Cloning and Characterisation of *Ifi206*: A New Murine HIN-200 Family Member

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Abstract   HIN-200 proteins are interferon-inducible proteins capable of regulating cell growth, senescence, differentiation and death. Using a combination of in silico analysis of NCBI EST databases and screening of murine C57BL/6 cDNA libraries we isolated novel murine HIN-200 cDNAs designated *Ifi206*$_S$ and *Ifi206*$_L$ encoding two putative mRNA splice variants. The p206$_S$ and p206$_L$ protein isoforms have a modular domain structure consisting of an N-terminal PAAD/DAPIN/Pyrin domain, a region rich in serine, threonine and proline residues and a C-terminal 200 B domain characteristic of other HIN-200 proteins. *Ifi206* mRNA was detected only in the spleen and lung of BALB/c and C57BL/6 mice and expression was up-regulated by both types I and II IFN subtypes. p206 protein was predominantly expressed in the cytoplasm and addition of LMB, a CRM1 dependent nuclear export inhibitor, caused p206 to accumulate in the nucleus. Unlike other human and mouse HIN-200 proteins that contain only a single 200 amino acid domain, overexpression of p206 impaired the clonogenic growth of tumour cell lines. Thus, p206 represents the newest HIN-200 family member discovered. It has distinct and restricted pattern of expression however maintains many of the hallmarks of HIN-200 proteins including the presence of a characteristic 200 X domain, induction by interferon and an ability to suppress tumour cell growth. J. Cell. Biochem. 103: 1270–1282, 2008.   © 2007 Wiley-Liss, Inc.

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Interferons (IFNs) are potent anti-viral, anti-tumour and immunomodulatory agents capable of regulating cell cycle control, differentiation and cell survival [Stark et al., 1998]. Their cellular functions are mediated by IFN-stimulated genes, the protein products of which induce biological responses. The HIN-200 (Hemopoietic, IFN-inducible, Nuclear proteins with a 200 X domain) proteins encoded by a cluster of genes located on chromosome 1q21–23 in the human and mouse genome regulate a range of biological responses consistent with an IFN response [Johnstone and Trapani, 1999; Ludlow et al., 2005]. HIN-200 proteins have a modular domain structure with one or two copies of a characteristic C-terminal 200 X domain (designated A, B and C) [Ludlow et al., 2005] which mediates protein–protein interactions. For example binding of IFI 16 to p53 [Johnstone et al., 2000; Aglipay et al., 2003], p202 to p53BP1 [Datta et al., 1996], c-Myc [Wang et al., 2000] and NF-κB [Ma et al., 2003] and p204 with UBF1 [Liu et al., 1999], Rb [Hertel et al., 2000] and Id2 [Liu et al., 2002] all require the 200 X domains. These protein interactions involve several conserved sequences such as the MFHATVAT motif which mediates interactions between p202 and p53BP1 [Datta et al., 1996] and homo-dimerisation of p202 [Koul et al., 1998]. All HIN-200 proteins, except p202, have an N-terminal α-helical PAAD/DAPIN/Pyrin domain that

mediates interactions between various proteins involved in apoptosis, cancer, inflammation, autoimmune disease and immune responses [Pawlowski et al., 2001; Staub et al., 2001]. Indeed, the PAAD/DAPIN/Pyrin domain is crucial for myeloid cell nuclear differentiation antigen (MNDA) self-association [Xie et al., 1997] and homo- and hetero-dimerisation of IFI 16 [Johnstone et al., 1998].

All HIN-200 proteins have been detected in the nuclei of cultured cells by virtue of an N-terminal nuclear localisation signal (NLS) ubiquitous to the family, with the exception of p202. Moreover, IFI 16 and p204 were prominent in the nucleolus [Choubey and Lengyel, 1992; Dawson and Trapani, 1995]. Although lacking an NLS, p202 is found in the nucleus, as well as the cytoplasm of IFN treated cells [Choubey and Lengyel, 1993]. The only other HIN-200 protein found in the cytoplasm was human Absent in Melanoma 2 (AIM2) which, when overexpressed, was detected in the cytoplasm of murine cells [Choubey et al., 2000]; however, endogenous AIM2 was found in the nucleus of human cell lines [Cresswell et al., 2005].

HIN-200 proteins are expressed in specific hemopoietic cell lineages and at different stages of lineage commitment. In the myeloid compartment, IFI 16 is more highly expressed in hemopoietic progenitor cells than mature cells [Dawson et al., 1998; Wagner et al., 2004], p204 is expressed from the promyelocytic stage onward [Gariglio et al., 1998] and *Ifi205* is induced during granulocyte and monocyte differentiation [Weiler et al., 1999]. In the lymphoid compartment Notch1 transcriptionally regulates *Ifi204* and *Ifi205* during single positive T cell maturation [Deftos et al., 2000]. p202 and p204 can both regulate skeletal muscle precursor myoblast differentiation to myotubes by regulating the transcription factors MyoD and Id [Datta et al., 1998; Liu et al., 2000; Liu et al., 2002]. Together these data suggest a role for HIN-200 proteins in differentiation.

When overexpressed, HIN-200 proteins can inhibit tumour cell proliferation by regulating progression of the cell cycle through the G1/S phase transition [Landolfo et al., 1998; Johnstone and Trapani, 1999; Asefa et al., 2004; Ludlow et al., 2005]. This biological effect appears to be mediated through functional interactions of HIN-200 proteins with various oncoproteins and tumour suppressors including E2F1 [Choubey et al., 1996; Yan et al., 2003],

E2F4 [Choubey and Gutterman, 1997], c-Myc [Wang et al., 2000], NF-κB [Ma et al., 2003] and c-Jun [Min et al., 1996]. Most importantly, p202 and p204 bind hypo-phosphorylated pRb via consensus LxCxE motifs located within the 200 X domains [Choubey and Lengyel, 1995; Lembo et al., 1998; Hertel et al., 2000] whereas IFI 16 can bind to and enhance the transcriptional activity of p53 [Johnstone et al., 2000; Fujiuchi et al., 2004; Xin et al., 2004].

To date, five murine (*Ifi202a*, *If202b*, *Ifi203*, *Ifi204*, *Ifi205*) and four human (*IFI 16*, *MNDA*, *AIM2*, *IFIX*) HIN-200 genes have been identified. We hypothesised that additional HIN-200 genes remained to be identified and their cloning and characterisation might provide important insight into the molecular, biochemical and biological characteristics of this family of proteins. Herein, we identified and cloned a novel cDNA encoding a new member of the HIN-200 family designated *Ifi206*. The p206$_S$ and p206$_L$ protein products of *Ifi206* mRNA splice isoforms contained the highly conserved N-terminal PAAD/DAPIN/Pyrin domain, a unique Serine/Threonine/Proline (S/T/P)-rich region and a C-terminal 200 B domain. The *Ifi206* mRNA transcript was most strongly expressed in spleen and lung of BALB/c and C57BL/6 mice and *Ifi206* mRNA was induced by types I and II IFNs. Constitutively expressed p206 was located predominantly in the cytoplasm, however, unlike p202 which translocated from the cytoplasm to the nucleus following IFN treatment, IFN-induced p206 remained localised predominantly to the cytoplasm. To assess the effect of p206 on cell proliferation and/or survival, we performed clonogenic assays and found that like p202 both p206$_S$ and p206$_L$ suppressed tumour cell growth.

MATERIALS AND METHODS

cDNA Cloning

DNA sequence encoding the N-terminal 88 amino acids of IFI 16 was used to screen the murine National Centre for Biotechnology Information (NCBI) EST sequence database. A 539 bp EST clone (EST accession number: mr27d07.r1) was used to screen a C57BL/6 spleen lambda gt11 cDNA library (Stratagene, LaJolla, CA) and clone 6.2.1 of 456 bp encoding a putative protein of 97 amino acids displaying 50% amino acid identity and 65.6% similarity to IFI 16 was identified. Radiolabelled clone 6.2.1

was used to screen a C57BL/6 spleen lambda ZAP II cDNA library (Stratagene) and clone 19 (2,531 bp) and clone 22 (2,105 bp) that overlapped with the 3′ end of clone 6.2.1 were identified. Clone 19 and 22 were identical except for an insertion of 426 bp in clone 19. Splice overlap PCR was used to produce the two full length *Ifi206* cDNA species designated *Ifi206$_S$* and *Ifi206$_L$* that were subcloned into the pCR2.1 plasmid (Invitrogen, Groningen, Netherlands) and sequenced. For protein over-expression studies cDNA encoding full length p206$_S$ and p206$_L$ were amplified by PCR incorporating Bam HI restriction endonuclease sites. PCR products were subcloned in-frame into the Bam HI site of the pKH3-HA vector (kindly provided by Donna Dorow, Peter MacCallum Cancer Centre, East Melbourne, Australia) incorporating an N-terminal triple influenza virus HA epitope tag and were sequence verified. For cell proliferation experiments, HA epitope tagged *Ifi206$_S$* and *Ifi202a* cDNA was subcloned into the pcDNA3.1 plasmid (Invitrogen).

Cell Culture and Animals

Murine embryonic fibroblast cell lines; NIH 3T3 and BALB/c 3T3, the C57BL/6-derived prostate carcinoma cell line; RM-1 and 293 embryonic kidney carcinoma cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (CSL, Parkville, Australia), 2 mM glutamine (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin and 100 μg/ml streptomycin (GibcoBRL, Life Technologies, Grand Island, NY). For IFN-induction experiments recombinant mouse IFN-α1 (kindly provided by Catherine Owczarek, Monash Institute of Reproduction and Development, Monash Medical Centre, Australia) and IFN-β (kindly provided by Stephen Ralph, Griffith University, Australia) and IFN-γ (Roche, Manniheim, Germany) were added to cell lines at a final concentration of 500 U/ml for the time points specified. Leptomycin B (LMB) used to study the nuclear export of p206 was added at 10 ng/ml for 5 h prior to cell fixation. Inbred C57BL/6 and BALB/c mice were purchased from The Walter and Eliza Hall Institute of Medical Research (WEHI, Melbourne, Australia). The New Zealand White rabbit used for anti-sera production was housed at The Austin Research Institute (ARI, Heidelberg, Australia).

Northern Blotting

Total cellular RNA was prepared from cell lines and tissues using TRIZOL (GibcoBRL, Life Technologies) which was followed by poly A+ RNA extraction using Dynabeads Oligo (dT)$_{25}$ (DYNAL Biotech, Oslo, Norway) following manufacturers instructions. RNA samples were electrophoresed on 1% denaturing agarose gels and RNA was transferred onto Hybond^{TM}-N+ nucleic acid transfer membranes (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) in 10× SSC. These Northern blots and the CLONTECH multiple tissue Northern blot containing 2 μg of poly A+ RNA per lane derived from eight different BALB/c mouse tissues (CLONTECH, Palo Alto, CA) were probed with ^{32}P-αATP radiolabelled cDNA overnight at 42°C. The temperature and SSC concentration were altered to regulate the washing stringency. Blots were stripped prior to reprobing by washing twice for 10 min in boiling 0.1% SDS and exposed to film overnight to ensure stripping efficiency.

RT-PCR

RNA (1 μg) from cell lines and tissues was reverse transcribed using Superscript II (GibcoBRL, Life Technologies) according to supplier's instructions. The *Ifi206* oligonucleotides generated 1,096 and 1,521 bp dual RT-PCR products that corresponded to the *Ifi206$_S$* and *Ifi206$_L$* splice variants, respectively. Annealing took place at 60°C (β2-microglobulin) and 48°C (*Ifi206*) for 30 cycles using Taq DNA polymerase (Promega, Madison, WI). The oligonucleotides were specific as they were unable to amplify products from other mouse HIN-200 cDNA templates. Oligonucleotide pairs used were as follows: β-2-microglobulin (β2m) forward 5′-GTCGCTTCAGTCGTCAGCATGG-3′, reverse 5′-CATTGCTATTTCTTTCTGCGTGCAT-3′ generating a 480 bp product and *Ifi206* forward 5′-TCCAGCAGCACTCAGACCA-3′, reverse 5′-ATGTCATATGTGAATGGCTC-3′ (position 646–1,742 bp in *Ifi206$_S$* and position 646–2,168 bp in *Ifi206$_L$* spanning cDNA encoding the S/T/P-rich region).

Production of p206 Rabbit Polyclonal Anti-Serum

A PCR fragment encoding the N-terminal region of p206 (1–951 bp) was subcloned in frame into the Not I site of the pGex4T2 GST fusion plasmid (Amersham Pharmacia Biotech).

The resulting fusion protein encoding GST and residues 1-317 of p206 was expressed in *E. coli* and purified using glutathionine sepharose. A New Zealand White rabbit was immunised primarily with 200 μg of recombinant protein in complete Freunds adjuvant (BD Biosciences, San Jose, CA) followed by 2×200 μg boosts in incomplete Freunds adjuvant.

Transfection of Eukaryotic Cells

Constructs were transiently transfected using Lipofectamine reagent (Invitrogen) and Opti-MEM I reduced serum media (Gibco, Grand Island, NY) or metafectine (Biontex Laboratories, Munich, Germany) according to manufacturer's instructions.

Western Blotting

Cells were lysed in RIPA buffer [150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% SDS and 50 mM Tris pH 8.0] supplemented with complete protease inhibitor (Roche) and the supernatant fraction was collected by centrifugation at 4°C for 15 min. Proteins were separated under reducing conditions on a 10% SDS polyacrylamide gel and electro-blottted onto Immobilon membrane (Millipore Corp., Bedford, MA). Blots were probed with the anti-HA (Cell Signalling Technology, Beverly, MA) and α-tubulin (Sigma Chemical Co., St. Louis, MO) monoclonal antibodies followed by the anti-mouse HRP conjugated secondary antibody (Dako, Carpinteria, CA) and visualised by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Immunofluorescence Microscopy

Transfected or IFN-induced cell lines were grown on coverslips, fixed (3.7% paraformaldehyde, 0.2% Triton X-100 in PBS), blocked in 5% skim milk for 30 min and incubated with the anti-HA monoclonal antibody (Cell Signalling Technology), rabbit 206 anti-sera, IFI-202 (S-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit 204 anti-sera (kindly provided by Divaker Choubey, Loyola University, Chicago) or control rabbit preimmune sera, goat sera and anti-HA monoclonal antibody isotype control. Bound antibody was detected with alexa (488)-conjugated anti-rabbit or anti-goat immunoglobulin (Molecular probes, Eugene, OR). The nuclei were counter-stained with propidium iodide (PI), mounted and examined for fluorescence using immunofluorescence microscopy.

Colony Assays

Two hundred ninety-three cells (2×10^5) were seeded into six-well plates 24 h prior to transfection as described above. Twenty-four hour post-transfection cells were plated at 5×10^3 cells per 10 cm plate and selected by addition of geneticin® (GibcoBRL, Life Technologies, 800 μg/ml). At this time, the transfection efficiency of pcDNA3.1-HA206$_S$, pcDNA3.1-HA206$_L$ and pcDNA3.1-HA202a was determined by immunofluorescence microscopy. The media containing 800 μg/ml geneticin® was replenished every second day and 9 days later colonies were stained with crystal violet and counted.

RESULTS

Cloning and Genomic Organisation of Ifi206

To identify a novel murine HIN-200 family member, EST NCBI databases were screened using sequence (264 bp) encoding amino acids 1-88 of IFI 16 encoding the N-terminal PAAD/DAPIN/Pyrin domain. A 539 bp EST clone (EST accession number: mr27d07.r1) isolated from C57BL/6 spleen was identified and found to encode a polypeptide 74% identical to IFI 16. Mr27d07.r1 was used to screen a C57BL/6 spleen cDNA library. A single clone (6.2.1) of 456 bp in length that overlapped with mr27d07.r1 and contained an ORF encoding 97 amino acids was identified. The 6.2.1 cDNA clone was then used to screen a second C57BL/6 spleen cDNA library, which resulted in the isolation of clones 19 (2,531 bp) and 22 (2,105 bp). The three partially overlapping cDNA clones; 6.2.1 and clone 19, and 6.2.1 and clone 22 encoded two putative mRNA splice variants that were designated *Ifi206$_L$* and *Ifi206$_S$*, respectively to follow the *Ifi200* nomenclature given to other murine HIN-200 genes. Splice overlap PCR was used to generate the full-length cDNA for both isoforms.

We next defined the position of the *Ifi206* gene in the HIN-200 locus and determined the exon/intron organisation of the gene using Mapview software (www.ncbi.nlm.gov/mapview) and an assembly of the murine genome (Build 33.1). The *Ifi206* gene was located at the distal end of the HIN-200 locus (spanning 6,087 kb) on mouse chromosome 1q21−23 between *Ifi204* and Serum amyloid P component (*Sap*) (Fig. 1A). *Ifi206* spans 38 kb and contains seven
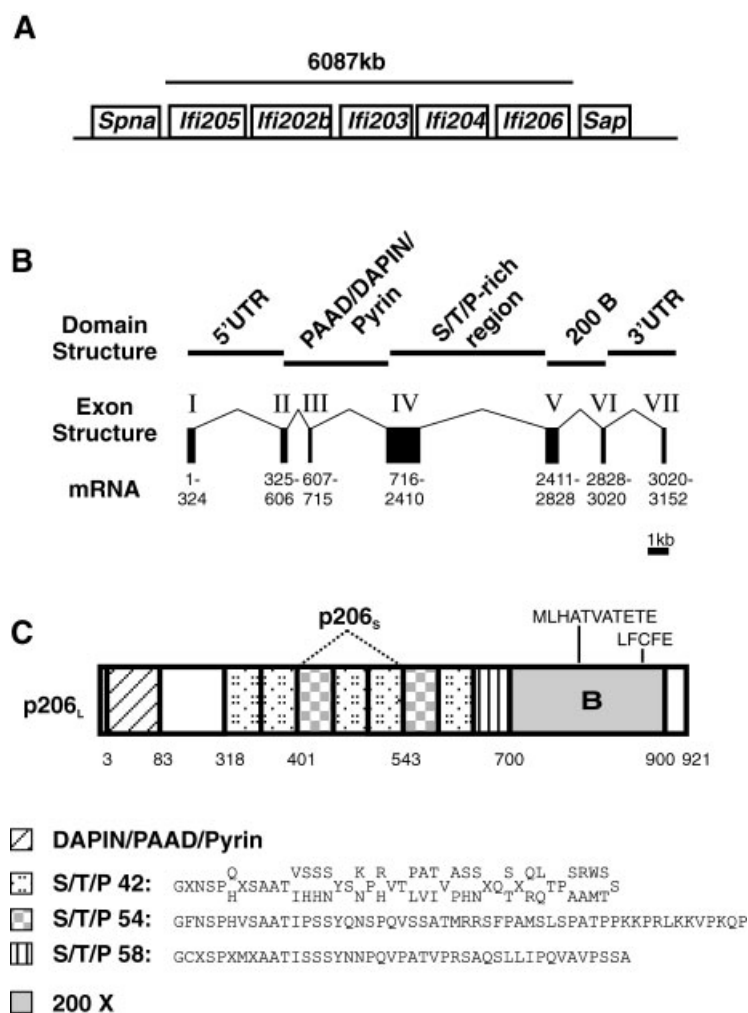
Fig. 1. Genetic and structural characteristics of *Ifi206*. **A:** The location of *Ifi206* in relation to other HIN-200 genes within the HIN-200 locus on chromosome 1q21–23 was established using the NCBI map viewer program created using Build 33.1 C57BL/6 murine genomic sequence (http://www.ncbi.nlm.nih.gov/map-view/). The map is not to scale. **B:** The *Ifi206* gene structure was determined by comparing the experimentally determined *Ifi206* mRNA against contigs assembled from the Celera database (http://www.celera.com/). *Ifi206* is encoded by seven exons indicated by black boxes and numbered with roman numerals. The domains of p206 and untranslated regions corresponding to each exon are illustrated by horizontal bars above the gene structure. Numbers located below diagrams indicate nucleotide positions within the mRNA in base pairs. The map is to scale indicated by the 1 kb scale bar. **C:** Putative protein structure of p206$_L$. The diagonal striped box represent the N-terminal PAAD/DAPIN/Pyrin domain, three classes of S/T/P-rich motifs are denoted by black and white hatching (S/T/P 42), grey and white hatching (S/T/P 54) and vertical stripes (S/T/P 58). p206$_L$ contains five copies of S/T/P 42, two copies of S/T/P 58 and one S/T/P 54 motif while p206$_S$ contains three copies of S/T/P 42, and one copy each of S/T/P 58 and S/T/P 54. The 200 B domain is coloured grey. Numbers located below the diagram represent amino acid positions.

exons (Fig. 1B) and genomic Southern blots using DNA from C57BL/6 splenocytes confirmed the gene size and organisation (data not shown). All of exon I and 16 nucleotides of exon II encode the 5′ untranslated region (UTR). Transcription factor binding sites that may confer IFN-induction were identified in exon and intron sequences upstream from the putative ATG start codon using the MatInspector/Transfac 4.0 program [Quandt et al., 1995]. Twelve consensus gamma-activated sequence elements, three IFN response factor-1 and two IFN response factor-2 consensus motifs were identified within this region however, no consensus IFN-stimulated response element motifs were present. Exons II, III and 583 bp of exon IV encode the N-terminal region of *Ifi206*, with the remainder of exon IV and 33 bp of exon V encode the S/T/P-rich region. Exon IV contains cryptic splice sites flanking a 426 bp sequence such that *Ifi206* gene is predicted to encode two splice isoforms p206$_L$ and p206$_S$. The 200 B domain is

encoded by exon V and 142 bp of VI while the 3′ UTR is within the remainder of exons VI and VII (Fig. 1B).

Predicted Protein Structure of p206

The primary amino acid sequences of $p206_L$ (921 amino acids) and $p206_S$ (779 amino acids) indicated that they contain a characteristic N-terminal PAAD/DAPIN/Pyrin domain, followed by a unique S/T/P-rich region preceding a C-terminal 200 B domain (Fig. 1C). The p206 N-terminal region does not contain a characteristic NLS found in other HIN-200 proteins [Choubey and Lengyel, 1992; Gribaudo et al., 1997; Briggs et al., 2001; Dermott et al., 2004; Ding et al., 2004]. p206 has a large S/T/P-rich region spanning 382 amino acids in $p206_L$ and 240 amino acids in $p206_S$ which separates the N-terminal region from the 200 B domain (Fig. 1C). This region encodes three conserved motifs of 42 (S/T/P 42), 54 (S/T/P 54) and 58 (S/T/P 58) amino acids repeated throughout (Fig. 1C). These S/T/P-rich regions were not present in other murine HIN-200 proteins and analysis of the NCBI database did not detect the presence of these domains in other mouse proteins. However, the 200 A and B domains of IFI 16 are separated by one, two or three copies of a 56 amino acid S/T/P-rich region, encoded by exons VI, VII and VIII of IFI 16 which are alternatively spliced to generate the isotypic variants IFI 16A, IFI 16B and IFI 16C, respectively [Johnstone et al., 1998]. The sequences encoding the p206 and IFI 16 S/T/P-rich regions share little sequence identity but are both rich in serine, threonine and proline residues.

p206 has a single 200 B domain that is characteristic of the HIN-200 family (Fig. 1C). This domain contains the MFHATVAT motif common to all 200 X domains [Ludlow et al., 2005] and an LXCXE motif at amino acids 895–899 of $p206_L$ that is conserved in 200 B domains and was shown to mediate binding of other HIN-200 proteins to the pRb tumour suppressor protein [Choubey and Lengyel, 1995; Hertel et al., 2000]. Therefore, $p206_L$ and $p206_S$ contain many of the structural hallmarks of HIN-200 proteins, specifically a PAAD/DAPIN/Pyrin domain at the N-terminus and a characteristic 200 B domain at the C-terminus. Like IFI 16, p206 also contains a region rich in serine, threonine and proline residues that is alternatively spliced to generate multiple protein isoforms.

HIN-200 mRNA Tissue Expression

We analysed the tissue expression profile of *Ifi206* and compared this to other murine HIN-200 genes by Northern blot. Radiolabelled full length $Ifi206_L$ (Fig. 2) hybridised with mRNA of approximately 3.4 kb in BALB/c spleen and lung. Similar expression patterns were seen using radiolabelled $Ifi206_S$, clone 19, clone 22 and 6.2.1 cDNA as probes and the same restricted tissue distribution pattern was observed using RNA from C57BL/6 mice (data not shown). By comparison *Ifi202* and *Ifi203* were more widely expressed with transcripts being detected in heart, spleen, lung, liver and kidney (Fig. 2). *Ifi204* and *Ifi205* were each detected in heart, spleen and kidney. RT-PCR assays were performed using an oligonucleotide primer set that produced a product which included the spliced region of *Ifi206* and therefore produced different sized products for $Ifi206_S$ and $Ifi206_L$ isoforms and were therefore capable of distinguishing between them. As
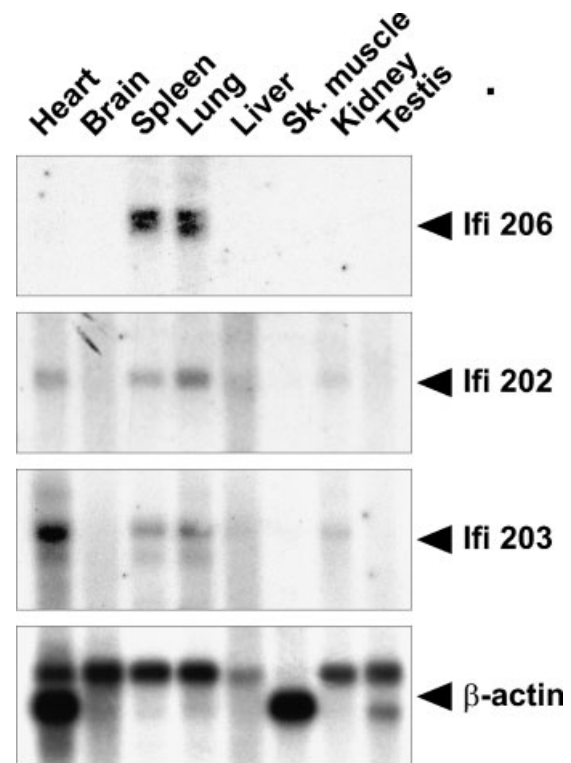

Fig. 2. Tissue expression pattern of *Ifi206* mRNA compared to other murine HIN-200 transcripts. Northern blot with poly A+ RNA isolated from BALB/c heart (**lane 1**), brain (**lane 2**), spleen (**lane 3**), lung (**lane 4**), liver (**lane 5**), skeletal muscle (**lane 6**), kidney (**lane 7**) and testis (**lane 8**). The Northern blot was probed with radiolabelled cDNA corresponding to *Ifi206*, *Ifi202*, *Ifi203* and β-*actin*.

shown in Figure 3A, both splice variants were detected in mRNA from the spleen of C57BL/6 and BALB/c mice (Fig. 3A) and, consistent with Figure 2, neither *Ifi206* isoform was detected in the brain of Balb/c mice (Fig. 3B).

IFN-Induced Expression of *Ifi206*

A characteristic of HIN-200 genes is their induction by types I and II IFN. To determine if *Ifi206* was induced by type I IFN, Northern blotting was performed using poly A+ RNA extracted from NIH 3T3 cells that had been treated with IFNβ for various times up to 48 h (Fig. 4A). *Ifi206* levels were low in un-stimulated NIH 3T3 cells and were induced following

IFNβ treatment, peaking after 12 h of induction (Fig. 4A, lane 4) and declining slowly thereafter. In contrast, *Ifi202* was more rapidly induced by IFNβ, with strong expression seen following 4 h treatment (Fig. 4A, lane 2) and peaking after 24 h of treatment (Fig. 4A, lane 6). To determine if *Ifi206* was induced by type II IFN, Northern blot assays were performed using polyA+ RNA extracted from RM-1 cells stimulated with IFNγ for times up to 48 h (Fig. 4B). *Ifi206* mRNA was slightly increased following the 4–24 h treatment with IFNγ with maximum induction seen after 48 h (Fig. 4B, lane 7). *Ifi204* was similarly induced by IFNγ over the time course (Fig. 4B, lanes 3–7). Thus *Ifi206* mRNA was induced by both types I and II IFN.
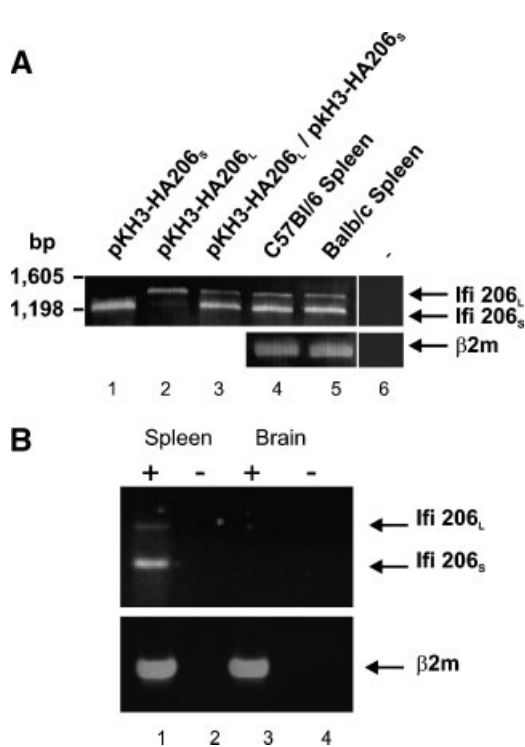

Fig. 3. Both *Ifi206$_L$* and *Ifi206$_S$* isoforms can be detected in murine tissues. **A:** RT-PCR was performed on mRNA samples from C57BL6 (**lane 4**) and Balb/c (**lane 5**) spleen using primers predicted to form a product spanning the alternatively spliced region of *Ifi206* or complementary to β2m. Products were compared to those from recombinant plasmid DNA containing *Ifi206$_S$* (**lane 1**), *Ifi206$_L$* (**lane 2**) or a mixture of the two plasmids (**lane 3**). PCRs were also performed on reverse transcription reactions performed in the absence of template (**lane 6**). **B:** RT-PCR was performed on mRNA samples from Balb/c spleen (**lanes 1 and 2**) and brain (**lanes 3 and 4**) using primers predicted to form a product spanning the alternatively spliced region of *Ifi206* or complementary to β2m. Reverse transcription reactions were performed either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of reverse transcriptase enzyme.
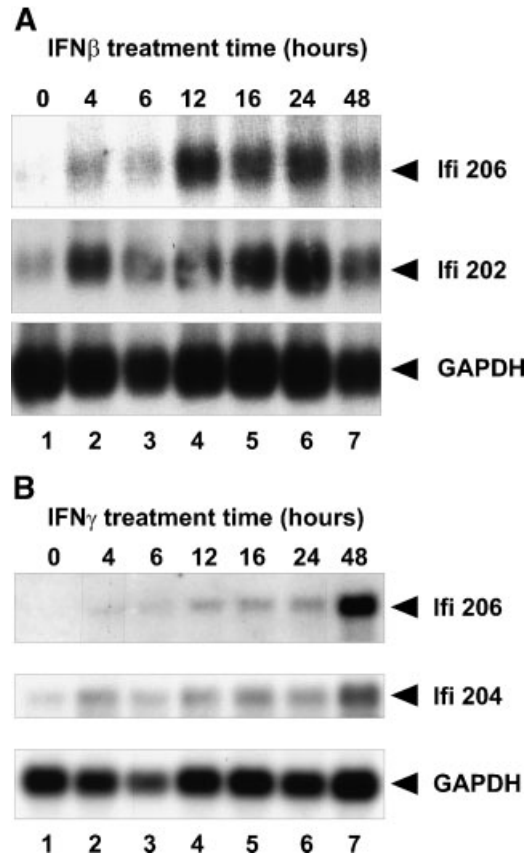

Fig. 4. Induction of *Ifi206* by types I and II IFN. **A:** Northern blot was produced from poly A+ RNA extracted from NIH 3T3 cells treated with 1,000 U/ml of recombinant mouse IFNβ for 4 h (**lane 2**), 6 h (**lane 3**), 12 h (**lane 4**), 18 h (**lane 5**), 24 h (**lane 6**) and 48 h (**lane 7**) or media alone (0 time point, **lane 1**) and probed with ^{32}P-labelled *Ifi206*, *Ifi202* and *GAPDH* cDNA. **B:** A Northern blot was produced from poly A+ RNA extracted from RM-1 cells treated with 1,000 U/ml of recombinant mouse IFNγ for 4 h (**lane 2**), 6 h (**lane 3**), 12 h (**lane 4**), 18 h (**lane 5**), 24 h (**lane 6**) and 48 h (**lane 7**) or media alone (0 time point, **lane 1**) and probed with ^{32}P-labelled *Ifi206*, *Ifi204* and *GAPDH* cDNA.

Sub-Cellular Localisation of p206

To determine the subcellular localisation of the p206$_L$ and p206$_S$ splice isoforms, HA epitope tagged p206$_L$ and p206$_S$ were expressed in NIH 3T3 cells by transient transfection and immunofluorescence assays performed using an anti-HA monoclonal antibody. Over-expressed HA206$_L$ and HA206$_S$ were detected predominantly in the cytoplasm (Fig. 5Ai,iv) while no staining was observed in untransfected cells (Fig. 5Avii). In each of these experiments cells were counterstained with propidium iodide to image the

nucleus. Merging images of HA-staining were with those of propidium iodide staining revealed that p206$_S$ and p206$_L$ were found predominantly in the cytoplasm and generally excluded from the nucleus. Consistent with previous reports [Choubey and Lengyel, 1993] transfected p202 was detected in the cytoplasm and nucleus of transfected NIH 3T3 cells (data not shown). We also assessed the localisation of HA-tagged proteins by isolating fractions from the cytoplasm and nucleus of transfected 293 T cells overexpressing HA-206$_L$, HA-206$_S$ and HA-202 and analysing them by western blot
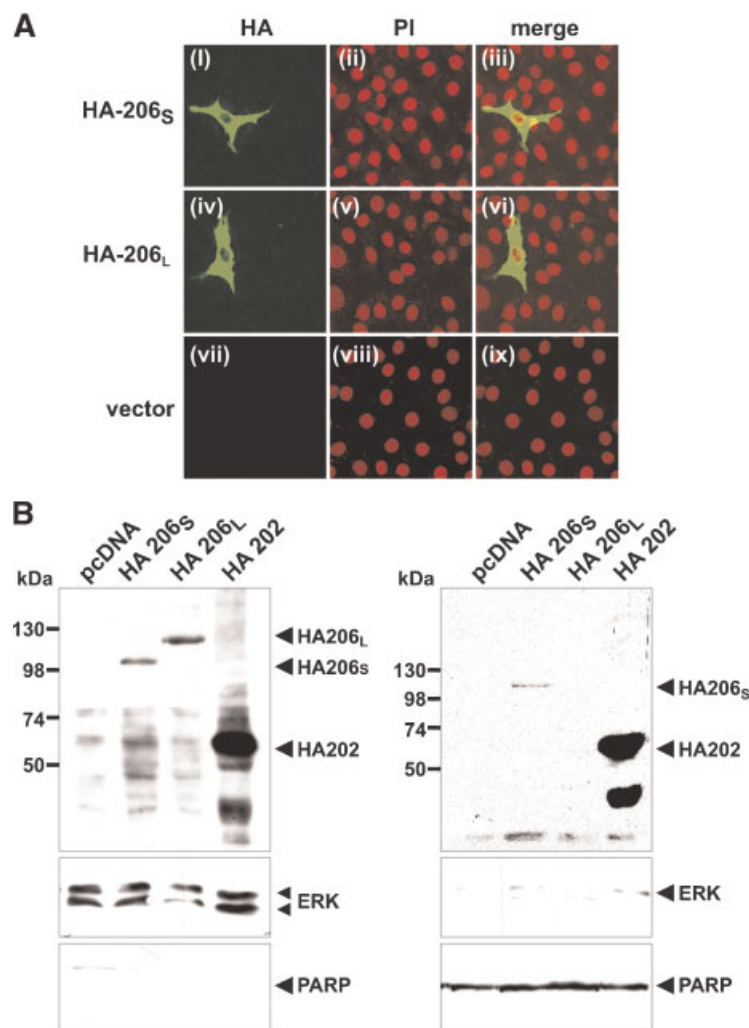

Fig. 5. Subcellular localisation of transfected p206. **A**: pKH3-HA206$_S$ (**i–iii**), pKH3-HA206$_L$ (**iv–vi**) and pKH3 (**vii–ix**) were transiently transfected into NIH 3T3 cells seeded onto coverslips. After transfection (48 h) cells were fixed, permeabilised and probed with anti-HA monoclonal antibody (i,iv,vii) and alexa-488 anti-mouse secondary antibody (shown in green). In each experiment nuclei were counterstained with propidium iodide (ii,v,viii) (shown in red). Images were obtained by confocal microscopy. Images of HA-stained images (HA) and propidium iodide (PI) counterstains were overlayed to generate merged images (Merge). **B**: pcDNA-HA206$_L$, pcDNA-HA206$_S$ and pcDNA-HA202 and pcDNA were transiently transfected into 293T cells and western blots of nuclear and cytoplasmic extracts were performed using antibodies specific for HA-tag, p42/p44ERK and polyADP ribose polymerase. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

using anti-HA antibodies. These experiments revealed that p206$_S$ and p206$_L$ were prominent in cytoplasmic extracts but that p206$_S$ was also weakly detectable in the nuclear fraction. As in immunofluorescence analysis p202 was detected in both cytoplasmic and nuclear fractions. Integrity of cytoplasmic and nuclear fractions was confirmed using antibodies recognising p42/44 ERK kinase (cytoplasm) and polyADP ribose polymerase (PARP, nucleus).

Analysis of the primary amino acid sequence of p206 revealed the presence of a putative nuclear export sequence (NES) within the N-terminus (Fig. 6A). To determine if the cytoplasmic localisation of p206 was due to net nuclear export, the nuclear export inhibitor LMB was used. LMB blocks the binding of the importin β family member CRM1 to proteins containing a NES resulting in nuclear accumulation [Fukuda et al., 1997]. BALB/c 3T3 and NIH 3T3 cells (data not shown) were transiently transfected with pHK3-HA206$_L$ followed by treatment with LMB or vehicle (Fig. 6B). The addition of LMB resulted in the accumulation of p206 in the nucleus (Fig. 6Bii,iv) compared to transfected cells treated with vehicle where p206 was predominantly present in the cytoplasm (Fig. 6Bi,iii). These data are consistent with p206 being synthesised in the cytoplasm, translocating to the nucleus and being rapidly exported from the nucleus to the cytoplasm via an active nuclear export process.

IFN-Induced p206 is Expressed in the Cytoplasm

It has previously been reported that endogenous p202 was expressed predominantly in the cytoplasm prior to IFN treatment and rapidly redistributed to the nucleus following IFN stimulation [Choubey and Lengyel, 1993]. To assess the subcellular expression of p206 following IFN stimulation, NIH 3T3 and RM-1 cells were treated with IFN-α or IFN-γ, respectively, for 24 h and expression of p206 assessed by immunofluorescence using anti-sera raised against p206 (Fig. 7A,B). Untreated NIH3T3 (Fig. 7Ai) or RM-1 cells (Fig. 7Bi) stained only weakly with anti-p206 anti-serum, but after treatment with IFN-α (Fig. 7Aii) or RM-1 with IFN-γ (Fig. 7Bi,ii), strong and predominantly cytoplasmic staining was induced. Preimmune sera did not immunostain either treated or untreated NIH 3T3 cells (Fig. 7Aiii,iv) or RM-1 cells (Fig. 7Biii,iv). As a control for the IFN-α
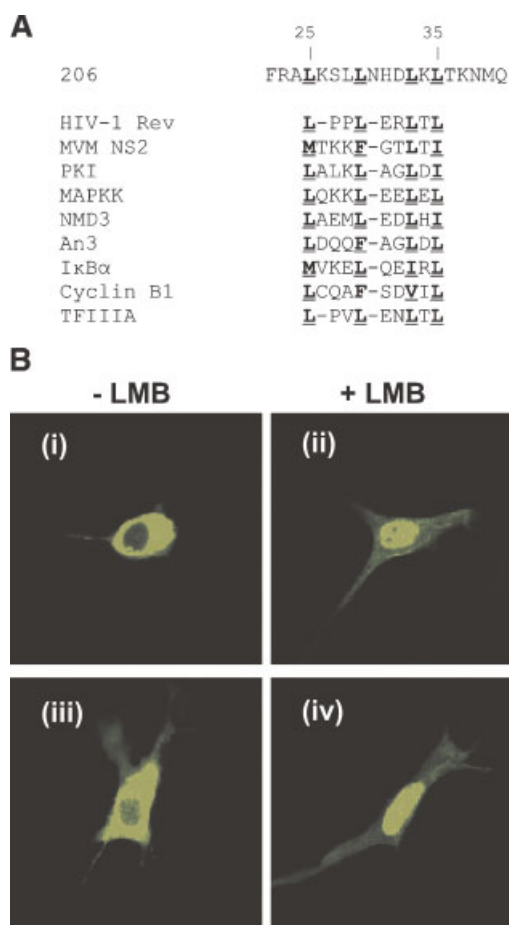

Fig. 6. Nuclear export of p206. A: Sequence of the NES positioned at the p206 N-terminus compared to known nuclear export motifs derived from other proteins [Kutay and Guttinger, 2005]. The consensus NES consists of a core tetramer (LX$_{(2-3)}$LX$_{(2-3)}$LXL) although leucine residues can be substituted by hydrophobic residues [Kutay and Guttinger, 2005]. B: BALB/c 3T3 cells transiently transfected with pKH3-HA206$_L$ were treated with 10 ng/m; LMB (ii,iv) or media alone (i,iii) for 5 h. Cells were fixed and permeabilised then immunofluorescence was performed using the anti-HA monoclonal antibody and alexa-488 anti-mouse secondary antibody (shown in green). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment the cells were stained with an anti-202 goat polyclonal antibody. Following IFN stimulation, p202 was detected in the nucleus of most NIH 3T3 cells, however, in some cells the staining was predominantly cytoplasmic (Fig. 7Av,vi). As a control for the IFN-γ treatment RM-1 cells were stained with an anti-204 rabbit polyclonal antibody. Following IFN-γ stimulation, p204 was detected predominantly in the nucleus of RM-1 cells (Fig. 7Bv,vi). Thus expression of p206 is robustly induced following treatment with both types I and II IFNs but
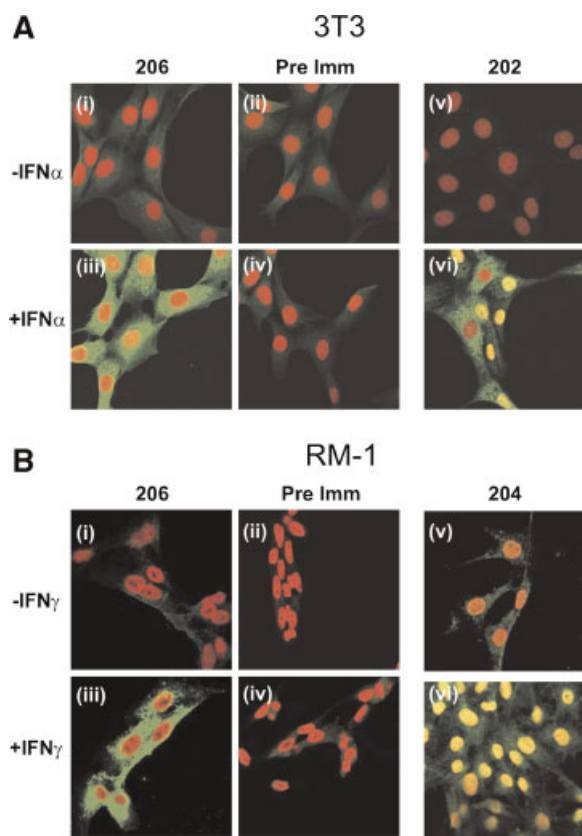
Fig. 7. Induction of p206 by types I and II IFN. **A**: NIH 3T3 cells were grown on coverslips and treated in the presence (**iii**,**iv**,**vi**) or absence (**i**,**ii**,**v**) of 1,000 U/ml of recombinant mouse IFNα for 24 h then stained with rabbit polyclonal anti-sera against p206 (i,iii), preimmune rabbit sera (ii,iv) or anti-202 goat polyclonal antibody (v,vi). Followed by Alexa488 conjugated secondary antibodies (shown in green). Samples were counterstained with propidium iodide (shown in red) and images were merged. **B**: RM-1 cells were grown on coverslips and treated in the presence (**iii**,**iv**,**vi**) or absence (**i**,**ii**,**v**) of 1,000 U/ml of recombinant mouse IFNγ for 24 h then stained with rabbit polyclonal anti-sera against p206 (i,iii), preimmune rabbit sera (ii,iv), anti-204 rabbit polyclonal antibody (v,vi). Followed by Alexa488 conjugated secondary antibodies (shown in green). Samples were counterstained with propidium iodide (shown in red) and images were merged. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

unlike p202, p206 does not redistribute to the nucleus.

Expression of p206 Affects the Clonogenic Capacity of Transfected Cells

Overexpression of HIN-200 proteins can result in decreased cell proliferation and a block in cell cycle progression at the G_1-S phase transition [Lembo et al., 1995, 1998; Dermott et al., 2004; Ding et al., 2004; Raffaella et al., 2004]. To determine if p206 could affect cell proliferation, pcDNA3.1-HA206$_L$, pcDNA3.1-HA206$_S$, pcDNA3.1-HA202a and pcDNA3.1 were transfected into 293 cells and colony assays were performed. Expression of these proteins in transfected cells was confirmed after 48 h by performing immunofluorescence analysis using the anti-HA monoclonal antibody (data not shown). Forty-eight hours after transfection, cells were selected in geneticin and 9 days later colonies were stained with crystal violet. Six separate experiments were performed and the relative colony number of cells transfected with the various constructs was calculated (Fig. 8). As previously reported [Lembo et al., 1995], expression of p202 resulted in a significant reduction of colony formation (Fig. 8) and a similar decrease in colony formation was observed in cells transfected with pcDNA3.1-HA206$_L$ and pcDNA3.1-HA206$_S$ (Fig. 8). These data indicate that like other HIN-200 proteins, p206 is capable of suppressing cell growth.

DISCUSSION

We have identified p206 as a new member of the murine HIN-200 family of proteins and added *Ifi206* to this gene family based on the genetic, biochemical and biological characteristics of the molecule. *Ifi206* spans approximately 22 kb, consists of seven exons and is found within the HIN-200 locus on mouse chromosome 1q21–23. Two protein isoforms p206$_S$ and p206$_L$ derived from *Ifi206* mRNA splicing within exon IV are produced. p206$_L$ and p206$_S$ proteins contain many of the structural
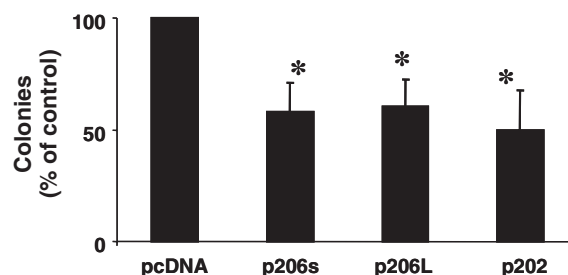

Fig. 8. Effect of p206 on colony formation. Two hundred ninety-three cells were transfected with pcDNA3.1-HA206$_L$ pcDNA3.1-HA206$_S$, pcDNA3.1-HA202a and pcDNA3.1 were selected in geneticin® for 9 days and colonies stained with crystal violet. Colony numbers from cells transfected with pcDNA3.1-HA206$_S$, and pcDNA3.1-HA202a were counted and plotted as a percentage of colony formation compared to the number of colonies formed when cells were transfected with pCDNA3.1. Results are the mean and standard deviation from six independent experiments. *$P < 0.0005$.

hallmarks of HIN-200 proteins, specifically a PAAD/DAPIN/Pyrin domain at the N-terminus and a characteristic 200 B domain at the C-terminus. p206 also contains a region of eight S/T/P-rich motifs, three of which are deleted in the p206$_S$ isoform. The presence of the S/T/P-rich region is unique to p206 among the murine HIN-200 proteins, however, human IFI 16 also contains a S/T/P-rich region that is also subject to a change in composition due to mRNA splicing [Johnstone et al., 1998].

In contrast to other murine HIN-200 genes expressed throughout the hemopoietic system [Ludlow et al., 2005] and in solid organs particularly in heart and kidney, the tissue expression pattern of *Ifi206* was more restricted. *Ifi206* was detected in the spleen and lung only and both mRNA splice variants were expressed. Induction by types I and II IFN is a common characteristic of all HIN-200 genes and *Ifi206* mRNA was up-regulated by1 IFNβ and IFNγ. The kinetics of *Ifi206* induction differed in response to types I and II IFN as the response to IFNβ was rapid and robust compared to the weaker, delayed response observed following IFNγ stimulation. A novel rabbit polyclonal anti-sera raised against the N-terminal region of p206 that did not cross-react with any other murine HIN-200 proteins (data not shown) was used to determine the subcellular expression of p206. HA-tagged transfected and endogenous p206 induced following treatment with types I or II IFN was expressed predominantly in the cytoplasm. Consistent with this finding, a putative NES was identified in the N-terminal DAPIN/PAAD/Pyrin domain of p206. The cytoplasmic localisation of p206 was due to net nuclear export as addition of LMB, an inhibitor of CRM1-mediated nuclear export resulted in nuclear accumulation suggesting that p206 may traffic to the nucleus, but is then actively translocated to the cytoplasm in a Crm1-dependent manner. Beclin 1, a putative tumour suppressor protein that also does not contain an NLS has similar subcellular localisation as it is normally found in the cytoplasm, but accumulates in the nucleus following LMB treatment [Fabbro and Henderson, 2003; Liang et al., 2001]. Even though most HIN-200 proteins contain an NES sequence similar to that of p206 they accumulate primarily in the nucleus and nucleolus. This could be because the residues flanking the NES also contribute to CRM1 binding affinity and are therefore also

important determinants of trafficking [Kutay and Guttinger, 2005]. However, the NES can have functional importance because p204, a regulator of myoblast differentiation, migrates from nucleus to cytoplasm during myotube fusion [Liu et al., 2000]. HIN-200 proteins have been detected in other subcellular compartments under other circumstances. For example p202 initially accumulates in the cytoplasm and subsequently translocates to the nucleus following prolonged IFN treatment [Choubey and Lengyel, 1993] and a GFP-p202 fusion protein co-localises with a mitochondrial dye [Choubey et al., 2003]. In addition, six single nucleotide polymorphisms within IFI 16 caused it to be mislocalised to the cytoplasm and rendered inactive [Xin et al., 2003].

Similar to the effects of overexpression of p202, expression of p206$_S$ and p206$_L$ resulted in decreased colony formation. Based on studies using p203 that contains only a single 200 B domain, it had been proposed that HIN-200 proteins containing only a single 200 X domain may not regulate cell growth whereas those with two 200 X domains do so [Gribaudo et al., 1999]. However, it has since been shown that murine p205 and human family members AIM2 and IFIX which all contain just one 200 X domain are capable of suppressing colony formation in studies similar to ours presented herein [Choubey et al., 2000; Dermott et al., 2004; Ding et al., 2004; Asefa et al., 2006]. Clearly HIN-200 proteins can suppress cell growth whether they are expressed predominantly in the nucleus (i.e. IFI 16, p204) or predominantly in the cytoplasm (i.e. p202, p206) consistent with the notion that HIN-200 proteins can affect multiple cell cycle regulatory pathways [Ludlow et al., 2005]. HIN-200 proteins have been shown to directly or indirectly regulate the activity of key tumour suppressor and cell cycle regulatory proteins such as p53, E2F and pRb [Ludlow et al., 2005]. As p206 is expressed almost exclusively in the cytoplasm it is unlikely that interaction with p53 or pRb is the mechanisms by which p206 regulates tumour cell growth. Exactly how p206 suppresses colony formation remains to be determined.

In conclusion, we have cloned and characterised p206, a new member of the HIN-200 family of proteins. Ifi206 is the first mouse HIN-200 family member demonstrated to have mRNA splice isoforms and we demonstrated that the

p206$_S$ and p206$_L$ protein isoforms have equivalent biochemical and functional characteristics. p206 exhibited many of the hallmarks of other HIN-200 proteins including conserved structural motifs (PAAD/DAPIN region, 200 X domain), induction by types I and II IFNs, hemopoietic expression, and *Ifi206* is located within the HIN-200 locus on chromosome 1q21–23. However, p206 contains a number of unique characteristics including predominant cytoplasmic localisation, the presence of a large stretch of serine, threonine and proline residues and a functional NES. Like other HIN-200 proteins, p206 can affect cell growth and its functional and physiological role remains to be determined.

ACKNOWLEDGMENTS

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