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Cytoplasmic localization of the interferon-inducible protein that is encoded by the *AIM2* (absent in melanoma) gene from the 200-gene family

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Abstract While interferons (IFNs) (α, β and γ), a family of cytokines, have the ability to exert the growth-inhibitory effect on target cells, the molecular mechanism(s) by which IFNs inhibit cell growth remains to be identified. Because IFN-inducible 'effector' proteins mediate the biological activities of IFNs, characterization of IFN-inducible proteins is critical to identify their functional role in IFN action. One family (the 200-family) of IFN-inducible proteins is encoded by structurally related murine (*Ifi202a, Ifi202b, Ifi203, Ifi204* and *D3*) and human (*IFI16, MNDA* and *AIM2*) genes. The proteins encoded by genes in the family share a unique repeat of 200-amino acids and are primarily nuclear. The *AIM2* gene is a newly identified gene that is not expressed in a human melanoma cell line. Here we report that AIM2 is estimated to be a 39 kDa protein and, unlike other proteins in the family, is localized primarily in the cytoplasm. Interestingly, overexpression of AIM2 in transfected cells retards proliferation and, under reduced serum conditions, increases the susceptibility to cell death. Moreover, AIM2 can heterodimerize with p202 in vitro. Together, these observations provide support to the idea that AIM2 may be an important mediator of IFN action.

Key words: Interferon; 200-Protein family; AIM2; Cell growth

1. Introduction

Interferons (IFNs) (α, β and γ) are a family of cytokines with the ability to exert the growth-inhibitory effect on target cells [1,2]. While the IFNs are being used in the clinic to treat various human diseases, including cancers, the molecular mechanisms by which IFNs exert the growth-inhibitory effect remain to be identified. Because IFN-inducible 'effector' proteins mediate the biological activities of the IFNs [1], the identification of the functional role(s) of IFN-inducible proteins in IFN action is likely to improve our ability to use IFNs effectively.

One family of IFN-inducible proteins (the 200-family proteins) includes p202a (previously p202), p202b, p203, p204 and D3 [3–6]. These proteins are encoded by six or more structurally related murine genes, which form a tight cluster (the 200-gene cluster) in the q21–q23 region of chromosome 1 [7,8]. The family also includes three highly homologous human proteins: IFI 16, myeloid nuclear differentiation antigen (MNDA) and AIM2 (Absent In Melanoma) [3,6]. The proteins in this family share a unique repeat of 200-amino acids. While p202, p204 and IFI 16 proteins contain two repeats of 200-amino acids (one **a**-type and one **b**-type), other proteins in the family, including AIM2, contain only one repeat (**a**-type or **b**-type). Additionally, a short sequence (MFHATVAT), which is found in 200-amino acid repeats of some 200-family proteins (for example, two repeats of p202 and **a**-type repeat of AIM2), was found to be sufficient for homo- and heterodimerization of the proteins in vitro [9]. While p203, p204, IFI 16 and MNDA proteins have a consensus sequence for nuclear localization and are primarily nuclear [3,6], p202 does not have a consensus sequence for nuclear localization and is detected both in the cytoplasm and nucleus [10].

The *AIM2* gene was identified as one of several genes not expressed in a human tumorigenic melanoma cell line, but was expressed upon introduction of a normal chromosome 6, thus resulting in the suppression of the malignant phenotype [11,12]. Interestingly, the steady-state levels of the AIM2 RNA increase in HL-60 cell line after IFN-γ treatment [12], raising the possibility that the IFN-induced levels of AIM2 protein may have a role in IFN action. The isolation and sequencing of the AIM2 cDNA revealed that it contains an open reading frame of 1032 bp corresponding to a putative polypeptide of 344 amino acids with a predicted molecular weight of 39.4 kDa [12]. Additionally, AIM2 protein contains only one repeat (**a**-type) of 200-amino acids and shares strong sequence similarity with 200-family proteins (for example, IFI 16, MNDA and p204) [6,12]. However, AIM2 protein remains to be characterized with respect to its biochemical and biological functions.

To identify the possible functional role of AIM2 in IFN action, we have characterized the AIM2 protein. Here we report that AIM2 is estimated to be a 39 kDa protein. In transfected cells, AIM2 protein is primarily localized in the cytoplasm. Interestingly, the ectopic expression of AIM2 in murine fibroblasts retards cell proliferation. Additionally, overexpression of AIM2 in murine fibroblasts, under reduced serum conditions, increased the susceptibility to cell death. Furthermore, AIM2 binds to the full-length p202, but not to a deletion mutant of p202 lacking the heterodimerization motif, in vitro. Together, these observations provide support to the idea that AIM2 may exert the growth-inhibitory effect of IFN.

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2. Materials and methods

2.1. Cell lines

The AKR-2B (originally a gift from Dr. H.L. Moses, Vanderbilt University, Nashville, USA) was grown in Dulbecco's modified Eagle medium (with high glucose) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO_2. If so indicated, cells were incubated in growth medium supplemented with 0.1% FBS for the indicated time.

2.2. Plasmids and generation of stable cell lines

To express AIM2 protein (tagged with V5 and 6×His epitopes towards the C-terminus of AIM2) in vitro or in transfected cells, plasmid pcDNA3.1/AIM2 was used. The addition of two epitope tags to AIM2 protein that is expressed from the above plasmid results in addition of 33 amino acid residues. Murine AKR-2B cells were transfected with plasmid pcDNA3.1/AIM2 or, as a control, with equal amounts of empty vector (pcDNA3.1). The transfected cells were selected in Zeocin (200 μg per ml) for Zeocin-resistance. After 2 weeks, Zeocin-resistance colonies (>300) were pooled and the cultures were maintained in 100 μg per ml of Zeocin. For experiments, cells were cultured without Zeocin.

2.3. In vitro transcription and translation

The RNA encoding AIM2 protein was transcribed in vitro (from plasmid pcDNA3.1/AIM2) using T7 RNA polymerase and translated in rabbit reticulocyte lysate either supplemented with [^{35}S]methionine or cold methionine as described previously [13]. To detect radiolabeled AIM2 in rabbit reticulocyte lysates, the lysates were subjected to SDS–PAGE followed by fluorography. The unlabeled AIM2 was detected by immunoblotting using monoclonal anti-V5 epitope antibody (purchased from Invitrogen Corp., Carlsbad, CA, USA).

2.4. Cell cycle analyses

To determine cell cycle distribution, cells (1×10^6) were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol and stored at −20°C. For staining, the fixed cells were washed with PBS and incubated for 5 min at room temperature in buffer containing 0.2% Triton X-100, 1 mM EDTA in PBS. The cells were stained for 1 h at room temperature with buffer containing 50 μg per ml propidium iodide and 50 μg per ml of RNase. The samples were subjected to flow cytometry (FACStar PLUS, Becton and Dickinson) and analyzed using ModFit LT version 2.0 software (Variety Software House, Topsham, ME, USA).

2.5. Immunodetection of AIM2

To detect AIM2 levels by immunoblotting, transfected AKR-2B cells were collected from plates in PBS and resuspended in modified RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (leupeptin, 50 μg per ml; pepstatin A, 50 μg per ml; PMSF, 1 mM), and incubated at 4°C for 30 min. Lysates were sonicated briefly before centrifugation at 14 000 rpm in a microfuge for 10 min. Supernatants were collected and equal amounts of proteins were processed for immunoblotting as described previously [10]. The monoclonal anti-V5 epitope antibody conjugated to horseradish peroxidase (HRP) and monoclonal anti-His epitope antibody conjugated to HRP were purchased from Invitrogen Corp. (Carlsbad, CA, USA) and used at 1:5000 dilution.

To test if AIM2 is phosphorylated in vivo, AIM2 was immunoprecipitated using anti-V5 or anti-His epitope antibodies from lysates prepared from AKR-2B cells transfected with AIM2 expression plasmid. The immunoprecipitated proteins were immunodetected by a mixture (1:1) of biotinylated phosphoserine and phosphothreonine-specific monoclonal antibodies.

2.6. Binding of AIM2 to glutathione-Sepharose beads loaded with GST fusion proteins

Aliquots (10 μl) from the reaction mixtures in which ^{35}S-labeled AIM2 protein had been translated in vitro were incubated with washed glutathione-Sepharose beads (loaded with the indicated GST fusion protein) in binding buffer (50 mM HEPES, 150 mM NaCl and 1% NP-40) at room temperature for 30 min. The beads were washed five times in binding buffer and the bound proteins were released by boiling in protein sample buffer and subjected to SDS–PAGE followed by fluorography.

3. Results and discussion

3.1. AIM2 is estimated to be a 39 kDa protein

As predicted from its sequence [12], the full-length AIM2 protein, when translated in a rabbit reticulocyte lysate from a transcript transcribed in vitro, migrated between 47.5 and 32.5 kDa size protein markers (Fig. 1, lanes 2 and 6) (it is of note that large amounts of protein in rabbit reticulocyte lysate made it difficult to estimate the size of AIM2 protein). While the origin of two additional protein bands seen in lanes 2 and

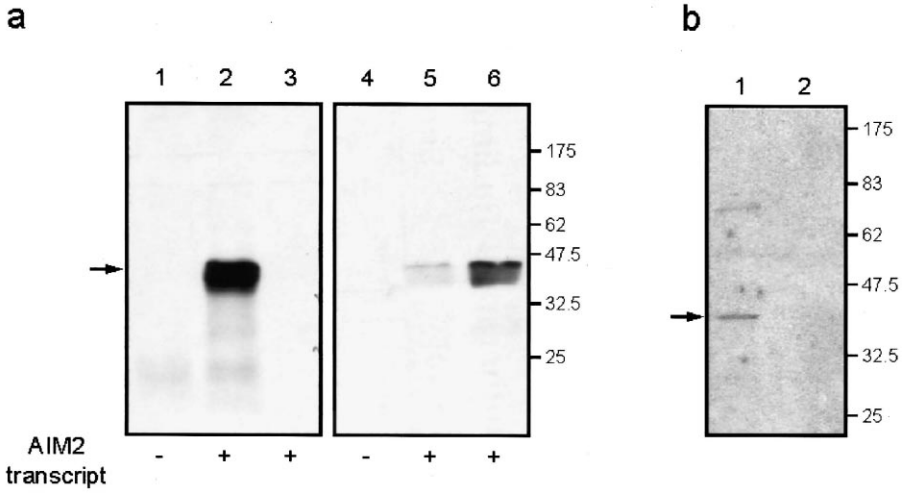

Fig. 1. AIM2 is a 39 kDa protein. AIM2 protein was translated (from a transcript generated in vitro) in rabbit reticulocyte lysate supplemented with either [^{35}S]methionine (lanes 1, 2, 4 and 5) or cold methionine (lanes 3 and 6). The translation reaction mixtures without any transcripts added (lanes 1 and 4) or after addition of the AIM2 transcript (lanes 2, 3, 5 and 6) were analyzed by SDS–PAGE followed by either fluorography (lanes 1–3) or immunoblotting using monoclonal anti-V5 epitope antibodies. The full-length AIM2 protein band is indicated by an arrow. Total cell extracts prepared from murine AKR-2B cell lines stably transfected with the empty vector (lane 2) or AIM2 expression plasmid (lane 1) were analyzed for the expression of AIM2 by SDS–PAGE followed by immunoblotting using monoclonal anti-V5 epitope antibodies. The full-length AIM2 protein band is indicated by an arrow.

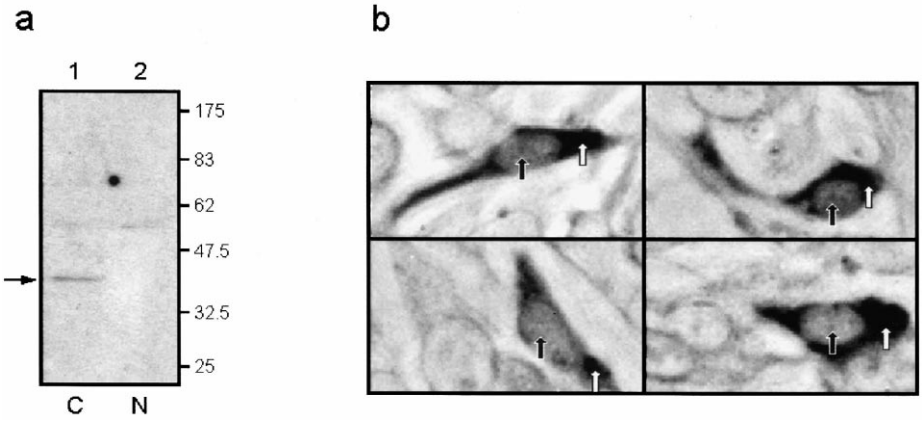

Fig. 2. In transfected cells, AIM2 is primarily localized in the cytoplasm. (a) Murine AKR-2B cells stably transfected with AIM2 expression plasmid were fractionated into the cytoplasmic (lane 1) and nuclear fraction (lane 2). The fractions containing equal amounts of protein were analyzed by SDS–PAGE followed by immunoblotting using monoclonal anti-V5 epitope antibodies. The AIM2 protein band is indicated by an arrow. (b) Murine AKR-2B cells were transiently transfected with AIM2 expression plasmid and the transfected cells were immunostained using monoclonal anti-V5 epitope antibodies conjugated to HRP. The cytoplasmic location of AIM2 is indicated by a white arrow.

6 (Fig. 1a) remains to be established, it is possible that these two fast migrating protein bands correspond to the translation products whose translation is initiated at the internal initiator codons in AIM2 RNA. Consistent with this prediction, we find that the AIM2 cDNA sequence contains two potential internal translational initiation sites to initiate the translation of AIM2 protein (which could give rise to two additional translation products of the sizes seen in lanes 2 and 6).

Because in vivo posttranslational modifications of proteins can contribute to the molecular weight, we determined the size of AIM2 protein that was expressed in mammalian cells. As shown in Fig. 1b (lane 1), AIM2 protein in extracts prepared from transfected cells migrated as approximately 39 kDa protein, providing support to the idea that in vivo posttranslational modifications do not apparently contribute to a significant change in the mobility of the AIM2 protein.

Because the amino acid sequence of AIM2 contains a potential consensus sequence for phosphorylation by several protein kinases [12], we tested if AIM2 is phosphorylated in vivo. In cycling AKR-2B cells, we could not detect phosphorylation of Ser and Thr residues in AIM2. Further work will be needed to determine if AIM2 is phosphorylated on tyrosine residues.

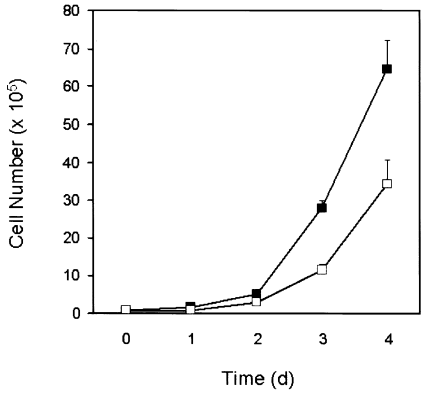

Fig. 3. Overexpression of AIM2 protein retards proliferation of murine AKR-2B fibroblasts. Cells stably transfected with the empty vector (black squares) or AIM2 expression plasmid (white squares) were seeded in a six well plate (in duplicates) in growth medium and cells were harvested at the indicated time to count cells. Each sample was counted three times. The standard error bars are indicated.

3.2. Cytoplasmic localization of AIM2

The analyses of the AIM2 amino acid sequence by the PSORT II program [14], to predict the subcellular location, revealed that AIM2 is predicted to be primarily localized in the cytoplasm and not in the nucleus. Additionally, it is predicted to localize in the cytoplasmic membranous organelles, including mitochondria. Therefore, we chose to test if AIM2 is a cytoplasmic protein. To determine the subcellular location of AIM2, we stably overexpressed AIM2 in murine AKR-2B cells and performed cell fractionation into the cytoplasmic and nuclear fraction. As shown in Fig. 2a, AIM2 was detected only in the cytoplasmic fraction and not in the nuclear fraction. To confirm this observation, we transfected AIM2 encoding plasmid into murine AKR-2B cells and stained the transfected cells with anti-V5 antibodies. As shown in Fig. 2b, AIM2, when overexpressed in transfected cells, is primarily localized in the cytoplasm. Together, these observations indicate that AIM2 is the first protein in the 200-family proteins to be localized primarily in the cytoplasm. Based on its cytoplasmic localization, it is conceivable that AIM2 may have functions different than the other members of the family.

3.3. Overexpression of AIM2 inhibits cell growth

Because 200-family proteins, including p202, participate in

Table 1
Constitutive overexpression of AIM2 in AKR-2B cells and cell cycle distribution

		G_0/G_1	S	G_2/M
Control	Exp. 1	27.0	63.6	9.3
Control	Exp. 2	37.1	47.5	15.3
AIM2	Exp. 1	35.8	54.2	9.9
AIM2	Exp. 2	35.6	51.2	13.1

AKR-2B cells, stably transfected with pCDNA3.1 (control) or pCDNA3.1/AIM2 (AIM2) plasmid, were subjected to flow cytometry as described in Section 2. The percentages of cells in a particular phase of the cell cycle were determined for each sample (in duplicates) in two experiments and an arithmetic mean of two readings is shown for each experiment.

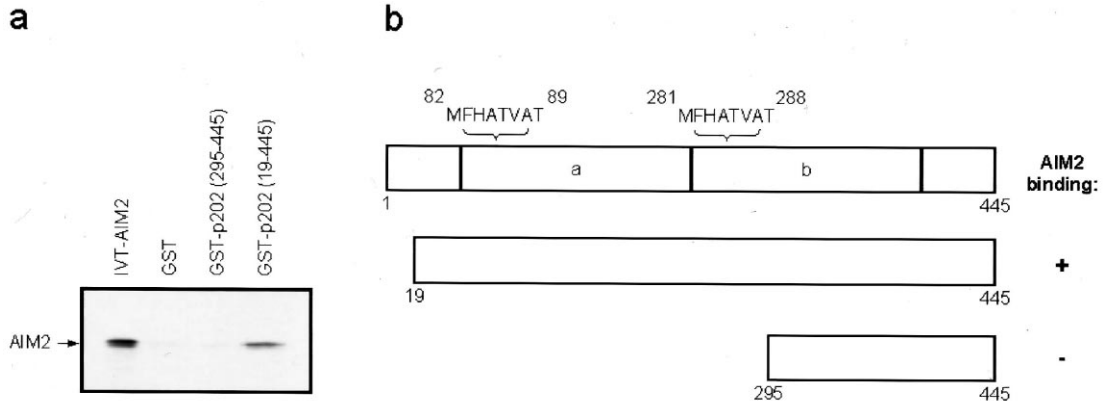

Fig. 4. AIM2 translated in vitro binds to GST–p202 fusion protein. (a) Binding of AIM2 to GST–p202. Affinity chromatography. In vitro-translated labeled AIM2 was incubated with glutathione-Sepharose beads loaded with GST (lane 2), GST–p202(295–445) (lane 3) or GST–p202(19–445) (lane 4). The bound proteins were analyzed by SDS–PAGE followed by fluorography. As a control, an aliquot (2 μl) of translation reaction mixture was loaded in lane 1. The full-length AIM2 protein band is indicated by an arrow. (b) Schematic representation of p202, its segments and their abilities to bind to AIM2 in affinity chromatography. The thin vertical lines indicate the borders of the 200-amino acid repeat regions **a** and **b**. The inserts indicate the positions of the conserved sequence MFHATVAT. The numbers of the N- and C-terminal aminoacyl residues of these sequences are indicated. +, binding; −, no detectable binding.

the regulation of cell proliferation [6,15,16], we tested if expression of AIM2 affects cell proliferation. For this purpose, murine AKR-2B cells (we chose these cells because in these cells the ectopic expression of p202, a 200-family protein, was found to inhibit cell growth) [15,16] were either transfected with a plasmid (pCDNA3.1/AIM2) encoding AIM2 or, as a control, empty vector (pCDNA3.1). The transfected cells were selected for Zeocin-resistance. Interestingly, transfection of AIM2 encoding plasmid did not result in a significant decrease in colony formation as compared to cells transfected with the empty vector (control cells) (376 colonies versus 400 colonies, respectively). However, we found that cells which

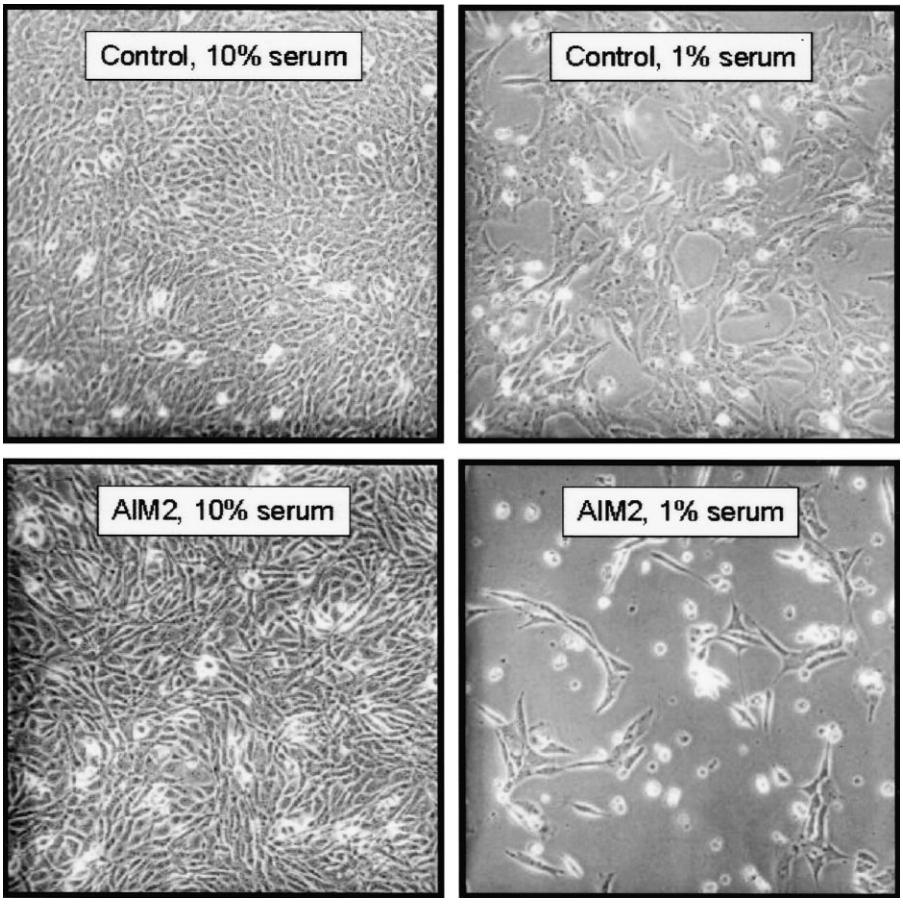

Fig. 5. Overexpression of AIM2 in murine AKR-2B cells increases the susceptibility to cell death under reduced serum conditions. Cells stably transfected with vector (control cells) or AIM2 expression plasmid (AIM2) were incubated in growth medium supplemented with either 10% or 0.1% FBS for 2 days. After 2 days, cells were photographed for alterations in their morphology and a decrease in cell number.

overexpressed AIM2 exhibited a decreased growth rate as compared to the control cells (Fig. 3). These cells did not appear to accumulate in a particular phase of the cell cycle in two independent experiments (see Table 1). This observation is consistent with our previous observation in which we found that overexpression of p202 also decreased the cell growth rate without accumulation of cells in a particular phase of the cell cycle [16].

While it has been reported that the growth suppression by the 200-family proteins requires the presence of at least two 200-amino acid repeats (AIM2 has only one repeat) [17], it is interesting that overexpression of AIM2 in AKR-2B cells slows cell proliferation. Although it remains to be seen how AIM2 regulates cell growth, our observations raise an interesting possibility that the 200-family proteins with only one repeat of 200-amino acids can also negatively regulate cell growth.

3.4. AIM2 can heterodimerize with p202

Because the 200-amino acid repeat in AIM2 protein contains the conserved sequence MFHATVAT, which is sufficient for heterodimerization of p202 with other proteins in the family [9], we hypothesized that AIM2 regulates cell growth by binding to other proteins in the family. Therefore, we tested if AIM2 can bind to p202. As shown in Fig. 4, the full-length p202, but not the N-terminal truncated p202, which lacks the dimerization motif (MFHATVAT) in p202, selectively bound to in vitro-translated AIM2. This observation is consistent with the possibility that binding of AIM2 to p202 depends on the presence of the sequence MFHATVAT. It is of note that substitution of the His residue in the sequence MFHATVAT by Phe in p202 repeats abrogates the ability of p202 to interact with 53BP protein and inhibit cell growth [18]. While the significance of binding of AIM2 to p202 remains to be explored, this observation raises the possibility that AIM2 functionally interacts with p202 and other proteins to exert the growth regulatory effects of IFN.

3.5. Overexpression of AIM2, under reduced serum conditions, increases the susceptibility to cell death

Because decreased levels of p202, under reduced serum conditions, were found to increase the susceptibility of AKR-2B cells to cell death by apoptosis [19], we tested if overexpression of AIM2 (which can bind to p202 in vitro) has any affect on cell death. As shown in Fig. 5, overexpression of AIM2 in AKR-2B cells, under reduced serum conditions, increased the susceptibility to cell death. While it remains to be seen how AIM2 influences apoptosis, it is tempting to speculate that interaction of AIM2 with p202 may overcome p202's ability to prevent apoptosis under reduced serum conditions [19]. Further work is needed to examine this possibility.

p202a (previously p202) is one of the most characterized proteins in the family with respect to its biochemical and cell growth regulatory functions and has a clear potential to participate in the regulation of the cell cycle [15,20], differentiation [21] and apoptosis [19]. p202 exerts some of its growth-inhibitory effects, in part, by binding and modulating the activity of known growth regulatory proteins. These proteins include the transcription factors (for example, E2F, AP-1, MyoD and myogenin) and members of the 'pocket' family of proteins (for example, retinoblastoma tumor suppressor protein) [3,6]. However, it remains to be seen how AIM2 exerts its growth regulatory effects.

AIM2 is a new member of the 200-gene family [6,12]. It was identified as one of several genes not expressed in a human tumorigenic melanoma cell line, but expressed upon introduction of normal chromosome 6, resulting in the suppression of the malignant phenotype. Our observations described herein that overexpression of AIM2 in murine fibroblasts retards cell proliferation provide support to the notion that AIM2 can negatively regulate cell growth. Additionally, our observation that in transfected cells AIM2 is primarily localized in the cytoplasm and not the nucleus raises an interesting possibility that in cytoplasm, AIM2 interacting proteins, such as p202, may functionally interact with AIM2.

Our observations reported here will facilitate the identification of the functional role of AIM2 in cell growth regulation. Further studies are in progress to examine the possibility if the lack of AIM2 expression contributes to tumorigenesis.

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