A Novel Repressor Domain Is Required for Maximal Growth Inhibition by the IRF-1 Tumor Suppressor*

Mirjam Eckert1, Sarah E. M. Meek2, and Kathryn L. Ball3

From the Cancer Research UK (CRUK) Interferon and Cell Signalling Group, Cell Signalling Unit, The University of Edinburgh Cancer Research Centre, Edinburgh EH4 2XR, United Kingdom

Interferon regulatory factor-1 (IRF-1) is a transcription factor and tumor suppressor that can regulate gene expression in a manner requiring either its sequence specific DNA binding activity or its ability to bind the p300 coactivator. We show that IRF-1-mediated growth inhibition is dependent on the integrity of a C-terminal transcriptional enhancer domain. An enhancer subdomain (amino acids 301–325) that differentially regulates IRF-1 activity has been identified and this region mediates the repression of Cdk2. The repressor domain encompasses an LXXLL coregulator signature motif and mutations or deletions within this region completely uncouple transcriptional activation from repression. The loss of growth suppressor activity when the Cdk2-repressor domain of IRF-1 is mutated implicates repression as a determinant of its maximal growth inhibitory potential. The data link IRF-1 regulatory domains to its growth inhibitory activity and provide information about how differential gene regulation may contribute to IRF-1 tumor suppressor activity.

IRF-1 has been described as a tumor suppressor as its loss can increase susceptibility to transformation by oncogenes (8) and expression of IRF-1 can revert the phenotype of cells transformed with c-Ha-ras or c-myc (9). Furthermore, deletion of the IRF-1 gene has been linked to the development of leukemia, myelodysplastic syndrome (10), and solid phase tumors of the gastrointestinal tract (11, 12). In mice loss of IRF-1 function does not lead to a significant increase in spontaneous tumor formation; however, the absence of IRF-1 has a marked effect on previous tumor predisposition initiated by c-Ha-ras or by the loss of p53. Thus, the number of tumors and the tumor spectrum in IRF-1/p53-double null mice is dramatically different from that of p53-null animals (13). These results suggest that in mice IRF-1 displays the activity of a tumor susceptibility gene. The mechanism(s) used by IRF-1 to suppress tumor development are poorly understood, but its growth inhibitory activity does not rely on an autocrine feedback loop (14). Furthermore, increased tumorigenesis associated with the loss of IRF-1 is independent of immunological disorders (13). Rather, IRF-1 tumor and growth suppressor activity seems to be intimately linked to intracellular signaling pathways that modulate the expression of specific genes (14). Known IRF-1 target genes, which are involved in growth control, include, in addition to p21, 2–5A synthetase (15) and the RNA-dependent protein kinase PKR (16).

IRF family members share a highly conserved N-terminal DNA binding domain (aa 1–120), which is characterized by five tryptophan repeats (17, 18). Within the DNA binding domain there is also a region required for homodimerization (aa 90–115 (19)) and a putative nucleic acid localization sequence (aa 116–139 (20)). The transactivation domain resides within the C-terminal half of the protein (aa 185–256) and is predicted to form a helix-loop-helix structure that is critical for IRF-1 activity (20–22). Adjacent to the activator domain is a region involved in enhancing transcription capacity (23). The C terminus of IRF-1 is also involved in interactions with the CBP/p300 coactivator (5, 24). However, it is not known how the domain structure of IRF-1 is utilized to differentially regulate gene activation versus gene repression. Furthermore, the relationship between IRF-1 structure and its function(s) in growth inhibition and tumor suppression remains to be fully elucidated.

Based on the observation that enhancer domain mutants of IRF-1 are severely compromised in their ability to suppress cell growth we have identified novel regulatory motifs within the C-terminal 25 amino acids of the protein which are involved in the differential modulation of gene activation and repression. We have used this information to generate mutant IRF-1 con-

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3 Supported by a CRUK program grant. To whom correspondence should be addressed: E-mail: kathryn.ball@ed.ac.uk.
4 The abbreviations used are: IRF-1, interferon regulatory factor-1; IFN, interferon; aa, amino acids; mAb, monoclonal antibody; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; wt, wild-type.

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structs in which transcriptional activation is completely uncoupled from Cdk2 gene repression. This has allowed us to demonstrate that inhibiting the expression of key downstream target genes contributes significantly to IRF-1 activity as a growth inhibitor. Thus, proteins that bind to, or modify, the extreme C terminus of IRF-1 are likely to modulate its activity. The data presented here shed light on how IRF-1 structure and function relate to its growth inhibitory activity and begins to define domains which may be important for IRF-1 mediated tumor suppression.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—Cdk2 was detected using mAb D-12 (Santa Cruz Biotechnology) and mAb IRF-1 was from BD Biosciences; both antibodies were used at 1 μg/ml. Cycloheximide (Supelco) was dissolved in H₂O to 5 mg/ml and used at a concentration of 30 μg/ml in cell culture medium.

**IRF-1 Mutants**—Oligonucleotides were designed containing the stop codon insertions and alanine substitutions as required (Sigma-Genosys). The QuickChange site-directed mutagenesis kit (Stratagene) was used, and the manufacturer’s instructions were followed. pcDNA3.1/IRF-1 was used as the plasmid template. Additional steps included a PCR purification (Qiagen) after DpnI digestion of the PCR product. Mini-plasmid preparations (Qiagen) were performed of selected transformed colonies, which were then confirmed by sequencing. Generation of the IRF-1YL/A mutant in which Tyr109, Leu112, and Pro113 were mutated to Ala has been described previously (5).

**Cell Culture and Transfections**—HCT116/HCT116 (p53⁻/⁻) cells, H1299 cells, and SAOS2 cells were cultured in McCoy’s 5A, RPMI 1640, or Dulbecco’s modified Eagle’s medium, respectively. The medium was changed 4 days after seeding. Dishes were prepared by washing with PBS followed by fixation of cells with 100% methanol (20 °C) for 0.5 h at room temperature. Methanol was discarded and cells were stained with 10% Giemsa stain (Sigma) for 15 min at room temperature. Dishes were thoroughly washed with water and left to dry. For each assay duplicate samples were prepared for every condition and several independent experiments performed on different batches of cells. For the data given the relative standard deviation was within 10%.

**Luciferase Reporter Assays**—Cells were harvested 24 h later, RNA was extracted using a RNeasy mini kit with Qiashredders (Qiagen). RNA was reverse-transcribed using the Omniscript RT kit (Qiagen). PCR was carried out using HotStarTaq (Qiagen) for 20 cycles for actin, 25 cycles for ISG20, and 45 cycles for TRAIL, using an annealing temperature of 55 °C. The primer sequences were as follows: TRAIL, ATGGCTATGTAGGGTTCCAG/TTGCTCTGCA- ATCTGCTTCCAG; ISG20, CTAACAACTCAGACAGGTA- GCCACT/GATTCTCTGGGAGATTTGAGC.

**EMSAs**—IRF-1-DNA binding assays were carried out as described previously (5) using wt and mutant IRF-1 protein (3 μl/assay) expressed using a coupled reticulocyte lysate system (Promega) together with an IRF-1-specific oligonucleotide probe (IRFE) labeled with biotin and an EMSA gel-shift kit (Panomics).

**RESULTS**

**IRF-1 Suppresses Cell Growth in Both the Presence and Absence of Functional p53**—It has been suggested that the stimulation of IRF-1 activity can lead to inhibition of cell growth by impeding cell cycle progression or by engaging a cell death pathway (4, 8, 25–27). In the current study we used colony formation assays to determine the activity of IRF-1 as a growth suppressor. The ability of IRF-1 to inhibit cell growth was assessed in two cell lines with differing p53 status; HCT116 colorectal cancer cells have a well characterized functional p53 pathway (28), whereas H1299 cells are null for p53 (29). Assays were set up and the conditions adjusted so that the number and density of colonies was optimal when the cells were transfected with an empty vector control (pcDNA3.1; Fig. 1a). When either HCT116 or H1299 cells were transfected with a vector containing wild-type human IRF-1 (pcDNA/IRF-1) the number of colonies was significantly reduced (p < 0.001 in both cases) giving a growth suppression of 76 + 4.2% (n = 3) and 87.3 + 2.2% (n = 3), respectively, when compared with the vector only controls (Fig. 1, a and b). Thus, IRF-1 suppresses growth in both the absence and presence of p53.
IRF-1-dependent Growth Suppression

We recently showed that IRF-1 could potentiate p53-dependent cell cycle arrest through induction of the growth suppressor protein p21 in a manner that required IRF-1 to interact with p300 to stimulate p53 acetylation (5). The fact that IRF-1 can inhibit cell growth in the absence of p53 (Fig. 1, a and b) suggests that it is able to suppress growth by two distinct mechanisms dependent on the cellular status of p53. We therefore sought to establish whether IRF-1-dependent inhibition of colony growth in the absence of p53 required its activity as a transcription factor.

Inhibition of Cell Growth by IRF-1 in the Absence of p53 Requires Sequence-specific DNA Binding and the Transcriptional Enhancer Domain—Mutant IRF-1 constructs that are defective in DNA binding (IRF-1YLP/A; has a decreased affinity for the IRFE in an EMSA (5)) or in which the transcriptional enhancer domain has been deleted (IRF-1Δenh; a truncation mutant in which the C-terminal 70 amino acids, 256–325, were deleted) are defective in their ability to activate expression from the IFNβ-promoter (Fig. 2a) (5, 23). Whereas the IRF-1YLP/A mutant is a very poor activator of the IFNβ reporter, IRF-1Δenh partially retains activity in this assay. In addition to acting as a transcriptional activator IRF-1 has also been reported to suppress the expression of some genes. When a reporter construct was used to determine expression from the Cdk2-promoter, wild-type IRF-1 was shown to be an efficient repressor, whereas both the IRF-1YLP/A and IRF-1Δenh mutants had lost the ability to repress expression from this promoter (Fig. 2b) despite the fact that they were expressed at higher levels than the wild-type protein (Fig. 2c). These experiments were carried out using H1299 cells and confirmed using HCT116 (p53−/−) cells (data not shown).

To determine whether defects in the transactivation and repressor activity of the IRF-1YLP/A and IRF-1Δenh mutants affect their ability to suppress cell growth, they were assessed in colony formation assays. The IRF-1YLP/A mutant was substantially impaired as a growth suppressor relative to the wild-type protein (with only 16% of wild-type activity; Fig. 2, d and e). Similar to IRF-1YLP/A, the IRF-1Δenh mutant was severely compromised as a growth inhibitor. In colony formation assays IRF-1Δenh retained only 20% of the wild-type proteins inhibitory activity (Fig. 2, d and e). The data suggest that the enhancer domain and sequence specific DNA binding are important for IRF-1-mediated gene activation, gene repression, and growth inhibition. In addition, the data show a good correlation between the ability of IRF-1 to activate and repress transcription with its ability to inhibit H1299 cell growth. Thus, colony formation assays provide a system to investigate the link between IRF-1-dependent modulation of transcription and its activity as a growth inhibitor.

Uncoupling IRF-1-dependent Transactivation from Cdk2 Repression Reveals a Role for Gene Repression in IRF-1-dependent Growth Inhibition—Although the enhancer domain has been defined as a region that has no intrinsic activation capacity but which can synergize with the activator domain of IRF-1 to give optimal transcription (23), it has not been studied in the context of full-length IRF-1 nor in terms of IRF-1 growth suppressor function. As deletion of the enhancer domain affected both IRF-1-dependent transcriptional activation and repression, as well as IRF-1-mediated growth control (Fig. 2), we wished to further define its function in the control of cell growth. First we addressed whether the determinants required for gene activation were the same as those required for gene repression.

When the C-terminal 25 amino acids of IRF-1 were deleted (aa 301–325; IRF-1ΔC25), there was no loss of transcriptional activity as measured using the IFNβ-reporter (Fig. 3a), in fact there was a 1.6-fold increase, which may be explained by an increase in protein expression level (Fig. 3c). On the other hand, there was a striking decrease in the ability of the IRF-1ΔC25 mutant to repress transcription from the Cdk2-promoter (Fig. 3b). To confirm the importance of the C terminus of IRF-1 in regulation of its transcriptional activity, we looked at the effect of the enhancer and C-terminal 25 amino acid regions on the expression of endogenous IRF-1 promoter targets. When mRNA expression for two validated IRF-1 targets, TRAIL (27) and ISG20 (30), were examined the results supported those obtained using reporter assays (Fig. 3d). TRAIL mRNA levels were barely present in cells transfected with a vector only control whereas cells expressing exogenous IRF-1 had readily detectable TRAIL mRNA (Fig. 3d, upper panel: lane 1 versus lane 2). As predicted from the experiments described above (Fig. 2), the IRF-1YLP/A and the IRF-1Δenh mutants were
unable to promote up-regulation of TRAIL mRNA (lanes 3 and 4), whereas in keeping with results from the IFNβ reporter assays IRF-1ΔC25 enhanced TRAIL mRNA levels to a greater degree than the wt protein (lane 5 versus lane 2). ISG20 mRNA levels showed a similar trend to those of TRAIL (Fig. 3d, upper panel), although the basal levels of ISG20 mRNA in the absence
IRF-1-dependent Growth Suppression

**Figure a:**
- Bar graph comparing RLU (IFN-γ-Luc/p10) for I-2BI and IRF-1ΔC25.

**Figure b:**
- Bar graph comparing RLU (Cdk2-Luc/protein) for control, IRF-1, and IRF-1ΔC25.

**Figure c:**
- Immunoblot showing IRF-1 and β-actin levels.

**Figure d:**
- RT-PCR analysis of TRAIL, ISG20, and Actin using vector, IRF-1, YLP/A, Δ enh, Δ C25, and control.

**Figure e:**
- Immunoblot analysis of Cdk2 with vector, IRF-1, YLP/A, Δ enh, Δ C25, IRF-1Δ enh, and IRF-1ΔC25.

**Figure f:**
- Petri dish images showing control, IRF-1, and IRF-1ΔC25.

**Figure g:**
- Bar graph showing relative Cdk2 protein levels for vector, IRF-1, YLP/A, Δ enh, Δ C25, IRF-1Δ enh, and IRF-1ΔC25.
of exogenous IRF-1 were higher. To support the data showing IRF-1 as a repressor of Cdk2, we measured Cdk2 protein levels (Fig. 3e). Wild-type IRF-1 reduced Cdk2 protein levels by ~60%, whereas the IRF-1-YLP/A, IRF-1-ΔC25, and IRF-1-Δenh mutants had no significant effect on levels of Cdk2 protein. The IFNβ and Cdk2 reporter assays therefore reflect changes in the expression from endogenous IRF-1 target promoters.

Thus, deletion of 25 amino acids from the C terminus of IRF-1 completely uncouples its Cdk2 repressor activity from transcriptional activation, suggesting that the enhancer domain contains distinct functional motifs that differentially regulate gene expression. As the IRF-1-ΔC25 mutant has lost the ability to repress the Cdk2-promoter while retaining activity against the IFNβ-promoter, we were able to use this mutant to ask whether the selective loss of Cdk2 repressor function affected IRF-1-mediated growth inhibition. In fact the IRF-1-ΔC25 mutant has a significant defect in its ability to inhibit cell growth when compared with the wild-type protein (Fig. 3, d and e). There was double the number of colonies on the plates with mutant IRF-1 (IRF-1-Δ25) compared with plates with wild-type protein (Fig. 3e; p < 0.01). Thus, loss of IRF-1 activity as a repressor of Cdk2 expression correlates with a decrease in IRF-1-dependent inhibition of cell growth.

The C Terminus of IRF-1 Contains a Negative Regulatory Domain for IFNβ Expression—The data presented in the previous section suggests that elements within the last 25 amino acids of IRF-1 are important for the control of IRF-1 activity in gene repression and growth inhibition. To investigate the C terminus further a series of C-terminal truncation mutants of IRF-1 were generated in which stop codons were introduced sequentially throughout the last 25 amino acids (Fig. 4a). To characterize the mutant proteins reporter assays were carried out in HCT116 (p53−/−) cells (all results were confirmed using H1299; data not shown). The results show similar activation of the IFNβ-promoter by wild-type IRF-1, IRF-1-ΔC4, and IRF-1-ΔC8 (Fig. 4b). An increase in transcriptional activity was then detected with the deletion of a further 4 amino acids (IRF-1-ΔC12; Fig. 4b). The increase was sustained with the loss of additional residues up to aa 301 (IRF-1-Δ12 to IRF-1-ΔC25; Fig. 4b). This is in contrast to removal of the C-terminal 70 residues (IRF-1-Δenh), which reduces IRF-1 transcriptional activity (Fig. 2a). All the truncation mutants were expressed at similar levels except for IRF-1-ΔC4, which was present at lower levels. Further investigation revealed that differences in the levels of expressed protein were not due to variation in mRNA or in protein solubility (data not shown) but were the result of more rapid protein turnover. Thus, whereas the half-life of IRF-1 wild-type protein in cyclohexamide-treated cells was 30–45 min, the t_{1/2} for IRF-1-ΔC4 was ~15 min (data not shown). Truncation mutants IRF-1-ΔC15, IRF-1-ΔC20, and IRF-1-ΔC25 all had elevated transcriptional activity against the IFNβ-promoter; this could be explained, at least in part, by an accompanying increase in protein levels, consistent with the idea that there is a degradation motif within the C terminus (31). However, it is also possible that the region between amino acids 311–317 of the protein has a negative effect on IRF-1-dependent activation of IFNβ expression that is revealed when these amino acids are deleted.

To confirm the role of the C terminus in negative regulation of IRF-1-dependent transcription we generated a set of Ala mutants that showed less variability in expression levels (Fig. 3e). The results show that substitution of residues from 311–313 (IRF-1-M1Ala) had the most striking effect (2.4-fold increase) on IRF-1-dependent transcription from the IFNβ-promoter (Fig. 4b), whereas IRF-1-M1Ala had decreased activity, most likely due to the fact that, like IRF-1-ΔC4, this mutant is present at low levels due to rapid degradation (data not shown). There was a reproducible increase in activity for IRF-1M2Ala, IRF-1M3Ala, and IRF-1M5Ala suggesting that residues surrounding 311–314 contribute to negative regulation, whereas IRF-1M6Ala (301–305) did not have a statistically significant effect on IRF-1 activity compared with the wild-type protein.

Taken together the data from the truncation and alanine substitution mutants suggest that residues centered around 311–317 of IRF-1 are involved in either intra- or intermolecular interactions that negatively regulate IRF-1 transcriptional activity.

An LXXLL Motif in the C Terminus of IRF-1 Is Linked to Gene Repression—Studies presented above (Fig. 3) suggested that residues from within the C-terminal 25 amino acids had a profound effect on the ability of IRF-1 to act as a repressor of Cdk2 gene expression and also impact on its growth inhibitory activity (Fig. 3). Using the set of mutant constructs described in the previous section (Fig. 4), we sought to pinpoint the residues that were key for gene repression. Using the Cdk2-reporter assay in HCT116 (p53−/−) cells, we found that deletion of amino acids between 311–321 (IRF-1-ΔC4, IRF-1-ΔC8, IRF-1-ΔC12, and IRF-1-ΔC15) did not produce any decrease in the activity of IRF-1 against the Cdk2-promoter (Fig. 5a). However, deletion of 20 amino acids (IRF-1-ΔC20) significantly reduced the activity of IRF-1, and in agreement with results in Fig. 3, IRF-1-ΔC25 failed to give any significant repression compared with the control. Thus, residues 306–310 containing an LXXLL coregulator signature motif and residues N-terminal of this motif appear to be pivotal in IRF-1-dependent repression.

**FIGURE 3. Uncoupling IRF-1 gene activation from gene repression.** a, H1299 cells were transfected with 1 μg of IFNβ-Luc together with 0.5 μg of empty pcDNA3.1 (control), IRF-1, or IRF-1-ΔC25. Cells were harvested after 24 h; reporter activity is expressed as the ratio of luciferase/[protein] relative to the vector only control. The results are for duplicates in four independent experiments standardized using and internal control, b, same as described for a except using Cdk2-Luc; relative reporter activity is expressed as the ratio of luciferase/[protein], c, H1299 cells were transfected with 0.5 μg of empty pcDNA3.1 (control), IRF-1, or IRF-1-ΔC25. Cells were harvested after 24 h and analyzed by SDS-PAGE followed by immunoblot. IRF-1 was detected using anti-IRF-1 monoclonal antibody, and β-actin is shown as a loading control. d, H1299 cells were transfected with 1 μg of IRF-1, IRF-1-YLP/A, IRF-1-Δenh, and IRF-1-ΔC25 or vector only. Cells were harvested after 24 h and extracted for protein or RNA. The upper panel shows an reverse transcription-PCR analysis for TRAIL and ISG20 mRNA using actin as a control. The lower panel shows an immunoblot for expressed IRF-1 protein developed using IRF-1 mAb. The results are representative of three such experiments. e, SAOS2 cells were transfected as described for d; the cells were harvested after 24 h, and the endogenous levels of Cdk2 or transfected IRF-1 were determined by immunoblot (upper panel). The Cdk2 levels were analyzed by densitometry and are expressed (lower panel) as relative Cdk2 protein (percent) normalized to the vector alone control and are representative of two independent experiments. f, H1299 cells were transfected with 1 μg of either pcDNA3.1 (control), IRF-1, or IRF-1-ΔC25. Geneticin-resistant colonies were selected and the colonies were stained using Giemsa; g, graphical depiction of the data in d, plotted as the number of colonies/plate. The data in d and e are representative of three independent sets of experiments. RLU, relative light units.
The results of Cdk2-reporter assays using the alanine mutation series support the above conclusions. Thus, whereas mutation of residues between 314 and 325 does not prevent repression, mutation of amino acids 306–310 (IRF-1M5Ala) produces a mutant that has lost the ability to repress Cdk2 giving a value similar to that obtain with an empty vector control (Fig. 5b). While IRF-1M4Ala (aa 311–313) and IRF-1M6Ala (aa 310–305) also failed to repress Cdk2 expression, they appear to have some dominant negative activity as increased Cdk2-reporter activity was detected in these cases.

There is currently little information about the structural relationship between the C-terminal region of IRF-1 and the DNA-binding domain. As the IRF-1YLP/A mutant is defective in gene repression, it is possible that mutations within the
C-terminal enhancer domain may affect gene repression by preventing IRF-1 sequence specific DNA binding. To test this a selection of C-terminal mutants (IRF-1ΔC4, IRF-1ΔC12, IRF-1ΔC25, and IRF-1Δenh) were translated in vitro using a T7-coupled reticulocyte lysate system, and binding to labeled IRFE was determined using an EMSA. This showed that the C-terminal deletion mutants bound to the IRFE with a similar affinity as wild-type IRF-1 (Fig. 5c, upper panel: lane 1 versus lanes 3–6). Thus, in agreement with previous studies, suggesting that the enhancer domain does not take part in intramolecular interactions with the DNA binding domain and that deletion of the C terminus does not affect DNA binding (22, 23), mutations within the enhancer domain did not inhibit IRF-1-IRFE interactions.

In conclusion, an LXXLL motif located between amino acids 306 and 310 and residues to both the C and N terminus of this define a novel domain required for IRF-1-mediated repression of Cdk2 gene expression.

Growth Inhibition Correlates with the Loss of IRF-1 Repressor Activity—Finally, to confirm the relationship between the ability of IRF-1 to repress expression from the Cdk2-promoter and to suppress cell growth, we tested the deletion mutants in growth suppression assays. The ability of each mutant to inhibit colony formation was determined by introducing them into H1299 cells (Fig. 6a). In good agreement with the data on Cdk2 repression (Fig. 5a), the loss of amino acids between 311–325 had no significant effect on IRF-1-dependent inhibition of colony growth. In contrast, the loss of an additional 5 amino acids (306–310; IRF-1Δ20) resulted in a 1.8-fold increase in the number of colonies, with IRF-1ΔC25 giving 2.1-fold more colonies (the difference between wt and IRF-1ΔC25 has a p value < 0.01, Fig. 6b). Last, we determined the ability of IRF-1M6Ala and IRF-1M4Ala, two alanine substitution mutants that showed loss of Cdk2 repression, to inhibit colony growth compared with wild-type and IRF-1ΔC25. Like IRF-1ΔC25, both IRF-1M6Ala and IRF-1M4Ala had reduced activity as growth suppressors (Fig. 6, c and d). Thus, loss of growth inhibitory activity correlates with decreased Cdk2 repression rather than increased transcriptional activity as IRF-1M6Ala has IFNβ-reporter activity comparable with wild-type IRF-1 but is unable to repress Cdk2 expression. We conclude that amino acids 301–313 play a role in maintaining IRF-1 maximal growth suppressor activity.

**DISCUSSION**

IRF-1 has been shown to inhibit the growth of both transformed and non-transformed cells, and it has been hypothesized that this activity is likely to underpin its function as a tumor suppressor. The ability of IRF-1 to modulate gene expression, either as a sequence specific DNA-binding protein and direct transcriptional activator (32, 33) or as a factor that can recruit CBP/p300 to gene promoters (5, 24), appears to be intimately linked to its growth inhibitory activity. In the current study we have identified gene repression as an
additional factor contributing to the activity of IRF-1 as a growth suppressor.

The C terminus of IRF-1 has previously been described as an enhancer domain (aa 257–325 (23)), and the current data support this as removal of 70 aa from the C terminus (IRF-1\text{enh}) led to a partial loss of IRF-1 activator function (Fig. 2). One reason why this domain is required for maximal transcriptional activity is that it contains a binding site for the coactivator p300 (Fig. 7 (5)). We have previously shown that IRF-1 cooperates with p53 by enhancing the recruitment of p300 to the \textit{p21-} promoter, and one of two p300 interaction sites (CT2) in IRF-1 is located between amino acids 271–290 of the enhancer domain, with a second p300 binding site lying within the activator domain (5). Deletion of CT2 produces an IRF-1 mutant protein, which is partially compromised in its ability to respond to p300 (5). Strikingly, we show that removal of the enhancer domain severely affects the activity of IRF-1 as a growth inhibitor, despite the fact that this protein partially retains transcriptional activity, suggesting that stable binding to p300 contributes to maximal growth inhibitory activity. In fact, IRF-1\text{enh} and the DNA binding mutant (IRF-1\text{YLP/A}) are similarly compromised in their ability to inhibit colony formation. Interestingly, when C-terminal truncations of up to 25 residues (aa 301–325) were made outside the CT2-p300 binding site, transcriptional activity was not inhibited, suggesting that the enhancer domain is confined to aa 257–300. Moreover, the use of a series of deletion and alanine mutant constructs showed that amino acids 311–317 were involved in the negative regulation of IRF-1 activity against the \textit{IFN-}\text{H9252-} promoter (Fig. 7). Studies using fragments of IRF-1 fused to the Gal-4 DNA binding domain have suggested that the N-terminal DNA binding region of IRF-1 also exerts an inhibitory effect on the activation domain (23). Thus, although IRF-1 is described as a weak transaction factor, this is due to the influence of at least two intrinsic negative

**FIGURE 6. Loss of repressor activity correlates with decreased IRF-1-mediated growth suppression.** a and c, the ability of IRF-1 to inhibit cell growth was determined using colony formation assays in H1299 cells transfected with 1 µg of either pcDNA3.1 (control), IRF-1, or IRF-1 mutant constructs as detailed in the figure. Geneticin-resistant colonies were selected and the colonies were stained using Giemsa. The data are representative of duplicate assays in 3 independent experiments. b and d, graphical depiction of the data in a and c, plotted as the number of colonies/plate.
regulatory domains suggesting that factors, which bind to, or modify, these domains under physiological condition, could promote transcriptional activity.

As well as being involved in transcriptional activation, IRF-1 has also been implicated in the repression of certain genes. SLPI, a low molecular mass elastase inhibitor involved in the inflammatory response, was pulled out as an IRF-1 target using RNA fingerprinting (7). Increased expression of IRF-1 leads to an inflammatory response, was pulled out as an IRF-1 target using SLPI, a low molecular mass elastase inhibitor involved in the inflammatory response (7). The presence of the LXXLL motif in the repressor domain may indicate that IRF-1 is involved in the assembly of a repressor complex at the Cdk2 promoter or potentially at the SP1 promoter. This would be analogous to the role of PIASy in the repression of STAT1, where the LXXLL motif in PIASy, although not required for STAT1 binding, is essential for STAT1 repression in a manner that is independent of PIASy protein E3 SUMO ligase activity (37, 38). It has therefore been proposed that the LXXLL motif is likely to be involved in the assembly of a PIASy-containing corepressor complex for STAT1 (38). The binding activity of LXXLL motifs has been demonstrated to be regulated by reversible phosphorylation. For example, binding of p300 to an LXXLL motif between amino acids 22 and 26 of p53 is stabilized by phosphorylation at either the Thr18 or Ser20 sites (39). As the signature motif in the C terminus of IRF-1 is in close proximity to a number of putative phospho-acceptor sites, it is possible that corepressor binding to the LXXLL motif could be inhibited or stimulated by post-translational modification.

The enhancer domain of IRF-1 can now be divided into a number of sub-domains that are intimately linked to the function of IRF-1 in transcriptional control and growth regulation (Fig. 7). These include, (i) a negative regulatory domain that suppresses IRF-1 mediated transcription, (ii) a p300 binding domain that is required for enhanced transcription (5), and (iii) a repressor domain that is essential for IRF-1-mediated repression of Cdk2 expression. Thus, further research is required to delineate the mechanism of action of the C terminus of IRF-1 and to define physiologically relevant signaling molecules that target this domain to modulate IRF-1 function.

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