

ORIGINAL ARTICLE

Nucleolin is involved in interferon regulatory factor-2-dependent transcriptional activation

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We have previously shown that interferon regulatory factor-2 (IRF-2) is acetylated in a cell growth-dependent manner, which enables it to contribute to the transcription of cell growth-regulated promoters. To clarify the function of acetylation of IRF-2, we investigated the proteins that associate with acetylated IRF-2. In 293T cells, the transfection of p300/CBP-associated factor (PCAF) enhanced the acetylation of IRF-2. In cells transfected with both IRF-2 and PCAF, IRF-2 associated with endogenous nucleolin, while in contrast, minimal association was observed when IRF-2 was transfected with a PCAF histone acetyl transferase (HAT) deletion mutant. In a pull-down experiment using stable transfectants, acetylation-defective mutant IRF-2 (IRF-2K75R) recruited nucleolin to a much lesser extent than wild-type IRF-2, suggesting that nucleolin preferentially associates with acetylated IRF-2. Nucleolin in the presence of PCAF enhanced IRF-2-dependent *H4* promoter activity in NIH3T3 cells. Nucleolin knock-down using siRNA reduced the IRF-2/PCAF-mediated promoter activity. Chromatin immunoprecipitation analysis indicated that PCAF transfection increased nucleolin binding to IRF-2 bound to the *H4* promoter. We conclude that nucleolin is recruited to acetylated IRF-2, thereby contributing to gene regulation crucial for the control of cell growth.

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Introduction

Interferon regulatory factors (IRFs) have been studied in the context of host defense and oncogenesis (Taniguchi *et al.*, 2001) and transcriptional regulation (Schaffer *et al.*, 1997). Interferon regulatory factor-2 (IRF-2) has generally been described as a transcriptional

repressor, and is thought to function by competing with the transcriptional activator IRF-1. However, IRF-2 can act as a positive regulator for interferon stimulated response element (ISRE)-like sequences such as the promoters *H4* (Vaughan *et al.*, 1998; Xie *et al.*, 2001), vascular adhesion molecule-1 and gp91phox (Luo and Skalnik, 1996) as well as Fas ligand (Chow *et al.*, 2000). Biologically, IRF-2 plays an important role in cell growth regulation, and has been shown to be a potential oncogene (Yamamoto *et al.*, 1994). A recent report indicated that IRF-2 drives megakaryocytic differentiation through regulation of the thrombopoietin receptor promoter (Stellacci *et al.*, 2004). It has been reported that many transcription factors, such as MyoD, β -catenin, p53, Tat and CTIIA regulate specific promoters associated with the coactivators p300 and p300/CBP-associated factor (PCAF), and that this regulation leads to specific biological functions (Lakin and Jackson, 1999; Deng *et al.*, 2000; Spilianakis *et al.*, 2000; Polwsskaya *et al.*, 2001; Wolf *et al.*, 2002). Certain IRF proteins interact with other transcription factors such as TFIIB, and the coactivators p300 and PCAF (Wang *et al.*, 1996; Yoneyama *et al.*, 1998; Masumi *et al.*, 1999) and these interactions lead to transcriptional activation or repression depending on the cell types involved. To clarify the regulatory functions of transcription factors, many investigators have studied the coactivators, such as p300 and PCAF.

There are many reports of the histone acetyltransferases such as PCAF, p300/CBP and GCN5 acting as co-activators (Benkirane *et al.*, 1998; Vassilev *et al.*, 1998; Hamamori *et al.*, 1999; Jiang *et al.*, 1999; Masumi *et al.*, 1999; Schiltz *et al.*, 1999; Harrod *et al.*, 2000; Lau *et al.*, 2000a,b; Li *et al.*, 2000; Trievel *et al.*, 2000; Yamauchi *et al.*, 2000; Vo and Goodman, 2001; Lang and Hearing, 2003; Patel *et al.*, 2004). A number of transcriptional factors associate with p300/CBP, originally known as the global co-activator, and with PCAF and GCN5. Recruitment of these histone acetylases is thought to alter chromatin structures, and is required as an integral part of transcriptional activation. As a result of interaction with histone acetylases, certain transcription factors become acetylated themselves, which often results in enhanced transcriptional activity (Sternner and Berger, 2000). We have previously reported that the

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transfection of PCAF-enhanced IRF-2-dependent *H4* promoter activity in NIH3T3 cells, (Masumi *et al.*, 1999) and that IRF-2 was acetylated by p300 and PCAF *in vivo* and *in vitro* (Masumi and Ozato, 2001), providing the first example of acetylation in the IRF family. Since then, additional IRF members IRF-3 and IRF-7, have been shown to be acetylated by p300 and PCAF, respectively, *in vivo* and *in vitro* (Caillaud *et al.*, 2002; Sahara *et al.*, 2002). Acetylation of IRF-2 leads to inhibition of histone acetylation by p300 *in vitro*, suggesting a possible mechanism for transcriptional repression by IRF-2 in U937 cells (Masumi and Ozato, 2001). In contrast, we demonstrated that acetylation of IRF-2 regulates cell growth by activation of the *H4* promoter (Masumi *et al.*, 2003). In NIH3T3 cells, IRF-2 associates with endogenous p300 and becomes acetylated, binds to an ISRE site, and activates *H4* promoter activity. Thus, we demonstrated that IRF-2 acts as repressor and activator through its acetylation. In this paper, which aimed to identify the protein that associates with acetylated IRF-2, we performed pull-down assay by using tagged a IRF-2 expression system and showed that IRF-2, acetylated by PCAF, recruits nucleolin and activates transcription. Nucleolin is reported to be a ubiquitously expressed multifunctional protein involved in ribosomal biogenesis and the regulation of nucleolar translocation of ribosomal proteins (Ginisty *et al.*, 1992; Srivastava and Pollard, 1999). Our results reveal a new function for nucleolin as an IRF-2-interacting partner and transcriptional activator.

Results

Exogenous p300/CBP-associated factor acetylates interferon regulatory factor-2 in 293T cells

We have demonstrated previously that IRF-2 acts as a transcriptional activator upon acetylation (Masumi *et al.*, 2003). To investigate IRF-2 acetylation by histone acetylases *in vivo*, flag-PCAF and flag-p300 were transfected into 293T cells with flag-IRF-2. Cells were labeled with ^{14}C -acetate 1 h before harvesting and a M2-agarose pull-down assay was performed (Figure 1). Western blot analysis of the immunoprecipitates using anti-flag M2 agarose indicated that these plasmid were expressed in 293T cells (Figure 1c). Western blot analysis of the immunoprecipitates using anti-flag M2 agarose showed that acetylation of flag-IRF-2 was enhanced by co-transfection with PCAF, and to a slightly lesser extent, by p300 (Figure 1a). The results from the incorporation of ^{14}C -acetate into p300, PCAF and IRF-2 in M2-agarose precipitates was in accordance with those seen with Western blotting (Figure 1b). The patterns of Western blotting with anti-acetyllysine antibody and the incorporation of ^{14}C -acetate in whole cell lysate transfected with any plasmid were almost similar between each lane (Figure 1a right and b right). To confirm that PCAF/histone acetyl transferase (HAT) acetylates IRF-2 in 293T cells, flag-PCAF and flag-PCAF Δ HAT were transfected into 293T cells with flag-IRF-2. The pattern of Western blotting

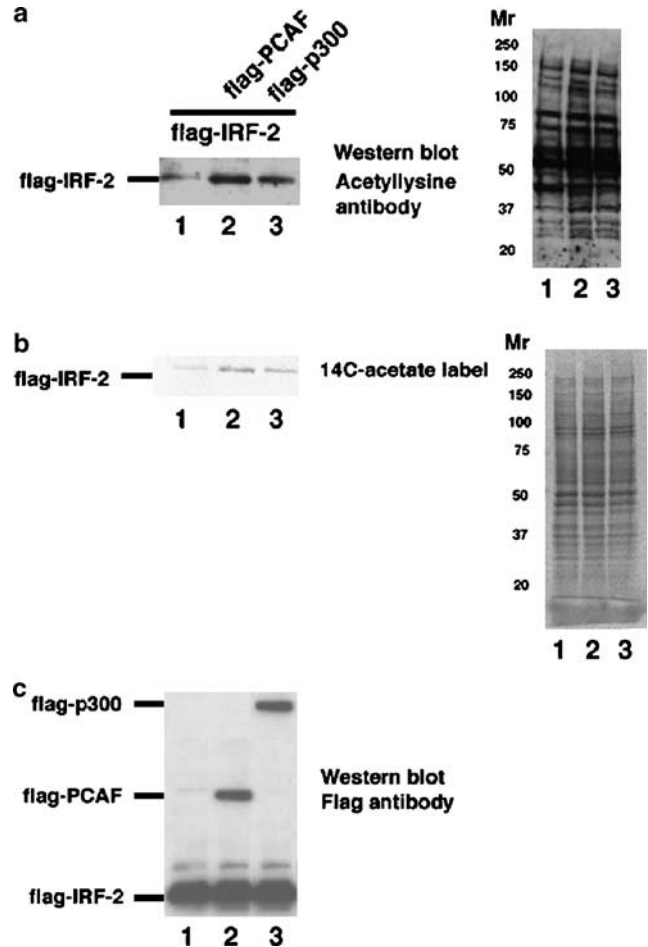


Figure 1 Interferon regulatory factor-2 (IRF-2) is acetylated by p300/CBP-associated factor (PCAF) and p300 in 293T cells. (a) 293T cells were transfected with flag-IRF-2 (1 μg) (lanes 1–3), together with flag-PCAF (1 μg) (lane 2) and flag-p300 (1 μg) (lane 3) plasmids. Of ^{14}C -acetate, 20 μCi were added 1 h before preparation of the cell lysate. Cell lysates from 293T cells were incubated with M2-agarose, and then the flag-peptide elution fraction was electrophoresed on SDS–10% PAGE and immunoblotted using an anti-acetyl lysine antibody (left). Whole lysate was electrophoresed on SDS–10% PAGE and immunoblotted with anti-acetyl lysine antibody (right) (b) Immunoblotted membranes from M2-agarose precipitates (left) and whole lysate (right) were reused for Image analysis using a Fuji BAS 2500 to visualize the ^{14}C -incorporated protein. (c) Anti-flag M2 agarose precipitates from 293T transfected cells transfected with above plasmids were electrophoresed on SDS–10% PAGE and immunoblotted with an anti-flag antibody.

for whole cell lysates did not significantly affect the results (Figure 2b). Western blot analysis of the immunoprecipitates using anti-flag M2 agarose indicated that these protein were expressed in 293T cells (Figure 2a bottom). An M2-agarose pull-down assay showed that compared to the control vector, the acetylation level of IRF-2 was increased by transfection of full-length PCAF, but not by PCAF lacking HAT activity (Figure 2a top). We detected PCAF autoacetylation as described earlier (Santos-Rosa *et al.*, 2003). These results indicate that IRF-2 acetylation is enhanced by PCAFHAT *in vivo*.

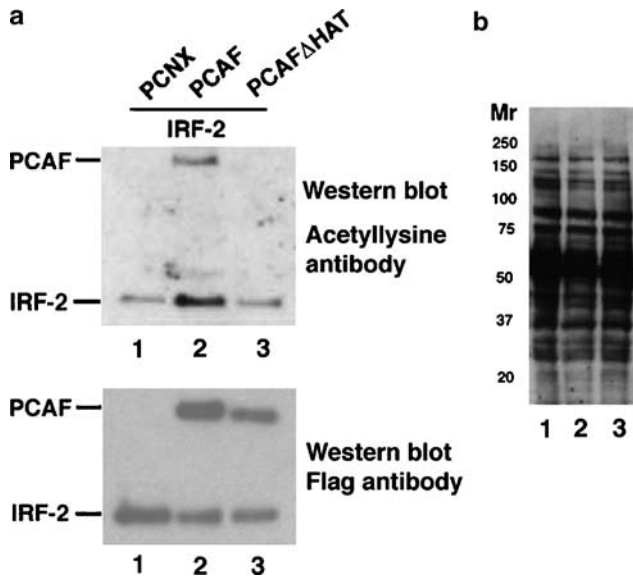


Figure 2 Western blot analysis of 293T cells transfected with interferon regulatory factor-2 (IRF-2) and p300/CBP-associated factor (PCAF). (a) 293T cells were transfected with PCNX control vector (lane 1), flag-PCAF (lane 2) and flag-PCAFΔhistone acetyl transferase (HAT) (lane 3) with flag-IRF-2. An M2-agarose-purified fraction from cell lysate was separated on SDS-10% PAGE and immunoblotted with an anti-acetyllysine (upper panel) or anti-flag antibody (bottom panel). (b) Whole cell lysates from transfected 293T cells were electrophoresed and immunoblotted with an anti-acetyllysine antibody. Full-length PCAF was auto-acetylated as shown in lane 2.

Interferon regulatory factor-2 recruits nucleolin in the presence of p300/CBP-associated factor

Our previous report suggested that it is the involvement of cellular proteins associated with acetylated IRF-2, which results in its transcriptional regulation (Masumi *et al.*, 2003). To identify those proteins that associate with acetylated IRF-2, we performed an M2-agarose pull-down assay using 293T cells transfected with flag-IRF-2 and flag-PCAF. An M2-agarose-purified fraction was subjected to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue (Figure 3a). As shown in Figure 3a, proteins of approximately 110 and 70 kDa were observed in the immunoprecipitate from cells transfected with both flag-IRF-2 and flag-PCAF, but not in the immunoprecipitate from cells transfected with flag-PCAF alone. To identify these proteins, the bands were cut from the acrylamide gel, digested by trypsin and analysed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Mass spectrometric analysis revealed that nucleolin was included in the 110-kDa band and that the heat shock protein 70 family was included in the 70 kDa band. As the heat shock protein 70 family is also included in the band in the PCAF-only transfected cells (Figure 3a, lane 1), we focused on nucleolin in this study. In Figure 3b, we investigated the nucleolin recruitment to IRF-2 using Western blotting. 293T cells were transfected with flag-IRF-2 in the presence or

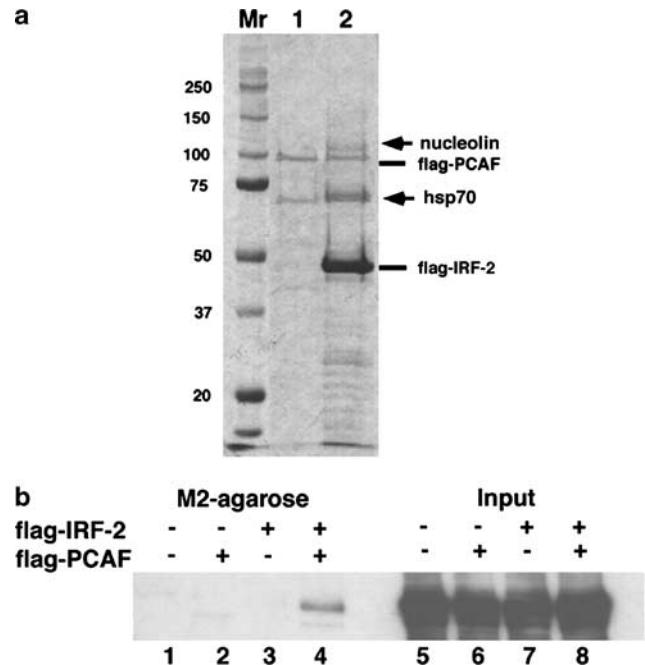


Figure 3 Coomassie blue staining pattern of the proteins co-precipitated with flag-interferon regulatory factor-2 (IRF-2). (a) 293T cells were transfected with 5 μg flag-p300/CBP-associated factor (PCAF) (lane1) alone and 5 μg flag-IRF-2 together with 5 μg flag-PCAF (lane 2). Whole cells were lysed in a buffer B and incubated with M2-agarose, and then the flag-peptide-eluted fraction was separated on SDS-10% PAGE and the gel was stained with Coomassie brilliant blue. The two bands indicated with arrows were cut and trypsinized, and then liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed. Nucleolin was included in the upper band and the hsp70 family was included in the lower band. (b) 293T cells were transfected with control vector (lane 1), flag-PCAF (lane 2), flag-IRF-2 (lane 3), and flag-IRF-2 and flag-PCAF (lane 4) as described in (a). Cell lysates from 293T cells were incubated with M2-agarose, and then the flag-peptide elution fraction was prepared. Whole cells (right) and M2-agarose precipitates (left) were immunoblotted with anti-nucleolin antibody.

absence of flag-PCAF, and then cell lysate was incubated with anti-flag M2-agarose. The flag-peptide-eluted fraction was immunoblotted with anti-nucleolin antibody. Nucleolin expression level was not altered in any plasmid-transfected 293T cells, and nucleolin was detected most clearly in the flag-peptide-eluted fraction from cells transfected with both flag-IRF-2 and flag-PCAF (Figure 3b).

We investigated whether the histone acetylase activity of PCAF was required to recruit nucleolin to IRF-2. Flag-IRF-2 was cotransfected with flag-PCAF or flag-PCAFΔHAT into 293T cells and precipitated with M2-agarose, then analysed for the amount of recruited nucleolin by Western blotting. There was no difference of the amount of nucleolin in the lysate of 293T cells transfected with any cDNA (Figure 4a). Nucleolin was clearly identified in the affinity-purified complex from IRF-2/PCAF-transfected cells, but not in the precipitates from IRF-2/PCAFΔHAT-, IRF-2 alone-, or PCAF alone-transfected cells (Figure 4a), consistent with the

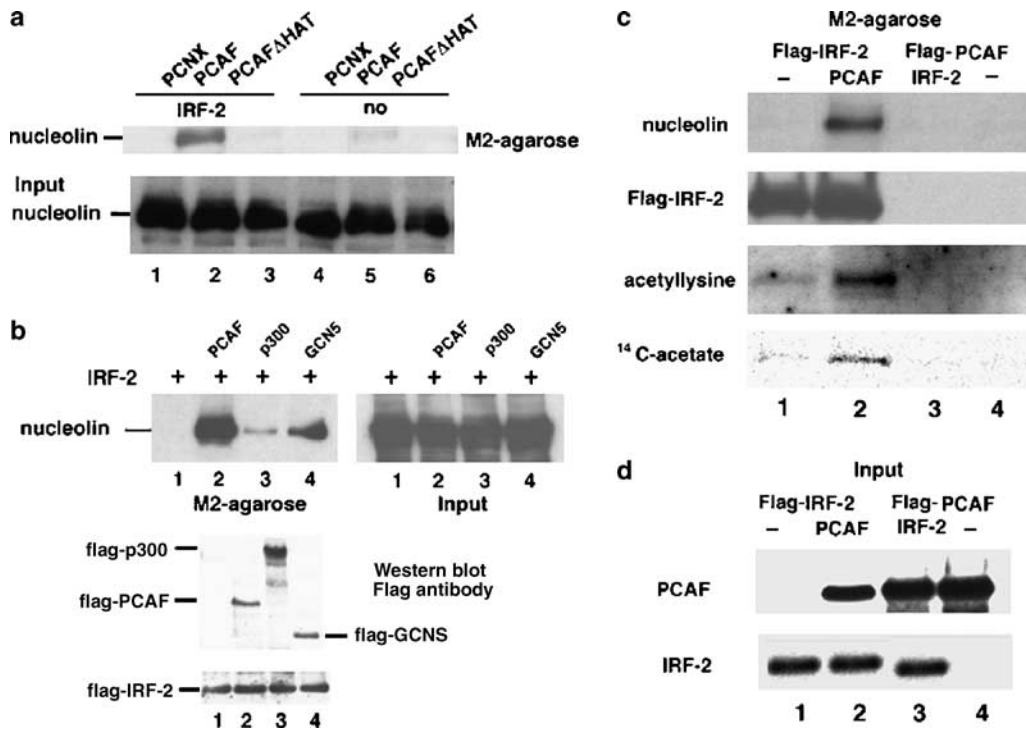


Figure 4 Nucleolin is involved in an interferon regulatory factor-2 (IRF-2)-binding complex. (a) Cell lysates from 293T cells transfected with PCNX (lanes 1 and 4), flag-p300/CBP-associated factor (PCAF) (lanes 2 and 5) and flag-PCAF Δ histone acetyl transferase (HAT) (lanes 3 and 6), flag-IRF-2 (lanes 1–3) were incubated with M2-agarose and the flag-peptide-eluted fraction was separated on a SDS–10% PAGE and immunoblotted with anti-nucleolin antibody (upper panel). Whole cell lysates from transfected 293T cells were separated on SDS–10% PAGE and immunoblotted with an anti-nucleolin antibody (upper right panel). (b) 293T cells were transfected with PCNX empty vector (lane 1), flag-PCAF (lane 2), flag-p300 (lane 3) and flag-GCN5 (lane 4) in the presence of flag-IRF-2. Cell lysate was incubated with M2-agarose and the flag-peptide-eluted fraction was separated on a SDS–10% PAGE and immunoblotted with anti-nucleolin antibody (upper left panel). Whole cell lysates from transfected 293T cells were separated on SDS–10% PAGE and immunoblotted with an anti-nucleolin antibody (upper right panel). (c) Acetylated IRF-2 preferentially recruits nucleolin in 293T cells. Flag-IRF-2 was transfected into 293T cells in the absence or presence of PCAF (without tag) (lanes 1 and 2) or flag-PCAF was transfected with or without IRF-2 (without tag) (lanes 3 and 4). 14 C-acetate was added to the 293T culture 1 h before harvesting. Cell lysates from 293T cells were incubated with M2-agarose and the flag-peptide fraction was electrophoresed and immunoblotted with anti-nucleolin, anti-flag, anti-acetyllysine antibodies. The membrane was reused for analysis with a BAS 2500 image analyzer to visualize 14 C-labeled protein. (d) Western blot analysis of PCAF and IRF-2 for whole 293T cells transfected with flag-IRF-2 (lanes 1 and 2), flag-PCAF (lanes 3 and 4), IRF-2 (lane 3) and PCAF (lane 2) was performed.

results of Figure 3b. To test whether IRF-2 recruits nucleolin in the presence of other histone acetylases, the same amount of p300 and GCN5 was transfected into 293T cells with IRF-2. As shown in Figure 4b, transfection of flag-p300 and flag-GCN5 also induced nucleolin recruitment to flag-IRF-2, although p300 recruited nucleolin to a much lesser extent compared to other histone acetylases. For comparative nucleolin recruitment to IRF-2, greater amounts of p300 may be required because of its larger molecular size. Nucleolin is not detected in the flag-peptide-eluted fraction from cell lysate transfected with only flag-IRF-2 although IRF-2 is acetylated at basal level in the absence of exogenous PCAF in 293T cells (Figures 1–4). Detectable basal acetylation level of IRF-2 does not have enough binding affinity with nucleolin in 293T cells.

To further investigate these results, PCAF without a flag-tag was transfected with flag-IRF-2 into 293T cells and then an M2-agarose pull-down assay was performed. Nucleolin was recruited more potently to

affinity-purified precipitates of both flag-IRF-2 and PCAF-transfected cells than that of the cells transfected with flag-IRF-2 alone (Figure 4c). This result is similar to that was shown in Figure 4a. In addition, we detected an increase in IRF-2 acetylation in PCAF-transfected cells, consistent with the results in Figure 2. In contrast, when flag-PCAF was cotransfected with IRF-2 (without the flag-tag) into 293T cells, nucleolin was hardly detected in the anti-flag M2-agarose precipitates (Figure 4c).

We investigated whether an increased amount of PCAF transfection led to an increase in the recruitment of nucleolin to IRF-2. Differing amounts of PCAF was transfected into 293T cells with flag-tag IRF-2 and cell lysate was incubated with anti-flag M2 agarose, and the flag-peptide-eluted fraction was then immunoblotted with anti-acetyllysine, anti-nucleolin antibodies. Acetylation of IRF-2 and nucleolin recruitment increased parallel to amount of PCAF in 293T cells (Figure 5).

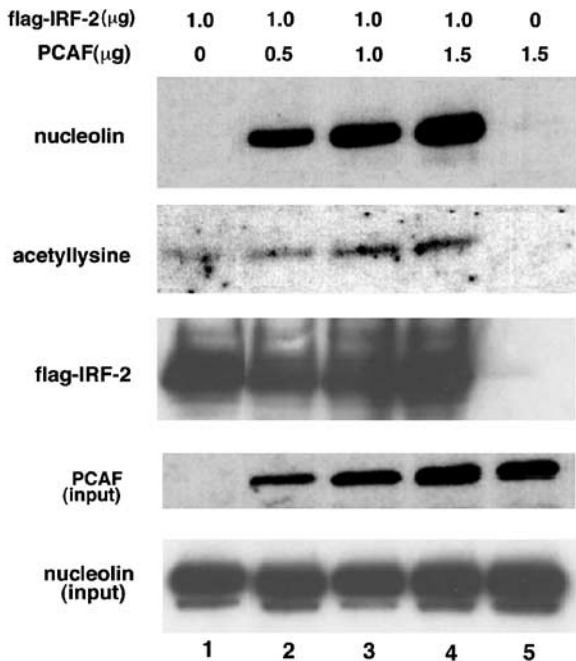


Figure 5 Interferon regulatory factor-2 (IRF-2) associates nucleolin in the presence of p300/CBP-associated factor (PCAF). 293T cells were transfected with several indicated amount of PCAF (lanes 1–5) and flag-IRF-2 (1 μg) (lanes 1–4). Cell lysate were incubated with anti-flag M2 agarose and flag-peptide eluted fraction were immunoblotted with anti-nucleolin, anti-acetyllysine and anti-flag antibodies. Whole cell lysate of transfected cells were immunoblot with anti-PCAF and anti-nucleolin antibodies.

We examined the localization of IRF-2 and nucleolin. HeLa cells transfected with flag-IRF-2 or a mutant flag-IRF-2K75R partially defective in acetylation (Masumi *et al.*, 2003) with or without PCAF were fixed with paraformaldehyde and immunostained with an anti-flag antibody conjugated to cy3, and then an anti-nucleolin antibody linked with fluorescein isothiocyanate (FITC). Immunostained cells were visualized by laser scanning confocal microscopy. As shown in Figure 6a, there was no significant difference between wild-type IRF-2 and IRF-2K75R mutant-transfected HeLa cells, with nucleolin localized mainly in the nucleolus and to a lesser extent in the nucleus. Although IRF-2 localized predominantly in the nucleus, some was also localized in nucleolus. We observed that IRF-2 colocalized with nucleolin in a peri-nucleolar location. p300/CBP-associated factor transfection with both wild-type IRF-2 and K75R mutant did not change the colocalization of IRF-2 and nucleolin significantly.

To confirm if acetyltable IRF-2 recruits to nucleolin, a protein–protein interaction assay was performed using IRF-2 stably transfected cells. Cell lysates were prepared from HeLa and K562 cells stably transfected with flag-tagged wild-type IRF-2 or IRF-2K75R (Masumi *et al.*, 2003). Lysates were incubated with anti-flag M2-agarose, and the precipitates were subjected to Western blot analysis using an anti-nucleolin antibody. In both HeLa and K562 cells, appreciable amounts of PCAF and p300 were detected (data not shown). As shown in

Figure 6b, nucleolin–IRF-2 interaction was observed in both HeLa and K562 cells that expressed wild-type IRF-2. However, in cells that expressed the K75RIRF-2 mutant (Masumi *et al.*, 2003), the nucleolin interaction was markedly diminished. These results suggest that IRF-2 is acetylated by histone acetylases such as PCAF and p300 in these cells, and that acetylated IRF-2 preferentially associates with nucleolin.

Nucleolin transactivates interferon regulatory factor-2-enhanced H4 promoter activity

Interferon regulatory factor-2 functions as an activator for the H4 gene promoter in NIH3T3 cells (Masumi *et al.*, 2003). To examine the functional role for nucleolin in IRF-2-dependent transcription, an H4 gene reporter plasmid was transfected into NIH3T3 cells with IRF-2, PCAF and nucleolin. As shown in Figure 7a, transfection of nucleolin and PCAF both increased IRF-2-induced H4 promoter activation. Co-transfection of nucleolin with PCAF further enhanced IRF-2-induced H4 promoter activity (Figure 7a). In NIH3T3 cells, endogenous p300 may also induce IRF-2-dependent transactivation through acetylation, resulting in its interaction with nucleolin. In addition, co-transfection with HAT-deficient PCAF had no effect on nucleolin/IRF-2 activity. (Figure 7a). Co-transfection of the K75RIRF-2 mutant with PCAF/nucleolin resulted in a much lower activation of the H4 promoter in NIH3T3 cells than with wild-type IRF-2 (Figure 7b). To examine nucleolin contribution to IRF-2-mediated H4 promoter activation, we performed luciferase reporter assay using nucleolin small interfering RNA (siRNA). NIH3T3 cells were transfected with nucleolin siRNA to knock-down endogenous nucleolin and then transfected with IRF-2, PCAF with H4 promoter-conjugated luciferase reporter. Compared to the control siRNA transfection, nucleolin siRNA transfection reduced the endogenous nucleolin protein in NIH3T3 cells (Figure 7d) and downregulated the IRF-2/PCAF-mediated H4 promoter activation (Figure 7c). These results confirm that nucleolin contributes to IRF-2/PCAF-mediated transcriptional activation in NIH3T3 cells.

We have shown previously that acetylation of IRF-2 is related to cell growth (Masumi *et al.*, 2003) and have therefore investigated whether IRF-2 is associated with nucleolin in growing NIH3T3 cells. For confocal analysis, we detected that IRF-2 and nucleolin were localized in nuclei and nucleoli in both growing and growth-arrested NIH3T3 cells. There was no significant difference between either type of NIH3T3 cell (Figure 8a). We performed a DNA affinity binding assay with biotinylated H4 promoter oligonucleotides that had been conjugated to magnetic beads. Nuclear extracts from growing and growth-arrested cells were incubated with the beads-conjugated H4 promoter DNA. Interferon regulatory factor-2 was detected in an eluted fraction from beads incubated with growing cell nuclear extract as reported earlier (Figure 8b) (Masumi *et al.*, 2003). We found that while a similar level of nucleolin was detected in both growing and growth-arrested cells, H4 promoter DNA was bound to

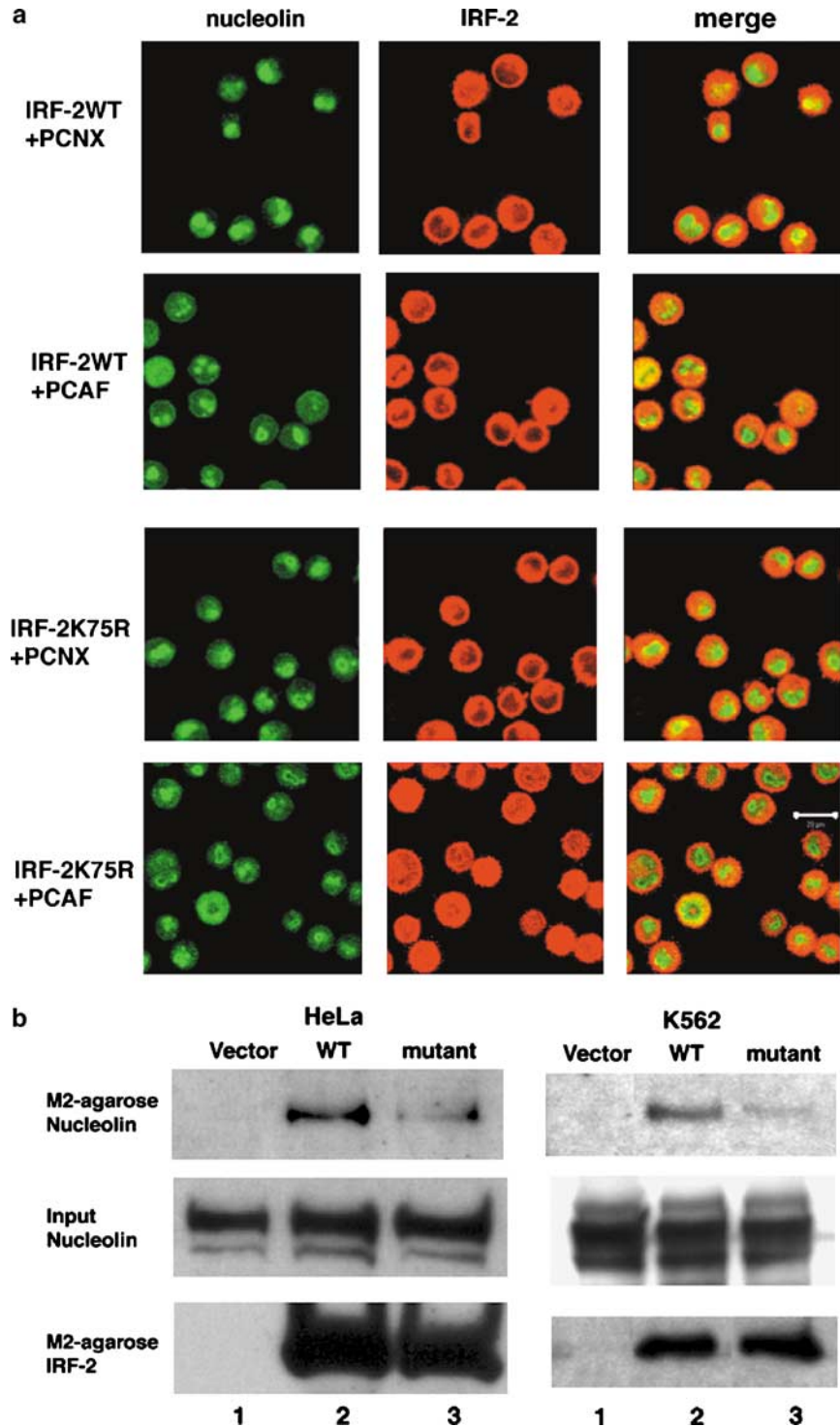


Figure 6 Interferon regulatory factor-2 (IRF-2) colocalizes and associates with nucleolin. **(a)** Laser scanning confocal microscopy was carried out on HeLa cells transiently transfected with flag-IRF-2 and flag-IRF-2K75R with or without p300/CBP-associated factor (PCAF). The cells were fixed with paraformaldehyde 24 h after transfection and lysed with 0.2% TritonX-100 for 10 min. Then, cells were immunocytostained with an anti-flag conjugated to Cy3 (red fluorescence) antibody for 24 h, following which, washed cells were immunostained with anti-nucleolin linked with fluorescein isothiocyanate (FITC) (green fluorescence) for 24 h. These washed cells were covered with glycerol and examined by laser scanning confocal microscopy. Colocalization of proteins results in a merging of red and green fluorescence to produce a yellow image. **(b)** Cell lysate from HeLa (left) and K562 (right) cells stably transfected with an empty vector (lane 1), flag-IRF-2 (lane 2) and flag-IRF-2K75R mutant (lane 3) were incubated with anti-flag M2-agarose and flag-peptide-eluted fractions were separated on SDS-10% PAGE and immunoblotted with anti-nucleolin (top) and anti-IRF-2 (bottom) antibodies. Whole cell lysates were separated on SDS-10% PAGE and immunoblotted with an anti-nucleolin antibody (middle).

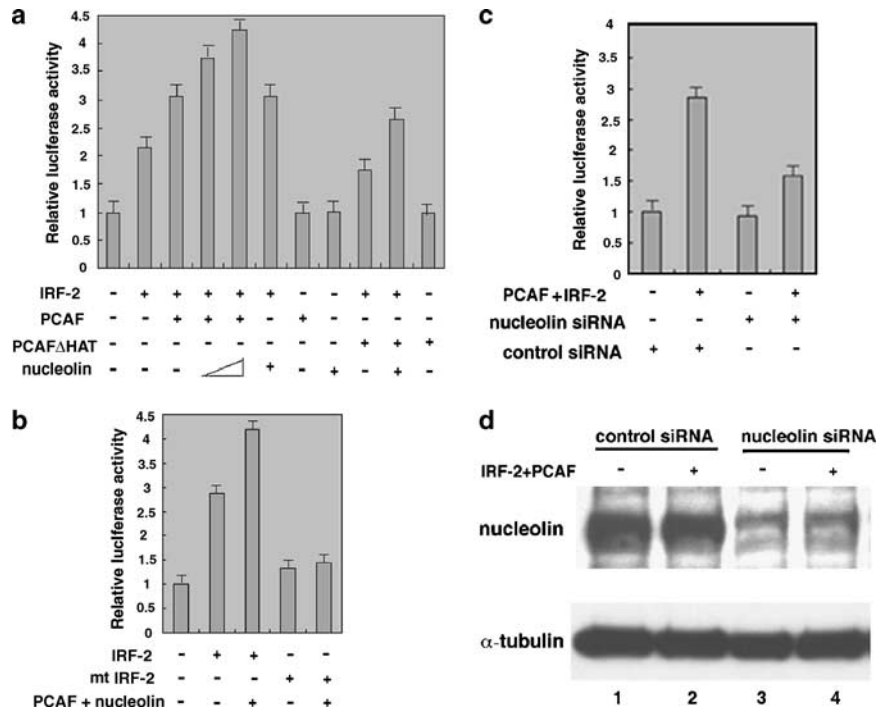


Figure 7 Nucleolin activates interferon regulatory factor-2 (IRF-2)-dependent *H4* promoter activity. (a) *H4* promoter reporter (400 ng), nucleolin (100 and 200 ng), p300/CBP-associated factor (PCAF)PCNX (100 and 200 ng) and PCAF Δ histone acetyl transferase (HAT)PCNX (200 ng) were transfected with IRF-2pcDNA3.1 (20 ng) into NIH3T3 cells. Luciferase activity was analysed 48 h after transfection. (b) Wild-type IRF-2 or mutant IRF-2 (IRF-2K75R) was transfected with nucleolin/PCAF with the *H4* promoter reporter as described in (a). Luciferase activity was analysed 48 h after transfection. The mean \pm s.d. from three separate experiments were calculated after normalization with TK Renilla activity. (c) Nucleolin small interfering RNA (siRNA) abrogates IRF-2/PCAF-induced *H4* promoter activation. Nucleolin siRNA was transfected into NIH3T3 cells and then PCAFPCNX and IRF-2pcDNA3.1 were transfected into NIH3T3 cells with *H4* promoter reporter. At 24 h after transfection of plasmids, luciferase activity was analysed. (d) Immunoblot analysis of NIH3T3 cell lysate transfected with nucleolin siRNA and plasmids as described in (c) using anti-nucleolin and anti- α -tubulin antibodies.

the nucleolin from growing cells only (Figure 8b). These findings are consistent with previous results, which support that the interaction of nucleolin and acetylated IRF-2 in growing cells mediate *H4* gene promoter activity (Masumi *et al.*, 2003).

From these results, it appears that the acetylation of IRF-2 rather than the change of colocalization of both factors is important for the interaction of IRF-2 and nucleolin in growing NIH3T3 cells. To confirm the association of nucleolin with IRF-2 on the *H4* promoter, chromatin immunoprecipitation analysis was performed. Chromatin was isolated from NIH3T3 cells transfected with PCAFPCNX and immunoprecipitated with anti-IRF-2 and anti-nucleolin and anti-PCAF antibodies. Immunoprecipitates were performed with polymerase chain reaction (PCR) using *H4* promoter primer as described earlier (Masumi *et al.*, 2003). As shown in Figure 8c, PCAF transfection slightly enhances the PCAF binding to *H4* promoter, however, a greater amount of nucleolin was bound to the *H4* promoter in the PCAF-transfected cells compared to control cells. In addition, a greater amount of IRF-2 was also bound to the *H4* promoter in PCAF-transfected cells compared to non-transfected cells. From these results it appears that, in NIH3T3 cells, IRF-2 and nucleolin bound the *H4* promoter more

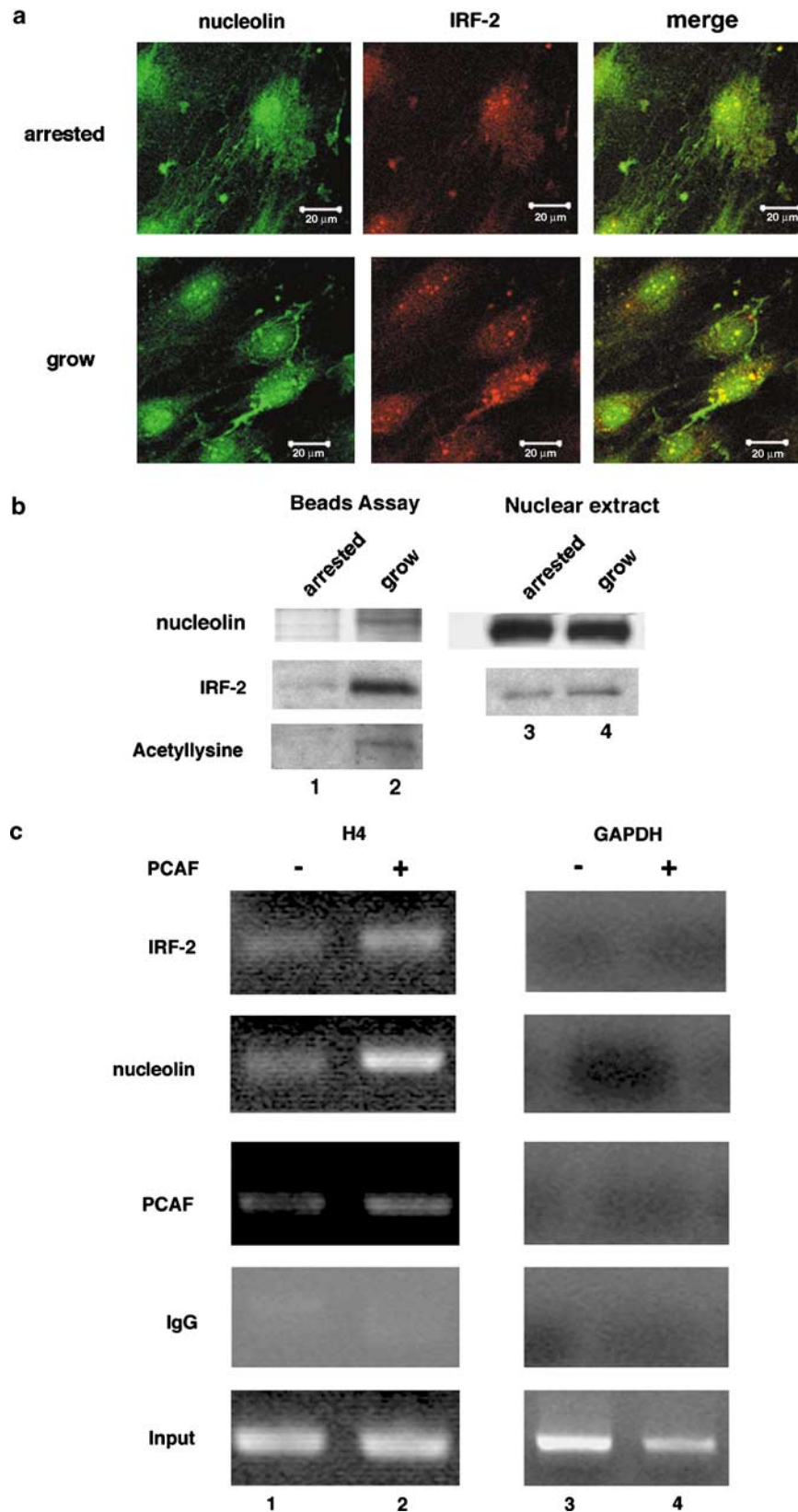
tightly following transfection with exogenous PCAF. We conclude that nucleolin binds to acetylated IRF-2 and IRF-2/PCAF/nucleolin complexes in turn stimulate the activation of gene transcription, which drives cell growth (Figure 9).

Discussion

In this study, we have demonstrated that nucleolin acts as a positive modulator of IRF-2-dependent transcriptional activation through an association with IRF-2. Nucleolin is one of the most abundant nucleolar proteins in rapidly growing eukaryotic cells. It is multifunctional and thought to be involved in many cellular processes, including ribosome biogenesis, the processing of ribosomal RNA, mRNA stability, transcriptional regulation and cell proliferation, and it is also a downstream target of several signal transduction pathways (Ginisty *et al.*, 1992; Srivastava and Pollard, 1999). Nucleolin has been shown by proteomic analysis to associate with various proteins, such as B23, Ku80, eIF2a and the RNA binding proteins (RNP) complex (Yanagida *et al.*, 2001). Ying *et al.* (2000) reported that the interaction of nucleolin with Myb downregulated Myb transcriptional activity. Recently, Grinstein *et al.*

(2002) reported that nucleolin is a key activator of the HPV18 oncogene transcription involved in chromatin structure regulation and thus identified nucleolin as a

cellular protein with oncogenic potential. From our study we can conclude that for *H4* gene regulation by IRF-2, nucleolin acts as an oncogenic activator via



transcriptional activation, suggesting an involvement in cell growth regulation.

Previously, we demonstrated in NIH3T3 cells that lysine residues 75 and 78 in the IRF-2 DNA-binding domain are the major acetylation sites and that the IRF-2K75R mutant showed reduced *H4* promoter activity. As reported in our previous paper, p300 acts as the main acetylase for IRF-2 in NIH3T3 cells because the level of PCAF expression is so very low. However, exogenous PCAF transfection induced IRF-2-dependent transcriptional activation (Masumi *et al.*, 1999), and exogenous PCAF might induce IRF-2 acetylation in NIH3T3 cells. We also found that transfection with another histone acetylase GCN5 induced IRF-2 acetylation (data not shown) and nucleolin-IRF-2 interaction in 293T cells. We previously concluded that in NIH3T3 cells acetylated IRF-2 binds to the *H4* promoter with p300 to

regulate the *H4* gene (Masumi *et al.*, 2003). However, in cells with a high amount of PCAF or GCN5, IRF-2 may be acetylated by both histone acetyltransferases, as well as p300, recruit nucleolin and thus regulate specific promoters. In the *H4* promoter assay, we demonstrated that PCAF acetyltransferase activity was required for efficient activation of transcription mediated by IRF-2/nucleolin. Interferon regulatory factor-2K75R mutant partially defective acetylation reduces the activation of the *H4* promoter in the presence of PCAF/nucleolin, consistent with the results of the poor association of IRF-2K75R with nucleolin in stable transfectants.

We observed that in HeLa cells, IRF-2 colocalized with nucleolin in the peri-nuclear region. Nucleolin has been reported to colocalize with p53 in a stress-dependent manner; it mobilizes between nucleoli, nuclei and the cytosol depending on the level of stress (Klibanov *et al.*, 2001; Daniely *et al.*, 2002). This mobilization depends on the cell condition, such as during various stage of growth or differentiation. In our confocal experiment, PCAF expression did not change the localization of either IRF-2 or nucleolin in HeLa cells. In growing and growth-arrested NIH3T3 cells, similar colocalization of nucleolin and IRF-2 is observed. p300/CBP-associated factor-mediated acetylation induces nucleolin-binding affinity to IRF-2 rather than colocalization of both factors.

Treatment with trichostatin A, a typical histone deacetylase inhibitor, enhanced both expression and acetylation of IRF-2 in IRF-2-stably transfected HeLa cells, but the association of IRF-2 with nucleolin was comparable between trichostatin A-treated and untreated HeLa cells (data not shown). Trichostatin A may affect other acetylatable transcription factors, which compete with IRF-2/nucleolin interaction. Alternatively, interaction of PCAF and p300 with IRF-2 may be required for the association of nucleolin and acetylated IRF-2. In fact, as IRF-2 binds PCAF or p300 *in vitro* and *in vivo* as reported earlier (Masumi *et al.*, 2003), acetylated IRF-2 may associate with nucleolin together with PCAF or p300. However, the nucleolin recruitment in anti-flag M2 agarose precipitate from flag-tagged PCAF-transfected cells was difficult to detect. From these results it can be concluded that nucleolin binds to IRF-2 directly, but not to PCAF. Acetylated IRF-2 could be detected at the basal level in

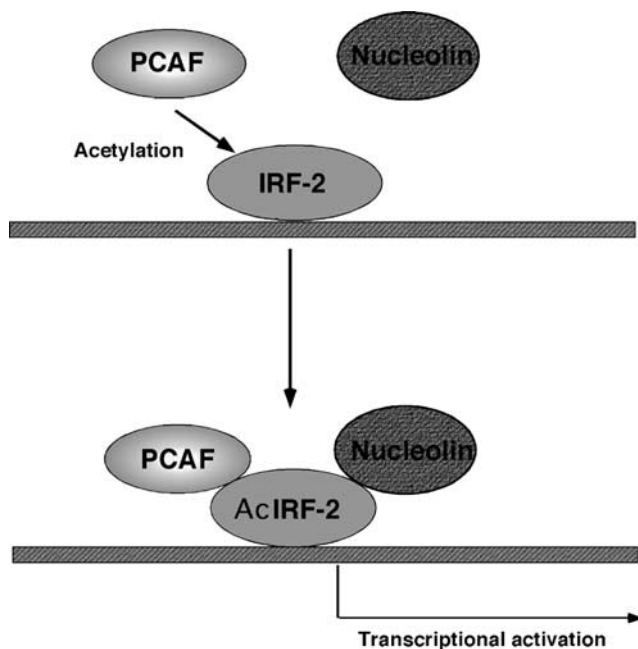


Figure 9 Schematic model of the interferon regulatory factor-2 (IRF-2)-binding complex for its transcriptional regulation. p300/CBP-associated factor (PCAF) acetylates IRF-2, and acetylated IRF-2 associates with endogenous nucleolin together with PCAF. IRF-2/nucleolin/PCAF transactivates the IRF-2-specific promoter.

Figure 8 Nucleolin binds *H4* promoter. (a) Laser scanning confocal microscopy was carried out on NIH3T3 cells. Growing or growth-arrested NIH3T3 cells were fixed with paraformaldehyde 24 h after transfection and lysed with 0.2% TritonX-100 for 10 min. Cells were then immunocytostained with a goat anti-interferon regulatory factor-2 (IRF-2) antibody at 4°C overnight and then immunostained with anti-goat second antibody conjugated to Alexa 488 for 2 h (green fluorescence). Following this, washed cells were immunostained with rabbit anti-nucleolin antibody and then anti-rabbit second antibody linked with Alexa 594 (red fluorescence) for 2 h. Washed cells were covered with glycerol and examined by Laser scanning confocal microscopy. Colocalization of proteins results in a merging of red and green fluorescence to produce a yellow image. (b) Nucleolin interacts with IRF-2 in growing NIH3T3 cells. Nuclear extracts were prepared from growth-arrested (lanes 1 and 3) and growing NIH3T3 cells (lanes 2 and 4), and incubated with magnetic beads conjugated to *H4* promoter. Bound materials (lanes 1 and 2) and whole nuclear extract (lanes 3 and 4) were analysed by immunoblot assay using anti-nucleolin, anti-IRF-2 and anti-acetyllysine antibodies. (c) NIH3T3 cells were transfected with p300/CBP-associated factor (PCAF) and crosslinked with 1% formaldehyde, chromatin was isolated as described under 'Materials and methods' and a chromatin immunoprecipitation assay of the *H4* promoter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using anti-IRF-2, anti-nucleolin, anti-PCAF and rabbit immunoglobulin G (IgG) was performed. Polymerase chain reaction (PCR) quantitation was carried out as indicated under 'Materials and methods.'

IRF-2-transfected 293T cells without transfection of PCAF (Figures 1–5). However, we could not detect significant nucleolin recruitment to flag-IRF-2 without transfection of PCAF. p300/CBP-associated factor transfection led to a great extent of nucleolin recruitment instead of slight increase of acetylation of IRF-2, although increasing the extent of PCAF transfection increased nucleolin recruitment and was consistent with IRF-2 acetylation (Figure 5). Acetylated IRF-2 may change its conformation and nucleolin may prefer to bind to acetylated IRF-2. Nucleolin has many acid residues such as glutamic acid and asparagic acid (Lapeyre *et al.*, 1987). In contrast, IRF-2 has 18 lysine residues in DNA-binding domain (Masumi *et al.*, 2003). We do not at present understand how they associate via their amino acids charge, but expect to be able to determine the binding form for both factors in the future. p300/CBP-associated factor transfection may not only enhance the acetylation but also the binding affinity of IRF-2 with nucleolin. According to the chromatin precipitation analysis, PCAF transfection enhanced nucleolin and IRF-2 binding to *H4* promoter, but PCAF binding to *H4* promoter was enhanced only slightly by PCAF transfection. Exogenous PCAF may contribute to the acetylation of IRF-2 rather than an association with *H4* promoter.

As shown in Figure 4b, p300 transfection into cells induced much less amount of nucleolin recruitment to IRF-2 compared to PCAF transfection. Although PCAF appeared to be a slightly better IRF-2 acetylase, transfection of p300 also resulted in substantial acetylation of IRF-2 (Figure 1a). It is not clear why p300 only induced modest recruitment of nucleolin to IRF-2 (Figure 4b), despite fairly high level of IRF-2 acetylation. p300 may acetylate other proteins which compete with IRF-2 for binding to nucleolin. We are currently searching for other acetylated proteins that associate with nucleolin when PCAF or p300 is transfected.

It has been shown that p300 and PCAF interact with and acetylate HIV Tat on distinct lysine residues (Kiernan *et al.*, 1999; Ott *et al.*, 1999). The acetylation of the activator domain of Tat by PCAF and p300 has different biological functions for Tat, and both events increase the activation of transcription from the LTR (Ott *et al.*, 1999, 49). In addition, Chen *et al.* (2002) demonstrated that acetylation of RelA at distinct sites differentially regulates various biological functions of NF- κ B. Martinez-Balbas *et al.* (2000) showed that acetylase PCAF, and to a lesser extent CBP and p300, can acetylate E2F1 *in vivo* and increase its DNA-binding ability and that the acetylation status of E2F1 is affected by the histone deacetylase associated with the RB–E2F1 complex. Thus, acetylation of transcription factors leads to changes in their biological activity in terms of DNA-binding affinity, transcriptional activity, interaction with other proteins, and intracellular protein stability (Bannister and Miska, 2000). In the case of IRF-2, we demonstrated that the same sites of IRF-2 were acetylated by PCAF and p300 and that acetylated IRF-2 bound to the promoter more efficiently than non-acetylated IRF-2 *in vivo* as shown earlier (Masumi *et al.*,

2003). Acetylated and non-acetylated IRF-2 appear to bind differently to cellular proteins. Acetylated IRF-2 binds to promoters more efficiently, probably by recruiting cellular factors, such as the nucleolin identified in this study.

Barlev *et al.* (2001) showed that acetylated p53 binds more tightly to the transcriptional cofactors transformation/transcription domain-associated protein (TRRAP) and CREB-binding protein than non-acetylated p53, although acetylated and non-acetylated p53 bind to the p21 promoter in the same manner. Levy *et al.* (2004) demonstrated that acetylated β -catenin associates preferentially with Tcf4 (T-cell factor/lymphoid enhancer factor) and that co-activation of β -catenin/Tcf by p300 is mediated in part by acetylation of β -catenin. In our study, we have confirmed that PCAF-acetylated IRF-2 forms a complex with nucleolin. Histone acetylases such as PCAF and p300 mediate IRF-2-dependent transcriptional activation through nucleolin–IRF-2 interaction. Our findings provide the biological evidence for a transcriptional regulatory mechanism which is effected via protein acetylation.

Materials and methods

Cell culture and transfection

NIH 3T3 cells were grown in Dulbecco's-modified Eagle's medium (DMEM) (Sigma, St. Louis, MI, USA) with 10% calf serum (GIBCO BRL, Rockville, MD, USA), penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C in 5% CO₂ and 95% air. NIH 3T3 cells were transfected with H4 reporter using lipofectamine (Invitrogen, Carlsbad, CA, USA) as described earlier (Masumi *et al.*, 2003). For making growth-arrested NIH3T3 cells, DMEM containing 0.5% calf serum was added to growing NIH3T3 cells, and cells were cultured for 48 h. HeLa and 293T cells were grown in DMEM with 10% fetal calf serum (Sigma). 293T cells were then transfected with IRF-2-pcDNA3.1, PCAFPCNX and p300pCI plasmids (Masumi *et al.*, 1999; Masumi and Ozato, 2001) using Fugene 6 (Roche Biochemicals, Indianapolis, IN, USA). At 24–48 h after transfection, cells were lysed in a buffer B (Tris-HCl, pH 8.0, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 100 mM NaCl, 0.1% NP-40), containing a protease inhibitor mix (Sigma). For some experiments, 20 μ Ci of ¹⁴C-acetate (Amersham, Piscataway, NJ, USA) were added 1 h before preparation of the cell lysate. Cell lysates were used for an anti-flag M2-agarose pull-down assay. K562 cells were cultured in RPMI medium (Sigma) with 10% fetal calf serum. To produce stable transfectants, HeLa and K562 cells were transfected with IRF-2 or IRF-2K75R (Masumi *et al.*, 2003) using Fugene 6 (Roche Biochemicals) and cultured for 2 weeks in the presence of 400 μ g/ml G418. G418-resistant cells were pooled and lysed for preparation of cell lysate. The nucleolin plasmid was a kind gift from Dr S Murakami (Hirano *et al.*, 2003).

Western blotting

Whole cell lysates were prepared in lysis buffer B, with the addition of a protease inhibitor cocktail (Sigma). The insoluble materials and whole cell lysates containing equal amounts of total proteins were suspended in an SDS sample buffer boiled, separated on SDS–10% PAGE, and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat dry milk

in a phosphate-buffered saline (PBS)-T buffer (PBS containing 0.5% Tween 20) for 1 h, incubated with anti-IRF-2 (Santa Cruz), anti-p300 (Santa Cruz), anti-acetyl lysine (New England Biology, Beverly, MA, USA) and anti-flag (Sigma) antibodies for 1 h, and washed in PBS-T. The antigen-antibody interaction was visualized by incubation in a chemiluminescent reagent (Perkin Elmer Co. Ltd) and exposure to X-ray film. Immunoblotted membranes were reused for Image analysis using a Fuji BAS 2500 (Fuji Film, Japan) to visualize ¹⁴C-incorporated protein.

Affinity DNA-binding assay

The DNA affinity-binding assay was performed as described (Masumi *et al.*, 2003). Briefly, nuclear extracts (500 μg of protein) were incubated with magnetic beads conjugated to biotinylated oligonucleotide from the H4 gene. Bound materials were immunoblotted with anti-nucleolin antibody.

Chromatin immunoprecipitation

A total 1×10^7 NIH3T3 cells were crosslinked with 1% formaldehyde for 15 min at room temperature. Cells were washed with PBS and resuspended in 1 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) plus a protein inhibitor mixture (Sigma), incubated on ice for 10 min, and sonicated to an average size of 500 bp by an ultrasonic cell disruptor (Ultra 5 homogenizer, TAITEC). Aliquots (100 μl) of sonicated chromatin were diluted in 1 ml of buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8) and precleared with 2 μg of sheared salmon sperm DNA and protein G-Sepharose (Invitrogen) for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with anti-IRF-2, anti-nucleolin (Santa Cruz) anti-PCAF (UBI) and rabbit immunoglobulin G (IgG) (Sigma). A 50-μl aliquot protein G-Sepharose, and 2 μg of salmon sperm DNA were added to each immunoprecipitation and incubated for 1 h. Precipitates were washed as described earlier and samples were extracted twice with elution buffer (1% SDS, 0.1 M NaHCO₃), heated at 65°C to reverse crosslinks, and DNA fragments were purified with phenol/chloroform. A 5-μl aliquot from a total of 30 μl was used in the PCR as described earlier (Masumi *et al.*, 2003).

Purification of interferon regulatory factor-2 precipitates and analysis of mass spectrometry

Cell lysates were prepared from 293T cells transfected with IRF-2 (flag-tag or no-tag) and PCAF (flag-tag or without tag) and incubated with 50–100 μl M2 agarose (Sigma) for 2 h with rotation. After washing with buffer B, bound proteins were eluted from M2 agarose by incubation for 5 min with 30 μl of the flag peptide (0.2 mg/ml) (Sigma) in the same buffer. Eluted protein was separated on SDS-PAGE and stained with Simply blue (Invitrogen). To identify the IRF-2-associated protein, a sliced band from the gel was digested with trypsin and peptides were analysed by LC-MS/MS using LCQ-Deca XP ion trap mass spectrometer (Thermo Electron Corp., Waltham, MA, USA).

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M2-agarose pull-down assay

For the M2-agarose pull-down assay, cell lysates from the 293T transfectants were incubated with M2 agarose (Sigma) in buffer B and washed three times. Bound materials were eluted by a 0.2 mg/ml flag peptide (Sigma), resolved on SDS-12.5% PAGE, and detected by Western blotting.

Small interfering RNA experiments

NIH3T3 cells were seeded at density of 3×10^5 cells per ml onto 24-well plate. After 16 h, cells were transfected with 100 mM siRNA oligonucleotides by RNAiFect Transfection Reagent (QIAGEN, Hilden, Germany) and the siRNA-containing medium was removed after 24 h of transfection, and then IRF-2pcDNA3.1, PCAFPCNX with H4 promoter luciferase reporter were transfected into NIH3T3 cells by lipofectamine (Invitrogen). Luciferase activity was analysed 24 h after transfection with plasmids. The sequences of siRNAs used here were as follows: nucleolin, GCUUAAAUCCU-GUAAUATT, negative control, non-silencing Alexa Flour 488 Labeled Control siRNA (QIAGEN).

Confocal microscopy

For laser scanning focal microscopy experiments, HeLa cells were cultured in a 35 mm glass bottom dish (Matsunami Glass Ind. Ltd, Japan). At 24 h after transfection, the cells were fixed with paraformaldehyde and lysed with 0.2% TritonX-100 in order to maintain the integrity of the cellular structures. They were then stained with appropriate antibodies as follows: cells transfected with flag-IRF-2 were stained with an anti-flag M2-Cy3 (Sigma). Cells were subsequently stained with anti-nucleolin-linked FITC (Santa Cruz 'sc-8023') for 16 h. NIH3T3 cells were stained with an anti-IRF-2 antibody (Santa Cruz) for 16 h and then with an anti-goat second antibody linked to Alexa 488 (Molecular Probes Inc., Eugene, OR, USA) for 2 h. Cells were subsequently stained with rabbit anti-nucleolin antibody (Santa Cruz), and then anti-rabbit IgG-linked Alexa 594 (Molecular Probe Co. Ltd). Stained cells were washed with Tris-buffer saline and mounted on glass slides with a mounting medium (glycerol-PBS). Fluorescent images were collected on a Zeiss Axiovert 100 confocal microscope using a Zeiss × 40 objective.

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