Interferon-inducible Myc/STAT-interacting Protein Nmi Associates with IFP 35 into a High Molecular Mass Complex and Inhibits Proteasome-mediated Degradation of IFP 35*

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Nmi is an interferon (IFN)-inducible protein homologous to IFN-inducible protein IFP 35. The homology consists of a novel Nmi/IFP 35 domain (NID) of 90–92 amino acids that is repeated in tandem in each protein and mediates Nmi-Nmi protein interactions and subcellular localization. In a yeast two-hybrid screen with a fragment of Nmi protein containing both NIDs, we identified an interaction between Nmi and IFP 35. Deletion derivatives of the proteins indicate that both NIDs are required for the interaction between Nmi and IFP 35. In mammalian cells, Nmi and IFP 35 co-immunoprecipitate and co-localize in large cytoplasmic speckles. Nmi and IFP 35 proteins associate into a high molecular mass complex of 300–400 kDa as determined by native gel electrophoresis and gel filtration. The association of Nmi and IFP 35 into a complex can be demonstrated in multiple cell lines and is not dependent on treatment with IFN. Short term and long term cultures of transfected HEK293 cells suggest that Nmi and IFP 35 proteins stabilize each other through complex formation. IFP 35 appears to be more labile because Nmi was stable in the absence of IFP 35, whereas IFP 35 was degraded in the absence of Nmi. A deletion analysis revealed that Nmi must interact with IFP 35 to prevent its degradation and that the amino terminus of Nmi is required, but not sufficient, for this function. Inhibition of the proteasome, but not other proteases, led to increased levels of IFP 35. Thus, we have shown that Nmi and IFP 35 associate into a protein complex, that IFP 35 is degraded in a proteasome-mediated process, and that a novel function of Nmi is to prevent IFP 35 degradation. The stabilization of IFP 35 by Nmi may serve to amplify the physiologic effects of IFNs.

Nmi was initially identified in a yeast two-hybrid screen with N-Myc as bait and was also shown to interact with c-Myc, Max (bHLH-Zip proteins), Daughterless (a bHLH protein), and Fos (a bZip protein) but not with Cdc2, cyclin, or TGF-β type I receptor (1). The amino terminus of Nmi, which has homology to coiled-coil proteins, was implicated in binding to Myc family members and other transcription factors (1). Myc proteins mediate cell growth, transformation, and apoptosis by functioning as activators or repressors of transcription through interaction with other proteins (2–8). Although Nmi was also shown to interact with c-Myc and N-Myc in mammalian cells, a role for Nmi in the modulation of Myc function has not yet been documented.

A portion of Nmi (from amino acids 102–288) was noted to have homology with IFP 35, an interferon (IFN)1-inducible protein (1, 9). We have previously shown that the homologous region consists of a novel Nmi/IFP 35 domain (NID) of approximately 90–92 amino acids that is repeated in tandem in each protein and mediates Nmi-Nmi protein interactions and subcellular localization (10). Based on the homology between Nmi and IFP 35, we showed that Nmi was also IFN-inducible (11). We found that of the numerous cell lines tested (derived from tumors of the liver, kidney, T-cell, B-cells, prostate, uterus, and cervix), all expressed endogenous Nmi and that Nmi was IFN-inducible (11). Among normal tissues that were investigated, we found that the highest levels of Nmi protein were in the thymus, spleen, and liver (11). Because IFNs have pleiotropic effects including the ability to restrict the growth of cells or induce apoptosis, the finding that Nmi was IFN-inducible suggested that it might be important for IFN function (12, 13).

More recently, Nmi was also isolated in a two-hybrid screen using a portion of Signal transducer and activator of transcription (STAT) 5b, as bait (14). STAT proteins function as activators or repressors of transcription through interaction with other proteins. STAT5 leading to augmentation of transcription from interleukin-2 and IFN-γ-inducible promoters (14).

In this study, we show that Nmi and IFP 35 associate into a

1 The abbreviations used are: IFN, interferon; NID, Nmi/IFP 35 domain; STAT, signal transducer and activator of transcription; HMMC, high molecular mass complex; DBD, DNA-binding domain; NIC, Nmi/IFP 35 complex; PBS, phosphate-buffered saline; HB, homogenizing buffer; FPLC, fast performance liquid chromatography; HA, hemagglutinin.

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high molecular mass complex (HMMC). Significantly, the association of Nmi and IFP 35 into a complex leads to stabilization of IFP 35 via inhibition of a proteasome-mediated pathway, thus defining a novel function for Nmi.

**MATERIALS AND METHODS**

**Yeast Two-hybrid Screening**—The entire NMI cDNA or various fragments were cloned into the two-hybrid vector pAS1, to produce Gal4 DNA-binding domain (DBD) fusions to Nmi (16). Fragments containing the amino terminus of Nmi activated transcription in yeast, excluding their use as baits. The Gal4-Nmi (58–307) fragment (an EcoRI-BamHI fragment of Nmi expressing amino acids 58–307 from Nmi) did not activate transcription and was therefore used for library screening.

Protein extracts prepared from Y153 transformed with the Gal4-Nmi (58–307) construct were analyzed by Western blotting with an anti-Nmi antibody to confirm that the expected Gal4DBD/Nmi (58–307) fusion protein was produced. The Gal4DBD/Nmi (58–307) was used to screen a human lymphoblastoid cDNA library expressed from the pACT vector (16). Y153 expressing the fusion protein were transformed with the pACT library and plated in the presence of 50 mM 3-aminoantizole to select for His3-expressing cells (16). 25 of 500,000 colonies that were screened grew in the presence of 3-aminoantizole and tested positive for β-galactosidase. DNA sequence was obtained from the 5′ end of each clone. pAS1 fusion constructions expressing various fragments of NMI have been previously described: NID1/2 constructs express Nmi amino acids 92–307, NDI1 constructs express amino acids 92–192, and NID2 constructs express amino acids 192–307 (10).

**Immunofluorescence Staining**—Cytospins were prepared from Jurkat cells (treated with or without 1000 units/ml of IFN-γ for 24 h). The cells were fixed with ice-cold methanol for 10 min followed by acetone for 10 min. Nmi was detected using an affinity purified anti-Nmi antibody and Alexa 594-labeled goat anti-rabbit as described previously (11). IFP 35 was detected using a mouse monoclonal anti-IFP 35 followed by fluorescein isothiocyanate-labeled goat anti-mouse. Nuclei were stained with Hoechst, 0.05 μg/ml for 1 min. Cells were visualized using a fluorescence microscope equipped with Hoechst, fluorescein isothiocyanate, and Texas red filters. Images were obtained by optical sectioning followed by deconvolution and processed using Adobe Photoshop (11).

**Coimmunoprecipitation**—For coimmunoprecipitation from transfected HEK293 cells, 2.5 × 106 cells/well were plated in six-well plates, grown overnight and transfected with 2 μg of plasmid DNA using SuperFect reagent according to the manufacturer’s instructions. After transfection, cells were washed with PBS and induced with 1000 units/ml IFN-γ in cultivation medium for 24 h. Cells were washed once and detached by using trypsin/EDTA, pelleted, and washed twice with PBS. Cells (2 × 106 cells/ml) were lysed for 1 h in lysis buffer (150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged for 15 min at 15,000 × g at 4 °C. 50 μl of supernatant, 50 μl of lysis buffer, and 1.4 μl of anti-FLAG antibody (2.8 mg/ml) were incubated at 4 °C for 2 h under shaking. 40 μl of 50% protein A-Sepharose equilibrated in lysis buffer was added and incubated for an additional 1 h. Immunocomplexes were washed four times with lysis buffer without Nonidet P-40, boiled in 50 μl of Laemmli buffer, electrophoresed on 9% SDS-polyacrylamide gels, blotted on nitrocellulose, probed with anti-FLAG and monoclonal anti-IFP 35 antibody, and developed using the ECL reagent plus Western blotting detection system. For immunoprecipitation from Jurkat cells, 10 million cells (with or without 1000 units/ml of IFN-γ treatment for 24 h) were lysed in 1 ml of buffer with 0.5% Nonidet P-40 and protease inhibitors as described (17). After centrifugation, 1 μl of rabbit anti-Nmi (1–67) or 1 μl of preimmune serum was added to 450-μl aliquots of supernatant for 1 h followed by protein A-agarose beads for 1 h. Beads were washed four times with 1 ml of lysis buffer and boiled in 50 μl of Laemmli buffer. Samples were electrophoresed on two 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with anti-Nmi or anti-IFP 35 antibodies.

**Subcellular Fractionation**—Jurkat cells (~10⁶ cells/ml in 250 ml) either treated or not treated with IFN-α (1000 units/ml) were collected by centrifugation (1000 g for 5 min) and washed twice with cold PBS. The pellet was resuspended in 1.5 ml of homogenizing buffer (10 mM Hepes-Na, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Nonidet, and protease inhibitors). A Dounce homogenizer with a tightly fitting pestle was used for the homogenization, and cell disruption was evaluated by phase contrast microscopy. The homogenate was centrifuged at 1000 × g for 10 min, and the pellet was suspended in 1 ml of HB. Both the supernatant and the resuspended pellet were centrifuged again at 10000 × g before both pellets from this spin were combined and suspended in 1 ml of HB supplemented with 2 mM MgCl₂ and 2 mM CaCl₂. The subcellular fraction was further washed three times, and the final sample was labeled nuclear fraction (N). The supernatants collected from the above step were combined and centrifuged at 16,000 × g for 30 min. The pellet was washed three times with HB and centrifuged at 16,000 × g for 30 min, and the subcellular fraction was labeled mitochondria-lysosomal fraction (ML). The supernatants collected from the above step were combined and further centrifuged at 100,000 × g for 1 h. The pellet was washed once again and labeled microsomal fraction (Micro). The resulting supernatant after removal of the microsomal fraction was labeled S-100.

**Lysate Preparation and Native Gel Western Blotting**—Samples of Jurkat cells (with or without IFN-γ treatment, 1500 units/ml for 24 h) were lysed by Dounce homogenization in HB. High speed (100,000 × g) spin supernatants were loaded on nondenaturing polyacrylamide gels (either 4, 6, or 10% gel) that were run at low voltage in a cold room. Proteins were transferred to polyvinylidene difluoride membrane and probed with the antibodies specified in the text or figures. FPLC—Jurkat cell supernatants at a protein concentration of 5 mg/ml were filtered through a 0.22 μm Millipore filter and loaded on a Superdex 200 column (HR10/30; Amersham Pharmacia Biotech) eluted with PBS at a flow rate of 0.4 ml/min. Fractions of 1 ml were collected and analyzed by Western blotting. The column was calibrated with ferritin (440 KDa), catalase (232 KDa), and aldolase (158 KDa).

**Stabilization of Nmi and IFP 35 by Complex Formation and Proteasome Inhibition**—HEK293 cells were transfected with the plasmids specified in the text and figures using standard calcium phosphate precipitation technique. Lysates were prepared and blotted as described above. Proteasome inhibitors MG-132 (final concentration, 10 μM) and lactocystin (final concentration, 10 μM), calpain inhibitor E64D (final concentration, 10 μM), and lysosomal inhibitor chloroquine (final concentration, 10 μM) were added to cells for 14 h prior to harvest for protein analysis.

**RESULTS**

**Yeast Two-hybrid Screening with Nmi Identifies Interaction between Nmi and IFP 35**—The yeast two-hybrid system was used to identify Nmi-interacting proteins. The Gal4 BBD was fused to either full-length Nmi or various portions of the Nmi protein. We found that Nmi (1–67) or Nmi (1–307) fused to either full-length Nmi or various portions of the Nmi protein. We found that Nmi (1–67) or Nmi (1–307) fused to either full-length Nmi or various portions of the Nmi protein. The Gal4 BBD was fused to either full-length Nmi or various portions of the Nmi protein. We found that Nmi (1–67) or Nmi (1–307) fused to either full-length Nmi or various portions of the Nmi protein. We found that Nmi (1–67) or Nmi (1–307) fused to either full-length Nmi or various portions of the Nmi protein. We found that Nmi (1–67) or Nmi (1–307) fused to either full-length Nmi or various portions of the Nmi protein.

25 interacting clones were identified from a screen of 500,000 cDNAs. Sequence analysis revealed that four of them were Nmi, demonstrating that the two-hybrid screen was working appropriately. Two clones contained the entire Nmi coding region, the third clone begins at Nmi amino acid 94, and the fourth at Nmi amino acid 144 (Fig. 1A).

Six interacting clones were derived from the IFP 35 gene (9). One of the clones was missing a large portion of the leucine zipper region (three leucines of five) suggesting that an intact...
L-Zip region is not required for the interaction in yeast (Fig. 1B). Each of the IFP 35 clones contained the two NID repeats previously identified in Nmi and IFP 35. To delineate the region of Nmi important for this interaction, we used Gal4 DNA-binding domain fusions to truncated Nmi constructs expressing NID1/2, NID1, or NID2. Two hybrid interactions between these constructs and the clone of IFP 35 missing three of five leucines in the L-Zip region were ascertained and revealed that both NID repeats are necessary for strong interactions between the proteins (Table I). Intact amino terminus of neither protein are required for the interaction because the amino-terminal truncation of IFP 35 interacts strongly with Nmi amino acids 92–307 (Table I).

We then determined whether the amino-terminal truncated IFP 35 could homodimerize as has been described for the intact protein (9, 18). The truncated L-Zip IFP 35 clone was used to construct a Gal4DBD domain fusion to test interaction with Act-L-Zip truncated IFP 35. A strong interaction was observed, suggesting that an intact L-Zip region is not necessary for homodimerization (Table I). Our yeast two-hybrid data suggest that IFP 35 can homodimerize with itself and heterodimerize with Nmi through NID repeats.

**Interaction of Nmi and IFP 35 in Mammalian Cells**—Two hybrid interactions were confirmed by studies in mammalian cells. Both Nmi and IFP 35 are cytoplasmic proteins that are found in punctate granular structures when cells are induced with IFN (11, 19). To determine whether they reside in the same structure, immunofluorescence microscopy with optical sectioning was performed to demonstrate co-localization. Jurkat cells were untreated or treated with IFN, fixed, and probed with antibodies against Nmi (rabbit polyclonal) and IFP 35 (mouse monoclonal) and secondary reagents. The cells not treated with IFN showed little staining for Nmi or IFP 35 (data not shown). In IFN-treated cells, IFP 35 and Nmi appeared in granular structures as described previously (Fig. 2, A and B). Dual exposures of Nmi (red) and IFP 35 (green) show that the majority of large granules stain yellow (Fig. 2, C and D), demonstrating that the proteins co-localize. Some of the smaller and fainter granules did not appear to co-localize (Fig. 2, C and D). IFP 35 and Nmi also co-localize in IFN-treated HeLa, SW 1573 WISH, and SK-N-SH cells (data not shown).

Co-immunoprecipitations were performed on extracts of transfected HeLa cells. HeLa S3 cells were transfected with an expression construct coding for FLAG-tagged Nmi or the vector alone. Monoclonal anti-FLAG antibody was used to precipitate FLAG-tagged Nmi, and monoclonal anti-FLAG and monoclonal anti-IFP 35 antibody were used for detection in Western blotting. IFP 35 was coprecipitated with FLAG-tagged Nmi, whereas the vector transfected cells showed no co-immunoprecipitation of IFP 35 (Fig. 3, A and B). Co-immunoprecipitations were also performed on extracts from untreated and IFN-treated Jurkat cells. Rabbit antisera directed against the first 67 amino acids of Nmi, termed anti-Nmi(1–67), was used for the immunoprecipitation, and anti-Nmi(1–67) and anti-IFP 35 were used for detection by Western blotting. Both Nmi and IFP 35 were detectable in the untreated samples, and they increased significantly in the IFN-treated samples (Fig. 3B, compare lanes 1 and 6). Preimmune serum did not immunoprecipitate Nmi or co-immunoprecipitate IFP 35 (Fig. 3B, lanes 4 and 9). Immune serum directed against Nmi(1–67) immunoprecipitated Nmi and co-immunoprecipitated IFP 35 (Fig. 3B, lanes 5 and 10). Note on the Western blot that rabbit anti-Nmi (1–67) does not detect IFP 35 because they do not share homology in the amino-terminal regions. Probing the immunoprecipitates with anti-IFP 35 revealed the presence of IFP 35 only when Nmi was immunoprecipitated with the immune serum. These results show that the association between Nmi and IFP 35 is not dependent on IFN treatment.

**Subcellular Distribution of Nmi and IFP 35 Changes with IFN Treatment**—We previously showed that Nmi appears cytoplasmic by immunofluorescence but that some Nmi protein fractionates with crude nuclear preparations after IFN treatment (11). We examined the subcellular fractionation profile of both Nmi and IFP 35 more carefully to determine whether they co-fractionated as would be expected for proteins in a complex. We fractionated Jurkat cells into nuclear, mitochondrial/lysosomal, microsomal, and S-100 fractions. Analysis of β-glucuronidase and succinate iodonitrotetrazolium violet reductase, which are markers for lysosomes and mitochondria, showed the enzyme activities in distinct fractions (not shown). 10 µg of protein from each sample was subjected to Western blotting with anti-Nmi, anti-IFP 35 and anti-tubulin antibodies as a control. Following IFN treatment, we found a significant increase of Nmi protein in mitochondrial/lysosomal and microsomal fractions (Fig. 4A). IFP 35 showed a similar pattern to that of Nmi both before and after IFN treatment (Fig. 4B). Tubulin was found mainly in the S-100 fraction, as expected (Fig. 4C).

**Nmi and IFP 35 Form a HMMC in Jurkat Cells**—IFP 35 is reported to be in a HMMC of 200–440 kDa and Nmi in a HMMC of approximately 300 kDa (10, 19). We reasoned that Nmi and IFP 35 would be in the same complex because the proteins co-localize and co-immunoprecipitate. We examined complex formation by native polyacrylamide gel electrophoresis. Supernatant fractions from Jurkat cells with and without

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**Table I**  
*Interaction between Nmi and IFP 35 in yeast two-hybrid assay*

<table>
<thead>
<tr>
<th>ACT-IFP35</th>
<th>Strong</th>
<th>None</th>
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<tr>
<td>ACT-IFP35*</td>
<td>Strong*</td>
<td>None</td>
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*IFP35 construct is missing 21 amino acids including three of five amino-terminal leucines.

*Interactions were scored using a lacZ reporter and were determined using color intensity.

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**Figure 2.** Nmi and IFP 35 speckles co-localize in mammalian cells. Jurkat cells were treated with 1000 units/ml of IFN-α for 18 h. They were fixed with 50% methanol, 50% acetone and probed with a rabbit anti-Nmi and mouse anti-IFP 35. IFP 35 is visualized in green (goat anti-mouse fluorescein isothiocyanate; A, C, and D), and Nmi is visualized in red (goat anti-rabbit Alexa594; B, C, and D); overlays of IFP 35 and Nmi are shown in C and D. Nuclei were stained with Hoechst (blue; D).
IFN treatment were prepared and analyzed by Western blotting after running 6 or 10% native polyacrylamide gels (n=4). As we previously reported, Nmi is found in a HMMC of approximately 300 kDa (Fig. 5A). The blot was stripped, shown to be free of signal, and reprobed with anti-IFP 35, which was found to be in the same molecular mass band with a similar shape when the films were superimposed (Fig. 5B). To rule out an artifact of blotting, a previously unprobed blot was cut in half, and one side was probed with anti-Nmi and the other with anti-IFP 35, which showed that each was in a HMMC. When the blots were aligned, the HMMC had the same mobility (data not shown). On the native gel blots of the Jurkat cell extracts, we found no evidence for free monomeric proteins for either Nmi or IFP 35, indicating that the vast majority of Nmi and IFP 35 were in a HMMC.

To show more definitively that both proteins are in the same complex, we treated Jurkat cell extracts (prepared from cells treated with IFN or untreated) with an antibody specific for Nmi or a preimmune serum obtained from the same rabbit prior to immunization. The proteins were then separated on native gels and probed with an anti-IFP 35 antibody. The preimmune antibody did not shift the IFP 35-containing complex, whereas the anti-Nmi antibody caused a large shift of the IFP 35 complex into two higher molecular mass bands (Fig. 6A). Using a chicken anti-Nmi antibody, we were able to demonstrate that the anti-Nmi antibody also supershifted the Nmi-containing complex as would be expected (Fig. 6B). These results imply that the vast majority of Nmi and IFP 35 are complexed with each other.

To confirm the native gel blots and the gel shift, we resolved the complex from Jurkat cells by FPLC. Fractions were screened by slot blotting, and those containing Nmi and IFP 35 proteins were subjected to Western blotting. We found that Nmi eluted in fraction 13 without IFN treatment but in fraction 12 after IFN treatment (Fig. 7). Similarly, IFP 35 eluted in fraction 13 without IFN treatment but in fraction 12 after IFN treatment (Fig. 7). As a control, the IFN-inducible protein MxA did not show a shift in the peak of the elution profile after IFN treatment. The altered elution profile suggests that some characteristic (composition or modification) of the Nmi/IFP 35 complex may change after IFN treatment, although this difference cannot be detected by native gel electrophoresis.
Nmi Inhibits Proteasome-mediated Degradation of IFP 35

**Fig. 6.** Nmi/IFP 35 complex can be supershifted by anti-Nmi antibody. Cytosolic extracts of Jurkat cells (with or without IFN treatment for 24 h) were prepared as described under “Materials and Methods,” treated with rabbit preimmune serum or rabbit anti-Nmi antibody, and separated on 6% nondenaturing polyacrylamide gels. A, probed with mouse anti-IFP 35 antibody. B, probed with chicken anti-Nmi. HMMC and shifted complex are indicated.

**Fig. 7.** Nmi and IFP 35 cofractionate by gel filtration chromatography. Cytosolic fractions of Jurkat cells minus and plus IFN were separated on a Superdex 200 HR 10/30 column using FPLC. Fractions were subjected to Western blotting with anti-Nmi, anti-IFP 35, and anti-MxA antibodies.

Nmi and IFP 35 Are Present in a High Molecular Mass Complex in Many Different Types of Cells—HMMC was also assessed in multiple other cell lines including HEK293EBNA (embryonal kidney), 041 (Li-Fraumeni fibroblast), SW620 (colon carcinoma), HT1080 (fibrosarcoma), and Raji (Burkitts leukemia) using native gel electrophoresis. HMMC containing Nmi was detected in each of the cell lines with or without IFN treatment (in SW620 cells not treated with IFN, Nmi complex could only be detected after prolonged ECL exposure) (Fig. 8). In HEK293EBNA cells, the Nmi HMMC appeared as a doublet with or without IFN treatment, and there was also a lower molecular mass band that represents free Nmi outside of the HMMC (Fig. 8). To determine the correlation with IFP 35 expression and complex formation, we probed a duplicate blot for the presence of IFP 35. We found IFP 35 in a HMMC of the same size as that seen in Jurkat cells in each of the cell lines except the HEK293EBNA where IFP 35 was almost undetectable (in SW620 cells not treated with IFN, Nmi complex could only be detected after prolonged ECL exposure) (Fig. 8). It is notable that the only cell line that has Nmi outside of the HMMC also contains very low levels of IFP 35, suggesting that higher levels of IFP 35 are required to enhance the amount of Nmi in a HMMC. Also notable is that Nmi or IFP35 complex did not appear inducible in either HEK293EBNA or HT1080 cells after IFN treatment. Probing the same samples for the IFN-inducible STAT1 protein showed that STAT1 was up-regulated in all IFN-treated cells (Fig. 8).

Co-transfection of Nmi and IFP 35 Leads to HMMC Formation—293T cells, which express little endogenous Nmi or IFP 35 (similar to the 293EBNA cells in Fig. 8), were transfected with HA-IFP35 or co-transfected with HA-IFP35 and Nmi.

Lysates were analyzed for complex formation using native gel electrophoresis and anti-IFP 35 antibody. In the absence of transfected Nmi, no HA-IFP 35 was found in a HMMC (Fig. 9A, lane 2); however, in the presence of Nmi, IFP 35 was found in a HMMC (Fig. 9A, lane 3). The size of the HMMC is consistent with that found in normal Raji cells induced with IFN (Fig. 9A, lane 1).

Complex formation after transfection of Nmi or IFP 35 or co-transfection with both was also demonstrated with an anti-Nmi antibody. In the absence of transfected Nmi, Nmi cannot be detected in a HMMC (Fig. 9B, lane 2). Co-transfection with both Nmi and IFP 35 leads to detectable complex formation (Fig. 9B, lane 3). Transfection with Nmi in the absence of IFP35 leads to an indistinct pattern, suggesting that Nmi may multimerize but cannot form a discrete complex when IFP 35 is not expressed (Fig. 9B, lane 4). The size of the HMMC appears to be the same as that of the endogenous complex found in Jurkat cells not stimulated with IFN (Fig. 9B, lane 1). These results suggest that co-transfection of Nmi and IFP 35 is sufficient to form a HMMC.

Stabilization of IFP 35 and Nmi by Complex Formation—An interaction between Nmi and IFP 35 may stabilize one or both proteins. To test this hypothesis, we co-transfected HEK293T cells with an HA epitope-tagged IFP 35 (expressed from a cytomegalovirus promoter) and an empty vector (CEP4) or with HA-IFP 35 and increasing amounts of a CEP4-Nmi. We found that the transfection with the Nmi-expressing vector significantly increased the levels of HA-IFP 35 compared with the empty vector (Fig. 10A, panel α-HA). Because HA-IFP 35 cannot be detected without Nmi transfection, pulse-chase experiments to determine the half-life of IFP 35 in the absence of Nmi could not be performed. It is unlikely that Nmi is acting at a transcriptional level or stabilizing proteins in general because it did not increase the amount of green fluorescent protein expressed from a cytomegalovirus promoter (Fig. 10A, panel α-GFP).

A similar experiment was done to determine whether IFP 35 expression can stabilize Nmi. Cells were co-transfected with Nmi and an empty vector (pDCR) or with Nmi and increasing amounts of pDCR-HA-IFP 35. We found that the transfection with IFP 35 expressing vector increased the levels of Nmi compared with the empty vector (Fig. 10B, panel α-Nmi). The increase in expression of Nmi was not as dramatic as the increase in expression of IFP 35 (Fig. 10A), suggesting that IFP 35 may be the more labile component of the complex. Transfection with IFP 35 did not increase the expression of co-transfected green fluorescent protein (Fig. 10B, panel α-GFP).
Nmi Inhibits Proteasome-mediated Degradation of IFP 35—HEK293 cells were transfected with combinations of CEP4 and pDCR, CEP4-Nmi and pDCR, CEP4 and pDCR-IFP 35, or CEP4-Nmi and pDCR-IFP 35 and selected with hygromycin and G418 for 2–3 weeks to establish long term cultures. Extracts of the pools were probed with anti-Nmi and anti-IFP 35 antibodies (Fig. 11A). Nmi was expressed in cells transfected with the combination of CEP-Nmi and pDCR and CEP-Nmi and pDCR-IFP. However, IFP 35 was detected only when pDCR-IFP 35 was co-transfected with CEP-Nmi (Fig. 11A). These results are consistent with the transient transfections showing that IFP 35 is unstable in the absence of Nmi.

To determine whether IFP 35 expression could be restored by coexpressing Nmi, we transiently transfected the HEK293 cells stably transfected with CEP4 and pDCR-IFP, HEK293-(CEP/pDCR-IFP 35), with CEP4-Nmi, control plasmids, and a deletion derivative of Nmi lacking the first 73 amino acids (NΔ73). Nmi and NΔ73 were expressed to similar levels, but increased amounts of IFP 35 were only detected when the cells were transfected with full-length Nmi (Fig. 11B). As a control, HEK293 cells stably transfected with CEP4 and pDCR vectors did not express IFP 35 after transient transfection with Nmi (Fig. 11B). To determine whether the amino terminus of Nmi is sufficient to stabilize IFP 35, we transfected various derivatives expressing the amino terminus of Nmi into the HEK293-(CEP/pDCR-IFP 35) cells. Derivatives expressing the first 68, 92, or 125 amino acids of Nmi did not stabilize IFP 35, demonstrating that the amino terminus of Nmi is necessary but not sufficient to stabilize IFP 35 (Fig. 11C). Furthermore, an Nmi derivative lacking both NID repeats, which are essential for the Nmi/IFP 35 interaction, did not stabilize IFP 35, suggesting that an interaction between the two proteins is necessary for the stabilization (Fig. 11C).

Because many unstable proteins are degraded by a proteasome-mediated pathway, we determined whether IFP 35 was degraded in a proteasome-mediated process. We treated cultures of HEK293(CEP/pDCR-IFP 35) with protease inhibitors MG-132, lactocystin, and chloroquine. IFP 35 expression increased significantly only after treatment with proteasome inhibitors MG-132 and lactocystin (Fig. 11D). Treatment of HEK293(CEP/pDCR) cells did not result in detectable endogenous IFP 35 protein (Fig. 11D). Therefore, when IFP 35 is
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expressed without concurrent expression of Nmi, it is rapidly degraded by a proteasome-mediated pathway.

DISCUSSION

Using the yeast two-hybrid system with a portion of Nmi containing both NID repeats as bait, we identified interacting clones that expressed Nmi or IFP 35. A strong interaction between Nmi and IFP 35 required both NID repeats. The interaction between Nmi and IFP 35 is significant because both Nmi and IFP 35 1) contain NID repeats, which mediate Nmi homodimerization (10); 2) are up-regulated by IFNs, with the majority of protein appearing in extranuclear cytoplasmic structures (11, 19); and 3) are components of high molecular mass complexes (10, 19).

We showed that Nmi and IFP 35 proteins interact in mammalian cells by co-immunoprecipitation and co-localization studies. The interaction does not appear to be dependent on treatment of cells with IFN because interactions can be demonstrated by co-immunoprecipitation and high molecular mass complex formation in the absence of IFN treatment of cells. Prior to IFN treatment, Nmi and IFP 35 are found primarily in the cytoplasmic (S-100) fraction, whereas after IFN treatment, there is a significant increase of the proteins in the mitochondrial/lysosomal and microsomal fractions. The association of Nmi and IFP 35 proteins with mitochondria/lysosomal and microsomal fractions does not imply a physical interaction of Nmi or IFP 35 with mitochondria, lysosomes, or microsomes but rather suggests that the fractionation properties of the proteins change after IFN treatment. Indeed, IFP 35 does not appear to associate with mitochondria, peroxisomes, endoplasmic reticulum, lysosomes, endosomes, or Golgi complex (19).

Nmi and IFP 35 associate into a HMMC, the Nmi/IFP 35 Complex (NIC), of approximately 300–400 kDa as demonstrated by native gel electrophoresis and gel filtration. NIC can be demonstrated with or without IFN treatment of cells that express Nmi and IFP 35 proteins. NIC can be reconstituted in HEK293 cells (expressing low or undetectable levels of endogenous Nmi and IFP 35) after transfection with Nmi and IFP 35 expression vectors. Our ability to reconstitute a complex indistinguishable from that seen in normal cells by co-expressing Nmi and IFP 35 suggests either that Nmi and IFP 35 are the only components of the NIC or that the other subunits are constitutively expressed. Interestingly, when Nmi is overexpressed without concomitant overexpression of IFP 35, it is found in a diffuse pattern rather than in a distinct band as demonstrated by native gel electrophoresis. These results imply that Nmi is associating with itself or numerous other cellular proteins but that IFP 35 is necessary for association of Nmi into a discrete complex.

Transient transfection of expression vectors for Nmi and IFP 35 demonstrated that Nmi and IFP 35 can co-stabilize each other through complex formation. IFP 35 appears to be the more labile of the two proteins. Although Nmi can be readily detected in transiently transfected and stably selected HEK293 cells in the absence of IFP 35 expression, IFP 35 can only be detected in both transient transfections and stably selected cells when Nmi is co-expressed. The inability to detect IFP 35 in cultures of cells transfected with an IFP 35 expression vector is due to degradation of the protein for the following reasons. First, transfection with an Nmi expressing vector restores IFP 35 expression. Second, a deletion analysis revealed that the amino terminus of Nmi is required but not sufficient to increase the level of IFP 35. Expression of a deletion derivative of Nmi lacking the NID repeats, which are necessary for Nmi to interact with IFP 35, also did not increase levels of IFP 35 protein, suggesting that Nmi has to bind to IFP 35 to stabilize it. Third, inhibition of the proteasome, but not other proteases, led to increased levels of IFP 35, suggesting that Nmi inhibits degradation of IFP 35 through a proteasome-mediated pathway. It is possible that the amino terminus of Nmi is required to physically impede the association of IFP 35 with the proteasomal degradation machinery. Thus, we have shown that Nmi and IFP 35 form a protein complex, that IFP 35 is a labile protein degraded in a proteasome-mediated process, and that a novel function of Nmi is to prevent IFP 35 degradation.

The association of Nmi and IFP 35 into a proteasome-resistant complex may serve to amplify the physiologic effects of IFN treatment. Because complex formation between Nmi and IFP 35 increases the amount of both proteins, it is likely that complex formation will potentiate the function of Nmi or IFP 35 (or both) rather than acting to sequester or down-regulate their function(s). Because IFP 35 appears to be extremely labile (often undetectable except after IFN treatment), we favor a model in which Nmi stabilizes IFP 35 and enhances IFP 35 function. This would be similar to the situation where elongin BC complex prevents proteasome-mediated degradation of von Hippel-Lindau tumor suppressor protein (20). While this manuscript was being revised, Zhou et al. (21) independently described the association of Nmi and IFP 35 into a complex. They also found that IFP 35 is phosphorylated and that complex formation correlates with IFP 35 dephosphorylation (21). The proteasomal degradation of many important cellular proteins including c-Myc, keratins, and Bcl-2 is modulated by their phosphorylation status (22–24). Therefore, IFP 35 levels and Nmi/IFP 35 complex formation and function may be regulated by both phosphorylation and proteasomal degradation.

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REFERENCES

Interferon-inducible Myc/STAT-interacting Protein Nmi Associates with IFP 35 into a High Molecular Mass Complex and Inhibits Proteasome-mediated Degradation of IFP 35

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