The antiproliferative activity of the murine interferon-inducible Ifi 200 proteins depends on the presence of two 200 amino acid domains

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Abstract Interferon-inducible proteins, p200, have a modular organization consisting of one (p203) or two (p202 and p204) 200 amino acid motifs, designated as type *a* or *b* domains. The relationship between this domain organization and the antiproliferative activity was investigated by generating a hybrid protein with the 204 *a* domain upstream from the 203 *b* domain. This 204*a*/203*b* protein inhibits the proliferation of transfected cells, delays G_0/G_1 progression into S phase following serum restimulation, and inhibits the E2F-mediated transcriptional activity. These results demonstrate for the first time that both *a* and *b* domains are needed for inhibition of proliferation by the Ifi 200 proteins.

Key words: Interferon; Ifi 200 protein; p203; p204; p204*a*/p203*b* hybrid protein; Antiproliferative activity

1. Introduction

Interferons (IFNs) are proteins involved in host resistance against infections and tumors through their exertion of antimicrobial, immunomodulatory and cell growth regulatory activities [1–3]. Following their binding to specific cell surface receptors, they induce the expression of a defined set of genes that elicit a number of cellular responses [4–6]. In vivo and in vitro studies have demonstrated that IFNs inhibit the growth of both normal and transformed cells by activating multiple pathways, namely induction of G_0/G_1 arrest, lengthening of S phase, oncogene and cyclin down-regulation, maintenance of pRb hypophosphorylation and inhibition of E2F activities [7–15]. However, the precise molecular mechanisms that are regulated by IFNs and responsible for these effects are still poorly defined.

The IFN-inducible (Ifi) 200 protein family is one of the few examples of a clear link between inhibition of cell proliferation and activation of specific IFN-induced proteins [16,17]. It is encoded by a cluster of structurally related genes located in the q21–23 region of the murine chromosome 1, that are transcriptionally activated following IFN treatment [18]. Three human genes (MNDA, IFI16 and AIM2) located on human chromosome 1 within a linkage group highly conserved between mouse and human [19–21] share amino acid similarity with the murine proteins and are regarded as their human homologues [22]. The common features of all these proteins are: (1) a modular organization consisting of one or two 200 amino acid long conserved motifs, designated as the type *a* and type *b* domains; (2) nuclear translocation upon IFN treatment.

The best defined member of this family, p202, is a 52 kDa phosphoprotein [23]. Its constitutive expression in transfected AKR-2B and L929 cells inhibits colony formation, whereas in NIH3T3 cells it delays their exit from G_0/G_1 phase following serum starvation [24,25]. Consistent with its growth regulatory activity, p202 binds the retinoblastoma tumor suppressor protein family (pRb) and the transcription factors E2F-1 and E2F-4 [26–28]. Moreover, upon direct binding, p202 modulates the activity of several transcription factors, namely NF-κB p50 and p65, AP-1, c-Fos and c-Jun, p53 and MyoD [24,29,30]. Since all these proteins play some roles in the regulation of cell growth, this modulation may account for p202 growth inhibitory functions, although a formal functional evidence for this activity has not yet been provided.

p204 is a 72 kDa protein that undergoes phosphorylation and translocates partly into the nucleus upon IFN treatment. In cell focus assays, it inhibits the colony formation of normal cells and arrests their progression from G_0/G_1 to the S phase [31,32]. Moreover, high levels of p204 expression impair normal embryo development, since total body overexpression of p204 is compatible with development to the four-cell stage, whereas no viable animals with an intact copy of the transgene are obtained [31]. These results show that p204 overexpression inhibits cell growth, but do not explain the molecular mechanisms responsible for this activity.

We have recently cloned a 3.8 kb cDNA corresponding to the murine 203 IFN-inducible mRNA, another member of the Ifi 200 gene family [33]. The 203 cDNA encodes a 48 kDa nuclear protein structurally similar to p202 and p204, but with only a type *b* domain. Comparison of p203 amino acid sequence with that of the other members of the family revealed that p203 is most similar to 204. In p203, the carboxy-terminal type *b* domain is similar (81% of the residues are identical) to that of p204, and despite the absence of the seven amino acid repeats in the NH_2-terminal segment of p204, about half of the 100 residues of the two NH_2-terminal sequences are identical [33]. Like p202 and p204, p203 can be induced by IFN-α in different cell lines regardless of their histological origin, whereas its expression in vivo seems to be more tissue-specific and dependent on the host genotype compared to p204 [34,35].

To investigate whether the 203 gene, like 202 and 204, functions as a negative regulator of cell proliferation, p203 was overexpressed in NIH3T3 or murine embryo fibroblast cells. Cell focus assays and analysis of the cell cycle progression demonstrated that it is not endowed with growth inhibitory activity. Since p203 has only the type *b* domain, we have now investigated the relationships between the domain organ-

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ization of Ifi 200 proteins and their antiproliferative activity on a hybrid protein generated by inserting the 204 *a* domain upstream from the 203 *b* domain. Here we show that this 204*a*/203*b* protein, like p204, inhibits the proliferation of transfected cells, delays G_0/G_1 progression into S phase following serum restimulation, and inhibits the activity of E2F, as measured by the inhibition of transcription of a reporter gene. By contrast, a p204 deletion mutant, obtained by removing the *b* domain and defined as pΔ*b*204 failed to exert any of these activities.

Taken as a whole, these results demonstrate for the first time that both *a* and *b* domains are needed for inhibition of proliferation by the Ifi 200 proteins.

2. Materials and methods

2.1. Cells and culture conditions

NIH3T3 (murine fibroblasts) from American Type Culture Collection (ATCC) and B6MEF cells (embryonic fibroblast cell line derived from C57BL/6 mice and immortalized through several passages in culture) were grown as monolayers in DMEM (Gibco/BRL) supplemented with 10% calf serum (Gibco/BRL; NIH3T3) or 10% fetal calf serum (Gibco/BRL; B6MEF). All media were supplemented with glutamine, penicillin and streptomycin (Gibco/BRL).

Cells were arrested in G_0/G_1 by allowing them to grow to about 60% of confluence and then incubating for 50 to 60 h in medium containing 0.5% serum. They were stimulated to reenter the cycle by replating at subconfluent density in DMEM containing 10% serum.

2.2. Interferon

Human recombinant IFN-α A/D, used as substitute for murine IFN-α, is a hybrid molecule composed of residues 1–62 from human IFN-α A and residues 64–166 from human IFN-α D. Its antiviral activity is similar on human, bovine, rat, mouse and feline cells [36]. On murine L929 fibroblasts, its specific activity is 4×10^8 IU/mg protein. The IFN stock was a generous gift from Michael Brunda (Hoffman, LaRoche).

2.3. Plasmids

Plasmid pRcRSV204 (1–632) was constructed by ligation of the blunt-ended 2060 bp *Eco*RI-*Xba*I fragment from pSVK3/204, containing the full-length 204 cDNA, into the blunt-ended *Hin*dIII site of the expression vector pRcRSV (Invitrogen) [31].

Deletion construct pRcRSVΔ*b*204 (1–466) (Fig. 1) was constructed from pRcRSV204 by removing the *b* domain (466–632) using the internal restriction sites *Nde*I and *Xba*I. After agarose gel electrophoresis, the termini of the largest DNA segment were filled using the Klenow fragment of *Escherichia coli* DNA polymerase I, and ligated with T4 DNA ligase.

Plasmid pRcRSV203 (1–408) was constructed by cloning the 203 cDNA [32] into the *Spe*I site of pRcRSV.

Plasmid pRcRSV 204*a*/203*b* (Fig. 1) was constructed by first obtaining the *a* domain (231–431) from 204 cDNA by PCR amplification with specific primers (forward: 5′-GAAATCACAACCCCAGAATCAG-3′; reverse: 5′-GCTTGATGAAACTGTGTTCTCC-3′). The corresponding 600 bp band was then gel purified, phosphorylated and inserted into the *Sty*I site (743 nt) of 203 cDNA. The resulting 2161 bp fragment (corresponding to 203 cDNA plus the *a* domain of 204 cDNA inserted just upstream from the *b* domain) was then inserted into the *Hin*dIII and *Spe*I restricted pRcRSV. The correctness of all the constructs was confirmed by double stranded DNA sequencing.

2.4. Preparation of protein extracts from murine cell lines and immunoblotting

Whole cell extracts from cultured cells were prepared by resuspending pelleted cells in cold 3% SDS lysis buffer (125 mM Tris/Cl pH 6.8, 3% SDS, 10 mM dithiothreitol) with the addition of 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 4 μg/ml leupeptin and 4 μg/ml aprotinin. After a brief sonication, samples were boiled for 5 min and insoluble materials were removed by centrifugation at 15 000 rpm. Supernatants were quantified for protein concentration with a BioRad Dc protein assay kit (BioRad Laboratories) and stored at −80°C in 10% glycerol.

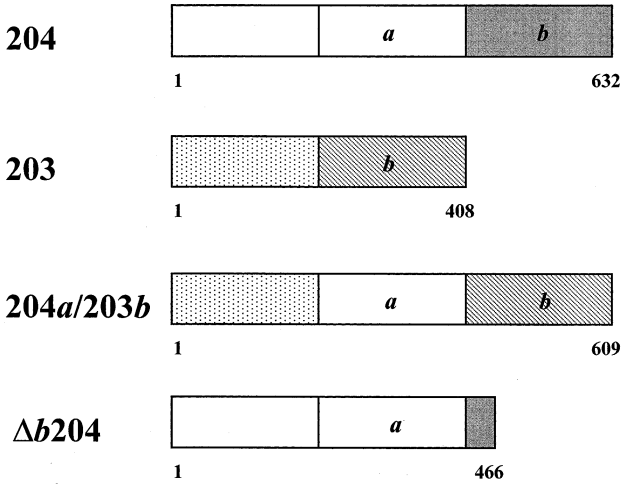

Fig. 1. Schematic representation of 204, 203, 204*a*/203*b* and Δ*b*204 proteins. The empty box represents the NH_2-terminal protein segment; the box designated as *a*, the type *a* 200 amino acid segment; the shaded box, the type *b* 200 amino acid segment from the 203 gene; the grey box, the type *b* 200 amino acid segment from the 204 gene.

After a 10% SDS-PAGE, the proteins were transferred to a PVDF membrane (Millipore) by electroblotting in transfer buffer (25 mM Tris/Cl pH 8.3, 150 mM glycine, 20% v/v methanol). Filters were then blocked in 5% non-fat dry milk in 10 mM Tris/Cl pH 7.5, 100 mM NaCl, 0.1% Tween-20 and immunostained with anti-p203 polyclonal antibody (diluted 2000-fold) [33] or with anti-p204 polyclonal antibody (diluted 1000-fold) at RT for 1 h [34]. Goat anti-rabbit IgG horseradish peroxidase conjugate (Amersham) (diluted 4000-fold) was used to detect the immunocomplex and then visualized by enhanced chemiluminescence (ECL, Amersham). Anti-actin mouse monoclonal antibody (Boehringer) was used as internal control.

2.5. Cell focus assay

To assay the effect of 203, 204, 204*a*/203*b* and Δ*b*204 expression, NIH3T3 and B6MEF cells were transfected with the expression vectors (pRcRSV203, pRcRSV204, pRcRSV204*a*/203*b* or pRcRSVΔ*b*204) or with the pRcRSV empty vector. All plasmids were purified by cesium chloride centrifugation. Briefly, the day before transfection cells were plated in growth medium at a density of 2.5×10^5 cells/60 mm diameter dish. The medium was changed 4 h before transfection. Cells were transfected by the calcium phosphate procedure [37] with 10 μg of expression vector. The DNA calcium precipitates were added to culture medium and the cells were incubated for 18 h. Thereafter, single-cell suspensions were formed by trypsinization and 5×10^3 cells were plated onto 100 mm tissue culture dishes. The transfected cells were cultured for 2 weeks in medium supplemented with G418 (500 μg/ml B6MEF; 750 μg/ml NIH3T3). Then, foci were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and stained with 0.1% crystal violet, 20% ethanol and the number of foci with a diameter of at least 2 mm were counted.

2.6. Transfections

For transient expression of p203, p204, p204*a*/203*b* and pΔ*b*204, the day before transfection NIH3T3 cells (10^6/100 mm diameter dish) were plated in growth medium and this was changed 4 h before transfection. Cells were transfected using the calcium phosphate precipitation method [37], with 10 μg of pRcRSV203, pRcRSV204, pRcRSV204*a*/203*b*, pRcRSVΔ*b*204 or with the empty pRcRSV respectively. The DNA calcium precipitates were added and the cells were incubated for 18 h. Thereafter, transfectants were washed twice with DMEM and cultured in growing medium. After 48 h whole cell extracts were prepared as described above.

For flow cytometric analysis, the day before transfection NIH3T3

cells (10^6/100 mm diameter dish) were plated in growth medium and this was changed 4 h before transfection. Cells were transfected using the calcium phosphate precipitation method with 10 μg of plasmids encoding 203, 204, 204*a*/203*b*, Δ*b*204 or the empty vector and 2 μg of pCH110 (a β-galactosidase expression vector driven by the SV40 enhancer and promoter). The DNA calcium precipitates were added and cells incubated for 18 h. Thereafter, transfectants were washed twice with DMEM and synchronized into G_0/G_1 by serum starvation. After 48 h, stimulation medium (10% serum) was added and cells harvested for flow cytometric analysis at the indicated time point.

For reporter gene experiments, NIH3T3 cells were plated in growing medium at a density of 2.5×10^5 cells/60 mm diameter dish and transfected with 2 μg of pE2WTX4-CAT (a CAT expression vector driven by four copies of E2F-specific sequences) [38], and with plasmids encoding 203, 204, 204*a*/203*b*, Δ*b*204 proteins or the empty pRcRSV plasmid in the amount specified. The inert pBluescript SK was used to balance the total amount of DNA to 10 μg. The cultures were harvested at 48 h and the extracts were assayed for CAT and β-galactosidase activity with equal appropriate amounts of proteins [37]. Thereafter, acetylated products were separated by thin-layer chromatography, visualized by autoradiography, excised from the plates and counted in a liquid scintillation counter. CAT activity was expressed as the percentage of input chloramphenicol converted to the 1′- and 3′-monoacetylated forms.

Reporter gene activity was also normalized to the amount of plasmid DNA introduced into recipient cells by determining either β-galactosidase activity or by DNA dot blot hybridization analysis as described by Abken and Reifenrath [39].

2.7. Flow cytometric analysis

Floating cells were collected and attached cells were detached with phosphate-buffered saline/EDTA 0.1%, pooled with floating cells, washed in phosphate-buffered saline and divided in two aliquots. The first cell aliquot was assayed for β-galactosidase activity to estimate transfection efficiency. The second was resuspended in 1.5 ml hypotonic fluorochrome solution (Propidium Iodide 50 μg/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma Chemical Co) with the addition of 0.5 ml RNAse A (1 mg/ml) [40]. The mixed cells were incubated in the dark at RT for 15 min and kept at 4°C in the dark overnight before flow cytometric analysis. The propidium iodide fluorescence of samples was measured by a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, USA). The flow rate was set about 200 cells/s and at least 10^4 cells of each sample were analyzed. The percentages of cells within the G_1, S and G_2/M phases of cell cycle were determined by analysis with the FACScan computer program provided by Becton and Dickinson.

3. Results

To determine whether p203 inhibits cell proliferation, NIH3T3 or B6MEF cells were transfected with a p203 expression vector or a control vector and assayed for growth suppression response by a transient focus assay. pRcRSV203 did not significantly reduce the number of foci compared with cells transfected with the empty vector (data not shown).

To examine whether duplication of the conserved 200 amino acid domain contributes to the growth inhibitory phenotype of Ifi 200 proteins, we generated a hybrid protein, 204*a*/203*b* (Fig. 1), in which the 204 type *a* domain was inserted just upstream from the 203 type *b* domain. As a control, a deletion mutant (designated Δ*b*204, Fig. 1) was prepared from p204 by deleting almost all of the *b* domain. To evaluate their expression, the constructs were transiently transfected into NIH3T3 cells, and the protein extracts were examined with rabbit polyclonal monospecific antibodies against the p203 (7–196) [33] or the p204 amino-terminal fragment (42–205) [34]. As shown in Fig. 2, the anti-p203 antibodies (upper panel) recognized a protein with an apparent mol. wt. of 48 kDa corresponding to p203 in both the extracts from NIH3T3 transfected with pRcRSV203 vector (lane 3) and cells treated with IFN-α

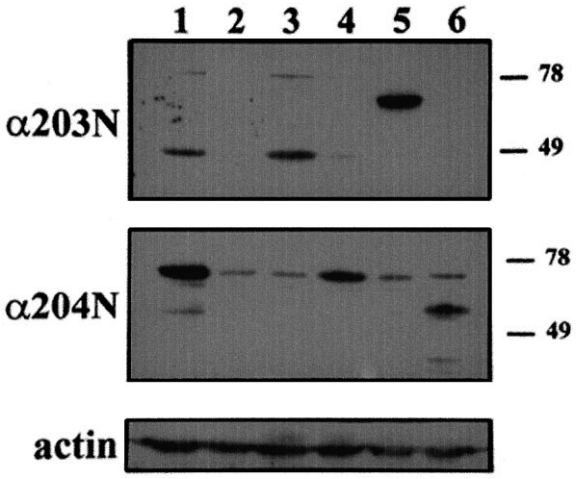

Fig. 2. Transient expression of 203, 204, 204*a*/203*b* and Δ*b*204 proteins in NIH3T3 transfected cells. Total cell extracts (50 μg protein/lane) from NIH3T3 cells stimulated with IFN-α (1000 IU/ml for 18 h) (lane 1) or transiently transfected with the empty vector pRcRSV (lane 2) or with the expression vector pRcRSV203 (lane 3), pRcRSV204 (lane 4), pRcRSV204*a*/203*b* (lane 5) or pRcRSVΔ*b*204 (lane 6) were fractionated by SDS-PAGE and analyzed by immunoblotting with the anti-p203 antibodies (α203N, upper panel) and with the anti-p204 antibodies (α204N, middle panel). The membrane was then incubated with goat anti-rabbit Ig horseradish peroxidase conjugate as secondary antibody and visualized with an ECL kit (Amersham). Immunodetection of actin monoclonal antibody was performed as an internal control. The size markers were 125 kDa, 78 kDa, 49 kDa and 30 kDa, and the electrophoretic mobilities are indicated on the right.

(1000 U/ml for 18 h) (lane 1). In cells transfected with the 204*a*/203*b*-encoding vector, they recognized a protein with a mol. wt. of about 72 kDa (lane 5), corresponding to the size predicted from the amino acid sequence (Fig. 1). Moreover, they did not recognize proteins in extracts from NIH3T3 cells transfected with the empty vector (lane 2), nor in extracts from pRcRSV204 (lane 4) or pRcRSVΔ*b*204 transfected cells (lane 6).

When the same blot was probed with anti-p204 antibodies (Fig. 2, middle panel) an IFN-inducible 72 kDa protein (corresponding to p204) was revealed in protein extracts from NIH3T3 cells stimulated with IFN-α (lane 1). The faster migrating band of about 55 kDa stained in these extracts (lane 1) corresponds to the IFN-inducible protein D3, another member of the Ifi 200 family with a strong similarity to the amino-terminal region of p204 (about 99% of identity) [34,41]. In cells transfected with the pRcRSV204-encoding vector (lane 4), higher levels of the corresponding 72 kDa protein were observed compared with the basal levels in extracts from NIH3T3 transfected with the empty vector (lane 2), pRcRSV203 (lane 3) or pRcRSV204*a*/203*b* (lane 5). Lastly, these antibodies recognized a protein of about 55 kDa, in line with the size predicted from its amino acid sequence, in extracts from NIH3T3 transfected with pRcRSVΔ*b*204 (lane 6).

To evaluate their antiproliferative activity, these constructs were transfected into NIH3T3 and B6MEF cells and assayed for growth inhibition by a cell focus assay (Table 1). Transfection of both the empty vector pRcRSV and pRcRSV203 generated a conspicuous number of foci, whereas transfection of pRcRSV204*a*/203*b* or pRcRSV204 dramatically reduced

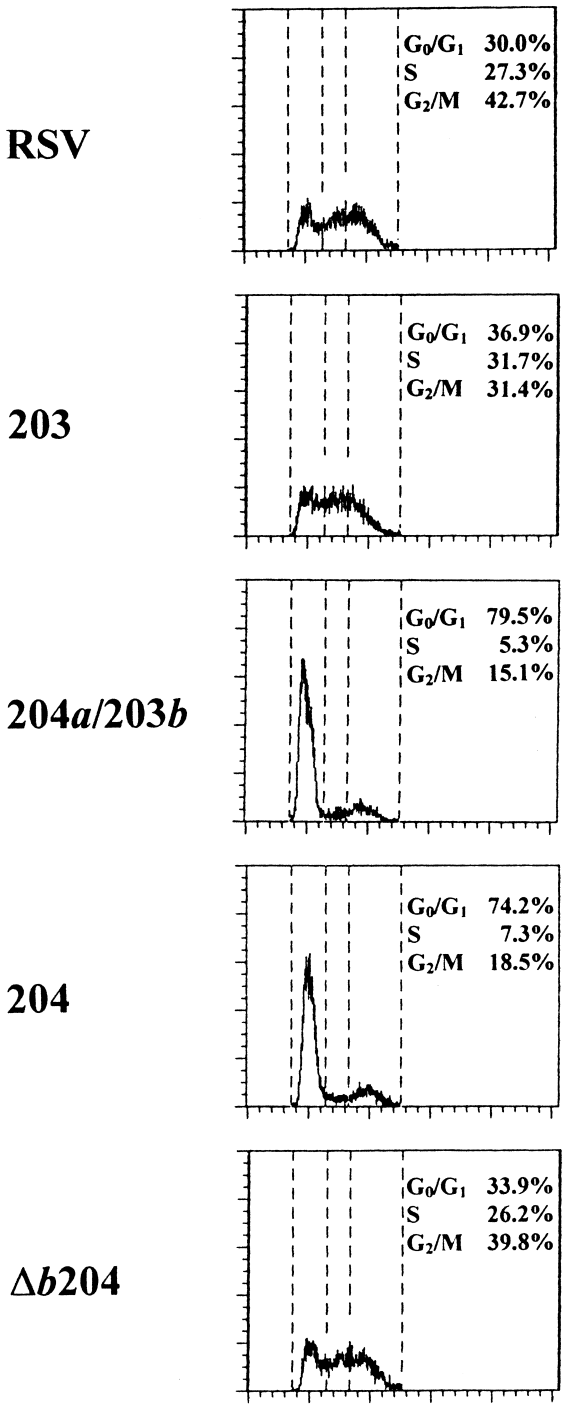

Fig. 3. Effect of p204*a*/203*b* expression on cell cycle phase distribution. NIH3T3 cells were transfected with 10 μg of plasmids encoding p203, p204, p204*a*/203*b*, pΔ*b*204 or the empty pRcRSV and 2 μg of pCH110βgal as an internal control. At 18 h after transfection, cells were synchronized by serum starvation into G_0/G_1 for 48 h, then stimulated with 10% serum and harvested at 18 h later. Thereafter, cells were divided in two aliquots: the first cell was assayed for β-galactosidase activity as a control for transfection efficiency. The second cell aliquot was examined for DNA content by propidium iodide staining and flow cytometry and each graph represents the analysis of 10^4 events.

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their number: about 4-fold reduction in NIH3T3 and more than 2-fold in B6MEF, respectively. By contrast pRcRSVΔ*b*204 failed to suppress cell growth. These results demonstrate that the Ifi 200 proteins require both domains to inhibit cell growth.

Previous studies have suggested that p202 and p204, though expressed at relatively low levels, affect cell cycle progression [24,25,31]. To determine whether this is also true for the 204*a*/203*b* hybrid protein, NIH3T3 clones constitutively expressing 204*a*/203*b*, 203, 204 or the Δ*b*204 proteins were generated. The presence of transfected cDNAs was determined by PCR amplification using two primers discriminating between amplification of the transgenes and the endogenous 203 and 204 genes. However, Western blot analysis performed on independent G418-resistant clones revealed that p203 and pΔ*b*204 were expressed at high levels, whereas p204*a*/203*b* and p204 were poorly expressed suggesting that transfected cells do not tolerate their high expression levels (data not shown). To circumvent this obstacle, we used transiently transfected cells in which p204*a*/203*b* and p204 were expressed at high levels (Fig. 2) to establish whether the impairment in proliferation was related to accumulation in a particular phase of the cell cycle. To this purpose NIH3T3 cells were transfected with plasmids encoding 203, 204, 204*a*/203*b*, Δ*b*204 or the empty vector, synchronized by serum starvation into G_0/G_1, stimulated to proliferate by serum addition and analyzed by flow cytometric assay. As shown in Fig. 3 (where the representative 18 h time point is reported), NIH3T3 transfected with RSV and 203 rapidly avanced through the G_0/G_1 and S phases, by contrast cells expressing either p204*a*/203*b* or p204 progressed more slowly. At 18 h, 79.5% of 204*a*/203*b* cells remained in G_0/G_1 compared with only 36.9% of the 203 cells. By 24 h, in contrast to 203 cells that have already begun to return to G_1, a significative percentage of 204*a*/203*b* cells (about 13%) were in S phase (data not shown). A similar pattern was observed for the 204 transfected cells, whereas the behavior of cells transfected with its truncated version Δ*b*204 resembled that of p203. The assay of β-galactosidase activity revealed equal transfection efficiency values among the different constructs. Thus, p204*a*/203*b* expression in contrast to p203 inhibits proliferation by slowing the progress of cells through the cycle. These results demonstrate that antiproliferative activity is phase-dependent when both domains are present.

Previous observations have demonstrated that Ifi 200 proteins inhibit cell growth by down-regulating E2F activity [27,28]. To see whether this is also true of p204*a*/203*b*, NIH3T3 cells were cotransfected with the pE2WTX4-CAT plasmid, in which the CAT expression is driven by an E2F-

Table 1
Analysis of the effects of p203, p204*a*/203*b*, p204 and pΔ*b*204 on cell growth by cell focus assays

Vector	No. of clones[a]	
	NIH3T3	B6MEF
pRcRSV	80 ± 7.5	44 ± 5.2
pRcRSV203	92 ± 8.1	37 ± 4.3
pRcRSV204*a*/203*b*	24 ± 2.4	22 ± 2.0
pRcRSV204	26 ± 2.4	18 ± 2.3
pRcRSVΔ*b*204	83 ± 9.0	52 ± 4.2

[a]Foci at least 2 mm in diameter. The data represent the means of four independent experiments for each cell line.

responsive motif repeated four times, and with 203, 204, 204*a*/203*b* or Δ*b*204 vectors. As reported in Fig. 4, transfection of p204 expression construct inhibited CAT activity in a concentration-dependent manner, whereas that of pΔ*b*204 abolished the suppression of E2F-mediated transcription. Transfection of the 204*a*/203*b* plasmid, like p204 also inhibited CAT activity, whereas p203 was ineffective. Inhibition by p204*a*/203*b* and p204 was specific, since the empty pRcRSV did not affect CAT activity.

These results further demonstrated that duplication of the conserved 200 amino acid domain, as occurring in the hybrid 204*a*/203*b* protein, confers a growth inhibition phenotype similar to that observed with the wild-type 202 and 204 proteins.

4. Discussion

The structural hallmark of the Ifi 200 protein family is a 200 amino acid motif, present singly or as a tandem repeat designated the type *a* and type *b* domains respectively. This study demonstrates that the antiproliferative activity of these proteins is closely dependent on the simultaneous presence of both domains because: (i) p203, which contains only one domain fails to exert antiproliferative activity; (ii) introduction of a second domain into p203 to create 204*a*/203*b* hybrid protein, restores it so that it is indistinguishable from that of p204; (iii) deletion of one of the p204 domains, as in pΔ*b*204, abolishes its antiproliferative activity.

The failure of p203 to inhibit growth is thus a consequence of its different molecular structure compared to those of p202 and p204, the only Ifi 200 proteins of this family with a documented antiproliferative activity [24,25,31]. Their structure is remarkably similar, and the sequence similarity between the *a* domain of p202 and that of p204 is 56%, whereas that of the *b* domain is 50%. By contrast, the homologies between the *a* and the *b* domain are 31% and 34% in p202 and p204 respectively. Homology in a given protein is consistent with repeated duplication during evolution of the 200 gene cluster. In this model, an ancestral gene encoding only one amino acid motif has duplicated to give rise to a double length transcription unit, whose two segments of this double unit then diverged before undergoing further duplication [42].

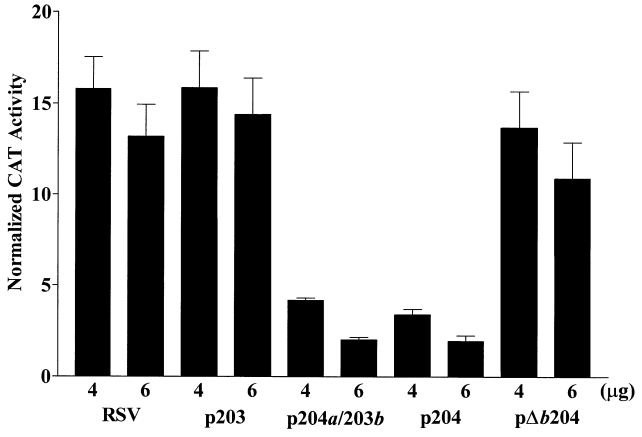

Fig. 4. Inhibition of E2F-dependent transcription by 204 or 204*a*/203*b* proteins. NIH3T3 cells were transiently transfected with 2 μg of the pE2WTX4-CAT indicator plasmid, 2 μg of pCH110βgal as an internal control, and, as indicated, with 4 or 6 μg of plasmids encoding p203, p204, p204*a*/203*b*, pΔ*b*204 or the empty pRcRSV. The inert pBluescript SK was used to balance the total amount of DNA to 10 μg. At 48 h after transfection, cell extracts were prepared and 15 μg of proteins were assayed for CAT and β-galactosidase activities as described in Section 2. CAT activity was normalized to β-galactosidase activity. The results shown are representatives of two independent experiments. The error bars represent the standard deviations.

The antiproliferative activity of p202 has been related to its ability to down-regulate E2F-dependent transcriptional activity by directly binding and inhibiting both E2F-1 and E2F-4 [27,28]. Since members of the E2F family regulate genes whose products are essential for progression through the cell cycle (e.g. DHFR, PCNA, cdc2, b-myb and c-myc), it is conceivable that p202 inhibits cell proliferation, at least in part, by inhibiting E2F activity. It has, in fact, been demonstrated that the transcription rates of the DHFR, PCNA and b-myb genes were reduced by about 60–80% in p202 overexpressing cell lines [27]. The present results show that introduction of an additional 200 amino acid segment in the backbone of a copy-bearing protein, namely p203, confers to the hybrid protein antiproliferative properties indistinguishable from those of the p204. Consistent is the observation that overexpression of MNDA, a human Ifi 200 homologue bearing only one copy of the 200 amino acid motif, does not inhibit cell growth in colony formation assays (R.C. Briggs, personal communication). It may be relevant to note that despite the occurrence in MNDA of a type *a* domain, the whole protein displays a remarkably similarity with the amino acid sequence of p203 (40.5% of the residues are identical).

The lack of an apparent antiproliferative activity on behalf of the natural p203 raises the question of its functions. Conservation of two one-copy-bearing Ifi 200 proteins, namely p203 and pD3 [33,41], indicates that these two genes exert functions related to the regulation of gene expression, since the ability of a single 200 amino acid segment to bind several transcription factors has been well established. Furthermore, the divergence in the amino-terminal segment of p203 and pD3 may reflect individual functions correlated with their cell- and tissue-specific expression patterns. The IFN-inducible expression of pD3 in macrophages may be involved in regulation of their differentiation [41].

We have recently observed that p203, pD3 and p204 are constitutively expressed in various degrees in myeloid and lymphoid organs [34,35], suggesting that their physiological levels may be high and not provoke an antiproliferative response. Inhibition of endogenous p202 expression unexpectedly did not increase cell proliferation. Instead, an increase in cell susceptibility to apoptosis in response to serum starvation was observed, consistent with the notion that basal levels of p202 may be needed for the correct regulation of cell proliferation [43]. These findings and the data concerning the antiproliferative function of Ifi 200 proteins suggest a model in which their constitutive expression contributes to the physiological regulation of cell proliferation, whereas, on IFN-mediated overexpression, they inhibit cell division and thereby limit tissue proliferation.

In conclusion, the present results indicate that duplication of the partially conserved 200 amino acid long region of the Ifi 200 proteins may determine the growth inhibition phenotype and thereby contribute to illustrate the molecular mechanisms exploited by IFNs to regulate progression throughout cell cycle.

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References

[1] DeMaeyer, E.M. and DeMaeyer-Guignard, J. (1988) Interferons and Other Regulatory Cytokines, John Wiley and Sons, New York.
[2] Landolfo, S., Gribaudo, G., Angeretti, A. and Gariglio, M. (1995) Pharmac. Ther. 65, 415–442.
[3] Sen, G.C. and Lengyel, P. (1992) J. Biol. Chem. 267, 5017–5020.
[4] Darnell Jr., J.E., Kerr, I.M. and Stark, G.R. (1994) Science 246, 1425–1431.
[5] Ihle, J.N. and Kerr, I.M. (1995) Trends Genet. 11, 69–74.
[6] Schindler, C. (1995) Annu. Rev. Biochem. 64, 621–651.
[7] Romeo, G., Fiorucci, G. and Rossi, G.B. (1989) Trends Genet. 5, 19–23.
[8] Balkwill, F. and Taylor-Papadimitriou, J. (1978) Nature 274, 798–800.
[9] Sreevalsan, T., Taylor-Papadimitriou, J. and Rozengurt, E. (1979) Biochem. Biophys. Res. Commun. 87, 679–684.
[10] Einat, M., Resnitzky, D. and Kimchi, A. (1985) Nature 313, 597–600.
[11] Melamed, D., Tiefenbrun, N., Yarden, A. and Kimchi, A. (1993) Mol. Cell. Biol. 13, 5255–5265.
[12] Tiefenbrun, N., Melamed, D., Levy, N., Resnitzky, D., Hoffmann, I., Reed, S.I. and Kimchi, A. (1996) Mol. Cell. Biol. 16, 3934–3944.
[13] Sangfelt, O., Erickson, S., Einhorn, S. and Grander, D. (1997) Oncogene 14, 415–423.
[14] Hokeiba, A.C., Etienne, W., Cruz, P.E., Subramaniam, P.S. and Johnson, H.M. (1998) Int. J. Cancer 77, 138–145.
[15] Gutterman, J.U. (1994) Proc. Natl. Acad. Sci. USA 91, 1198–1205.
[16] Landolfo, S., Gariglio, M., Gribaudo, G. and Lembo, D. (1998) Biochimie 80, 721–728.
[17] Landolfo, S., Gariglio, M. and Gribaudo, G. (1998) Rev. Clin. Exp. Hematol. 6, 21–34.
[18] Lengyel, P., Choubey, D., Li, S.J. and Datta, B. (1995) Sem. Virol. 6, 203–213.
[19] Trapani, J.A., Browne, K.A., Dawson, M.J., Ramsay, R.G., Eddy, R.L., Shows, T.B., White, P.C. and Dupont, B. (1992) Immunogenetics 36, 369–376.
[20] Briggs, J.A., Burrus, G.R., Stickney, B.D. and Briggs, R.C. (1992) J. Cell. Biochem. 49, 82–92.
[21] DeYoung, K.L., Ray, M.E., Su, Y.A., Anzick, S.L., Johnstone, R.W., Trapani, J.A., Meltzer, P.S. and Trent, J.M. (1997) Oncogene 15, 453–457.
[22] Dawson, M.J. and Trapani, J.A. (1996) J. Leukocyte Biol. 60, 310–316.
[23] Choubey, D. and Lengyel, P. (1993) J. Interferon Res. 13, 43–52.
[24] Min, W., Ghosh, S. and Lengyel, P. (1996) Mol. Cell. Biol. 16, 359–368.
[25] Lembo, D., Angeretti, A., Benefazio, S., Hertel, L., Gariglio, M., Novelli, F. and Landolfo, S. (1995) J. Biol. Regul. Homeost. Agents 9, 42–46.
[26] Choubey, D. and Lengyel, P. (1995) J. Biol. Chem. 270, 6134–6140.
[27] Choubey, D., Li, S.J., Datta, B. and Lengyel, P. (1996) EMBO J. 15, 5668–5678.
[28] Choubey, D. and Guttermann, J.U. (1997) Oncogene 15, 291–301.
[29] Datta, B., Li, B., Choubey, D., Nallur, G. and Lengyel, P. (1996) J. Biol. Chem. 271, 27544–27555.
[30] Datta, B., Min, W., Burma, S. and Lengyel, P. (1998) Mol. Cell. Biol. 18, 1074–1083.
[31] Lembo, M., Sacchi, C., Zappador, C., Bellomo, G., Gaboli, M., Pandolfi, P.P., Gariglio, M. and Landolfo, S. (1998) Oncogene 16, 1543–1551.
[32] Choubey, D. and Lengyel, P. (1992) J. Cell Biol. 116, 1333–1341.
[33] Gribaudo, G., Ravaglia, S., Guandalini, L., Riera, L., Gariglio, M. and Landolfo, S. (1997) Eur. J. Biochem. 249, 258–264.
[34] Gariglio, M., De Andrea, M., Lembo, M., Ravotto, M., Zappador, C., Valente, G. and Landolfo, S. (1998) J. Leukocyte Biol. 64, 608–614.
[35] Gribaudo, G., Riera, L., Hertel, L. and Landolfo, S. (1999) J Interferon Cytokine Res. 19, 129–136.
[36] Weck, P.K., Apperson, S., Stebbing, N., Gray, P.W., Leung, D., Shepard, H.M. and Goeddel, D.V. (1981) Nucleic Acids Res. 9, 6153–6166.
[37] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[38] Loeken, M.R. and Brady, J. (1989) J. Biol. Chem. 264, 6572–6579.
[39] Abken, H. and Reifenrath, B. (1992) Nucleic Acids Res. 20, 3527.
[40] Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) J. Immunol. Methods 139, 271–279.
[41] Tannenbaum, C.S., Major, J., Ohmory, Y. and Hamilton, T.A. (1993) J. Leukocyte Biol. 53, 563–568.
[42] Choubey, D., Snoddy, J., Chaturvedi, V., Toniato, E., Opdenakker, G., Thakur, A., Samanta, H., Engel, D.A. and Lengyel, P. (1989) J. Biol. Chem. 264, 17182–17189.
[43] Koul, D., Lapushin, R., Xu, H.J., Mills, G.B., Gutterman, J.U. and Choubey, D. (1998) Biochem. Biophys. Res. Commun. 247, 379–382.