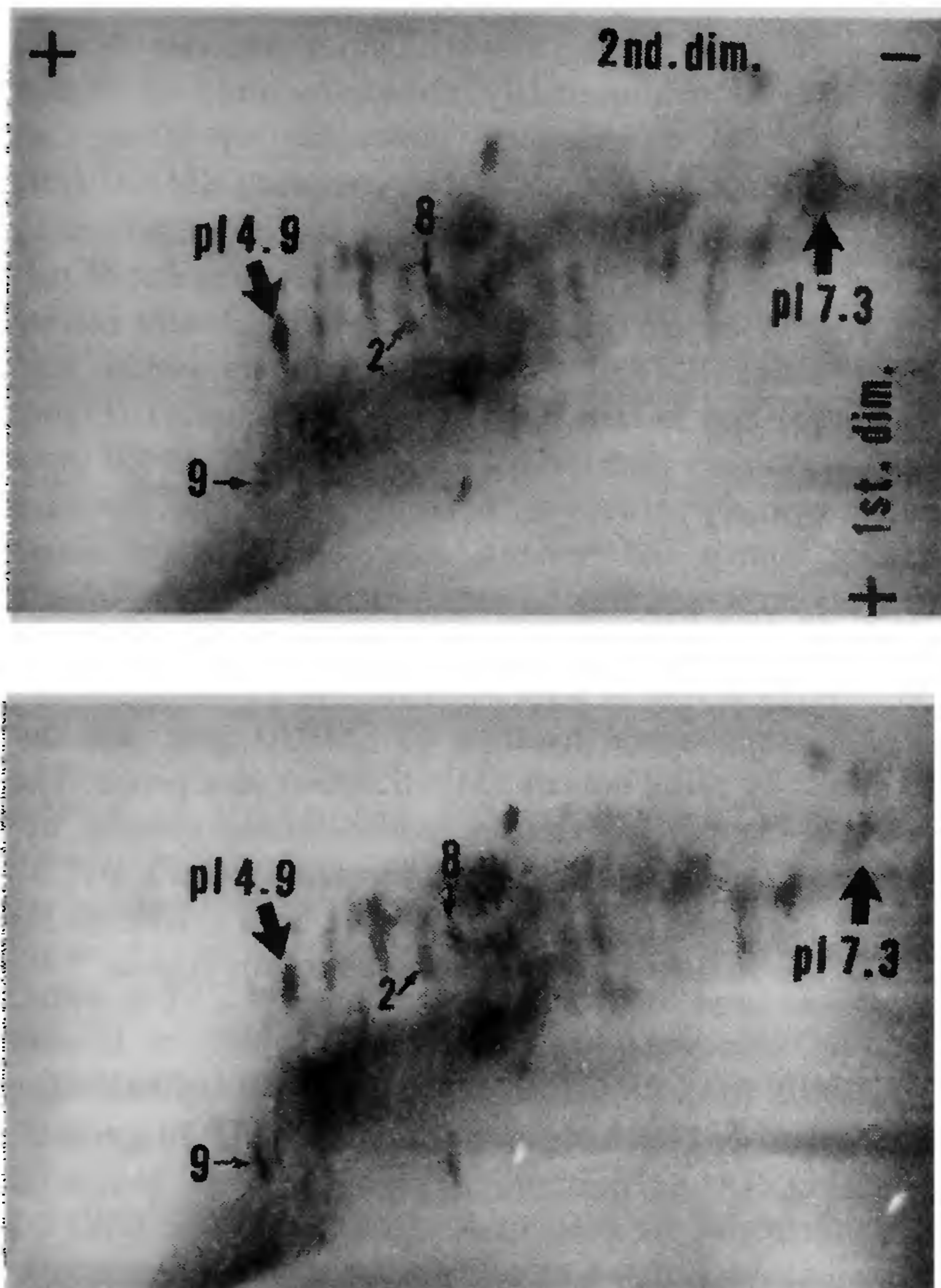


Figure 1. Two-dimensional electrophoretograms of soluble proteins from livers of adult (upper) 6-month-old and senescent (lower) 24-month-old male rats. Bold arrows indicate the proteins which decrease during aging: one is p/4.9 protein and the other is p/7.3 protein. Thin arrows, 2, 8, 9 indicate proteins that did not change quantitatively during aging. Proteins are visualized after Coomassie blue staining. 100 µg of proteins from animals of each age were subjected to 2D-CAME. (T. Fujita *et al.*, *Biochim. Biophys. Acta*, 1992, 1116, 122-128.)

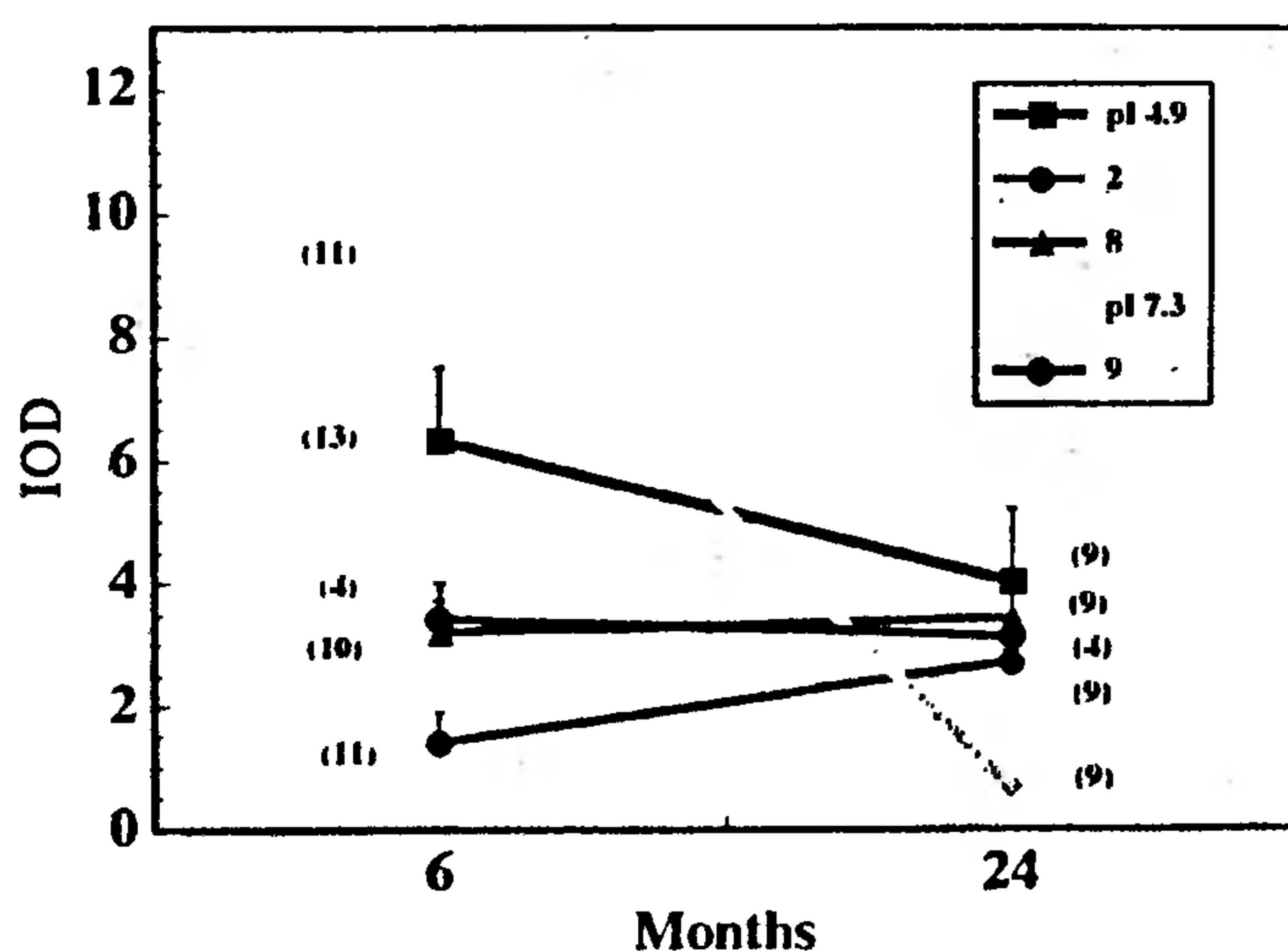


Figure 2. Quantification of protein spots on 2D-CAME from adult v.s. senescent male rat livers. Quantitative analyses are performed on each spot (p/4.9, p/7.3, 2, 8, 9), indicated in Figure 1 by computer-assisted two-dimensional densitometry after 2D-CAME. Values are shown as mean IOD + standard deviation and each sample number is shown in parentheses. IOD; Integrated optical density.

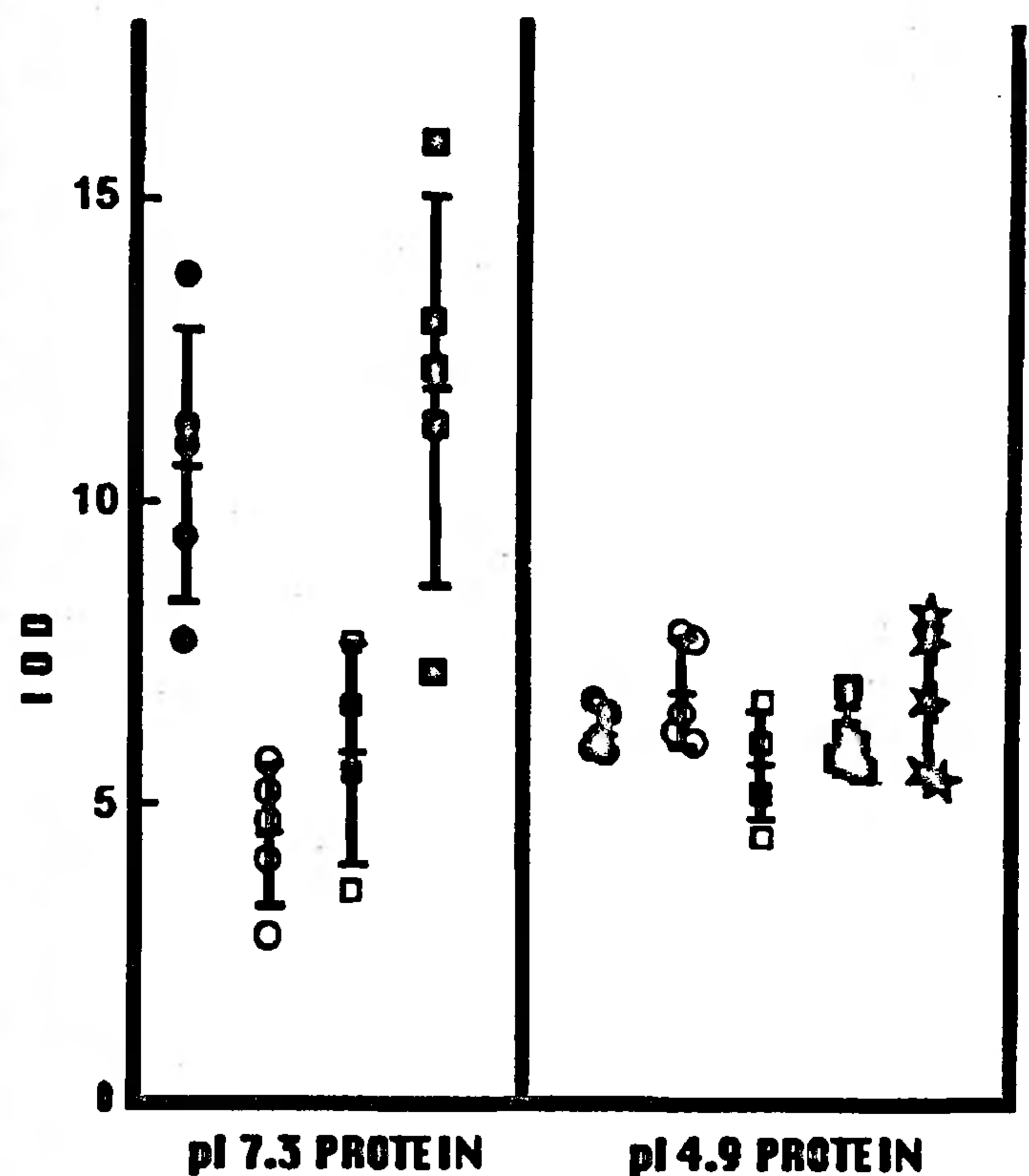


Figure 3. Effects of castration and post-operative administration of testosterone on the expression of p/4.9 and p/7.3 proteins. Wistar male rats were used. ●, control; ○, castration; □, treatment with sesame oil after castration; ■, treatment with testosterone propionate after castration; ◇, female (no treatment). 100 µg of proteins from animals of each age were subjected to 2D-CAME. The amounts of p/4.9 and p/7.3 protein spots were quantified by computer-assisted two-dimensional densitometry after 2D-CAME. IOD, Integrated optical density. (Fujita *et al.*, *Biochim. Biophys. Acta*, 1992, 1116, 122-128.)

pression of p/4.9 and p/7.3 proteins (Figure 3). The expression of p/4.9 protein was not affected by castration or by treatment with testosterone after castration. On the contrary, p/7.3 protein decreased after castration, but completely recovered after the administration of testosterone. We focused on the protein of p/4.9 because its expression is down-regulated in an androgen-independent manner in aged rat liver. We purified this protein from rat liver by biochemical methods. This soluble p/4.9 protein was a single polypeptide with molecular mass of 30 kDa and we designated it as senescence marker protein-30 (SMP30) (ref. 5).

In order to characterize the biochemistry and histochemistry of SMP30, we prepared a specific antibody for the detection of SMP30. We evaluated age-associated changes of SMP30 by quantitative rocket immunoelectrophoresis using anti-SMP30 serum. The results showed that 60-70% decrease of SMP30 occurs in both aged male and female livers as compared to the younger adults⁵. In addition, this quantitative analysis revealed that the amount of SMP30 is 2% of total soluble protein fraction in the liver of adult rats⁵.

To exclude the possibility that the detected amounts of SMP30 was influenced by post-translational modifications in aged rat liver, we developed a double staining method, a combined method of immunodetection and Coomassie blue-staining¹². In this method, a post-translational modification of SMP30 would shift its location in two-dimensional profile. However, only a single spot corresponding to SMP30 reacted with anti-SMP30 serum in double stained two-dimensional profile prepared from senescent livers (24 months), strongly suggesting that the decrease of SMP30 with age was due to the decrease of protein amount, and not due to translational modification⁵.

Primary structure of SMP30

We isolated and characterized a cDNA clone encoding rat SMP30 (ref. 6). The full length cDNA clone, 1,600 bp in length, had an open reading frame of 897 bp, which could encode 299 amino acids. The estimated molecular weight of deduced polypeptide was 33,387 and the estimated pI was 5.101. In its 3' untranslated region, two polyadenylation signals were found. The homologous sequence was searched against GenBank database, but no significant homology was found with known nucleic acid or amino acid sequences, suggesting that SMP30 is a novel protein. In 1993, Shimokawa and Yamaguchi reported a cDNA encoding a calcium-binding protein called regucalcin and found that it was identical to SMP30 (ref. 13). Since the primary structure of SMP30 failed to show known calcium-binding motifs such as EF-hand or other motifs, SMP30 represents a novel calcium-binding protein.

The genomic Southern hybridization analysis showed that SMP30 evolutionarily conserved only in higher animals⁶. To characterize molecular evolution, we cloned human and mouse cDNA encoding SMP30 (refs 7, 8). All cDNA clones showed a single open reading frame consisting of 299 amino acids. Alignment of primary structure revealed that rat SMP30 closely resembled human SMP30 (88.9% identity in amino acid sequence) and mouse SMP30 (94.3% identity) (Figure 4). The primary structure of SMP30 was conserved in its entire primary structure, including 9 cysteine residues among human and rodents, suggesting that the entire primary structure may be required for the physiological function of SMP30. These data suggest a conserved physiological role of SMP30 in various animals.

The chromosomal location of SMP30 gene was determined by using human SMP30 cDNA as a probe. The regional mapping in various rodent-human somatic hybrids indicated that the gene is located on the X p11.3-q.11.2 segment⁷. This gene thus may be a candidate for one of the X-linked diseases mapped to this region.

Genomic organization, transcription initiation sites, and 5' flanking sequence of SMP30 gene

To understand the regulatory mechanism of SMP30 expression, we isolated mouse genomic locus encompassing SMP30 gene, and characterized the putative promoter and *cis*-regulatory sequences in its 5' flanking region⁸. Analysis of the genomic clone revealed that the SMP30 gene consists of 7 exons and 6 introns, spanning approximately 17.5 kb. Primer extension analysis revealed that two major transcription initiation sites are



Figure 4. Alignment of SMP30 amino acid sequences of mouse, rat and human. Sequences highlighted in black are those conserved in mouse, rat and human. (T. Fujita *et al.*, *Biochim. Biophys. Acta*, 1996, 1308, 49-57.)

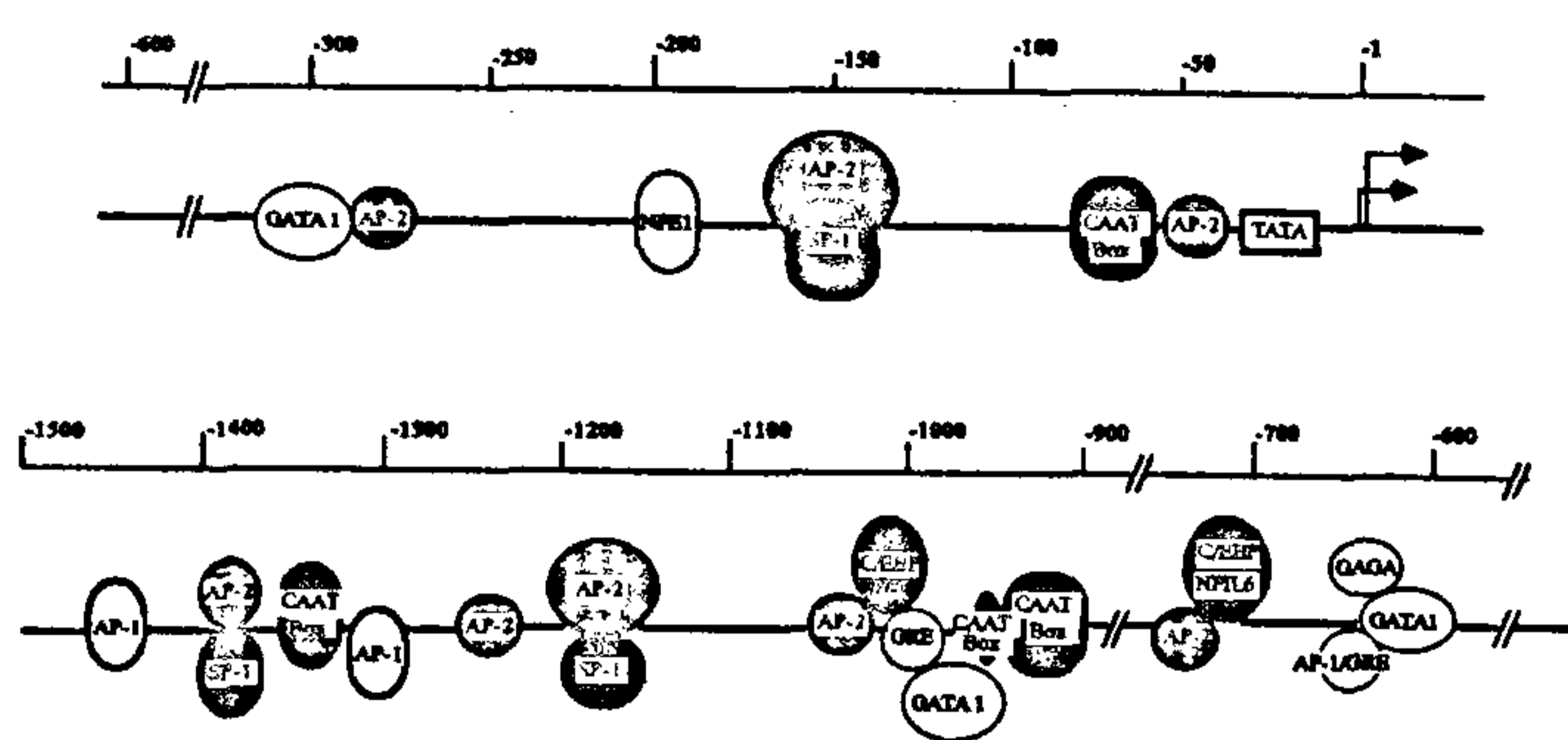


Figure 5. Putative transcription factor binding sites of mouse *SMP30* gene. Transcription initiation sites are indicated by arrows and referred to as +1 and +2.

located at 101 bp and 102 bp upstream from ATG translation initiation codon⁸. Putative transcription factor-binding sites of mouse *SMP30* gene are illustrated in Figure 5. The nucleotide sequence of 5' flanking region shows a TATA-like sequence, a CAAT box, and Sp-1 sites at nt -29, -72 and -169 in the promoter region, respectively. Interestingly, we find binding sites for two classes of C/EBP transcription factors which are highly expressed in the liver, in addition to AP-2, AP-1, GATA-1 and AP-1/GRE. These results provide important clues for the understanding of the regulatory mechanism(s) of *SMP30* and its relationship to aging.

Coordinated up-regulation of *SMP30* with tissue maturation and its down-regulation with aging

Northern blot and immunohistochemical analyses of rat tissues showed that *SMP30* was specifically expressed in the liver and kidney where its immunoreactivity was localized in the hepatocytes and the renal tubular epithelia⁶. Immunoelectron microscopic examinations showed that *SMP30* was restricted in the cytosol of these cells.

As mentioned earlier, the amount of *SMP30* in aged rat liver decreased significantly. However, its expression in the liver and kidney during the embryogenesis and maturation of these tissues had not been investigated. Therefore, we studied the expression of *SMP30* in embryonic, neonatal, young, adult and senescent rats (Figure 6)¹⁴. No transcript was detected in the liver and the kidney at embryonic day 18. Northern blot analysis showed a characteristic increase in *SMP30* mRNA in the livers of neonatal and young rats. Western blot analysis showed that the expression of *SMP30* protein started to increase at neonatal day 7 and rapidly reached a plateau at day 10. Substantial levels of protein and transcripts were maintained in adult livers until 3–6.5 months of

age. In the kidney, *SMP30* mRNA and protein started to increase at day 21 and reached a maximal level at day 35. The levels of transcript and protein were maintained at high levels in adult kidneys up to 3 months of age. As age progressed to senescent stages, the levels of transcript and protein significantly decreased both in the liver and the kidney. In this study, the higher expression of *SMP30* in the adult stages suggests that *SMP30* is required for the maintenance of differentiated hepatocytes and renal tubular epithelia.

Discussions and perspectives

Using a special two-dimensional electrophoresis (2D-CAME), we detected and isolated a hepatic protein which decreased in an androgen-independent manner with aging. We designated it as senescence marker protein-30 (*SMP30*) since this soluble protein was a single polypeptide with a molecular mass of 30 kDa. The first notable character of *SMP30* is its androgen-independent expression. Its level decreases with aging both in male and female livers. We described two prominent proteins that decreased with aging (Figures 1 and 2) that were identified by their p/values of 4.9 and 7.3. The amount of *SMP30* was not affected by castration, whereas the p/7.3 protein significantly decreased under this situation. This noteworthy decrease in p/7.3 protein agrees with several reports on sex differences in age-associated changes in gene/protein expression in the rat liver^{15,16}.

Next we isolated and characterized cDNAs encoding rat, mouse and human *SMP30*. The alignment of amino acid sequences in these animals showed the highly conserved primary sequence of *SMP30* from rodents to humans.

Tissue distribution of *SMP30* was specific in hepatocytes and renal tubular epithelia, suggesting a pivotal role of *SMP30* in the liver and the kidney. Following our findings, Shimokawa and Yamaguchi reported a cDNA clone encoding a rat hepatic protein which was identical to *SMP30*. They had characterized this protein as a calcium-binding protein and called it as regucalcin (RC)¹³. Regucalcin shows reversible effects in the activation and inhibition of various enzymes by Ca^{2+} in liver cells¹⁷. Their findings suggest that *SMP30*/RC may act as a modulator of liver cells that are regulated by Ca^{2+} . As would be expected from its characterization, we speculate that *SMP30* may play an important role in intracellular calcium homeostasis and in the modulation of effector molecules including some hepatic enzymes that require calcium for their action. Furthermore, the decrease in *SMP30* during the aging process may lead to senile alterations in the buffering capacity for calcium, and dysregulation of calcium signals in aged livers and kidneys. However, as for the physiological roles of *SMP30*, studies using hepatocytes or renal tubular epithelia had so far not been undertaken. Hence we

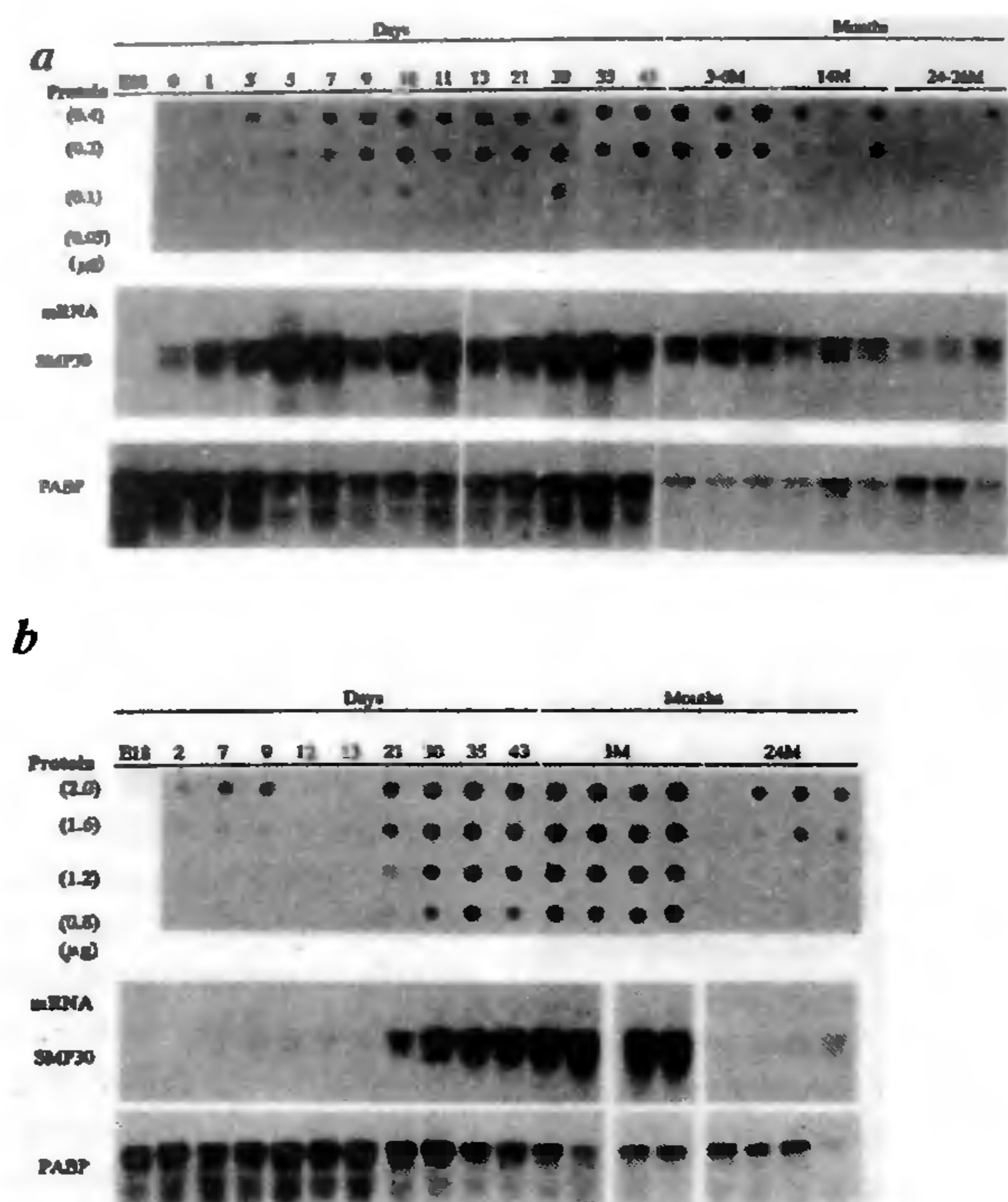


Figure 6. Protein and mRNA levels of SMP30 in rat livers (A) and kidneys (B) from embryo to senescence. *Upper*, the protein levels were analysed by immuno-dot blot analysis. *Lower*, mRNA levels were analysed by Northern blot analysis. The filter was reprobbed with poly(A)⁺ binding protein (PABP) as control. (Modified from T. Fujita *et al.*, *Mech. Ageing Dev.*, 1996, 87, 219–229.)

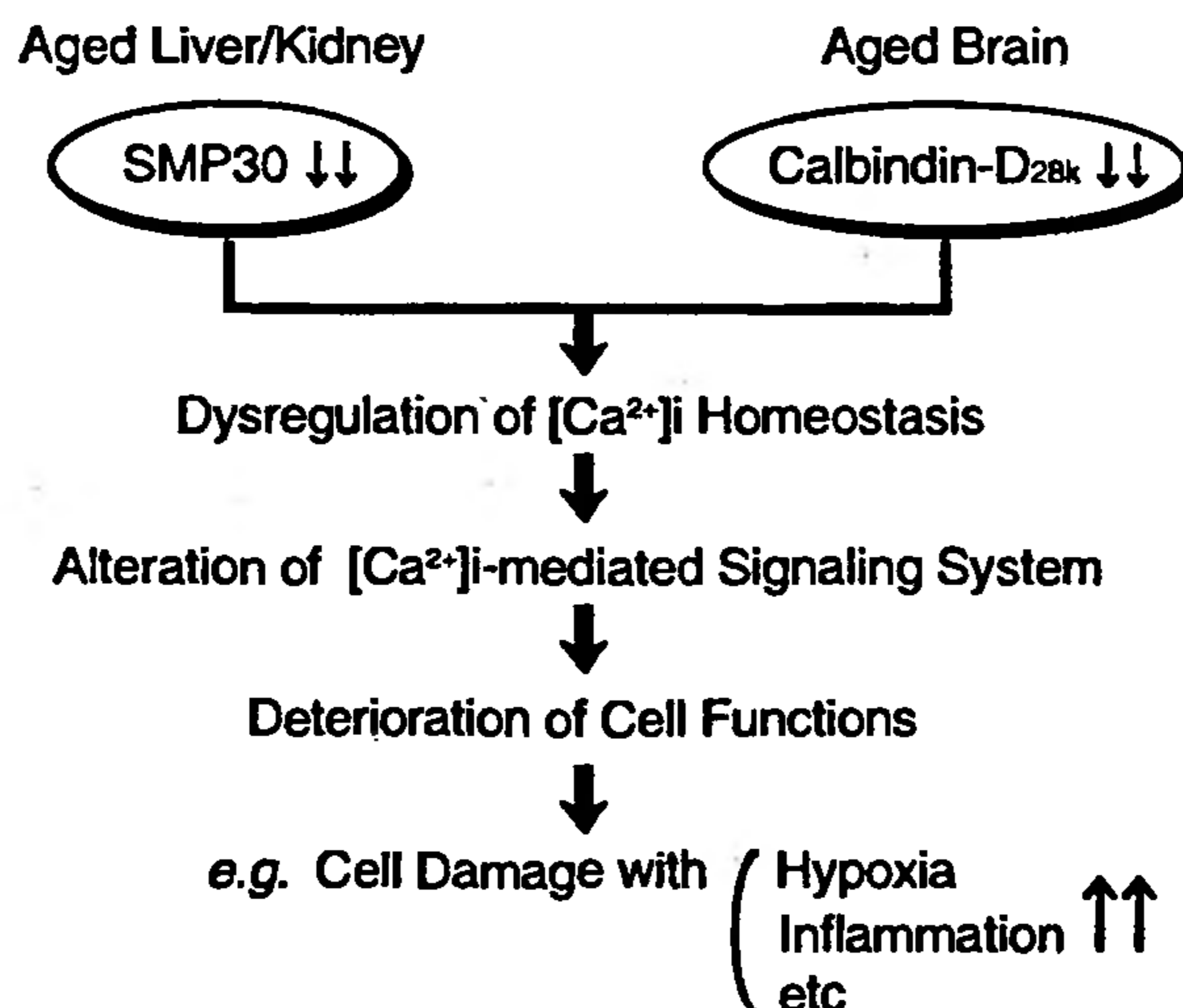


Figure 7. Hypothetical scheme for age-associated decrease in SMP30 or calbindin.

established stable HepG2 (a human hepatocellular carcinoma cell line) and LLC-PK1 (a swine renal tubular epithelial cell line) transfectants expressing high amounts of human SMP30. To assess its physiological roles in calcium homeostasis, we are investigating the effect of SMP30 on Ca²⁺ pump activity in plasma mem-

brane in these cells. Ca²⁺ efflux from both SMP30 transfectants was significantly enhanced in comparison to mock transfectants (data not shown), suggesting that SMP30 may modulate the activity of plasma-membrane Ca²⁺-pumping ATPases. In addition, SMP30 transfectants survived more efficiently than mock transfectants

when cell death was induced by Ca^{2+} influx with calcium ionophore (data not shown). These lines of evidence suggested that SMP30 may regulate the cytosolic Ca^{2+} levels by modulating the activity of the plasma-membrane Ca^{2+} -pumping ATPases in HepG2 and LLC-PK1 [Fujita *et al.*, in preparation].

Among calcium-binding proteins, we compared the expression of SMP30 with another tissue-specific calcium-binding protein, calbindin-D 28 kDa, preferentially expressed in brain and kidney. The amount of calbindin-D 28 kDa also decreased with aging in the cerebellum of rats and humans^{18,19}. Interestingly the extent of decrease in the aging process is quite comparable to SMP30. These two molecules shared similarities as follows; i) their expression is developmentally regulated in a tissue-specific manner, ii) overexpression of these molecules rescues the cells from apoptosis caused by calcium ionophore²⁰. These remarkable similarities between SMP30 and calbindin may contribute to the understanding of 'calcium homeostasis in aging'. Figure 7 represents our hypothetical scheme for age-associated decrease in SMP30 or calbindin. In aged tissues, expression of genes for SMP30 or calbindin is decreased. This age-dependent decrease may induce the dysregulation of calcium homeostasis, which may result in the alteration of signaling system in aged tissues. Consequently, such alterations may account for the age-associated deterioration of cellular functions in tissues and organ systems. Without proper calcium homeostasis, cells fail to respond properly to various harmful stimuli. Our results suggested that the age-associated decrease of SMP30 in liver and kidney may increase the tissue susceptibility for harmful stimuli in aged tissues.

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ACKNOWLEDGEMENTS. We thank Mr Y. Fujita and Ms H. Kaneko for their excellent editorial support. This study was supported by a Grant-in-Aid for Scientific Research (C) from Ministry of Education, Science, and Culture and CREST, Japan Science and Technology Corporation (JST).