Interferon-α Induces Nmi-IFP35 Heterodimeric Complex Formation That Is Affected by the Phosphorylation of IFP35

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Nmi and IFP35 are interferon (IFN)-induced proteins. In cells treated with IFN-γ, Nmi enhances the association of transcription co-activator CBP/p300 with signal transducer and activator of transcription proteins, and IFP35 forms a high molecular weight cytosolic complex of unknown constituents. Here we show that Nmi and IFP35 co-immunoprecipitate with an anti-keratin 19 antibody, which is due to cross-reaction of the antibody with Nmi, and suggests an Nmi-IFP35 physical association. In support of this, Nmi and IFP35 co-immunoprecipitate using anti-Nmi and anti-IFP35 antibodies, manifest enhanced colocalization as determined by immunofluorescence staining of IFN-treated cells, and form heterodimers as determined by chemical cross-linking. Nmi and IFP35 are primarily cytosolic proteins, and their interaction is increased after IFN-α treatment of cells as early as 1 h after exposure. Sucrose gradient sedimentation and size fractionation showed a shift of Nmi-IFP35 heterodimers toward a heavier fraction (100–200 kDa) in IFN-α-treated cells. This dynamic complex formation is reversed by pretreatment with okadaic acid. Two-dimensional gel analysis indicates that the IFN-induced complex formation correlates with IFP35 dephosphorylation. Our data demonstrate Nmi-IFP35 cytosolic localization and heterodimerization, and an IFN-α-regulated molecular event in which Nmi and IFP35 participate, reversibly and by a dephosphorylation dependent fashion, in a 100–200-kDa molecular complex formation.

The early cellular responses to IFN,1 in terms of signal transduction and immediate gene expression, have been studied extensively (reviewed in Refs. 1 and 2). The essential role of the molecular signaling complex of IFN receptors/Janus kinases (JAKs)/STATs in signal transduction has been demonstrated by many studies including mouse knock-out experiments (3–7). In general, the IFN receptor-preassociated kinase JAKs are activated by IFN-dependent IFN-α/β receptor dimerization or IFN-γ receptor tetramerization. JAKs then initiate a tyrosine phosphorylation cascade within the signaling complex, resulting in activation of STATs through tyrosine phosphorylation-dependent dimerization (e.g. STAT1/2 heterodimers for IFN-α/β, and STAT1 homodimers for IFN-γ). Activated STATs are then rapidly translocated into the nucleus by unknown mechanism(s) and bind to specific DNA sequences, then activate transcription of early responsive genes (8, 9). The known crystal structure of a tyrosine-phosphorylated and DNA-bound STAT1 homodimer highlights the molecular detail by which STAT-DNA binding is controlled by the intermolecular regulatory interactions of phosphorylated tyrosine-Src homology 2 domains between two STATs (10). More recently, human cDNA microarray analysis of cells treated with IFN identified regulated gene expression patterns that had some similarities as well as differences depending on the IFN (α versus γ) (11). These studies of IFN signal transduction provide insight to our understanding of cellular events by which IFN exert their biological effects, including antiviral and antiproliferative effects, which have not been fully elucidated.

Although it is thought that the biological effects of IFN are likely carried out by their induced proteins, many IFN-induced proteins are poorly characterized, and no specific IFN-induced gene products have been linked directly to antiproliferative activity (1). Two independently identified IFN-induced proteins are Nmi (N-Myc-interacting protein) and IFP35 (IFN-induced 35-kDa protein). IFP35 was identified by differential screening of cDNA libraries from HeLa cells treated with IFN-γ (12). IFP35 contains a N-terminal leucine zipper domain, and is translocated into a 200–440-kDa complex with unknown constituents in cells exposed to IFN-γ (13). The transcription factor B-ATF associates with IFP35, as demonstrated by yeast two-hybrid screening and in vitro pull-down experiments (14). Human IFP35 is located within a 500-kilobase region of chromosome 17q21 which also includes the BRCA1 gene (15). Nmi was first cloned as a Myc-interacting protein by yeast two-hybrid screening and is located within human chromosome 22q13.3 where translocations have been reported in some human leukemias (16, 17). Nmi contains two Nmi-IFP35 homolog domains (18, 19), interacts with all STATs except STAT2, and augments IFN-γ signal transduction (20).

In this study we show that IFN-α induces Nmi-IFP35 association. Identification of this association occurred as part of experiments aimed at characterizing proteins that associate...
with the epithelial specific intermediate filament protein, keratin polypeptide 19 (K19), using an anti-K19 monoclonal antibody (mAb) termed 4.62. Two proteins with apparent molecular masses of 38 and 35 kDa co-purified with K19. Microsequencing of these two proteins showed that they correspond to Nmi and IFP35, respectively. Further characterization of Nmi and IFP35 and their apparent association with K19 supported the conclusion that anti-K19 mAb 4.62 recognizes Nmi upon IFN treatment of cells rather than that Nmi/IFP35 bind to K19. However, we demonstrate using several modalities that Nmi and IFP35 do associate physically and form a 100–200 kDa complex upon IFN stimulation of cells. This complex formation is dynamic and correlates with IFP35 dephosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Constructs, Antibodies, and Reagents—**Human HT29 (colon), Molt-4 (leukemic T lymphocyte), and Jurkat (leukemic T lymphocyte) cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The antibodies used were: anti-keratin 19 mAbs 4.62 (Sigma), A53-1I2.26 (B2/A2, NeoMarkers, Union City, CA), and K19 (gift from Robert Webster Labs, Inc., Bloomington, IN); anti-keratin 35 mAbs C8K5 (Sigma), anti-IFP35 rabbit polyclonal and mAb RB53 and mAb355, respectively; Ref. 12; anti-Nmi rabbit Ab (RbNmi, Refs. 18 and 19); anti-lamin B1, mAb (Zymed Laboratories Inc., South San Francisco, CA); and anti-Ep-CAM mAb I4D4 which recognizes an epithelial-specific cell surface cell adhesion molecule (22). Anti-STAT1 (sc-646), c-Myc (9E10), CBF (sc-319), and c-Fos (sc-253x) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Disuccinimidyl suberate (DSS) and immobilized protein A-Sepharose resin were supplied by Pierce Chemical Co. (Rockford, IL). In the in vitro TNT TT quick coupled transcription/translation system was from Promega (Madison, WI). The Nmi cDNA (in the pcDNA3.1/IGS plasmid) was supplied by Invitrogen (Carlsbad, CA). Bacterial expressed maltose-binding protein (MBP) fused IFP35 plasmid pMALc IFP35 was generated by in-frame fusion strategy using the mAb355 clone (26). Keratin polypeptides from Escherichia coli were diluted (1:100) with Nonidet P-40 buffer then incubated with 10 µg/ml anti-IFP35 or Nmi antibodies. Ultracentrifugation of molecular complexes, which contained most of these proteins, were subjected to immunoprecipitation, sucrose gradient centrifugation, or size fractionation by centrifugal filters. The centrifugal filters used for size fractionation were Centricon™ YM-100 which retain >90% of proteins larger than 97 kDa (Millipore, Bedford, MA). Cytosolic fractions (500 µl) were purified through the filter by centrifugation (1,000 × g, 30 min, 4 °C) and washed twice with 1 ml of PBS by spinning (1,000 × g, 30 min, 4 °C). Both flow-through (24.2 µl) and retained fraction (100 µl) were recovered. The flow-through was further concentrated with Centricon YM-10 (5,000 × g spinning, 2 h, 4 °C) and the volume of retained material was adjusted to be equivalent to the retained material from Centricon™ YM-100. Equal portions of the flow-through and retained material were analyzed by Western blotting using anti-Nmi and anti-IFP35 antibodies. For some experiments, a 10-ml continuous 0 to 50% sucrose gradient (in PBS with 5 mM EDTA) was prepared using a gradient mixer. An HT29 cell cytosolic fraction (1 ml) was layered at the top of the gradient, followed by ultracentrifugation (38,000 rpm, 18 h, SW40 rotor). Fractions (1 ml/fraction for fraction numbers 1–7 in Fig. 6 and number 1–6 in Fig. 7, 0.5 ml/fraction for numbers 8–14 in Fig. 6 and numbers 7–15 in Fig. 7) were collected after puncturing the tube bottom with a 20-gauge needle. The average fraction (which contained most of Nmi and IFP35) were subjected to DSS cross-linking followed by SDS-PAGE, transfer to polyvinylidene difluoride membranes, then blotted with anti-IFP35 or Nmi antibodies. Ultracentrifugation of molecular weight reference proteins (catalase, human IgA, myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor) and fraction collection were similarly done, followed by SDS-PAGE and Coomassie staining to determine the migration position of the protein standards.

**Immunofluorescence Staining—**HT29 cells were seeded in an 8-chamber slide (Nalge Nunc, Naperville, IL) (10,000 cells/well) allowed to adhere, then cultured in the absence or presence of IFN-α (1000 IU/ml, 16 h), then fixed with methanol (3 min, −20 °C). After blocking with PBS containing 2% bovine serum albumin and 0.2 mg/ml RNase A (blocking buffer) for 30 min, cells were co-incubated with mAb35 (1.50 dilution) and RbNmi (1:100 dilution) in blocking buffer for 30 min. After washing three times, cells were incubated with 10% goat serum in blocking buffer for 15 min followed by a 20-min incubation with Texas Red-conjugated goat anti-mouse antibody (1:100 dilution), fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:100 dilution), and Texas red DNA staining dye (1:10,000 dilution), and then washed five times with PBS. All incubations were done at 25 °C. Images were captured using a Nikon TE300 microscope coupled to a Bio-Rad MRC1024 confocal microscope.

**RESULTS**

Co-immunoprecipitation of Nmi and IFP35 with Anti-K19 mAb 4.62—As part of characterizing potential keratin-associated proteins in human colon HT29 cells, we used HT29 cell Nonidet P-40 lysates to immunoprecipitate different keratin pairs. Keratins, the intermediate filament proteins of epithelial cells, are expressed as obligate noncovalent heteropolymers (27). Of the >20-member keratin family, enterocyties express...
preferentially keratin (K) polypeptides 8 and 18 (K8/18) with variable levels of K19 and K20. Immunoprecipitation of K8/18 (preferentially) using mAb L2A1 or CK5 also co-precipitates the already characterized associated proteins 14-3-3 and heat shock protein 70 that associate with K18 and K8, respectively (Fig. 1A, lanes 1 and 2) (28, 29). However, immunoprecipitation of K8/19 using mAb 4.62 (which preferentially recognizes K19; Ref. 30) co-precipitated in addition to HSP70 which associates with K8) two proteins with an apparent molecular mass of 38 and 35 kDa (Fig. 1A, lane 3, asterisks highlight p38 and p35). The co-precipitated p38 and p35 appeared to associate specifically with K19 since they were absent in anti-K18 immunoprecipitates of HT29 cell lysates, and from mAb 4.62 precipitates from Molt-4 cell lysates (Fig. 1A, lane 4) that lack keratins (not shown).

Microsequencing of peptides isolated from purified p38 and p35 afforded the sequences NVTEIPDTLREDQM (p38) and TRGGGEVآلTVVPQG and IPLVRFRGHTQQDPEVPK (p35) with 100% identity with residues 160–173 of Nmi and residues 219–234 and 272–287 of IFP35, respectively. Since Nmi and IFP35 are IFN-induced proteins (12, 18), we tested and showed that Nmi/IFP35 association with K8/19 precipitates is far more easily detectable upon treatment of HT29 cells with IFN-α (Fig. 2B). Further confirmation of p38 and p35 identity was obtained by immunoblotting K8/18 and K8/19 precipitates with the antibody that recognizes K19 or 4.62 (30). Nmi co-precipitates with K8/19 only upon using mAb 4.62 but not B/A2 (Fig. 2A) or KA4 (not shown but identical to mAb B/A2 results). This suggested that Nmi and/or IFP35 may be recognized by mAb 4.62 after IFN-α induction, or that their association with K19 blocked recognition by mAb B/A2 and KA4. To distinguish between these possibilities, we examined mAb 4.62 and L2A1 immunoprecipitates, obtained from Jurkat cell lysates, by immunoblotting with anti-Nmi/IFP35 antibodies. Of note, Jurkat cells express Nmi and IFP35 but not keratins. Interestingly, mAb 4.62 but not L2A1 (Fig. 2B) or mAbs B/A2 or KA4 (not shown) co-immunoprecipitates IFP35 (Fig. 2D) and Nmi (not shown) from Jurkat cell lysates. Furthermore, blotting of K8/19 immunoprecipitates, that were obtained from IFN-α-treated Jurkat and HT29 cells, with mAb 4.62 showed that it also cross-reacts with Nmi but not IFP35 (Fig. 2C, lane 1). In addition, Nmi and IFP35 co-immunopurify together upon precipitation using mAb 4.62 regardless of whether HT29 cells (K19 containing) or Jurkat cells (K19 lacking) are used (Fig. 2C, lanes 1–6). The recognition of Nmi by mAb 4.62 was further confirmed by the immunoprecipitation of in vitro translated and [35S]-labeled Nmi protein.

FIG. 1. Anti-K19 mAb 4.62 co-precipitates interferon-induced Nmi and IFP35 proteins. Panel A, near confluent HT29 or Molt-4 cells were solubilized using Nonidet P-40 buffer then used for immunoprecipitation with antibodies that recognize K8/18 (L2A1) and CK5, K8/19 (4.62) preferentially, or with normal ascites (Ig) as control. Immunoprecipitates were analyzed under nonreducing conditions by SDS-PAGE then Coomassie staining. Two proteins (mass of 38 and 35 kDa) that co-purified with K19 using mAb 4.62 (lanes 3) are highlighted with asterisks. Note that small amounts of K18 and K19 co-precipitate with mAb 4.62 followed by analysis with SDS-PAGE and Coomassie staining. Panel B, HT29 cells were cultured with or without 1,000 IU/ml IFN-α for 16 h, then used to immunoprecipitate K8/18 with mAb L2A1 or K8/19 with mAb 4.62 followed by immunoblotting with anti-Nmi or anti-IFP35 antibodies. Aliquots of Nonidet P-40 solubilized cell lysates were also analyzed concurrently.
with mAb 4.62 but not with mAb B/A2 (Fig. 2D). Taken together, these results indicate that mAb 4.62 recognizes Nmi by molecular mimicry, after IFN-α exposure of cells, and that Nmi associates with IFP35.

**Nmi and IFP35 Are Primarily Cytosolic Proteins and Their Association Is Induced by IFN**—We examined the cellular localization of Nmi and IFP35 by immunoblotting of four HT29 cellular fractions: cytosol, membrane (Nonidet P-40 soluble), cytoskeletal/nuclear (Emp soluble), and insoluble pellet (Fig. 3, A and B). Most of Nmi and IFP35 are found in the cytosol, but a small population is present in the Nonidet P-40 and Emp fractions (Fig. 3A). IFN-α induced a slight increase of Nmi/IFP35 in all fractions (Fig. 3A), and in some experiments the level of increase was more dramatic (e.g. Fig. 1C and not shown). Lack of significant contamination of the different fractions was verified by immunoblotting using antibodies to membrane associated (Ep-CAM) and nuclear (lamin B$_1$) markers (Fig. 3A). Similar results were obtained by immunoprecipitation of K8/19 from the cytosolic and Nonidet P-40 fractions of HT29 cells (Fig. 3B). The preferential recognition of the Nmi-IFP35 complex by mAb 4.62 after IFN-α treatment, in a fashion that is independent of Nmi/IFP35 protein levels, suggests that a modification and/or a conformational change of Nmi allow recognition by mAb 4.62. Such recognition occurs within 1 h upon HT29 cell exposure to IFN-α and peaks by 24 h (Fig. 3C). Exposure to IFN-α beyond 24 h gives similar results to the 24-h time point (not shown).

**Nmi and IFP35 Form Heterodimers**—In order to examine the nature of Nmi and IFP35 association, we performed pull-down, co-immunoprecipitation, and chemical cross-linking experiments. The MBP-IFP35 fusion protein, but not MBP, binds with in vitro translated and $^{35}$S-labeled Nmi (Fig. 4A). In addition, Nmi and IFP35 co-immunoprecipitate with each other, when using anti-Nmi or anti-IFP35 antibodies, from IFN-α-treated (Fig. 4B, lanes 5–8) or untreated cells (Fig. 4B, lanes 1–4). However, Nmi-IFP35 association becomes recognized by mAb 4.62 preferentially in cells after IFN-α treatment (Fig. 4B, compare lanes 4 and 8). Chemical cross-linking of HT29 cell lysates followed by immunoprecipitation using anti-Nmi and anti-IFP35 antibodies (RbNmi and Rb35, respectively) then blotting with anti-IFP35 mAb35 demonstrated that Nmi and IFP35 form heterodimers (Fig. 4C, note generation of a 75-kDa species after cross-linking with simultaneous loss of the 35-kDa IFP35 band). We were unable to obtain meaningful data if an identical membrane to that used in Fig. 4C is blotted with RbNmi due to the high background that results from two antibodies of the same species (i.e. rabbit antibody for both immunoprecipitation and blotting, not shown). Colocalization of Nmi and IFP35 in cells was also demonstrated by indirect immunofluorescence staining of HT29 cells with RbNmi and mAb35. As shown in Fig. 5, there is partial colocalization of Nmi and IFP35 in HT29 cells, and the extent of colocalization increases after IFN-α treatment commensurate with increased Nmi/IFP35 levels. Of note, fluorescence staining is likely to underestimate the colocalization since most of Nmi and IFP35 are soluble cytosolic proteins (Fig. 3A).

**IFN Induces Nmi-IFP35 Heterodimer Formation and Participation in a 100–200-kDa Complex**—The cross-linking and co-immunoprecipitation results shown in Fig. 4 clearly indicated that Nmi and IFP35 form heterodimers. We further investigated the size of this complex and whether IFN-α plays a role in modulating its size. For this we first used size exclusion filters (i.e. Centricon™ YM-100) that allow 90% retention of proteins >97 kDa, in order to distinguish Nmi or IFP35 monomers and Nmi-IFP35 heterodimers from other >100 kDa complexes containing Nmi or IFP35. As shown in Fig. 6A, the amounts of Nmi and IFP35 from IFN-treated and untreated HT29 cells were relatively similar in the flow-through (lanes 1, 2, 7, and 8), but were significantly increased in the retained fractions (lanes 3, 4, 9, and 10). This indicates that IFN may induce the formation of a >100 kDa complex that contains Nmi and IFP35 with or without other possible proteins. To address this further, we subjected the HT29 cell cytosolic fractions (obtained from cells with or without IFN-α treatment) to sucrose gradient centrifugation, followed by DSS cross-linking. As shown in Fig. 6B, immunoblotting of the sucrose gradient centrifugation fractions with mAb35 and RhNmi indicated that Nmi and IFP35 are mainly in fractions 10 and 11, which correspond to molecular mass in the range of 100–200 and 50–100 kDa, respectively. The Nmi and IFP35 in fraction number 11 likely represents the heterodimeric form, while species in fraction 10 correspond to higher mass complexes. RhNmi antibody does not recognize the 75-kDa cross-linked species (Fig. 6B), although it is able to recognize it by immunoprecipitation (Fig. 4C). Notably, both Nmi and IFP35 signals in IFN-α-treated cells shifted toward the heavier fraction 10, which

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on July 26, 2018)
which results in IFP35 hyperphosphorylation without any apparent effect on Nmi phosphorylation (Fig. 7B, panels c and f). Exposure of IFN-treated cells to OA does not affect the IFP-Nmi association as determined by cross-linking using DSS (not shown) or by co-immunoprecipitation (Fig. 7C, lanes 3 and 4), but does affect the ability of the anti-K19 4.62 mAb to recognize Nmi-IFP35 (Fig. 7C, lanes 1 and 2). The IEF profile of Nmi-IFP35 that is co-immunoprecipitated using mAb 4.62 (from IFN-treated HT29 cells) is similar to that obtained by direct IFP35 immunoprecipitation (not shown). Taken together, these data suggest that the IFN-induced 100–200 kDa complex formation and the recognition of IFP-Nmi by anti-K19 mAb 4.62 are associated with IFP35 dephosphorylation but inhibited by IFP35 hyperphosphorylation.

**DISCUSSION**

**Anti-K19 mAb 4.62 Cross-reacts with Nmi Upon IFN Induction**—As part of studying K19-associated proteins using anti-K19 antibodies, we encountered the Nmi and IFP35 proteins which co-immunoprecipitated with K19 using mAb 4.62 (Fig. 1). However, further characterization indicated that this apparent trimeric complex is due to anti-K19 mAb 4.62 recognition of Nmi by molecular mimicry after IFN-α treatment (Fig. 2), with pull down of IFP35 due to its physical association with Nmi (Fig. 4). Amino acid sequence alignment of Nmi and K19 shows no significant similarity (also true for IFP35 and K19), which suggests that Nmi recognition by 4.62 is likely to be conformation-dependent rather than primary amino acid sequence-dependent. To that end, the epitope of mAb 4.62 on K19 was mapped to K19 amino acids 346–359 (30). Within this region, the maximal homology between Nmi and K19 is VDYDQRS (Nmi) versus DYDERSQRQ (K19). We examined the possibility that these homology regions may represent the 4.62 recognition site on Nmi by testing the peptides EDRVDYDQRS and DYRADSERQNE (from K19) for their ability to block binding of mAb 4.62 to Nmi and/or K19. However, neither peptide had any measurable effect (not shown), thereby suggesting that Nmi linear amino acid sequence 192–203 is not involved in mAb 4.62 recognition. The preferential recognition of IFN-α-induced and IFP35-bound Nmi by 4.62 makes this antibody a useful reagent for studying Nmi dynamics upon IFN-α activation, particularly in non-epithelial cells that normally do not express K19. Although mAb 4.62 can recognize Nmi without IFN-α activation, the efficiency and stoichiometry of recognition increase dramatically even when taking into consideration the induction of Nmi protein levels by IFN-α (e.g. Fig. 1C, lanes 3 and 4 versus lanes 5 and 6 and Fig. 3, A and B). Another important point to make is a cautionary one in that co-immunoprecipitation studies that conclude protein-protein associations should be done with more than one antibody. Cross-reaction of anti-keratin antibodies with other proteins has been reported previously. For example, a mouse anti-rat K19 antibody (termed mAb RK7) cross-reacts with cytocentrin, a 102-kDa protein that associates with the pole of the mitotic apparatus (31). In addition, an anti-p53 antibody can also recognize epidermal keratins depending on the keratinoocyte differentiation state (32).

**Nmi-IFP35 Heterodimeric and Complex Formation**—The recently described role of Nmi in cytokine signaling, by enhancing STAT-3/CBP/p300 complex formation in cells treated with interleukin-2 or IFN-γ, raises the possibility that it may represent a general mechanism in cytokine signal transduction (20). IFP35 may interact with the transcription factor B-ATF (14) and form part of a 200–440-kDa complex in IFN-γ-treated HeLa cells, although its function is unknown (13). Our identification of Nmi association with IFP35 may shed light on the function of Nmi and IFP35 since their association by definition...
links at least one aspect of their function together. We show here that Nmi and IFP35 are primarily cytosolic proteins, although they are present in small amounts in the membrane and cytoskeletal/nuclear fractions of HT29 cells (Fig. 3A). The interaction of Nmi and IFP35, as shown by 4.62 co-immunoprecipitation, appears in IFN-α-treated cells as early as 1 h (Fig. 3C) and is likely due to an IFN-α-regulated modification that allows recognition by mAb 4.62. The IFN-α-induced Nmi-IFP35 interaction peaks at 24 h (Fig. 3C) and lasts up to 72 h after IFN-α treatment (not shown), also in part due to the IFN-α-induced expression of both proteins. This suggests that an IFN-α-regulated Nmi-IFP35 interaction occur as part of post-translational and translational contributions. Isoelectric focusing analysis of Nmi/IFP35 obtained by mAb 4.62 (not shown), RhNmi (Fig. 7B, panel d), and Rh35 (Fig. 7B, panel a) immunoprecipitations show that Nmi and IFP35 have multiple isoforms (two for Nmi and 4 for IFP35) that are (in the case of IFP35) modulated upon IFN or IFN/OA exposure.

The evidence for Nmi-IFP35 heterodimerization was demonstrated by the generation of a 75-kDa mAb35-recognized band in Nmi and IFP35 immunoprecipitates of DSS cross-linked HT29 cell lysates (Fig. 4C). This 75-kDa species likely consists of IFP35 (35 kDa) and Nmi (38 kDa). The interaction of Nmi and IFP35 is also supported by in vitro binding of the MBP-IFP35 fusion protein with 35S-labeled in vitro translated Nmi (Fig. 4A), by their reciprocal co-immunoprecipitation from HT29 cell lysates (Fig. 4B), by their co-immunoprecipitation using mAb 4.62 (Fig. 1), and by their in vivo co-localization (Fig. 5). It is important to emphasize that Nmi-IFP35 heterodimers occur under basal conditions (e.g. Fig. 4, B and C) but heterodimer formation also increases as Nmi and IFP35 are induced by IFN-α. In addition, IFN-α results in higher order complex formation (Fig. 6) that includes Nmi-IFP35 in association with a molecular event that allows the anti-K19 antibody mAb 4.62 to recognize Nmi-IFP35 (Fig. 1).

The size fractionation results (Fig. 6A) indicate that a sub-

![Fig. 5. INF-α induces partial colocalization of Nmi with IFP35 as determined by immunofluorescence staining.](http://www.jbc.org/)

![Fig. 6. INF-α induces Nmi and IFP35 complex formation. Panel A, cytosolic fractions of HT29 cells (with or without 1,000 IU/ml IFN-α treatment for 16 h) were obtained as described in the legend to Fig. 3. The fractions were allowed to pass through Centricron YM-100 filters by centrifugation. Aliquots from the flow-through (FT) and retained (Ret) subfractions, along with input cytosol prior to subfractionation (Input), were separated by SDS-PAGE followed by immunoblotting with RhNmi (anti-Nmi) or mAb35 (anti-IFP35) antibodies. Note the shift in intensity of the Nmi and IFP35 signals in the retained fractions (+ versus − IFN-α) as compared with the input and flow-through samples that gave more similar + IFN versus − IFN-associated signals. Panel B, cytosolic fractions of HT29 cells (with or without 1,000 IU/ml IFN-α-treatment for 16 h) were subfractionated over a sucrose gradient as described under “Experimental Procedures.” All subfractions were analyzed for the presence of Nmi/IFP35 and only subfractions 8–13 had any measurable Nmi/IFP35 (not shown). Subfractions (numbers 8–13) were further divided into two parts, one of which was cross-linked using DSS (as described in the legend to Fig. 4). The cross-linked and uncross-linked subfractions were analyzed by Western blotting with RhNmi (anti-Nmi) or mAb35 (anti-IFP35) antibodies. Asterisks highlight the cross-linked and migration-shifted IFP35 products. The anti-Nmi antibody does not recognize the cross-linked Nmi-containing product(s) by immunoblotting. The ranges of molecular sizes for each fraction are: numbers 8, >300 kDa; 9, 150–300 kDa; 10, 100–200 kDa; 11, 50–100 kDa; 12, 20–50 kDa; 13, <20 kDa as determined by processing of molecular reference markers under identical conditions.)
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However, the possibility of Nmi-IFP35 heterotetramer formation upon IFN-α stimulation cannot be ruled out. Given that IFP35 is found in a 200–440-kDa molecular complex in HeLa cells (13) and that Nmi associates with STATs and CBP/p300 in peripheral blood lymphocytes (20), it is possible that STATs, CBP/p300, or other reported Nmi and IFP35-interacting proteins such as Myc, Fos, and Jun may be involved in the observed 100–200-kDa Nmi-IFP35 complex in HT29 cells. Immunoprecipitation of HT29 cell (with or without IFN-α treatment) lysates (with or without DSS cross-linking) with RbNmi or Rb35 followed by immunoblotting with antibodies to STAT1, c-Myc, CBP, and c-Fos, and vice versa, failed to identify any associations between these proteins and Nmi/IFP35 (not shown).

An important feature of Nmi-IFP35 interaction is its regulation by IFN-α. The Nmi and IFP35 binding, as examined by RbNmi and mAb35 immunoblotting of mAb 4.62 immunoprecipitates with HT29 cell lysates, is not induced by insulin-like growth factor-1, hepatocyte growth factor, and epidermal growth factor (not shown). The molecular mechanism underlying the IFN-α-induced 100–200-kDa complex formation appears to involve IFP35 dephosphorylation (Fig. 7). Inspection of the IFP35 protein sequence (12) shows at least 7 Ser/Thr potential phosphorylation sites (based on proximity to Pro and Lys/Arg residues) whose in vivo identification may shed light on the precise regulation and function of the IFN-induced complex described herein. The IFN-α-regulated Nmi-IFP35 complex formation is likely to play a role in IFN-α signaling or cellular response(s), and links at least some aspects of Nmi and IFP35 functions together. Further characterization of the Nmi-IFP35 interaction and complex formation, and their regulation, should provide insight into the cellular response to IFN-α.

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