The p400 E1A-associated protein is a novel component of the p53 → p21 senescence pathway

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Adenovirus E1A-associated p400 belongs to the SWI2/SNF2 family of chromatin remodeling proteins. Here, we report that p400 is a component of the p53–p21WAF1/CIP1/Acid pathway, regulating the p21 transcription and senescence induction program. Acute depletion of p400 expression by shRNA (short hairpin RNA) synthesis led to premature senescence of untransformed human fibroblasts, whose features include G1 arrest, p21 induction, senescence-associated heterochromatic foci (SAHF), and β-gal staining. Importantly, p400shRNA-induced premature senescence phenotypes were rescued by coexpression of p53-shRNA or p21-shRNA. Furthermore, p400 complex colocalized with p53 on the p21 promoter. These data suggest that the p400 complex inhibits p53 → p21 transcription and the development of premature senescence.

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The N-terminal region of adenoviral E1A interacts with p400, a member of the SWI2/SNF2 chromatin remodeling family [Fuchs et al. 2001]. E1A mutants defective in interacting with p400 cannot transform primary cells efficiently when coexpressed with activated Ras [Deleu et al. 2001; Fuchs et al. 2001; Seger et al. 2002], suggesting that p400/E1A complexes participate in the transformation process. p400 interacts with SV40 large T antigen [Lill et al. 1997b], c-Myc [Fuchs et al. 2001], and the tumor suppressor, p53 [Lill et al. 1997a]. It is also a component of the human TIP60/NuA4 complex [Ikura et al. 2000; Fuchs et al. 2001; Cai et al. 2003; Doyon et al. 2004], which has specific histone H4/H2A acetyltransferase activity [Ikura et al. 2000; Doyon et al. 2004].

ChIP (chromatin immunoprecipitation) analysis has detected p400 complexes at certain c-Myc and E2F-regulated promoters [Frank et al. 2003; Taubert et al. 2004]. Their recruitment to such promoters correlated with an increase in histone acetylation in the surrounding chromatin and induction of transcription. These findings suggest that p400-containing complexes exert key chromatin-remodeling functions and participate in transcription regulation.

In the present study, retroviral-mediated shRNA was used to target p400 complexes and to investigate the outcomes of this perturbation in human cells. Specifically, acute depletion of p400 induced premature senescence in multiple, untransformed human fibroblasts. p400 shRNA-induced SAHF and other premature senescence phenotypes were rescued by coexpression of shRNA for p53 or p21, but not pRb. p400-depleted cells revealed elevated p21 mRNA and protein expression. In keeping with these findings, p400 was detected at or near the p53-binding site in the p21 promoter. These data suggest that p400 complexes inhibit p53 → p21 expression, and that deregulated p21 expression leads to senescence.

Results and Discussion

p400-shRNAs cause cell cycle arrest, SAHF, and premature senescence in untransformed human fibroblasts

Multiple retroviral vectors each encoding an shRNA directed against human p400 were constructed. The program for antibiotic selection and analysis of cells expressing these vectors is shown in Figure 1A. When IMR90 primary human diploid fibroblasts were transduced with shRNA-p400#2 or shRNA-p400#4, endogenous p400 protein expression was suppressed [Fig. 1B, cf. lanes 2, 4 and 1]. Moreover, shRNA-p400#2 and ShRNA-p400#4 effectively suppressed p400 protein expression in HeLa, T98G, telomerase-immortalized BJ fibroblasts [BJ-tert], U2OS, and a primary human lung fibroblast strain [Supplementary Fig. 7; data not shown]. ShRNA-p400#3 did not significantly affect the p400 protein level [Fig. 1B] and was used as a negative control [Fig. 1D]. Suppression of p400 expression was associated with G1 arrest [Fig. 1D, graph i], decreased BrdU incorporation [Fig. 1D, graph ii], and it inhibited proliferation of IMR90 [Fig. 1C]. Similar results were observed in BJ-tert (data not shown). None of these effects was detected when experiments were performed on two transformed human cell lines in which p53 function is compromised, including HeLa and T98G (Supplementary Fig. 2).

Unlike naïve IMR90, p400 shRNA-treated IMR90 became elongated and spindle shaped. When their DNA-staining properties were examined by fluorescence microscopy, ~40%–60% of the culture revealed clustered DAPI-dense regions [Fig. 2A, panel 3]. These structures were virtually absent in control cells [Fig. 2A, panel 1] and are decidedly unusual in human tissue culture cells in general. Spots of this type were recently described in IMR90 undergoing replicative senescence or experiencing activated Ras expression [Narita et al. 2003]. They were referred to as senescence-associated heterochromatic foci (SAHF) [Narita et al. 2003]. SAHF were also observed following p400 depletion in normal diploid lung fibroblasts and in telomerase-immortalized BJ fibroblasts [data not shown]. In contrast, T98G and HeLa cells failed to display SAHF before or after p400 knockdown [Supplementary Fig. 2, data not shown]. Where tested, the above-noted, DAPI-dense regions contained with an
antibody to methylated histone H3 Lys 9 (Fig. 2A, panel 4), consistent with earlier findings (Narita et al. 2003). The development of a SAHF phenotype following p400 depletion led to a further analysis of whether p400 shRNA-treated cells, like their activated Ras-producing counterparts, enter premature senescence. Indeed, p400 shRNA-treated IMR90 (Fig. 2B) displayed senescent β-gal staining, again suggesting that they were senescent. Two independent shRNAs designed against two different sequences of p400 suppressed p400 protein expression and cell cycle progression (Fig. 1D). Each also induced SAHF (Fig. 2A, panel 3 for shRNA-p400#4; data not shown) and senescent β-gal staining (Fig. 2B), linking this phenotype to the specific targeting of p400.

To determine whether knockdown of other components of the p400 complex lead to similar phenotypes, shRNAs were designed against TIP48 and TIP49, two subunits of this complex (Fuchs et al. 2001). Depletion of TIP48 or TIP49 expression by shRNA recapitulated the phenotypes of shRNA-p400 in IMR90 and BJ-tert, including G1 arrest, decreased BrdU incorporation, and the development of SAHF [Supplementary Fig. 1]. Taken together, these data imply that acute loss-of-function of the p400 complex is associated with premature senescence.

Thus far, we have not detected a spontaneous decrease of expression of the p400 complex in cells undergoing replicative senescence (data not shown). If a perturbation of p400-complex function is required for this outcome, it must involve other molecular alteration[s] than simple protein depletion.

**shp400-infected IMR90 has elevated p21 expression**

Since oncogenic Ras overexpression and p400 depletion both induced premature senescence and SAHF (Serrano et al. 1997; Narita et al. 2003), we compared the molecular features of these two states in search of similarities and differences. Both perturbations led to maximum senescent β-gal staining at approximately day 10 post-infection. Activated Ras overexpression had no effect on p400 protein level, and shRNA-p400 transduction did not activate endogenous Ras expression (Fig. 2C). Moreover, in Ras-expressing cells, there was up-regulation of p16 (Fig. 2C), and some enhancement of p53 Ser15 phosphorylation (Fig. 2C), consistent with previous reports (Serrano et al. 1997; Ferbeyre et al. 2000; Narita et al. 2003). However, shRNA-p400 did not affect the p16 protein level or p53 Ser15 phosphorylation, but was associated with p21 up-regulation (Figs. 2C, 3B). By quantitative RT–PCR, significant increases in the p21 mRNA level were reproducibly detected in shRNA-p400-treated cells (Fig. 2D), suggesting an enhancement of p21 transcription.

In keeping with its function as a cdk inhibitor [Sherr and Roberts 1999], p21 [also known as sdi [senescent cell-derived inhibitor]] is highly expressed in senescent cells and inhibits DNA synthesis [Noda et al. 1994]. Indeed, it can elicit this phenotype on its own, for overexpression of p21 can result in premature senescence [McConnell et al. 1998] and SAHF [M. Narita and S.W. Lowe, unpubl.]. Moreover, it serves as a lifespan regulator, because knocking out p21 can extend the lifespan of human fibroblasts [Brown et al. 1997].
Inactivation of p53/p21, but not pRb, rescues p400 shRNA-induced premature senescence in IMR90

Ras-induced SAHF can be partially rescued by suppressing the pRb/E2F pathway [Narita et al. 2003], while inactivation of p53 had no such effect [Narita et al. 2003]. In light of these findings, we asked whether the pRb or p53 pathways contribute to shRNA p400-induced premature senescence. Consistent with a previous report [Narita et al. 2003] and as a positive control, it was possible to use shRNA-pRb to partially rescue Ras-induced SAHF [data not shown]. However, shRNA-pRb had no effect on shRNA-p400-induced SAHF, cell cycle arrest, or senescent β-gal staining (Fig. 3A, Exp1; Supplementary Fig. 3). Its knockdown of pRb expression is shown in Supplementary Figure 4A. Furthermore, overexpression of cyclin E or cdk4 [R24C] [Supplementary Fig. 4B], both predicted to relieve pocket protein-mediated transcription, one wonders whether p400 contributes to shRNA-p400-induced premature senescence. Down-regulation of p21 expression by shRNA-p21 (Fig. 3C, top, cf. lanes 1 and 3, and lanes 2 and 4) substantially reduced the incidence of shRNA-p400-induced senescent phenotypes [Fig. 3A, Exp3]. A large fraction of cells treated with shRNA-p400 revealed SAHF and strong p21 staining (for examples, cf. Supplementary Fig. 6). Both SAHF and p21 staining were greatly reduced in cells doubly treated with shRNA-p21 and shRNA-p400 [Supplementary Fig. 6]. Importantly, the shRNA p21 reagent did not overtly compromise the ability of shRNA p400 to suppress p400 expression in the cells doubly infected with viruses encoding shRNAs for p21 and p400 [Fig. 3C, bottom]. Overall, we conclude that the p53–p21 pathway contributes to shRNA-p400-induced premature senescence.

It is understandable that no phenotype was observed when shRNA-p400 was introduced into HeLa and T98G cells [Supplementary Fig. 2], since p53 function in these cells is compromised by the presence of HPV E6 and of a genomic loss of function mutation, respectively [Hall et al. 1998; Goodwin and DiMaio 2001]. To further test the above-noted hypothesis, shRNA-p400 was transduced into U2OS cells, an osteosarcoma cell line carrying wild-type p53 alleles. In p400-depleted U2OS cells, increased p21 expression and G1-arrested cells were observed (Supplementary Fig. 7A,B). Consistent with the observation in IMR90 [Fig. 2C], p400 depletion in U2OS cells did not affect the p53 expression level (Supplementary Fig. 7C). Unlike IMR90, however, SAHF were not observed in U2OS following p400 depletion, or exogenous p21 overexpression [data not shown]. Therefore, elevated expression of p21 is not sufficient to cause SAHF or premature senescence in U2OS. This notwithstanding, p21 overexpression or p400 depletion were not inert events in these cells, since both led to marked inhibition of DNA synthesis [Supplementary Fig. 7D].

p400 complexes colocalize with p53 on the p21 promoter

p21 expression is normally up-regulated by p53. One outcome is G1 arrest [el-Deiry et al. 1993; Macleod et al. 1995; Vogelstein et al. 2000]. Given that p400 depletion induces p21 synthesis, one wonders whether p400 plays a direct role in down-regulating p53-dependent transcription of p21. In this regard, using ChIP analysis, we detected p400 bound to a region that overlaps the distal p53-binding site within the p21 promoter (Fig. 4B, #1, cf. lanes 8 and 3,7). Consistently, TIP49, a p400 complex member [Fuchs et al. 2001], was also detected in the same region (Fig. 4C). p400 binding at this site was specific, since no p400 was detected at the proximal TGFβ response element-containing region within the p21 promoter (Fig. 4B, #2), nor was it bound to a region ~3 kbp downstream of the p21 transcription initiation site (Fig. 4B, #3).

By performing ChIP with an anti-

Figure 3. Inactivation of p53/p21, but not pocket family proteins, rescues shRNA-p400-mediated premature senescence. (A) Summary of SAHF and senescent β-gal staining in IMR90 exposed to the indicated perturbations. (B,C) IB and IP-IB of the indicated IMR90 proteins after the indicated perturbations.
p400 Ab and then reimmunoprecipitating with anti-p53 Ab [DO-1], a PCR-amplified signal was detected from the distal p53-binding region [Fig. 4D, #1], but not from the region 3 kbp downstream of the transcription initiation site [Fig. 4D, #3]. Therefore, both p53 and p400 were colocated within the distal p53-binding/regulatory region of the p21 promoter. These data are consistent with a model in which interplay between p53 and p400 contributes to normal regulation of the p21 promoter. While p53 exerts a positive effect on this element, p400 appears to exert a negative one. As a result, depletion of p400 elicited p53-dependent transcription of the p21 gene, and elevated p21 expression can lead to premature senescence [McConnell et al. 1998].

By quantitative ChIP analysis, multiple experiments demonstrated 60%–70% more p53 bound to site #1 in IMR90 cells depleted of p400, compared with control IMR90-pSuper cells [Fig. 4E]. This phenomenon is specific to the p21 promoter, since there was no increase in p53 binding to the puma or bax promoters in the same experiments [data not shown]. Furthermore, no p400 was detected at the p53-binding region of the puma or bax promoters [data not shown]. Thus, it is possible that increased p53 binding to the p21 promoter in p400-depleted IMR90 contributed to the enhanced p21 expression observed.

In conclusion, p400, an SWI2/SNF2 chromatin-remodeling factor, plays a role in regulating p53 → p21 transcription and eliciting a cellular senescence program. Acute depletion of p400 or components of the p400 core complex in primary cells led to G1 arrest, SAHF, induction of p21, and senescence β-gal staining, all features indicative of cells entering senescence [Narita et al. 2003]. By epistatic analysis, p400 shRNA-mediated senescence has been linked to the p53/p21 pathway, but not the pRb pathway. This is the opposite of the mechanism leading to Ras-induced SAHF [Narita et al. 2003]. These findings elicit speculation that p21 and p16 participate in distinct senescence pathways that respond to different cues [Fig. 5; Herbig et al. 2004].

Cellular senescence has been hypothesized to serve as a barrier against tumor development. The ability to overcome senescence is a prerequisite for tumor formation [Sager 1991; Hanahan and Weinberg 2000]. We have observed that both E1A and c-Myc up-regulate p400 expression [data not shown]. Furthermore, p400 is required for E1A-dependent apoptosis [A. Samuelson, M. Narita, and S.W. Lowe, unpubl.]. Given the results reported above, one wonders whether E1A or c-Myc, by up-regulating p400 expression, override p53/p21-dependent cell cycle arrest, an outcome that was suggested to sensitize cells to p53-dependent apoptosis [Seoane et al. 2002]. If so, one would argue that c-Myc and/or E1A do not inactivate p400. Rather, one wonders whether c-Myc/p400 or E1A/p400 complexes exhibit a qualitative alteration in p400 function or even its activation. Since senescence is suspected of serving as a major component of tumor suppression and is associated, in at least one setting, with suppression of p400 complex function, it is conceivable that up-regulation of p400 expression/function by certain oncogenes [and, quite possibly, its binding to the N-terminal region of E1A [Fuchs et al. 2001]] contributes to neoplastic transformation. An analysis of whether p400 function is deregulated in certain human tumors now seems timely.

Materials and methods

Cell culture, cell cycle analysis, and plasmids

All cells were cultivated at 37°C in a 10% CO2-containing atmosphere, 293T, IMR90, HeLa, U2Os, and T98G were maintained in DMEM.
supplemented with 10% FBS and antibiotics. BJ-Tert cells were cultivated in 4:1 DMEM/medium 199 (Invitrogen), supplemented with 15% FBS and antibiotics. Retroviral production and infection protocols were as described (Serrano et al. 1997; Narita et al. 2003). When cells were doubly infected by viruses, cells were first selected with Puromycin (20 μg/ml) for 2 d, followed by Hygromycin (75 μg/ml) for 2 d. Routinely, 60–80% of the cells survived selection. The cell growth assay and senescence β-gal-staining protocol have been described (Serrano et al. 1997; Narita et al. 2003). For FACS and BrDU incorporation assays, 4 × 106 cells were seeded on 10-cm plates 30–36 h before harvesting. Ten micromolar of BrDU was added to the culture medium for 45 min before harvesting. BrDU-free cells [with stained with α-BrDU Ab] were included as negative controls. The following virus combinations were used in the experiment presented in Figure 3, Exp1 [pSuper-shRNA-Rb(Hygro) + pSuper-shRNA-p400#3(Puro)]; pSuper(shRNA-Rb-Hygro) + pSuper-shRNA-p400#4(Puro); pSuper[Hygro] + pSuper-shRNA-p400#3(Puro); pSuper[Hygro] + pSuper-shRNA-p400#4(Puro); in Exp2, [pSuper-shRNA-p400#4(Hygro) + pSuper(Puro)]; pSuper-shRNA-p400#4(Hygro) + pSuper-shRNA-p53(Puro); pSuper[Hygro] + pSuper[Hygro] + pSuper-shRNA-p53[Puro]; In Exp3, [pSuper(Puro) + pSuper-shRNA-p400#4(Hygro) + pSuper-shRNA-p21(Puro); + pSuper-shRNA-p400#4(Puro)] + pSuper[Hygro]. In Exp5, [pSuper[Puro] + pBabe(αe); pSuper-shRNA-p400#4(Puro) + pBabe(αe); pSuper[Puro] + pBabe(αe); pSuper[p53DD](Neo); pSuper-shRNA-p400#4(Puro); + pBabe-P53D(Xe); pBabe(Puro-cyclin E, pBabe(Puro)-cdk4 (R24C) and pBabe(Puro-RasV12 have been described (Serrano et al. 1997); pBabe-P53D (Shaullian et al. 1992) was a kind gift of Dr. W. Hahn (Dana-Farber Cancer Institute, Boston, MA). shRNAs against p53 and p21 were described in Voorhoeve and Agami (2003). shRNAs against p53, and TIP49 were cloned into pSuperRetro-(Puro) or pSuperRetro-(Hygro)-based vectors (OligoGene). Sequence information can be obtained upon request.

Immunofluorescence (IF)

Cells were fixed with 4% paraformaldehyde and subjected to DAPI staining. The IF procedure for anti-dimethyl H3K9 (Upstate Biotechnology) and p21 [c-19, Santa Cruz] Ab was described previously (Ganesan et al. 2002).

Antibodies

Antibodies against c-myc (N262), max (c-17), pRb (c-15), and p21 (c-19, Santa Cruz) Ab was described previously (Ganesan et al. 2000). The IF procedure for anti-dimethyl H3K9 (Upstate Biotechnology) and p21 (c-19) was described previously (Ganesan et al. 2000). Antibodies against c-myc (N262), max (c-17), pRb (c-15), and p21 (c-19) were from Santa Cruz Biotechnology. Antibodies against c-myc (N262), max (c-17), pRb (c-15), and p21 (c-19) were described previously (Ganesan et al. 2000). For quantitative RT-PCR using primers GCTCTGATTGGCTTTCTG were used. This set of primers did not yield primer dimers in PCR reactions. The method for quantitating the results of ChIP experiments has been described (Frank et al. 2001).

Quantitative RT-PCR

Total cellular RNA was harvested by RNeasy (Qiagen). First-strand cDNA synthesis was carried out using the Superscript first-strand synthesis system (Invitrogen). Quadruplicate samples were subjected to quantitative PCR using iCycler (Bio-Rad), and the primers were described (Kaaser and Igo 2002). PCR for actin mRNA was used as an internal control. The relative abundance of p21 mRNA was calculated after normalization using actin mRNA.

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