Subcellular localization and mechanisms of nucleocytoplasmic distribution of p202, an interferon-inducible candidate for lupus susceptibility

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Abstract Increased expression of p202 (52 kDa), an interferon (IFN)-inducible murine protein, in splenic cells (B- and T-cells) derived from female mice of the lupus-prone strains is correlated with increased susceptibility to develop systemic lupus erythematosus. However, the molecular mechanisms remain unclear. Our previous studies have indicated that, in IFN-treated fibroblasts, p202 is detected both in the cytoplasm and in the nucleus. Moreover, in the cytoplasm, a fraction of p202 associates with a membranous organelle. Here we report that, in the cytoplasm, a fraction of p202 associated with mitochondria. Additionally, we found that the constitutive p202 is primarily detected in the cytoplasm. Remarkably, the IFN treatment of cells potentiated nuclear accumulation of p202. Our observations are consistent with the possibility that IFN signaling regulates p202 levels as well as its nucleocytoplasmic distribution. These observations will serve as a basis to elucidate the molecular mechanisms by which p202 contributes to lupus susceptibility.

Key words: Interferon; p202; Mitochondrial targeting; Lupus; Green fluorescence protein

1. Introduction

Interferon (IFN)-inducible p202 (52-kDa) is a member of the p200 protein family [1–5]. The family includes murine proteins, such as p202a (referred here as p202), p202b, p203, p204, and D3, and human proteins, such as IFI 16, MNDA, and AIM2 [2,3]. Proteins in the family share a partially conserved 200-amino-acid-long repeat (either a-type or b-type) [2]. Additionally, proteins, such as p202 [6], IFI 16 [7], and MNDA [8] are known to form homo- and heterodimers, raising the possibility that homo- and heterodimerization may be important for their cellular localization.

Proteins, such as p203, p204, IFI 16, and MNDA are known to contain a nuclear localization signal (NLS) and are shown to be primarily nuclear [3]. In contrast, AIM2 and p202 proteins do not contain a NLS [4,5]. Consistent with the lack of a NLS, AIM2 was detected primarily in the cytoplasm in transfected cells [9]. Interestingly, in IFN-treated murine AKR-2B fibroblasts, p202 is detected both in the cytoplasm and in the nucleus [10]. Moreover, in the cytoplasm, a fraction of p202 is associated with a fast-sedimenting ($20\,000 \times g$) membranous organelle [4,10], the identity of which remains unknown.

Increased expression of p202, p204, and IFI 16 is shown to retard cell proliferation [3,5]. Although, it remains to be determined how p200 family proteins inhibit cell growth, the ability of p202 (and other proteins in the family) to bind several proteins [5], including the transcription factors, makes it conceivable that the p200 proteins act as scaffolds to assemble large protein complexes, which may be important in the regulation of transcription of numerous genes.

Generation of mice congenic for the *Nba2* (derived from the NZB strain of mice) interval on C56BL/6 (B6) background, coupled with gene expression analyses, has identified p202 as a candidate for lupus susceptibility [11]. Studies also showed that increased expression of p202 in splenic cells (both B- and T-cells) correlated with splenomegaly and autoantibody production in female mice [11].

Remarkably, generation of NZB mice that are deficient in the IFN-α/β receptor did not result in a significant reduction in p202 levels in the spleens of old mice [12]. However, deficiencies in IFN type I signaling reduced lupus-like disease in these mice [12]. Although, these observations are consistent with the possibility that expression of the *Ifi202* gene can be regulated independent of type I IFN signaling in spleen cells, these observations also raised the possibility that the IFN signaling may be needed for functional 'activation' of p202. This prompted us to investigate whether type I IFN signaling has any effect on the nucleocytoplasmic distribution of p202. Here we report that, in the cytoplasm, p202 associated with mitochondria. Importantly, we found that treatment of cells with type I IFN potentiated nuclear accumulation of p202. These novel observations will serve as a basis to identify the molecular mechanisms by which p202 contributes to lupus susceptibility in certain strains of mice.

2. Materials and methods

2.1. Cell culture and transient transfections

The NIH 3T3 mouse fibroblasts (purchased from American Type Culture Collection) were grown on sterile glass cover slips in six-well plates. Subconfluent cultures of cells were transfected with the indicated plasmid using Ca-phosphate transfection kit (from Gibco-BRL). After 36–40 h of transfections, glass cover slips were mounted on a glass microscopic slide in phosphate-buffered saline and screened under the microscope for transfected cells exhibiting green fluores-

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cence protein (GFP) green autofluorescence (using a blue filter). When indicated, cells were incubated with MitoTracker Red (oxidized; purchased from Molecular Probes) as suggested by the supplier. Mitochondrial red autofluorescence was visualized under the microscope (Zeiss, Axioplan) using a green filter.

2.2. Indirect immunofluorescence

Mouse embryonic fibroblasts (MEFs), derived from the B6.Nba2 congenic strain of mice [11], were grown on glass cover slips, as described above. Subconfluent cultures of MEFs were either left untreated or treated with universal IFN-α (1 000 U ml^{-1}) for 24 h. Cells were fixed with methanol and p202 was detected by incubation with anti-p202 goat antibodies (Santa Cruz cat # sc-6054) as described previously [13]. These antibodies detect p202a better than p202b protein. The anti-goat secondary antibodies conjugated to rhodamine allowed the detection of anti-p202. Cells were examined under the microscope (Zeiss, Axioplan) using the green filter as described above.

2.3. Plasmids

Plasmids, allowing expression of p202, fused with GFP, were generated by polymerase chain reaction (PCR) amplification of 202 cDNA sequence, either corresponding to the full-length p202 or a desired protein segment in p202, such as p202(32–445) or p202(1–32). The PCR fragments were ligated directly into the NT-GFP fusion TOPO TA vector (using a kit purchased from Invitrogen, Carlsbad, CA, USA) to generate plasmid GFP-p202 (full length). Alternatively, the PCR fragment was ligated into the CT-GFP fusion TOPO TA cloning vector to generate plasmids encoding either p202(33–445)-GFP or p202(1–32)-GFP. The PCR inserts in expression vectors were sequenced for the generation of the correct open reading frame for the fusion protein.

2.4. Immunoblotting

Total cell extracts from subconfluent cultures of MEFs (at passage 6 or below) and from NIH 3T3 cells were prepared and analyzed by immunoblotting as described previously [10]. Polyclonal antibodies to p202 [10] or p204 [13] have been described.

3. Results and discussion

3.1. In the cytoplasm, p202 is targeted to mitochondria

Because immunostaining of cells for p202 localization indicated a punctate staining in the cytoplasm [10], a characteristic of membranous subcellular organelle [10], we analyzed the amino acid sequence of p202, using the recently available PSORT (prediction of protein sorting signals and localization sites in amino acid sequences) program, which can predict subcellular localization of a protein [14]. This analysis uncovered a potential mitochondrial targeting sequence in the N-terminus of p202 with a prediction (21.7%) for mitochondrial localization (see [14] for details). Therefore, using GFP fusion proteins, we investigated whether, in the cytoplasm, a fraction of p202 could be targeted to a membranous subcellular organelle. As shown in Fig. 1 (top right panel), expression of GFP-p202, but not GFP protein alone (top left panel), in NIH 3T3 cells gave punctate autofluorescence in the cytoplasm. Because the N-terminus of p202 contains a potential mitochondrial targeting sequence, we investigated whether de-

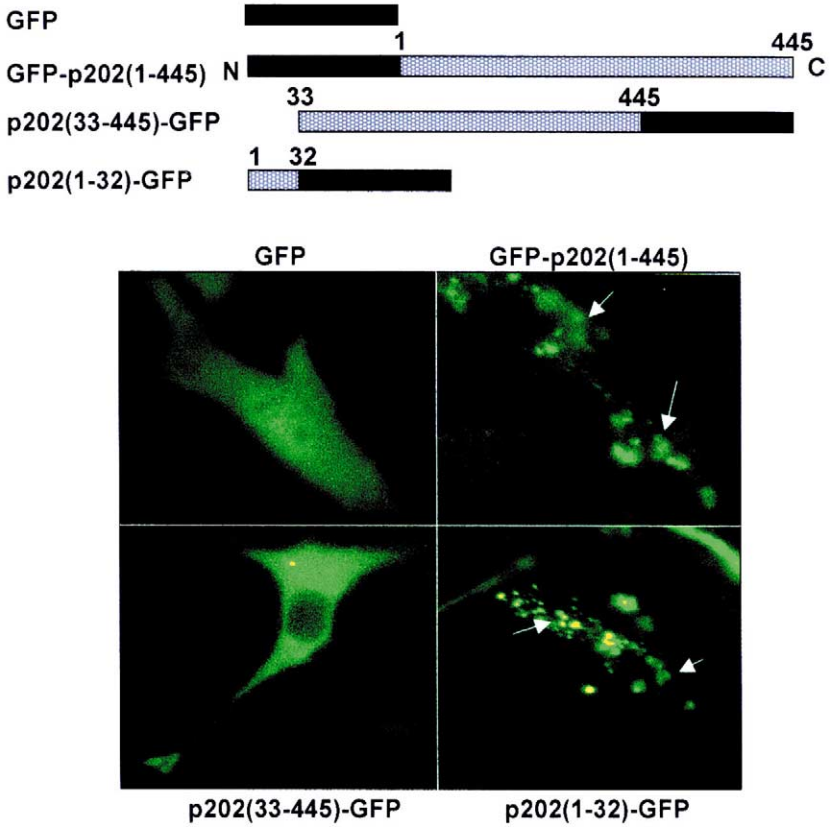

Fig. 1. In the cytoplasm, p202 associates with a membranous organelle. Upper panel: Schematic representation of the GFP fusion (the N- or C-terminal fusion) with the full-length p202 or its indicated segments. The numbers of the N- and C-terminal aminoacyl residues in the p202 segments are indicated above the line. Lower panel: NIH 3T3 cells (grown on glass cover slips) were transfected with plasmid allowing expression of GFP (top left panel), GFP-p202(1–445) (top right panel), p202(33–445)-GFP (bottom left panel) or p202(1–32)-GFP (bottom right panel). After 42–46 h of transfections, cells were examined for the expression of GFP or GFP fusion proteins (by autofluorescence using the blue filter) and its subcellular localization. The white arrows indicate the punctate green autofluorescence in the cytoplasm.

letion of this sequence could change the subcellular localization of p202. As shown in Fig. 1 (bottom left panel), expression of the N-terminal truncated p202(33–445)-GFP resulted in loss of cytoplasmic punctate autofluorescence. Importantly, expression of p202(1–32)-GFP fusion protein in which the N-terminal p202 (amino acids 1–32) was fused with GFP resulted in punctate autofluorescence in the cytoplasm. These observations indicated that the N-terminus of p202 contributes to the targeting of GFP-p202 fusion protein to a membranous subcellular fraction in the cytoplasm.

Next, we tested whether p202 is targeted to mitochondria. For this purpose, we expressed GFP-p202 in NIH 3T3 cells and labeled mitochondria with MitoTracker red. As shown in Fig. 2 (left panel), labeling of cells with MitoTracker gave punctate red autofluorescence in the cytoplasm. Interestingly, the green GFP autofluorescence was also detected in some of the same cytoplasmic subcellular organelles, suggesting that GFP-p202 was colocalized with the MitoTracker. Together, these observations indicated that a fraction of p202 associates with mitochondria in the cytoplasm.

3.2. IFN treatment potentiates nuclear accumulation of p202

We have reported previously that p202 levels are very low in murine AKR-2B fibroblasts [10]. However, IFN treatment of these cells results in several fold induction of p202. Interestingly, the induced p202 is first detected in the cytoplasm, and after a delay of about 30 h, it is detected in the nucleus [10].

Because splenic cells derived from B6Nba2 congenic strain of mice, but not the parental B6 strain of mice, express relatively high levels of p202 (but not p204) [11], we tested

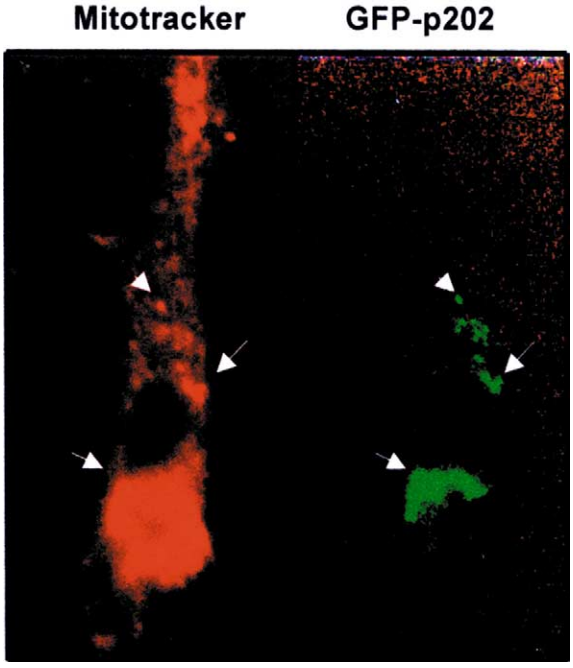

Fig. 2. GFP-p202 colocalizes with MitoTracker. NIH 3T3 cells were transfected with a plasmid allowing expression of GFP-p202 fusion protein. After 42 h of transfections, cells were briefly incubated with MitoTracker red as described in Section 2. Cells were examined for the localization of the GFP-p202 and the MitoTracker using the blue and green filters, respectively. The white arrows indicate colocalization of the red and the green punctate autofluorescence in the cytoplasm.

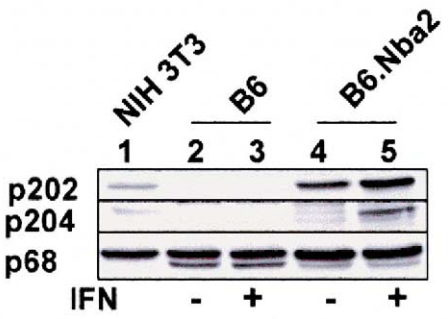

Fig. 3. MEFs derived from the B6.Nba2 congenic strain of mice express high constitutive levels of p202. Cultures of MEFs derived from the B6 (lanes 2 and 3) or B6.Nba2 (lanes 4 and 5) strain of mice were either left untreated (lanes 2 and 4) or treated with IFN-α, as described in Section 2. Total extracts were prepared from cells and analyzed by immunoblotting using antiserum to p202 or p204. As a positive control, we also loaded extracts from IFN-treated NIH 3T3 cells (lane 1), which express p202 and p204. Levels of p68 protein served as control for equal protein amounts [10].

whether MEFs derived from this lupus-prone strain of mice also express high levels of p202. As shown in Fig. 3, basal levels of p202 were readily detectable in MEFs derived from the B6.Nba2, but not B6, strain of mice (compare lane 4 with 2). As expected from our previous studies [5,10], IFN treatment of MEFs derived from B6.Nba2, but not B6, strain resulted in further increases in the levels of p202 (compare lane 5 with 4). As a control, we also compared basal and IFN-induced levels of p204 in these cells. Consistent with our earlier observations [11], basal levels of p204 were not detectable in MEFs derived from the B6 or B6.Nba2 strain of mice. However, IFN treatment of MEFs resulted in induction of p204 in MEFs derived from the congenic strain (compare lane 5 with 4). These observations indicated that MEFs derived from the B6.Nba2 strain of mice in culture express high levels of constitutive p202, but not p204.

Availability of MEFs from the B6.Nb2 strain of mice, which express high constitutive levels of p202, allowed us to determine the distribution of constitutively expressed p202 between the cytoplasm and nucleus. As shown in Fig. 4A, in these cells the bulk of p202 was detected in the cytoplasm. Furthermore, in the cytoplasm, staining was punctate, indicating an association of p202 with a membranous organelle. Interestingly, treatment of cells with IFN-α resulted in nuclear accumulation of p202, and the bulk of the p202 was detected in the nucleus (Fig. 4B). These observations indicated that in the absence of IFN signaling, the bulk of endogenous p202 is detected in the cytoplasm and the type I IFN treatment of cells potentiates nuclear accumulation of p202. Similarly, treatment of cells expressing GFP-p202 with IFN (type I) also resulted in nuclear accumulation of GFP-p202 (Fig. 4C,D). However, treatment of cells expressing GFP alone did not result in accumulation of GFP in the nucleus (data not shown).

Localization of the bulk of p202 or GFP-p202 in the cytoplasm is consistent with the lack of a NLS in the p202 sequence [2,4,5,10]. Notably, p202 does not contain a nuclear export signal [15]. Together, these observations indicate that the cellular distribution of p202 between the cytoplasm and the nucleus may depend on p202-binding proteins. Because p202 can form heterodimers with other p200 family proteins, such as p204 [6], it is conceivable that IFN-induced levels of

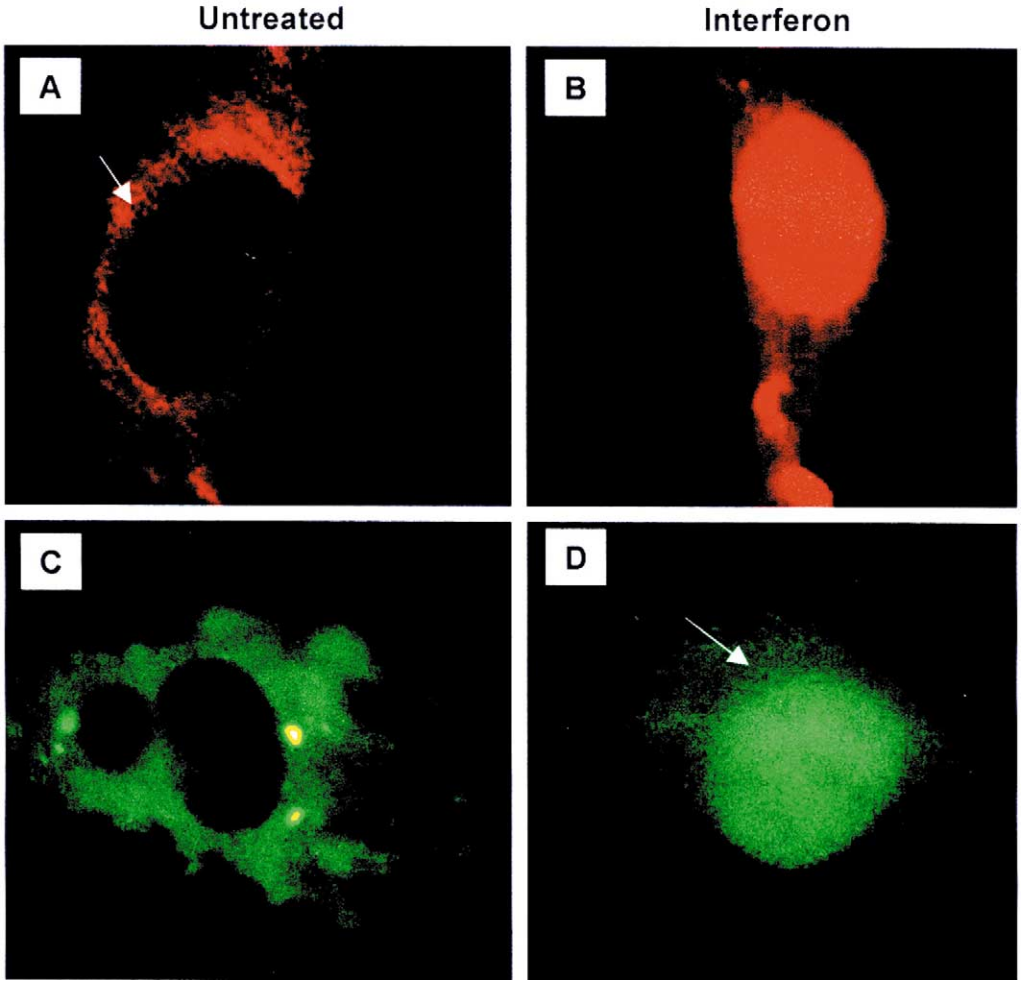

Fig. 4. IFN treatment potentiates nuclear accumulation of p202. Upper panel: MEFs derived from the B6.Nba2 congenic strain were grown on glass cover slips as described in Section 2. Cells were either left untreated (A) or treated with IFN-α (B) for 24 h. Cells were fixed and immunostained with antibodies to p202 as described in Section 2. The white arrow indicates the nucleus. Lower panel: NIH 3T3 cells (grown on glass cover slips) were transfected with plasmid allowing expression of GFP-p202. After 24 h of transfections, cells were either left untreated (C) or treated with IFN (D). Cells were examined for the expression of GFP and its localization. The white arrows indicate the nuclear accumulation of GFP-p202.

p204 contribute to nuclear localization of p202. This could account for potentiation of nuclear accumulation of p202 after IFN treatment of MEFs (see Fig. 4). Consistent with this idea, it has been shown that both p202 and p204 exhibit a similar nucleocytoplasmic distribution during differentiation of C2C12 myoblasts in vitro [16]. Because expression of both p202 and p204 in cells may depend on mouse strain [5], it is likely that the nucleocytoplasmic distribution of p202 depends on mouse strain.

Several transcription factors are known to bind p202 [5]. Therefore, it is likely that binding of these factors to p202 contributes to its nucleocytoplasmic distribution. Consistent with this idea, it has been reported that treatment of cells with PMA, which increases the nuclear translocation of p50 (a subunit of the transcription factor NF-κB), potentiated nuclear accumulation [17].

Our previous observations have revealed that p202 levels increase in cells growth arrested in the G_0/G_1 phase of cell cycle after serum starvation and serum growth factors negatively regulate p202 expression [18]. These observations raise the possibility that nucleocytoplasmic distribution of p202 could be regulated by cell cycle. Therefore, further work will be needed to determine whether the nuclear localization of p202 is cell cycle dependent.

The protein p202 is a phosphoprotein [10]. Moreover, p202 binds to several proteins, such as retinoblastoma protein (and other pocket protein) [19], whose phosphorylation is regulated in a cell cycle dependent manner. Therefore, it is conceivable that phosphorylation of p202 and/or its binding proteins, such as pRb, could also contribute to its nucleocytoplasmic distribution. Further work is in progress to test this possibility.

Increased levels of p202 inhibit p53-mediated apoptosis [20]. However, the molecular mechanisms remain unclear. Localization of p202 to mitochondria in our studies described here raises the possibility that p202 could inhibit p53-mediated apoptosis, which is transcription independent [21], by interacting with p53. Consistent with this possibility, we note that p202 binds to p53 in vitro and in vivo (D'Souza et al., unpublished data). Further work will be needed to determine whether a fraction of p202 colocalizes with p53 at the mitochondria.

In summary, our observations described here will facilitate elucidation of the molecular mechanisms by which the IFN-

inducible p202 contributes to increased lupus susceptibility in certain strains of mice.

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