[Frontiers in Bioscience 13, 2444-2451, January 1, 2008]

Multiple splicing results in at least two p203 proteins that are expressed in the liver and down-regulated during liver regeneration

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1. ABSTRACT

The interferon inducible p200 family proteins are expressed in a variety of cells and tissues. Intensive studies showed that they were involved in the regulation of cell proliferation and differentiation based on their ability to bind and modulate the activities of multiple transcription activators and inhibitors. Among p200 proteins, p203 has received the least attention and its function is unknown. In the present study, four multiple splicing isoforms of Ifi203, named temporally as Ifi203a/b-1, Ifi203a/b-2, Ifi203 a/b-3, and Ifi203a/b-4, were cloned from the interferon induced 10T1/2 cells. Anti-p203 antiserum was prepared and it could immunodetect a 46 kD and a 51 kD p203 proteins in a variety of cell lines. Unlike other p200 proteins, p203 was exclusively expressed in the liver in the adult C129/SvJ and C57BL/6 inbred strain mice. During the liver regeneration following a standard partial hepatectomy in C57BL/6 mice, the level of p203 decreased significantly in 6-24 h post-operation prior to the cell cycle progression through the G1/S transition.

2. INTRODUCTION

The interferon-inducible p200 (Ifi200) family of proteins is a group of homologous proteins that are induced by interferon treatment. The murine p200 family consists of at least four members: p202, p203, p204 and p205 (1-4). The homologous human p200 members are IFI16, MNDA, AIM2, and IFIX (5-9). Some new members or candidates of the family have been identified in recent studies (10). All the p200 proteins possess one or two 200-amino-acid segments at the C-terminus, which are grouped into two (a and b) or three (a, b and c) types based on their sequence similarities (3, 10). Among them, MNDA and AIM2 contain only one a segment, p203 contains only one b segment, while p202, p204 and IFI16 each consists of one a- and one b segment (3, 10). Most of the p200 proteins possess several conserved domains or motifs (11-17).

The p200 proteins are expressed and play important roles in a variety of cells and tissues (18). Intensive studies showed that the p200 proteins were

Table 1. Primer sequences

RT-PCR	Forward 5'-3'	Reverse 5'-3'
Splicing	GCGCAAGCTTGCATTCTGGTGTACTTTTTTGAGTC	GCCGCTCGAGCCAACAGTTAAAGGAGTTATAGAG
Antibody	CGGATCCCCGAGAAGGCAAAAGTTCAAGAG	AAGAATTCTTGTTTGAGGAACTGCTGGATGG
RNA level of Ifi203	AATGCAAGAAGATTATGATAGGAT	TGACTGAGTCTGGGTTGAGTGG
RNA level of G3PDH	ACCACAGTCCATGCCATCAC	TACAGCAACAGGGTGGTGGA

involved in the regulation of cell proliferation and differentiation based on their ability to bind and modulate the activities of multiple transcription factors and of some inhibitors of transcription factors (19-22). Over-expression of p200 family proteins will inhibit cell proliferation and block the cell cycle at the G1/S phase transition (9, 14, 23). The anti-proliferation function of p200 family proteins seemed to be implemented via their interaction with members of the p53/pRb/E2F pathway (10, 18, 24-27).

Among p200 family proteins, p203 (including p203a, p203b, and p203c subtypes), first described by Engel, D.A. *et al.* in 1988 (28), is less well known and the function of the protein is not clear (3, 4, 18). Gribaudo *et al.* reported that over expression of p203 has no significant effect on cell proliferation, while over expression of a p204a/203b fusion protein inhibits the cell proliferation and delays G0/G1 progression into S phase in NIH 3T3 and B6MEF cells. They suggested that the presence of both a and b segments of the p200 family was essential for their anti-proliferative activity (29, 30). However, this hypothesis was controversial, as two other members of p200 family, p205 and AIM2 which contain only a single a segment, possess anti-proliferative properties similar to p202 and p204 (14, 23). Deletional analysis of IFI16 showed that each of its 200-amino-acid segments is capable of transcriptional repressive activity (31). The reasons for this contradiction are not clear.

In this study, we started with identifying the multiple splicing variants of Ifi203 and characterizing its expression in various cell lines and organs as a basic investigation of p203 protein. In contrast to the previous report, we found that p203 is exclusively expressed in the liver. It is well known that the liver has enormous regenerative capacity. After partial hepatectomy, normally quiescent hepatocytes rapidly undergo one or two rounds of replication in a synchronized manner to restore the original liver mass, thus providing an excellent *in vivo* model for studying the mechanisms of cell proliferative regulation (31-34). Given that all other p200 members are implicated in cell proliferative regulation, high level of p203 in the adult mouse liver led us to examine the p203 expression during liver regeneration after partial hepatectomy. The results showed that p203 was dramatically down-regulated in the course of liver regeneration.

3. MATERIALS AND METHODS

3.1. Cells and interferon treatment

Balb/C 3T3, BLK, NIH 3T3, C2C12, AKR2B, and 10T1/2 cells were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum (GIBCO). Cells at around 60% confluency were treated with 1,000 IU/ml interferon alpha. Cytoplasmic and nuclear proteins were extracted for immunoblot 48 h after the treatment. Balb/C 3T3 and 10T1/2 cells were treated with interferon alpha and 16 h post-treatment total RNA was extracted and purified for RT-PCR using RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's protocol.

3.2. Tests of multiple splicing of Ifi203 in 10T1/2 cells

RT-PCR was performed with primers (Table 1) which were located in exon 1 and exon 7 of Ifi203 (GenBank Accession No AF022371). The PCR products were separated by 1% agarose gel. Each band was purified with Gel Extraction Kit (Qiagen), inserted into pcDNA3 to construct pcDNA3-p203 plasmids, and then sequenced. The sequence of the p203 cDNAs was analysed by comparing with mouse genomic sequence (GenBank Accession No AC008100). p203 mRNA sequences quoted from GenBank (Accession Nos L14559, AF022371, and BC008167) were used as circumstantial evidence.

3.3. Expression and purification of GST fusion protein

To generate anti-p203 antibody, a specific fraction of Ifi203 was amplified from total RNA of interferon alpha treated Balb/C 3T3 cells by RT-PCR (Table 1). The PCR product was digested with BamHI and EcoRI, and then inserted into pGEX-3X to construct a prokaryotic expression plasmid. After confirmed by sequencing, the plasmid was introduced into *E. coli* BL21-Dold (DE3) (Stratagene). A Glutathione S-transferase (GST) fusion protein which contains a 97-amino-acid segment of p203 (84-180 amino acids of the protein) was expressed and purified using glutathione-sepharose beads (Glutathione Sepharose High Performance, Amersham Biosciences) following the manufacture's instruction.

3.4. Preparation and purification of an anti-p203 antiserum

The purified fusion protein as antigen was entrusted to Stategic Biosolutions Company to generate rabbit anti-p203 antiserum. The anti-GST activity in the rabbit serum was depleted by using GST protein immobilized on glutathione-sepharose beads as previously described (19).

3.5. Subcellular localization of p203 and its expression in a variety of cell lines

About 1×10^6 cells treated or untreated with interferon alpha was collected. After washed 3 times with PBS, the cells were collected by centrifugation at 1,500 g for 5 min. The pellet was resuspended in 600 µl cold lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml pepstatin, 5 µg/ml leupeptin, and 10 µg/ml aprotinin. The cells were allowed to swell for 15 min on ice. The suspension was vigorously vortexed for 10 sec after 5µl

NP-40 was added. The suspension was centrifuged at 1,500 g for 30 sec at 4 ^{o}C. The resulting supernatant contained cytoplasmic proteins. The precipitate was briefly washed in lysis buffer and resuspended in equal volume of ice-cold nuclear extraction buffer containing 20 mM HEPES (pH 7.9), 0.8 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. After vigorous vortex at 4°C for 15 min, the extract was centrifuged at 12,000 g for 5 min at 4 ^{o}C. The supernatant was collected as nuclear protein fraction (35).

The BLK, NIH 3T3, C2C12, AKR2B, and 10T1/2 cells treated or untreated with interferon alpha was collected and the nuclear proteins were extracted as described above. All protein samples were quantified by DC Protein Assay Kit (Bio-Rad). 20 μg proteins were immunoblotted with anti-p203 antiserum as previously described (19). The signal was detected using enhanced chemiluminescence system (ECL, Amersham).

3.6. Transient transfection of p203 plasmids into NIH 3T3 cells

NIH 3T3 cells at around 60% confluency were transient transfected with pcDNA3-p203 plasmids. 48 h after transfection, cells were harvested by trypsinization, washed in PBS and pelleted by brief centrifuge. Cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 10 μg/ml aprotinin). 20 μg total proteins were immunoblotted with anti-p203 antiserum.

3.7. Assay of the tissue distribution of p203 in mice

Adult C129/SvJ and C57BL/6 mice were sacrificed. Samples of organs were collected and homogenized in an extraction buffer (20 mM Tris, pH7.5, 150 mM NaCl, 10 mM DTT, supplemented with 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice. The homogenates were clarified by centrifugation at 12,000 g for 20 min at 4°C. Supernatants were quantified for protein concentration and 20 μg of total protein per organ was immunoblotted, the signal was visualized using ECL system (19).

To compare the levels of Ifi203 mRNAs in several tissues, total RNA was extracted and purified from the samples of organs from C57BL/6 mice, and then RT-PCR was performed (Table 1), with primers which were located in the exon 2 and 7 of Ifi203 respectively. Mouse G3PDH (Glyceraldehyde-3-phosphate dehydrogenase gene, GenBank accession no XM 001474369) was amplified as an endogenous control (Table 1). The PCR product was separated by gel electrophoresis and confirmed by sequencing.

3.8. Partial hepatectomy

Standard 70% partial hepatectomy in mouse was performed as previously described (36). Briefly, C57BL/6 inbred mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). After opening the upper abdomen under the sterile condition, the left and medial lobes of the liver were individually ligated and excised resulting in removal of about 70% of the hepatic mass. The resected liver samples were immediately frozen in liquid nitrogen. At 3 h, 6 h, 24 h, 2 d, 4 d, 6 d, 8 d and 12 d post-operation, mice were sacrificed and the remnant liver was resected and frozen in liquid nitrogen. Three mice were studied per time point.

3.9. Immunoblotting assay of p203 from normal and regenerated livers

Total proteins from mouse liver samples were extracted as described above. To avoid the individual difference, protein extracted from the resected liver during the partial hepatectomy operation was regarded as an individual control sample while protein extracted from the remnant liver of the same mouse was regarded as a regenerated liver sample. Equal amount of protein samples were separated by sodium dodecylsulfate-10% polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was incubated with anti-p203 antiserum (diluted 1: 5000 in block solution) at 4°C overnight after blocked with block solution (10 % non-fat milk, 10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 % Tween-20) for 1 h, and then incubated with HRP-conjugated goat anti-mouse IgG (diluted 1:10,000) in block solution for another 1 h. The signal was detected by ECL.

4. RESULTS AND DISCUSSION

4.1. The multiple splicing isoforms of Ifi203 existed in 10T1/2 cells

Total RNA was extracted from 10T1/2 cells treated with interferon alpha. RT-PCR gave rise to four Ifi203 cDNA fragments. Each of the four cDNA fragments was inserted into pcDNA3 plasmid and transformed into the *E.coli* host strains, then several clones were picked and sequenced. By comparing the sequence of the four PCR products with the genomic sequence (GenBank Accession No AC008100) and p203 mRNA sequences (GenBank Accession Nos L14559, AF022371, and BC008167), four multiple splicing isoforms of Ifi203 were identified. Sequence analysis revealed that all four Ifi203 cDNA fragments consisted of both Ifi203a and Ifi203b. Ifi203b has a cytosine instead of a thymine in Ifi203a at the position of the 31st nucleotide in exon 3a. The changed codon specifies threonine in p203b instead of methionine in p203a at the 98th amino acid to the N-terminal (4). The four multiple splicing isoforms of Ifi203 were named temporally as Ifi203a/b-1, Ifi203a/b-2, Ifi203a/b-3, and Ifi203a/b-4 respectively (Figure 1). Interferon induced all 4 mRNAs of both the Ifi203a and the Ifi203b in 10T1/2 cells, though Ifi203a/b-3 mRNA was about ten times more than each of the other three rare isoforms (data not shown). In a previous report (28), five Ifi203-specific mRNAs were detected, but not sequenced, from cultured interferon-treated Ehrlich ascites tumor cells by Northern blotting. It remains to be seen whether these arose in consequence of multiple splicing, different polyadenylation sites (3) or both.

Among all variously spliced isoforms, only complete exons are present. No nucleotides are missing and

no additional nucleotides are present. In addition, the

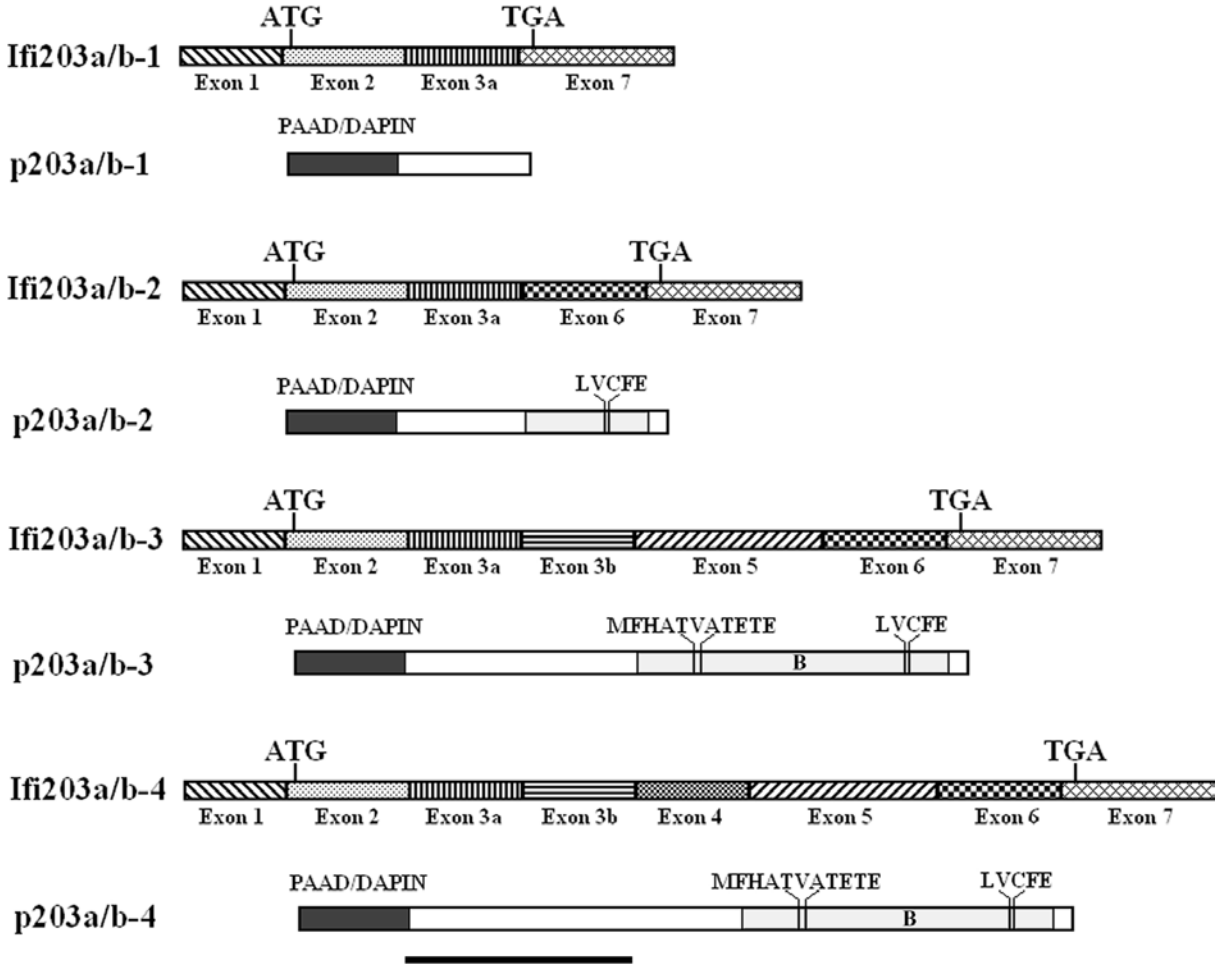

Figure 1. The multiple splicing isoforms of Ifi203. Four splicing isoforms of Ifi203 were cloned by RT-PCR from 10T1/2 cells treated with interferon, named temporally as Ifi203a/b-1, Ifi203a/b-2, Ifi203a/b-3, and Ifi203a/b-4. The shortest isoform Ifi203a/b-1 had an open reading frame encoding for a deduced protein with a total of 150 amino acids, named temporally as p203a/b-1. The primary structure of p203a/b-1 contained no typical domain for p200 protein other than a PAAD/DAPIN/Pyrin domain. Even the indicative b-type 200-amino-acid segment does not exist. Ifi203a/b-2 encoded for a deduced p203a/b-2 protein with 213 amino acids. p203a/b-2 contained a PAAD/DAPIN/Pyrin domain plus an incomplete fraction of the b-type 200-amino-acid segment in which a conserved LXCXE motif was present. The line below the schematic drawing of p203a/b4 indicated the position of the p203 segment (amino acid 84-180) against which the antiserum was prepared.

known full-length Ifi203 was composed of 8 rather than 7 exons reported previously (4). There are actually two exons, designated as exon 3a and exon 3b, between exon 2 and exon 4. The previously reported exon 3 is divided by an intron of 1,334 bp into exon 3a (122 bp) and exon 3b (168 bp). Among the four multiple splicing isoforms, Ifi203a/b-1 is the shortest, containing only exon 1, 2, 3a and 7. Since exons 5 and 6 encode the b type 200-amino-acid segment (4), p203a/b-1 does not have the b segment. Ifi203a/b-2 contains exon 1, 2, 3a, 6 and 7, and p203a/b-2 has an incomplete b segment. Ifi203a/b-3 is the most plentiful isoform, with exon 1, 2, 3a, 3b, 5, 6 and 7. Ifi203a/b-4 is the longest isoform, with exon 1, 2, 3a, 3b, 4, 5, 6 and 7. Both p203a/b-3 and p203a/b-4 with a complete b type 200-amino-acid segment were reported previously (4).

The newly identified Ifi203a/b-1 isoform and the predicted protein p203a/b-1 contain PAAD/DAPIN/Pyrin-only domain. The PAAD/DAPIN/Pyrin domain is reported to be associated with protein-protein interactions and be also sufficient for the DNA binding activity of IFI16 [26]. Since that p203a/b-1 protein lack any other known functional domains, it is possible that these PAAD/DAPIN/Pyrin-only proteins might act as dominant-negative regulators of p200 proteins via disrupting the protein-protein interactions and/or occupying the binding sites normally targeted by p200 proteins (10). p203a/b-2 contained a PAAD/DAPIN/Pyrin domain plus only a short fraction of the b-type 200-amino-acid segment and might function in a similar way as p203a/b-1. The multiple splicing was also seen with other p200 proteins and there existed splicing products encoding proteins lacking a 200-

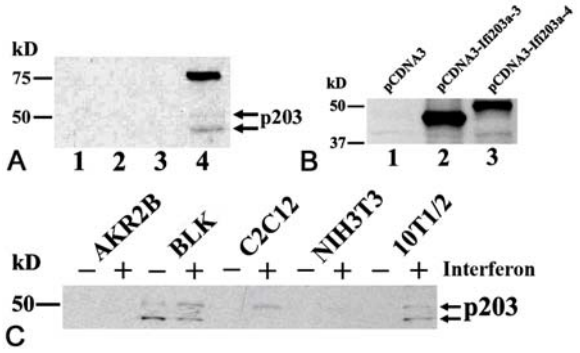

Figure 2. The expression of p203 in murine cell lines. (A) Cytoplasmic (lanes 1, 3) and nuclear (lanes 2, 4) proteins extracted from Balb/C 3T3 cells with (lanes 3, 4) or without interferon treated (lanes 1, 2) were immunoblotted with p203 antiserum. The 46 kD, 51 kD and 75 kD proteins were recognized by p203 antiserum. (B) After NIH 3T3 was transfected with plasmids pcDNA3, pcDNA3-Ifi203a-3 and pcDNA3-Ifi203a-4 respectively, the expressed p203 proteins (p203a-3 and p203a-4) could be detected by p203 antiserum and their electrophoretic mobility was similar to that of the interferon-inducible 46 kD and 51 kD proteins. As expected, these proteins were absent in the cells transfected with the pcDNA3 vector. (C) Extracts from several murine cell lines with (+) or without (-) interferon treatment were immuno-stained with p203 antiserum. The 46 kD and 51 kD p203 proteins are indicated by arrows.

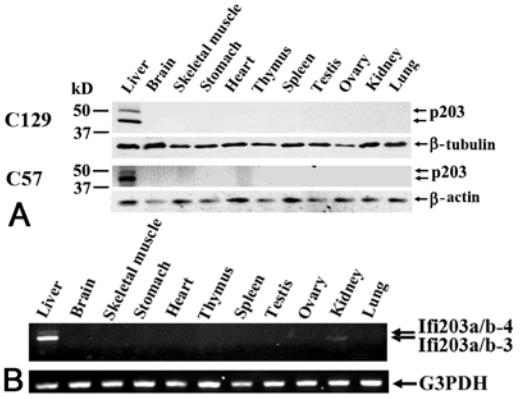

Figure 3. Tissue distribution of p203 in adult mice. (A) A multiple-tissue Western blotting assay. 20 μg protein samples from the indicated tissues of adult C129/SvJ and C57BL/6 mice were probed with anti-p203 antiserum. Arrows indicate the positions of 46 kD and 51 kD p203. The two strains of mice showed the same expression pattern of p203. (B) RT-PCR generated an Ifi203a/b-3 and an Ifi203a/b-4 fragment from the liver of the C57BL/6 mice, which corresponded to the 46 kD and 51 kD p203 proteins respectively. A weakly expressed Ifi203a/b-3 mRNA was detected in the kidney, although p203 protein was undetectable in this tissue. No significant Ifi203 mRNAs were detectable in other tissues.

amino-acid segment (9, 10, 37). The patterns reported previously are similar to those in the case of p203. In addition to PAAD/DAPIN/Pyrin domain, both p203a/b-3 and p203a/b-4 have a complete b segment with a conserved MFHATVATETE motif and a LVCFE motif. The conserved MF/LHATVAT/S motif was found in all known 200-amino-acid repeats of p200 proteins. This motif can mediate protein-protein interactions and was found to be sufficient for homo- and hetero-dimerization of the proteins *in vitro* (13, 14). The LXCXE motif or its similar forms (i.e., IXCXEE in IFI16 protein) were reported to allow p200 proteins to bind to Retinoblastoma protein (pRb) (15) and to be necessary for the inhibition of cell proliferation (16).

4.2. The subcellular localization of p203 and its expression in cell lines

In order to investigate the expression of p203 protein, an anti-p203 antiserum was prepared. Since p203 shows high homology with p202 and p204, the peptide used as antigen was carefully chosen. A stretch of 97 amino acids (84-180 amino acids of the protein) encoded by exon 3a and exon 3b which shared lower identity with p202 and p204 was qualified (Figure 1). The N-terminal sequence of p203 is avoided because it is highly similar to that of p204 (3, 4, 18).

The purified antiserum was used to study the subcellular localization of p203 in Balb/C 3T3 cells. Both cytoplasmic and nuclear proteins with or without interferon alpha treatment were extracted and detected by immunoblotting with purified anti-p203 antiserum. As shown in figure 2A, p203 was not detectable in the cytoplasmic and nuclear fractions of untreated control cells (Figure 2A, lanes 1 and 2). After interferon treatment, several interferon-inducible proteins were detected from nuclear fraction but not cytoplasmic fraction (Figure 2A, lanes 3 and 4). Among them, a 46 kD and a 51 kD proteins were in line with the size of the protein predicted from the cDNA of Ifi203a/b-3 and Ifi203a/b-4. These two proteins were subsequently confirmed as p203 proteins by transient transfection assay. After NIH 3T3, a cell line without endogenous p203, was transfected with plasmids pcDNA3-Ifi203a-3 and pcDNA3-Ifi203a-4, the expressed p203 proteins could be detected by the antiserum and their electrophoretic mobility were similar to that of the interferon -inducible 46 kD and 51 kD proteins immuno-stained in extracts from Balb/C 3T3 cells (Figure 2B). Thus the interferon-inducible 46 kD and 51 kD proteins were verified as p203 proteins. Furthermore, the anti-p203 antiserum did not recognize p202 and p204 (data not shown).

To explore the pattern of p203 expression in other cultured cell lines, nuclear proteins were extracted from interferon-treated and control cultures and assayed by Western Blotting with p203 antiserum. p203 proteins were absent from interferon-treated and control cultures of AKR2B and NIH3T3 cells (Figure 2C, lanes 1, 2, 7, 8). The 46 kD and 51 kD p203 proteins were constitutively expressed in control BLK cells and not further increased by interferon treatment (Figure 2C, lanes 3, 4). Both p203 proteins were induced by interferon treatment in 10T1/2 cells (Figure 2C, lanes 9, 10), showing similar pattern

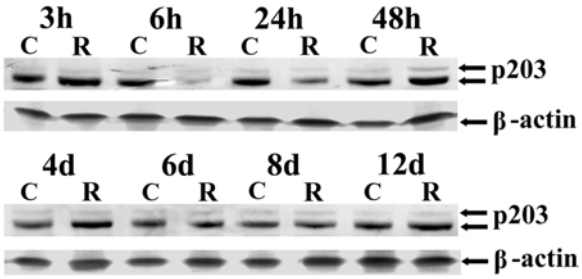

Figure 4. Imunodetection of p203 level during the liver regeneration after partial hepatectomy. Total protein from control (marked with C) and regenerated liver sample (marked with R) of the same mouse were extracted and immunoblotted with the purified anti-p203 antiserum. p203 (46 kD and 51 kD proteins) remained at the control level at 3 h post-operation, decreased significantly during 6 h to 24 h, and then restored to the control level from 2 d to 12 d.

of expression to Balb/C 3T3 (Figure 2A, lanes 3, 4). In C2C12 cells interferon induced only the 51kD band (Figure 2C, lanes 5, 6). These results reveal remarkable differences among the cell lines tested in their characteristics of p203 expression.

In addition, an unknown 75 kD protein was interferon inducible and strongly immuno-stained in the nuclear fraction of Balb/C 3T3 (Figure 2A) and other cells that expressed 46 kD and 51 kD p203 proteins. Interestingly, the level of the 75 kD protein was in concomitance with that of the 51 kD p203 protein. The 75 kD protein band was cut and processed for mass spectrometry, but was not characterized yet. This strongly immuno-stained 75 kD protein was also detected in previous reports (3, 29). Although the nature of it was not identified, the 75 kD protein is possibly an unknown p203 member.

4.3. Tissue distribution of p203 in C129/SvJ and C57BL/6 mice

A multiple-tissue Western blot with purified p203 antiserum was performed using C129/SvJ and C57BL/6 inbred strain adult mice. Results showed the C129/SvJ and C57BL/6 mice have the same expression pattern of p203, that is, both 46 kD and 51 kD p203 proteins are exclusively expressed in the liver (Figure 3A). The unknown 75 kD protein which was in concomitance with the 51 kD in the various cell lines was also detected in the liver. No significant immuno-stained band was detectable in other tissues, including brain, skeletal muscle, stomach, heart, thymus, spleen, testis, ovary, kidney, and lung. However these results were inconsistent with previous report (29). Gribaudo *et al.* identified the distribution of p203 with an anti-p203 N-terminal segment (7-196 amino acids) antiserum and reported that p203 was constitutively expressed in the thymus, bone marrow and spleen, but not liver in C57BL/6 mice (3, 29). The reasons for the discrepancy remained unclear.

To compare the levels of Ifi203 mRNAs in several tissues, a multiple-tissue RT-PCR was performed in the C57BL/6 mice with primers which were located in exon 2 and 7 of Ifi203. As shown in figure 3B, RT-PCR generated a significant band and a weak band from the liver. Sequence analysis indicated that they were the isoforms of Ifi203a/b-3 and -4, which corresponded to the 46 kD and 51 kD p203 proteins respectively. The two short isoforms Ifi203a/b-1 and -2, which were present in 10T1/2 cells, were not found in the liver yet. Furthermore, a weakly expressed Ifi203a/b-3 mRNA was detected in the kidney, although no p203 protein was immunodetected in the tissue. No significant Ifi203 mRNA was detectable in other tissues. The result of RT-PCR was in accordance with that of Western blotting assay.

4.4. The changes of p203 level during the liver regeneration after partial hepatectomy in mouse

The high level of p203 in the adult mouse liver led us to examine p203 expression pattern in the course of liver regeneration, an *in vivo* model for studying the cell proliferation (34). A standard 70% partial hepatectomy was performed in C57BL/6 inbred mice. About 8 d after the operation, the liver restored its mass to normal level (data not shown). Total proteins from control and regenerated liver sample of the same mouse were extracted and detected with the anti-p203 antiserum. The results showed that p203 (the 46 kD and 51 kD proteins) remained at the control level at 3 h post-operation. The p203, especially the 46 kD protein, decreased dramatically between 6 h and 24 h, and then restored to the control level from 2 d to 12 d (Figure 4).

After partial hepatectomy, highly differentiated and largely quiescent hepatic cells reenter the cell cycle in a synchronized manner to replace the lost functional mass (32, 33). The large number of genes involved in liver regeneration may be categorized into three networks: cytokine, growth factor and metabolic. Hepatocytes primed by the cytokines respond to growth factors and enter the cell cycle. The sequential activation of cytokine and growth factor receptors stimulates the signaling pathways implicated in cell proliferation. The increased metabolic body demands placed on hepatocytes presumably function to calibrate the regenerative response. In the immediate early phase within minutes to approximate 4 hours after partial hepatectomy in rodents, immediate early genes are activated (32, 33). Cytokines such as tumor necrosis factor and interleukin 6 have important roles in liver regeneration, whereas interferon gamma has been identified as an inhibitor (38, 39). The following delayed early gene response renders the hepatocytes responsive to growth factors that drive the quiescent cells into the G1 phase of the cell cycle. Members of the p53/pRb/E2F pathway regulate cell cycle progression through the G1/S transition. Hyperphosphorylated form of pRb appeared at 36-48 h after partial hepatectomy and a DNA synthesis peak followed (32, 33, 40). p202a and p204 could suppress cell proliferation via interaction with pRb mediated by the LXCXE motif in the 200-amino-acid segments (15, 16). Similarly, p203 contains the pRb protein binding motif LXCXE. The possibility that p203 interacts with pRb and suppresses cell proliferation remains to be determined.

After partial hepatectomy, p203 was markedly down-regulated in 6-24 h post-operation via an unknown pathway. Previous report indicated that the levels of other interferon-inducible, growth-inhibitory proteins changed similarly to that of p203 during liver regeneration after partial hepatectomy in rodents (41). It is conceivable that down-regulations of p203 and other interferon-inducible, growth-inhibitory proteins disrupted the suppression of cell cycle. As a consequence, the hepatocytes passed G1/S transition. Moreover, it was also possible that interferon gamma inhibited liver regeneration via inducing its target molecules, including p203.

After one or two round of cell division, the hepatocytes switch to cell growth and replace the liver mass after partial hepatectomy (32, 33). Consistent with this, p203 restored to the control level at 48 h after partial hepatectomy when the cells had passed into the S phase (32, 33, 40). Afterwards p203 kept at the basal level to exert its antiproliferative activity, thus blocked the cell division and prompted the cell growth.

Unlike other high differentiated cell type, hepatocytes possess enormous regenerative capacities to respond to the continuous risk of various injuries, such as chemical, traumatic, or infectious injuries (34, 42). The consistently present p203 in mouse liver might function to suppress the proliferation of a majority of hepatic cells and keep them quiescent under normal conditions. Thus p203 might play an important role in the liver regeneration after partial hepatectomy as well as in maintenance of the liver mass under normal circumstances.

5. ACKNOWLEDGEMENT

Drs Yanjun Zhang, and Qingyun Tian equally contributed to this work**.** This work was supported by the National Nature Science Foundation of China (30470653), Shandong Nature Science Foundation (Y2003D01) and a National Institutes of Health research grant (AI-12320) from the National Institute for Allergy and Infectious Diseases.

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6. REFERENCES

1. Samanta H., J. P. Dougherty, M. E. Brawner, H. Schmidt & P. Lengyel: Interferon action: cloning of cDNA segments complementary to messenger RNAs induced by interferons. *UCLA Symposia on Molecular and Cellular Biology* 25, 59-72 (1982)

2. Lengyel P., D. Choubey, S. J. Li & B. Datta: The interferon-activatable gene 200 cluster: from structure toward function. *Se*min *Virol* 6, 203-213 (1995)

3. Gribaudo G., S. Ravaglia, L. Guandalini, L. Rira, M. Gariglio & S. Landolfo: Molecular cloning and expression of an interferon-inducible protein encoded by gene 203 from the gene 200 cluster. *Eur J Biochem* 249, 258-264 (1997)

4. Deschamps S., J. Meyer, G. Chatterjee, H. Wang, P. Lengyel & B. A. Roe: The mouse Ifi200 gene cluster: genomic sequence, analysis, and comparison with the human HIN-200 gene cluster. *Genomics* 43, 34-46 (2003)

5. Briggs J. A., G. R. Burrus, B. D. Stickney & R. C. Briggs: Cloning and expression of the human myeloid cell nuclear differentiation antigen: regulation by interferon alpha. *J Cell Biochem* 49, 82-92 (1992)

6. Trapani J. A., K. A. Browne, M. J. Dawson, R. G. Ramsay, R. L. Eddy, T. B. Show, P. C. White & B. Dupont: A novel gene constitutively expressed in human lymphoid cells is inducible with interferon-gamma in myeloid cells. *Immunogenetics* 36, 369-376 (1992)

7. Ray M. E., Y. A. Su, P. S. Meltzer & J. M. Trent: Isolation and characterization of genes associated with chromosome-6 mediated tumor suppression in human malignant melanoma. *Oncogene* 12, 2527-2533 (1996)

8. DeYoung K. L., M. E. Ray, Y. A. Su, S. L. Anzick, R. W. Johnstone, J. A. Trapani, P. S. Meltzer & J. M. Trent: Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. *Oncogene* 15, 453-457 (1997)

9. Ding Y., L. Wang, L. K. Su, J. A. Frey, R. Shao, K. K. Hunt & D. H. Yan: Antitumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer. *Oncogene* 23, 4556-4566 (2004)

10. Ludlow L. E., R. W. Johnstone & C. J. Clarke: The HIN-200 family: more than interferon-inducible genes. *Exp Cell Res* 308, 1-17 (2005)

11. Staub E., E. Dahl & A. Rosenthal: The DAPIN family: a novel domain links apoptotic and interferon response proteins. *Trends Biochem Sci* 26, 83-85 (2001)

12. Dawson M. J. & J. A. Trapani: The interferon-inducible autoantigen, IFI16: localization to the nucleolus and identification of a DNA-binding domain. *Biochem Biophys Res Commu* 214, 152-162 (1995)

13. Koul D., N. U. Obeyesekere, J. U. Gutterman, G. B. Mills & D. Choubey: p202 self-associates through a sequence conserved among the members of the 200-family proteins. *FEBS Lett* 438, 21-24 (1998)

14. Choubey D., S. Walter, Y. Geng & H. Xin: Cytoplasmic localization of the interferon-inducible protein that is encoded by the AIM2 (absent in melanoma) gene from the 200-gene family. *FEBS Lett* 474, 38-42 (2000)

15. Choubey D. & P. Lengyel: Binding of an interferon-inducible protein (p202) to the retinoblastoma protein. *J Biol Chem* 270, 6134-6140 (1995)

16. Hertel L., S. Rolle, M. D. Andrea, B. Azzimonti, R. Osello, G. Gribaudo, M. Gariglio & S. Landolfo: The retinoblastoma protein is an essential mediator that links the interferon-inducible 204 gene to cell-cycle regulation. *Oncogene* 19, 3598-3608 (2000)

17. Briggs L. J., R. W. Johnstone, R. M. Elliot, C. Y. Xiao, M. Dawson, J. A. Trapani & D. A. Jans: Novel properties of the protein kinase CK2-site-regulated nuclear-localization sequence of the interferon-induced nuclear factor IFI16. *Biochem J* 353, 69-77 (2001)

18. Asefa B., K. D. Klarmann, N. G. Copeland, D. J. Gilbert, N. A. Jenkins & J. R. Keller: The interferon-inducible p200 family of proteins: a perspective on their roles in cell cycle regulation and differentiation. *Blood Cells, Molecules, and Diseases* 32, 155-167 (2004)

19. Liu C. J., H. Wang, Z. Zhao, S. Yu, Y. B. Lu, J. Meyer, G. Chatterjee, S. Deschamps, B. A. Roe & P. Lengyel: MyoD-dependent induction during myoblast differentiation

of p204, a protein also inducible by interferon. *Mol Cell Biol* 20, 7024-7036 (2000)
20. Wang H., B. Ding, C. J. Liu, X. Y. Ma, S. Deschamps, B. A. Roe & P. Lengyel: The increase in levels of interferon-inducible proteins p202a and p202b and RNA-dependent protein kinase (PKR) during myoblast differentiation is due to transactivation by MyoD: their tissue distribution in uninfected mice does not depend on interferons. *J Interferon Cytokine Res* 22, 729-737 (2002)
21. Liu C. J., B. Ding, H. Wang & P. Lengyel: The MyoD-inducible p204 protein overcomes the inhibition of myoblast differentiation by Id proteins. *Mol Cell Biol* 22, 2893- 2905 (2002)
22. Liu C. J., E. Chang, J. Yu, C. S. Carlson, L. Prazak, X. P. Yu, B. Ding, P. Lengyel & P. E. Di Cesare: The interferon-inducible p204 protein acts as a transcriptional coactivator of Cbfa1 and enhances osteoblast differentiation. *J Biol Chem* 280, 2788-2796 (2005)
23. Dermott J. M., J. M. Gooya, B. Asefa, S. R. Weiler, M. Smith & J. R. Keller: Inhibition of growth by p205: a nuclear protein and putative tumor suppressor expressed during myeloid cell differentiation. *Stem Cells* 22, 832-848 (2004)
24. Johnstone R. W., W. Wei, A. Greenway & J. A. Trapani: Functional interaction between p53 and the interferon-inducible nucleoprotein IFI16. *Oncogene* 19, 6033-6042 (2000)
25. Xin H., R. Pramanik & D. Choubey: Retinoblastoma (Rb) protein upregulates expression of the Ifi202 gene encoding an interferon inducible negative regulator of cell growth. *Oncogene* 22, 4775-4785 (2003)
26. Hertel L., S. Rolle, M. De Andrea, B. Azzimonti, R. Osello, G. Gribaudo, M. Gariglio & S. Landolfo: The retinoblastoma protein is an essential mediator that links the interferon-inducible 204 gene to cell cycle regulation. *Oncogene* 19, 3598-3608 (2000)
27. Choubey D., S. J. Li, B. Datta, J. U. Gutterman & P. Lengyel: Inhibition of E2F-mediated transcription by p202. *EMBO J* 15, 5668-5678 (1996)
28. Engel D. A., J. Snoddy, E. Toniato & P. Lengyel: Interferons as gene activators: close linkage of two interferon-activatable murine genes. *Virology* 166, 24-29 (1988)
29. Gribaudo G., L. Riera, L. Hertel & S. Landolfo: *In vitro* and *in vivo* expression analysis of the interferon-inducible 203 gene. *J Interferon Cytokine Res* 19, 129-136 (1999)
30. Gribaudo G., L. Riera, M. De Andrea & S. Landolfo: The antiproliferative activity of the murine interferon-inducible Ifi 200 proteins depends on the presence of two 200 amino acid domains. *FEBS Lett* 456, 31-36 (1999)
31. Johnstone R. W., J. A. Kerry & J. A. Trapani: The human interferon inducible protein, IFI16, is a repressor of transcription. *J Biol Chem* 273, 17172-17177 (1998)
32. Fausto N.: Liver regeneration. *J Hepatology* 32, 19-31 (2000)
33. Fausto N., J. S. Campbell & K. J. Riehle: Liver regeneration. *Hepatology* 43, S45-S53 (2006)
34. Fernández M. A., C. Albor, M. Ingelmo-Torres, S. J. Nixon, C. Ferguson, T. Kurzchalia, F. Tebar, C. Enrich, R. G. Parton & A. Pol: Caveolin-1 is essential for liver regeneration. *Science* 15, 1628-1632 (2006)
35. Schreiber E., P. Matthias, M. M. Muller & W. Schaffner: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 17, 6419 (1989)
36. Sekine S., P. J. Gutiérrez, B. Y. Lan, S. Feng & M. Hebrok: Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* 45, 361-368 (2007)
37. Staub E., E. Dahl & A. Rosenthal: The DAPIN family: a novel domain links apoptotic and interferon response proteins. *Trends Biochem Sci* 26, 83-85 (2001)
38. Sato Y., K. Tsukada, Y. Matsumoto & T. Abo: Interferon-gamma inhibits liver regeneration by stimulating major histocompatibility complex class II antigen expression by regenerating liver. *Hepatology* 18, 340-346 (1993)
39. Brooling J. T., J. S. Campbell, C. Mitchell, G. C. Yeoh & N. Fausto: Differential regulation of rodent hepatocyte and oval cell proliferation by interferon gamma. *Hepatology* 41, 906-915 (2005)
40. Albrecht J. H., R. Y. Poon, C. L. Ahonen, B. M. Rieland, C. Deng & G. S. Crary: Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene* 16, 2141-2150 (1998)
41. Smekens-Etienne M., J. Goldstein, H. A. Ooms & J. E. Dumont: Variation of (2'-5') oligo (adenylate) synthetase activity during rat liver regeneration. *Eur J Biochem* 130, 269-273 (1983)
42. Fausto N.: Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 39, 1477-1487 (2004)

Key Words: p203, Multiple Splicing, Anti-Proliferation, Liver Regeneration

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