Isotypic Variants of the Interferon-Inducible Transcriptional Repressor IFI 16 Arise through Differential mRNA Splicing†

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ABSTRACT: We recently demonstrated that IFI 16, a human member of a family of interferon-inducible nuclear proteins, can function as a potent repressor of transcription. All members of this family are found in the nucleus and contain 1 or 2 copies of a conserved 200 amino acid repeat domain. IFI 16 migrates on SDS−PAGE as three distinct protein species (IFI 16A, 16B, 16C) clustered at 85−95 kDa, and we therefore set out to determine the molecular mechanisms underpinning the production of these different isoforms. In the present report, we have used thermal cycling amplification of reverse-transcribed mRNA (RT-PCR) and Southern blotting of genomic DNA to show that the three protein isoforms result from translation of three separate mRNA species produced by differential mRNA splicing. This differential splicing gives rise to variability in the central ("hinge") domain of the molecule which separates the two 200 amino acid repeats. The longest mRNA (∼2.7 kb) encodes an open reading frame of 2355 bp and generates the IFI 16A isoform of 785 amino acids. It contains sequences from 11 exons, including a newly identified exon (7a) which appears to have arisen by tandem duplication of exon 7. The second isoform (IFI 16B, corresponding to the form reported previously) is the most abundantly expressed, and results from deletion of exon 7a (168 bp) to encode a protein of 729 amino acids. The smallest mRNA encodes the IFI 16C isoform (2019 bp), has deleted both exon 7 and exon 7a, and shortens the protein by a further 56 amino acids. Culture of IFI 16-expressing cells with tunicamycin and incubation of cellular lysates with endoglycosidase H suggested that neither IFI 16A nor IFI 16B is glycosylated; however, some IFI 16C molecules showed a minor degree of complex carbohydrate addition. Furthermore, immunoprecipitation and Western blotting indicated that all three IFI 16 isoforms are phosphorylated on serine and threonine residues, but not on tyrosine. Thus, the three IFI 16 protein isoforms arise due to alternative RNA splicing and not due to differential glycosylation or phosphorylation. Finally, IFI 16 isoforms can homo- and heterodimerize, and we have mapped the dimerization domain to the amino terminus which contains an imperfect leucine zipper domain.

IFI 16 belongs to a family of interferon (IFN)[1]-inducible human and mouse proteins known as the HIN-200 family (Hematopoietic IFN-inducible Nuclear antigens with 200 amino acid repeats) (*1*). IFI 16 nucleoprotein has been characterized using specific monoclonal and polyclonal antisera and reduced SDS−PAGE as a closely migrating cluster of three proteins of 85−95 kDa (*2*, *3*); however, the molecular basis of the three protein isoforms has not been explained. Expression of IFI 16 protein is restricted to the nuclei of hematopoietic cell subsets and fibroblasts, and both the mRNA and protein are strongly induced from very low basal levels in immature myeloid cell lines such as HL-60 and U937, when these cells are exposed to differentiating agents such as dimethyl sulfoxide and retinoic acid (*2*, *3*). In normal human bone marrow, IFI 16 expression is found in CD34+ pluripotent myeloid cell precursors and in their daughter cells destined for monocytic differentiation; however, expression is rapidly and completely down-regulated when CD34+ cells differentiate into neutrophils, eosinophils, or erythroid cells (*4*). On the basis of this restricted expression, together with IFI 16's nuclear localization and its ability to bind to DNA (*2*), it has been suggested that IFI 16 may function as a transcription factor involved in controlling differentiation in the myeloid lineage. IFI 16 shares a high degree of sequence homology with two other human genes: the myeloid cell nuclear differentiation antigen (MNDA) (*5*) and the recently identified AIM-2 (*6*), which have also been implicated in growth regulation and differentiation of myeloid cells and melanoma cells, respectively.

Mouse members of the HIN-200 family, designated p202, p204, and D3, have also been identified, and share a similar tissue distribution and pattern of inducibility by various interferons with their human counterparts (*1*, *7*). There is strong evidence that p202, in particular, can modulate the function of several known transcription factors; e.g., it can form stable complexes with p53 (*8*), pRb (*9*), E2F (*10*), c-Jun,

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[1] Abbreviations: MNDA, myeloid cell nuclear differentiation antigen; IFN, interferon; RT-PCR, thermal cycling amplification of reverse-transcribed mRNA; NLS, nuclear localization signal; bp, base pair(s); CKII, casein kinase II.

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c-Fos, and NFkB (*11*). Its binding to p53, E2F, and to members of the AP-1 family prevents cognate DNA binding of these transcription factors, resulting in a loss of their transcriptional activity (*8*, *10*, *11*). Thus, p202 can modulate transcription in a 'passive' manner by preventing the action of transcription factors. In contrast, we have recently shown that IFI 16 can actively repress transcription, and we have mapped the repression domains to regions outside of its DNA binding domain. In addition, we have demonstrated that IFI 16 can also bind to p53 and pRb both in vitro and in vivo and thus, like p202, may also affect the transcription function of these molecules (manuscript in preparation).

Like many transcription factors and transcriptional modulators, this family of proteins appears to be organized into modular functional units. All HIN-200 mouse and human proteins share a high degree of sequence homology in a characteristic 200 amino acid repeat region present either as a single copy (e.g., D3, MNDA) or in duplicate, as is the case in both IFI 16 and p202 (*1*, *7*). The function of these domains is unknown; however, both the A and B domains of IFI 16 are independently capable of transcriptional repressor function (*12*), and one short, highly conserved subregion is permissive for binding of p202 to p53, through the intermediary p53 binding protein (*8*). Other domains of IFI 16 include a basic amino-terminal domain which we have shown to subserve both nuclear localization and DNA binding functions (*2*), and a shorter "hinge" domain separating the "A" and "B" 200 amino acid repeat regions, which is unique to IFI 16, and whose function is unknown. We previously reported the organization of the *IFI 16* gene, and showed that the domain organization of the protein is closely mirrored in its intron−exon boundaries (*13*). The amino-terminal domain is represented on exons 2 and 3, while the A and B repeats are encoded on exons 4/5 and 8/9, respectively (*13*). IFI 16 appears to have arisen by a series of exon duplications followed by mutation, as exons 4/8 and 5/9 are almost identical in length and encode exactly corresponding portions of the 200 amino acid domains (*13*). We also found that exons 6 and 7, representing the hinge region, are imperfect repeats of identical length, each encoding 56 amino acids of the predicted 729 amino acid protein represented in the "full length" cDNA that had been identified at that time (*13*).

In the present study, we were interested in determining the molecular basis for the three closely migrating protein species representing IFI 16 in SDS−PAGE analysis, and ultimately to ascertain whether they have different functions. We had previously postulated that the isoforms may arise from alternatively spliced mRNAs, especially given the degree of exon duplication present in the *IFI 16* gene, and the appearance of a broad band of mRNA species seen on Northern blots, rather than a sharply defined single species (*14*). Moreover, due to the presence of long introns in the central portion of the gene, we were unable to define the exact length of the gene, and to determine whether other copies of exon 6/7 might exist. Here we report that the protein isoforms do indeed arise because of alternative mRNA splicing that involves the hinge domain, and further that a hitherto undetected exon (7a) contributes to this variation in size of the gene transcript and the resultant protein. The functional role of the different protein isoforms is unknown; however, we show that the isoforms can dimerize through the amino-terminal region of the molecule. This raises the possibility that a complex containing different combinations of IFI 16 isoforms may have distinct molecular or biological functions.

MATERIALS AND METHODS

Cells and Cell Lines. HeLa, Daudi, or 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin. Human peripheral blood leukocytes from normal donors were separated on Ficoll-Hypaque.

RT-PCR and Subcloning. Total cellular RNA was prepared using RNA-zol (Biotecz Laboratories, Houston, TX) according to the manufacturer's recommendations. First-strand cDNA was synthesized from 10 μg of RNA in 25 μL using 200 units of murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD), 0.25 mM each of dATP, dCTP, dGTP, and dTTP, and 0.5 μg of random hexamer oligonucleotides for 1 h at 37 °C. PCR reactions were performed with 0.25 unit of Taq polymerase (Boehringer Mannheim), 5 μL of first-strand cDNA, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 1 μM exon-specific oligonucleotides. For amplification of the full-length coding region of IFI 16, oligonucleotides to exon 2 (5′-CTTATGTCTGTAAAGATG-3′) and exon 10 (5′-ATCGTCAATGACATCCAG-3′) were used. To amplify the 5′ end, the middle portion and the 3′ end of the IFI 16 coding region of the following oligonucleotides were used: 5′ end, exon 2 (5′-CTTATGTCTGTAAAGATG-3′) and exon 4 (5′-TCCATTGAATTTCTCCTT-3′); middle portion, exon 4 (5′-GAGACCCCAGAAATGGAG-3′) and exon 8 (5′-CTGTTGCGTTCAGCACCA-3′); 3′ end, exon 8 (5′-CCAAGACTGAAGACTGAA-3′) and exon 10 (5′-ATCGTCAATGACATCCAG-3′). For amplification of genomic DNA, oligonucleotide primers corresponding to exon 6 (5′-CCTGAGAGCCATCTTCGG-3′) and exon 8 (5′-CTGTTGCGTTCAGCACCA-3′) were used in PCR reactions using Expand thermostable polymerase (Boehringer Mannheim) according to the manufacturer's instructions. PCR products were ligated into the pMosBlue vector as per manufacturer's instructions and sequenced using the dideoxy method (*15*). The original full-length IFI 16 cDNA was ligated into the *Xba*I site of the pRcCMV (Invitrogen) expression vector.

GST-IFI 16(1−729) was produced by transfecting full-length IFI 16 cDNA in-frame into the pGex4T1 vector (Promega). GST-IFI 16(127−729) and IFI16(478−729) were produced by digesting full-length IFI 16 cDNA with *Sac*I and *Pvu*II, respectively, and subcloning in-frame into pGex4T1. Production of GST-IFI 16(1−159) was described previously (*3*).

In Vitro Transcription/Translation and Expression in Eukaryotic Cells. In vitro transcription/translation reactions (Promega, Madison, WI) containing [^{35}S]methionine (1175 Ci/mmol) were programmed with pMOS-IFI 16A, -B, and -C, encoding the three identified mRNA splice variants or with pRcCMV-IFI 16. For expression in eukaryotic cells, 293 cells were transfected with 20 μg of either pRcCMV-IFI16 or pMOS-IFI 16A, -B, or -C, by the calcium phosphate precipitation method (*16*). Cells were harvested 48 h after addition of the precipitate.

Western Blotting/Immunoprecipitation. Forty-eight hours after transfection, cells were lysed in lysis buffer [25 mM HEPES (pH 7.0), 0.25 M NaCl, 2.5 mM EDTA, 0.5 mM DTT, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 mM phenylmethanesulfonyl fluoride, and 0.1% NP-40] for 30 min on ice. Proteins were separated by electrophoresis through a 10% polyacrylamide gel, transferred onto Immobilon PVDF membrane (Millipore, Bedford, MA), and probed with monoclonal IFI 16 antibody (*3*). Blots were incubated with HRP rabbit−mouse antibody (DAKO, Glostrup, Denmark), and immunoreactive proteins were visualized by chemiluminescence (Amersham, Buckinghamshire, U.K.).

For immunoprecipitation analyses, extracts were incubated with antibody overnight at 4 °C, and immune complexes were collected with protein A−Sepharose beads at 4 °C for 1 h. Antibodies used were rabbit anti-IFI 16 polyclonal serum (*3*) and monoclonal antibodies against phosphotyrosine, -serine, and -threonine (Sigma, St. Louis, MO) residues. The beads were washed 8 times with lysis buffer.

GST-Based Assays of IFI 16 Dimerization. GST fusion proteins were purified as described (*17*), and the yield of each protein was determined by SDS−PAGE analysis and Coomassie blue staining. GST proteins bound to glutathione−agarose beads (Sigma) were washed twice in NET-50 (20 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl) for 15 min at room temperature and were incubated in 200 mL of binding buffer [25 mM HEPES, pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 150 mM KCl, 1 mM dithiothreitol (DTT), 150 μg/mL bovine serum albumin (BSA)] for 10 min at room temperature. Five microliters of ^{35}S-labeled, in vitro translated protein was incubated with immobilized GST fusion proteins for 1 h at room temperature. The beads were washed 5 times with 1 mL of washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Bound proteins were eluted with Laemmli sample buffer, separated on a 10% SDS−polyacrylamide gel, and visualized by autoradiography.

Tunicamycin and Endoglycosidase H Treatment. For endoglycosidase H treatment, IFI 16 immunoprecipitates were resuspended in 50 mM potassium acetate, pH 5, and treated with 1 μL of endoglycosidase H (Sigma) for 16 h at 37 °C. 293 cells transfected with pMOS-IFI 16A, -B, or -C were cultured in the presence of 5 μg/mL tunicamycin (Sigma) for 24 h until harvested for Western blotting.

RESULTS

IFI 16 antigen expressed in the nucleus of myeloid cell lines and primary human cells consists of three protein species at 85−95 kDa; however, the reason for this differential migration on SDS−PAGE is unknown. Among the possibilities we considered were that IFI 16 protein is differentially and variably glycosylated (unusual for a nuclear protein), that the apparent size differences arise due to phosphorylation, and finally that the different sized molecules arise from different mRNA transcripts generated by differential pre-mRNA splicing.

Previously Described IFI 16 mRNA/cDNA Accounts for Only the 16B Protein Isoform. We have previously described "full length" IFI 16 mRNA and protein as encoding 729 amino acids on an open-reading frame of 2187 bp (*14*). When we translated this cDNA in vitro, we found that the protein

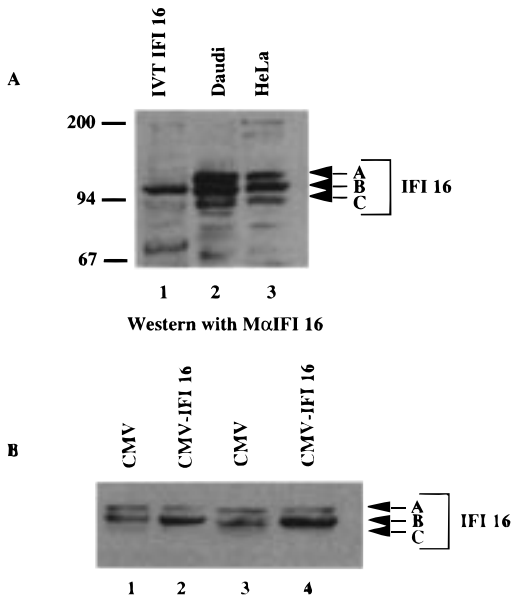

FIGURE 1: Identification of IFI 16 protein isoforms. (A) Whole cell lysates from Daudi (lane 2) or HeLa (lane 3) cells were run on 10% SDS−PAGE with a rabbit reticulocyte lysate programmed with pRcCMV-IFI 16 (lane 1). Proteins were separated and probed with an anti-IFI 16 monoclonal antibody. The IFI 16A, -B, and -C isoforms are indicated by arrows on the right, and molecular mass markers (kDa) are on the left. (B) HeLa cells were transfected with pRcCMV-IFI 16 (lanes 2 and 4) or with pRcCMV alone (lanes 1 and 3), whole cell lysates were prepared, and IFI 16 protein isoforms were detected as above. The IFI 16C isoform was present in all lysates upon longer development of the Western blot (data not shown).

product comigrated with the most abundant IFI 16B isoform identified in the nuclear lysates of Daudi and HeLa cells by Western blotting (Figure 1A). To examine whether overexpression of this isoform in mammalian cells could result in production of the other isoforms, we then transiently expressed the IFI 16B isoform in HeLa cells, under the control of a CMV promoter (Figure 1B). As HeLa cells constitutively express all three isoforms, we compared the expression levels of each isoform with those in cells transfected with vector DNA alone. In each of two independent experiments (lanes 1/2 and 3/4), the nuclear lysates showed a clear increase of the IFI 16B isoform, without any appreciable effect on the quantities of IFI 16A (the slowest migrating species) and IFI 16C (the fastest). This showed that one mRNA species, in this case IFI 16B, could encode only the corresponding protein isoform, and suggested that posttranslational modification of IFI 16 was not a major contributor to protein heterogeneity.

Multiple Copies of Exon 7 Account for IFI 16 Heterogeneity. We used RT-PCR to identify whether IFI 16 mRNA transcripts are heterogeneous. Initially, primers flanking the entire IFI 16B open-reading frame were used to amplify IFI 16 cDNA derived from human peripheral blood mononuclear cells. We identified three major species of cDNA clustered at approximately 2.2 kb (data not shown). We then used oligonucleotides spanning various portions of the IFI 16 transcript to map the heterogeneous region. Oligonucleotides corresponding to the 5′ (exons 2−4) and 3′ (exons 8−10) ends produced only single species; however, primers spanning the central region (exons 4−8) that encodes the hinge domain reproduced a similar pattern as the full-length RT-

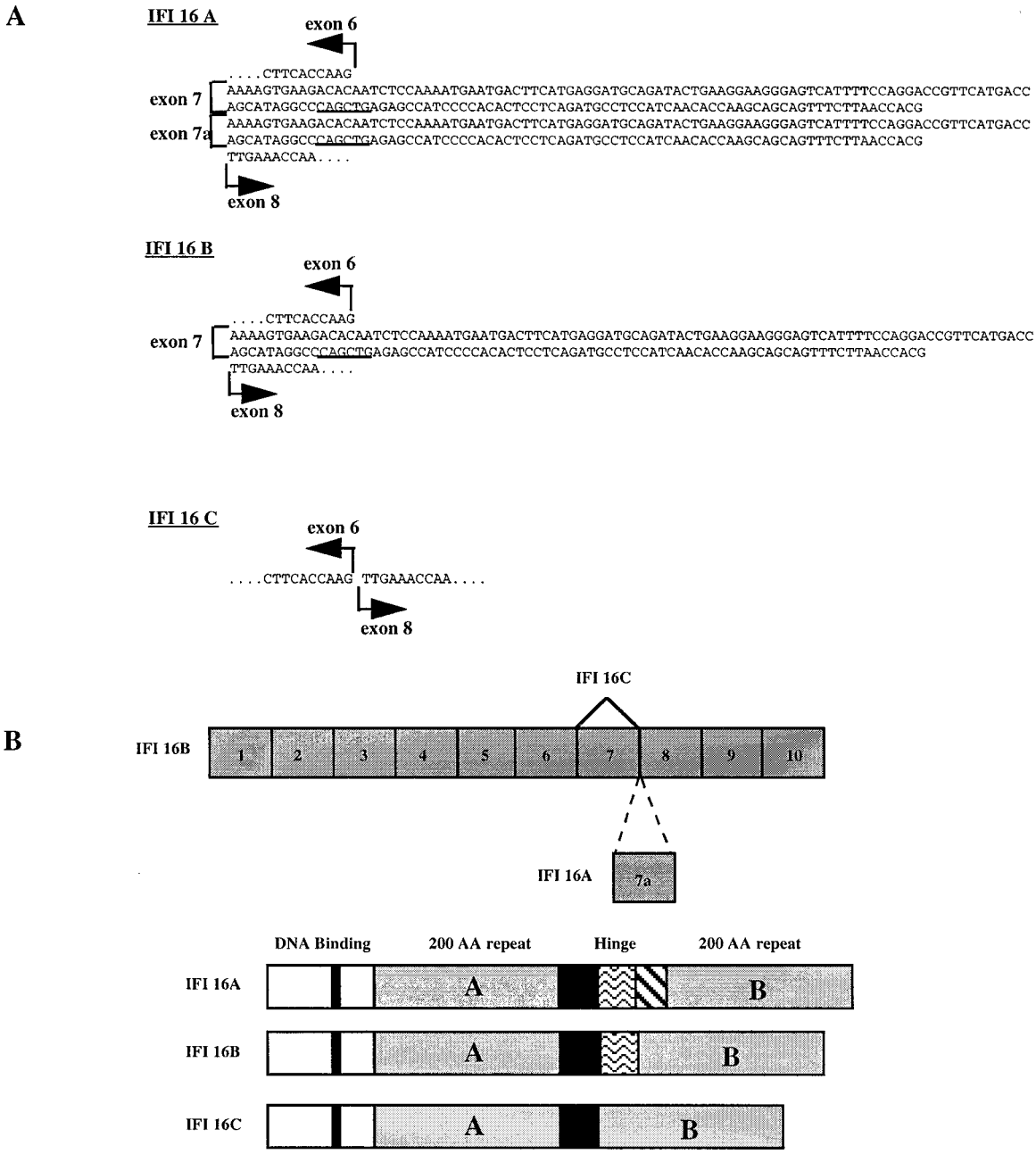

FIGURE 2: Cloning and sequencing of IFI 16 splice variants. (A) Nucleotide sequences of IFI 16A, -B, and -C splice variants were identified by RT/PCR using paired oligonucleotide primers corresponding to exons 2 and 10, and to exons 4 and 8. The exon structure and nucleotide sequences of IFI 16A, -B, and -C are identical except for the region between the end of exon 6 and the start of exon 8. The last 10 bp of exon 6 and the first 10 bp of exon 8 are shown as well as the full nucleotide sequences for exon 7 and exon 7a. The *Pvu*II sites within exons 7 and 7a are underlined. (B) Schematic representation of the three different IFI 16 mRNA (top) and protein (bottom) isoforms that arise due to RNA splicing. IFI 16A contains exons 1 through 10 including the newly identified exon 7a. IFI 16B arises due to a loss of exon 7a, while IFI 16C is produced after a loss of exons 7 and 7a. The protein products from the different splice variants are shown on the bottom. The amino-terminal DNA binding domain (white box) is shown with the nuclear localization signal sequence (black bar); the two, 200 amino acid repeats (A and B) are shaded and separated by a hinge region of variable length encoded by exons 6 (black box), 7 (wavy box), and 7a (striped box).

PCR reaction (data not shown). We then isolated, subcloned, and sequenced all three species (Figure 2A), and found that IFI 16B was encoded by the centrally migrating cDNA molecule. The smallest cDNA encoded a putative protein with a deletion of 56 amino acids, corresponding exactly with sequences encoded on exon 7. The largest cDNA was identical with IFI 16B, but additionally contained an in-frame 168 bp duplication of exon 7, resulting in a protein predicted to contain an additional 56 amino acids in the hinge region (Figure 2B). These findings strongly suggested that IFI 16 isoforms arise from three differentially spliced mRNA species with either 1, 2, or 3 copies of a modular 56 amino acid "hinge unit" encoded on a single copy of exon 6 and 2 copies of exon 7 (Figure 2B).

To investigate this possibility further, and to define the exact number of copies of exon 7 present in the genome, we amplified genomic DNA using primers corresponding to exons 6 and 8. We isolated a fragment of genomic DNA of approximately 10 kb, and confirmed that it hybridized specifically with an exon 7 cDNA probe (data not shown).

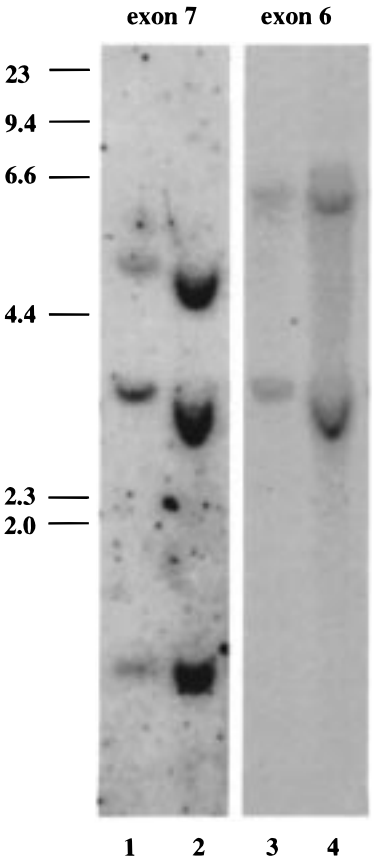

FIGURE 3: Multiple copies of exons 6 and 7 in the IFI 16 gene. Genomic DNA was extracted from peripheral blood lymphocytes from two donors. DNA was digested with *Pvu*II and transferred to a nylon membrane. The blot was probed with an exon 7-specific probe (lanes 1 and 2), then stripped and reprobed with an exon 6-specific probe (lanes 3 and 4). Size markers (kbp) are shown on the left.

When a *Pvu*II digest of the 10 kb fragment was hybridized with the same probe, three hybridizing bands of approximately 5.5, 3.5, and 1.5 kb were identified, and corresponded closely with the pattern of hybridizing bands seen with a *Pvu*II digest of whole genomic DNA (Figure 3). As both copies of exon 7 found in the 16A transcript include a single *Pvu*II site (Figure 2A), the presence of three hybridizing bands in both the PCR and genomic DNA samples indicated that there are two copies of exon 7 in the normal haploid genome. When *Pvu*II-digested genomic DNA was probed with an exon 6 probe (which does not cross-hybridize to exon 7/7a sequences under the stringent hybridization and washing conditions used), we were surprised to find two hybridizing bands. One corresponded with the 3.5 kb band seen with the exon 7-specific probe, indicating that both exon 6 and exon 7 are present on this 3.5 kb *Pvu*II genomic fragment. We have digested the 10 kb PCR fragment spanning exons 6−8 with *Pvu*II and will subclone and sequence the DNA fragments in order to verify the length and nucleotide sequence of the intervening introns. A second, fainter band was observed at approximately 6 kb (Figure 3), indicating the likelihood that like exon 7, exon 6 has undergone duplication. To date, however, we have not identified an mRNA or protein isoform with duplication of exon 6-encoded sequences.

We next expressed each of the three mRNA/cDNA sequences as in vitro transcription/translation products and in transient transfection assays in human 293 cells, which do not express endogenous IFI 16. As was seen previously with the IFI 16B isoform (Figure 1A), we found that the protein encoded by the largest mRNA species corresponded closely with the largest (IFI 16A) isoform seen in Daudi nuclear lysates (Figure 4). In contrast, however, the protein encoded by the smallest mRNA showed a minor degree of heterogeneity which was not evident in molecules expressed in the cell-free system (see below).

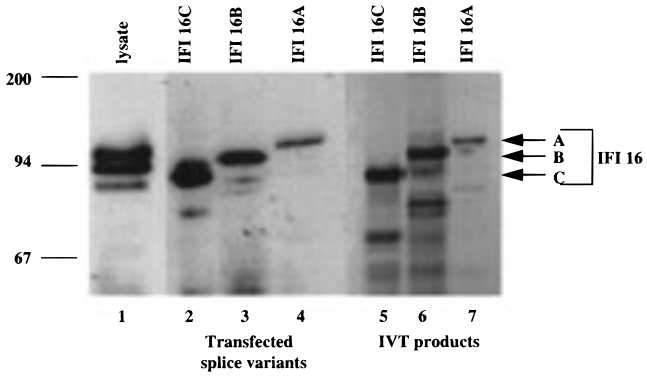

FIGURE 4: Expression of IFI 16 protein isoforms encoded by different RNA splice variants. IFI 16A, -B, and -C protein isoforms were expressed either as transfected products in 293 cells or as in vitro translated products in rabbit reticulocyte lysates. Proteins were separated by 10% SDS−PAGE and probed with an anti-IFI 16 monoclonal antibody. A control lysate from Daudi cells is shown on the left, and the position of the IFI 16A, -B, and -C protein isoforms is indicated on the right.

Glycosylation May Contribute to the Minor Heterogeneity of IFI 16C. Incubation of transfected cells in medium containing tunicamycin produced no change in the apparent molecular sizes of either IFI 16A or IFI 16B isoforms, indicating that both these isoforms are not glycosylated (Figure 5A). However, the heterogeneity of IFI 16C proteins was reduced to a single sharp band by tunicamycin treatment, indicating the addition of carbohydrate. In contrast, digestion of nuclear lysates with endoglycosidase H, followed by immunoprecipitation and Western blotting with anti-IFI 16 antisera, resulted in no appreciable change in the migration of any of the isoforms, confirming that high-mannose glycans do not contribute appreciably to molecular mass for any of the species (Figure 5B). Taken together, these results indicate that a minor degree of complex carbohydrate addition takes place, specifically on the IFI 16C isoform.

Differential Phosphorylation of IFI 16 Isoforms. We considered the possibility that phosphorylation might alter the migration of IFI 16 isoforms (Figure 6). Daudi nuclear cell lysate was immunoprecipitated with mouse monoclonal antibodies specific for phosphoserine, phosphothreonine, and phosphotyrosine and with a control monoclonal antibody, followed by Western blotting with anti-IFI 16 monoclonal antiserum. We found that all three IFI 16 isoforms are phosphorylated on serine and threonine, but not on tyrosine. Interestingly, an additional species highly phosphorylated on serine was also identified as a band migrating slower than the IFI 16A isoform (designated IFI 16D, Figure 6). The relationship of this isoform to A, B, and C is as yet undefined. Reciprocal immunoprecipitation/Western experiments using polyclonal anti-IFI 16 as the immunoprecipitating antibody, and anti-phospho-tyrosine, -serine, and -threonine antibodies for Western blotting, gave similar results (data not shown).

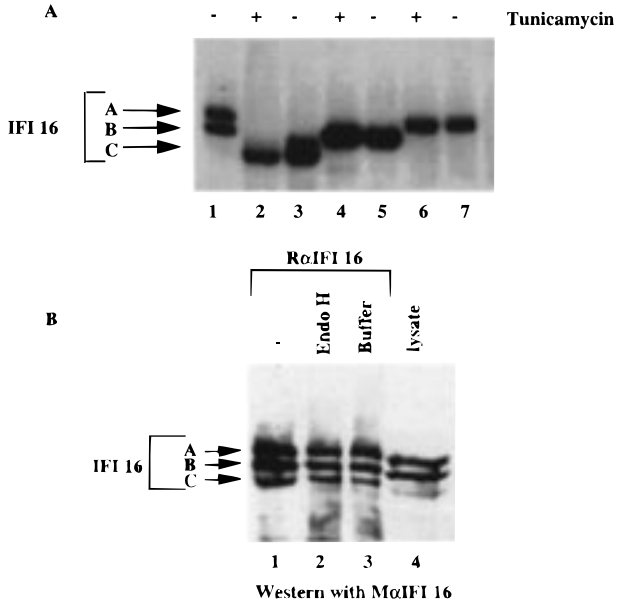

FIGURE 5: Glycosylation may contribute to the minor heterogeneity of IFI 16C. (A) 293 cells were transfected with plasmids expressing IFI 16A, -B, and -C isoforms in the presence (lanes 2, 4, 6) or absence (lanes 3, 5, 7) of 5 μg/mL tunicamycin. Proteins were separated by 10% SDS−PAGE and probed with an anti-IFI 16 monoclonal antibody. A control lysate from Daudi cells is shown in lane 1, and the position of the IFI 16A, -B, and -C protein isoforms is indicated on the right. (B) IFI 16 was immunoprecipitated from a Daudi cell lysate using a rabbit polyclonal antibody raised to the amino-terminal region of IFI 16. Immunoprecipitates were washed and incubated in buffer with (lane 2) or without (lane 3) endoglycosidase H. The control Daudi lysate is in lane 4, and the immunoprecipitate not incubated in endoglycosidase H buffer is in lane 1. Western blots were performed as in panel 5.

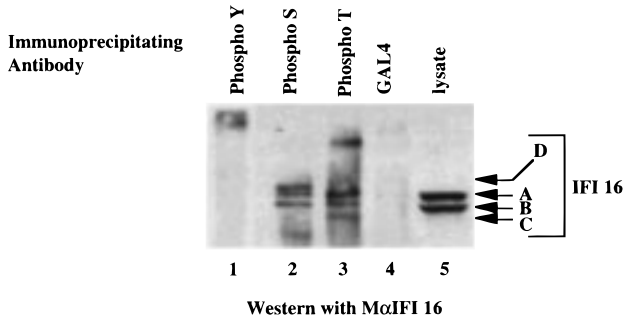

FIGURE 6: IFI 16 protein isoforms are phosphorylated on serine and threonine residues. Immunoprecipitations were performed on Daudi whole cell lysates with monoclonal antibodies against phosphorylated tyrosine (lane 1), serine (lane 2), and threonine (lane 3) residues, or with a control antibody against the yeast GAL4 DNA binding domain (lane 4). Immunoprecipitates were washed, proteins separated by 10% SDS−PAGE, and Western blots performed as in Figure 1A. The position of IFI 16A, -B, and -C and the newly identified D isoforms is indicated on the right.

Alternative IFI 16 Isoforms Can Form Heterodimers. Previously, it had been shown that the related human protein, MNDA, can homodimerize via its amino-terminal domain containing an imperfect leucine zipper and a basic region (*18*). As IFI 16 also contains an imperfect leucine zipper and basic regions within its amino terminus, we tested for homo- and heterodimerization of IFI 16 protein isoforms. As shown in Figure 7, using GST-IFI 16B and in vitro transcribed/translated ^{35}S-labeled IFI 16A, -B, and -C protein, IFI 16B isoform can homodimerize and can also form stable complexes with the A and C isoforms.

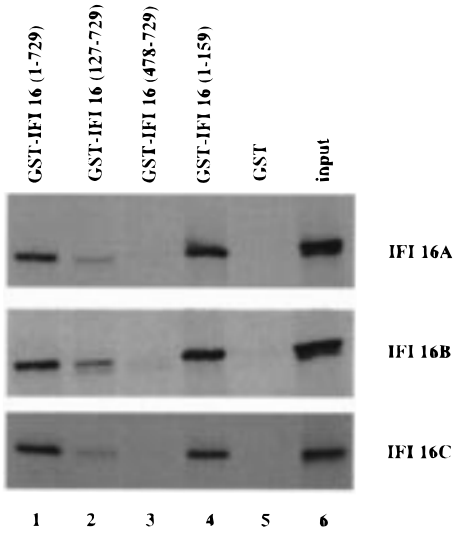

FIGURE 7: Alternative IFI 16 isoforms can form heterodimers. GST fusion proteins containing (lane 1) full-length IFI 16B isoform consisting of amino acids 1−729, (lane 2) amino acids 127−729, (lane 3) amino acids 478−729, (lane 4) amino acids 1−159, or (lane 5) GST alone were mixed with in vitro translated, ^{35}S-labeled IFI 16A (top panel), IFI 16B (middle panel), and IFI 16C (bottom panel). Beads were washed and bound proteins separated on 10% SDS−PAGE. Half the amount of in vitro translated product used in the binding reactions is shown in lane 6.

DISCUSSION

The data presented in this paper clearly indicate that the observed heterogeneity of IFI 16 protein isoforms results principally from alternative mRNA splicing. As a result, the three principal forms on SDS−PAGE differ from one another in the size of their hinge domain, which can consist of one, two, or three multiples of a 56 amino acid motif encoded by a single copy of exon 6, or two reiterated copies of exon 7 (now designated 7 and 7a). We have found evidence for a second copy of exon 6 (tentatively designated exon 6a), but as yet we have no evidence that it is transcribed. In addition, we have found that, as expected for a nuclear antigen, carbohydrate addition has little or no bearing on the apparent molecular size of IFI 16, as only the IFI 16C isoform contained minor quantities of complex carbohydrate addition. In contrast, IFI 16A and IFI 16B appear to lack carbohydrate totally and are more abundant than IFI 16C in most cells and cell lines tested. In addition, all three isoforms are constitutively phosphorylated on serine and threonine, but not on tyrosine.

The functional significance of the isoforms of IFI 16 is yet to be defined. It is noteworthy that the hinge regions encoded by exons 6, 7, and 7a are rich in serine, threonine, and proline residues, and that all three isoforms show evidence of serine and threonine phosphorylation. The IFI 16C isoform that appears to have the least degree of phosphorylation also lacks both exon 7 and exon 7a. It is thus tempting to speculate that the sites for serine−threonine phosphorylation map principally to exon 7/7a sequences, and that the "IFI 16D" isoform may represent a hyperphosphorylated form of IFI 16A which arises due to its "double dose" of critical serine residues mapping to exons 7/7a. However, no formal evidence exists to support this hypothesis at this time, and the functional significance of phosphorylation is unclear.

One possibility is that serine/threonine phosphorylation may play a crucial role in nuclear localization of IFI 16. Nuclear import for molecules larger than approximately 40 kDa is normally dependent on a stretch of basic amino acid residues analogous to the nuclear localization signal (NLS) of SV40 large T antigen (*19*). In addition, neighboring phosphorylation sites that are substrates for casein kinase II (CK II) or $p34^{cdc2}$ may modulate the rate of uptake into the nucleus, either in a positive or in a negative fashion. As all three IFI 16 isoforms including IFI 16C are present in the nucleus and none is found in the cytoplasm in appreciable quantities, it is unlikely that exons 7/7a encode phosphorylation sites that are crucial for this function. Indeed, this region is not a strong candidate to regulate nuclear import, as it encodes no appreciable stretches of basic residues that could act as an NLS. Exon 6 encodes ^{392}KKKTNPR, but this is not close to CK II consensus sites. The most likely NLS we have identified to date is the sequence ^{126}AQKRKK, which is encoded on exon 3. This region is strongly conserved in all members of the family which are constitutively present in the nucleus, but is absent from p202, whose entry into the nucleus is cell cycle dependent. We have shown that the adjacent sequence (^{132}STKE) is a site of phosphorylation by CK II and, in conjunction with a second stretch of basic residues at ^{140}KGSK, combines to modulate the rate of nuclear uptake (manuscript in preparation).

Another possibility is that the hinge domain may affect folding of the molecule as a whole, and determine the spatial relationship of the A and B 200 amino acid repeats. Part of the first Ser-Thr-Pro-rich domain (residues 418−444, mapping to exon 6) has strong similarity to residues 474−501, which map to exon 7. The identical amino acid sequence is then reiterated in the IFI 16A isoform at residues 530−557, encoded on exon 7a. These regions are similar to other Ser-Thr-Pro-rich regions found in the membrane proximal domain of CD8α (*20*) and CD46 (*21*), and between domains 1 and 2 of CD5 (*22*). It is believed that the high content of these residues confers rigidity to the sequence, to separate flanking domains from one another. If this is the case for IFI 16, it might be expected that the spatial relationships of the two 200 amino acid domains to one another would differ appreciably between the three isoforms.

We have shown previously that either the A or the B 200 amino acid repeat of IFI 16 is sufficient and necessary for transcriptional repression mediated by IFI 16. However, these studies also showed the conformation of the molecule may be important for the repression function of IFI 16 as deletion of the amino-terminal DNA binding domain (shown to be transcriptionally inert) results in a loss of transcriptional repression (*12*). Thus, an increase or decrease in the distance between the A and B 200 amino acid repeats might regulate the relative strength of transcriptional repression by IFI 16. Studies are currently underway to determine the repression properties of the A and C isoforms relative to the previously characterized B isoform. The fact that the hinge domain is unique to IFI 16 and is also of a variable length suggests it specifically confers a molecular function that is peculiar to this family member.

Finally, we have shown that IFI 16 can dimerize and that all three isoforms can interact with each other. The related protein, MNDA, has also been shown to homodimerize, and two distinct regions within the amino terminus are required for this interaction (*18*). The first region contains an imperfect leucine zipper, and a comparison of the amino acids forming this motif in MNDA with a similar region in IFI 16 (amino acids 52−82: MEKKFRGDAGLGKLIKIFE-DIPTLEDLAETL) shows that although the two molecules share only 45% identity within this domain, three of the four leucine residues implicated by Xie et al. (*18*) in forming the zipper are the same, and the fourth has a conserved isoleucine−leucine change. The second region identified as necessary for MNDA oligomerization is highly basic, and a comparison between MNDA and IFI 16 reveals a similarly basic amino terminus (33 basic residues within the first 143 amino acids) for IFI 16. Removal of the putative imperfect leucine zipper of IFI 16 greatly reduced the ability of IFI 16 to dimerize; however, like MNDA, IFI 16 has two regions within the amino terminus capable of dimerization. Thus, IFI 16 can also form multimeric complexes, and we have evidence to suggest that IFI 16 can interact with MNDA and with the other human family member, AIM 2, thereby adding another level of complexity to the possible functions of this family of proteins. It has yet to be determined if heterodimerization of different IFI 16 isoforms results in a change in repression function or if the presence of MNDA and/or AIM 2 modulates the transcriptional activity of IFI 16.

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