p53 Regulates Caveolin Gene Transcription, Cell Cholesterol, and Growth by a Novel Mechanism†

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ABSTRACT: Transcription of the human caveolin gene, directed by a TATA-less promoter, is downregulated in actively dividing cells during S-phase, together with free cholesterol (FC) efflux. It is upregulated by medium low density lipoprotein FC levels in quiescent cells. In this study, a common mechanism has been identified to coordinate the growth- and FC-dependent expression of caveolin. In human skin fibroblasts, transcription factors E2F/DP-1 and Sp1 bound to adjacent consensus sites at −151 to −138 bp of the caveolin promoter DNA sequence in a complex stabilized by tumor suppressor protein p53. Wild-type p53 also bound directly to DNA to a caveolin promoter sequence containing two consensus half-sites (−292 to −283 bp and −273 to −264 bp) for this transcription factor. SREBP-1, previously identified as a transcriptional regulator of caveolin expression in response to FC, mediated its effect via the same E2F/Sp1 site. Overexpression of E2F or p53 increased E2F binding to the −148 to −141 bp site, increased FC efflux, and inhibited cell division. The mutant protein $p53_{(143V\rightarrow A)}$ was inactive. Okadaic acid, previously shown to inhibit growth, FC efflux, and caveolin expression, inhibited E2F/Sp1 binding, while higher concentrations of extracellular FC increased it. The present findings provide a molecular link between the cell cycle and FC homeostatic effects of caveolin. These results also describe a novel mechanism of action for p53 in a TATA-less gene promoter and provide further evidence for a significant regulatory role for FC in cell cycle progression.

Caveolin 1 (hereafter referred to as caveolin) is a free cholesterol (FC)[1] binding protein present within the cell-surface caveolae expressed in most cells (*1*, *2*). It has been implicated both in FC transport to the cell surface and in FC efflux from the cell (*3*−*5*). Its expression is regulated by cell FC content (*6*, *7*). Caveolin mRNA levels are increased in cells exposed to low-density lipoprotein (LDL), the major FC-rich plasma lipoprotein, while they are down-regulated in lipoprotein-deficient media. A G/C-rich box at −395 bp was required for the FC-dependent regulation of caveolin mRNA levels. Electrophoretic mobility shift analysis (EMSA) identified SREBP-1 as the transcription factor binding to this site (*8*).

Caveolin is decreased in or absent from many (although not all) cancer and transformed cell lines (*9*, *10*). In caveolin-deficient cancer cells, transfection with caveolin cDNA was strongly growth-suppressive. In synchronized normal human fibroblasts, caveolin transcription and expression were down-regulated at S phase in the cell cycle. As a result, FC efflux was also decreased, while FC per cell doubled (*11*). In contrast, transfection of these cells with human caveolin cDNA stimulated FC efflux, decreased cellular FC, and blocked cell division. The cell cycle effects of caveolin expression were shown to require a DNA sequence within the caveolin gene promoter, consisting of adjacent consensus binding sites for the E2F and Sp1 transcription factors (*11*).

E2F, as a heterodimeric complex with transcription factor DP-1, plays a key role in the induction of a number of cell cycle genes, including several cyclin-dependent kinases (*12*), although it has not been previously implicated in FC homeostasis. Sp1 is a transcription factor that interacts with E2F in a number of promoters (*13*, *14*). SREBP regulates the expression of a number of genes involved in FC synthesis but has not been previously implicated in growth control, although SREBP-1, like E2F, interacts with Sp1 in the promoters of a number of genes of FC synthesis (*15*). Nevertheless, the regulation of FC homeostasis during the cell cycle (*11*) suggested the presence of a checkpoint, mediated at the transcriptional level of caveolin.

The caveolin gene promoter sequence[2] between −292 and −264 bp also includes sequences suggestive of half-sites for binding to tumor suppressor protein p53 (*16*, *17*). Dominant negative mutations of p53 have been identified in over 50% of human cancers (*18*). Two different mechanisms have been

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[1] Abbreviations: FC, free cholesterol; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; OKA, okadaic acid; STA, staurosporine; DME, Dulbecco's modified Eagle's medium.

[2] Caveolin promoter DNA sequences cited are GenBank AF019742 (*11*) and AC006159 and are numbered relative to the translational start site (+1).

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described for this transcription factor. p53 plays a key role in inhibiting cell cycle initiation and in the induction of apoptosis, through its activity as a transcriptional regulator of genes including cyclin G, bax, and mdm-2. In these promoters, p53 binds as a tetramer to half-sites on both strands and also to the TATA box binding protein complex, which forms part of the basic transcriptional machinery of these genes. In TATA-less promoters where p53 transcriptional dependence has been demonstrated, DNA-binding sites could not be identified, and in these cases p53 was considered to act indirectly, by binding to other nuclear proteins, including Sp1 or E2F (*19*−*21*).

In the present research, we explored the possibility that a common mechanism might mediate cell cycle- and FC-mediated effects on transcription and that these involved the p53 consensus sites, despite the absence of a TATA box in the caveolin gene promoter.

EXPERIMENTAL PROCEDURES

Materials. Rabbit or goat monoclonal antibodies to human trancription factors E2F, Sp1, DP-1, SREBP-1 and p53 were from Santa Cruz Biotechnology. An expression plasmid for full-length human E2F-1 was donated by Dr. Ali Fattaey, Onyx Pharmaceuticals, Richmond, CA. A human Sp1 expression plasmid was the gift of Dr Robert Tjian, University of California, Berkeley, CA. Plasmids coding for wild-type and mutant ($143_{V\rightarrow A}$) p53 proteins (*22*) were gifts from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD. A plasmid expressing codons 1−440 of human SREBP-1 was donated by Dr. Timothy Osborne, University of California, Irvine. [1,2-^{3}H]Cholesterol and [*methyl*-^{3}H]-thymidine were from NEN, Boston, MA. Okadaic acid (OKA) and staurosporine (STA) were from Calbiochem, San Diego, CA. Protamine−agarose covalent complex and methyl-β-cyclodextrin were from Sigma, St Louis, MO.

Synthesis of Caveolin Mutant Promoter Sequences. The 705 bp human caveolin 1 promoter fragment previously characterized (*8*) was subcloned into the *Bgl*II site of the pGL3 luciferase vector (Promega) to generate pGL3-cav. Deletions within this fragment were made by site-directed mutagenesis with QuikChange kits (Stratagene, La Jolla, CA). Each modified DNA fragment was sequenced by the dideoxy method prior to cloning into pGL3. TTTGGCGG (−151 to −144 bp) was modified to TTAAGCGG to inhibit E2F binding (*23*). The sequence from bp −143 to −138 (GCGGCC) was modified to ATATCC to inhibit Sp1 binding (*24*). A mutant lacking the G/C-rich box at −395 bp or the entire potential E2F/Sp1 site (−151 to −138 bp) was prepared as previously described (*8*, *11*). Finally, a truncated promoter (−207 to +1 bp) was generated by PCR and then subcloned into the *Kpn*I−*Hin*dIII site of the pGL3 vector.

Transfection. Normal human skin fibroblasts were plated at a density of 1.3×10^5 cells per 6 cm dish in DME−10% fetal bovine serum (FBS). The cells were transfected with pGL3-cav or with mutant caveolin promoter sequences ligated into pGL3 (10 μg of DNA in each case) by a calcium phosphate method (Profectin) (Promega). Cotransfection with pSV-β-galactosidase (Promega) provided an internal control used to normalize for transfection efficiency between dishes (*11*). In other experiments, plasmids coding for E2F, Sp1, SREBP, or wild-type or mutant p53 proteins in pcDNA3 were cotransfected with pGL3-cav. Twenty-four hours after transfection, a double block with aphidicolin, an inhibitor of DNA polymerase, was carried out to arrest the cells at the G_1/S interface (*11*). The cells were washed with PBS and assayed for luciferase and galactosidase according to the manufacturer's instructions. In some experiments, aphidicolin was removed and the cells were allowed to enter the cycle before processing and analysis.

Electrophoretic Mobility Shift Assays. These assays were carried out with Bandshift kits from Pharmacia (Parsippany, NY). Extracts were prepared from the purified nuclei of synchronized human skin fibroblasts (*25*). Except where indicated, buffers contained a cocktail of phosphatase and protease inhibitors (*26*). The following oligonucleotide probes were used in these studies. A 25 bp probe (−157 to − 133 bp of the wild-type caveolin promoter) overlapped the entire E2F/Sp1 consensus site. A −165 to −133 bp probe had bp −149 to −147 (TGG) replaced by AAG. A −165 to −133 bp probe had −143 to −140 (GCGG) replaced by ATAT. Finally, a 39 bp probe extending from −297 to −259 bp included two 10-bp repeats of potential p53 DNA binding sequence (*17*) separated by 8 bp. Complementary oligonucleotides were ^{32}P-end-labeled and annealed. A total of 0.5 pmol of probe and 4 μg of nuclear extract protein were used in each reaction in a volume of 20 μL. To compete for nonspecific DNA binding, 4 μg of poly(dI·dC) was included in each reaction. In competition assays a 200-fold molar excess of homologous unlabeled double-stranded oligonucleotide was added 5 min before the addition of labeled probe. Reaction mixtures were incubated at room temperature for 30 min. In some experiments, antibodies to human E2F, DP-1, Sp1, or p53 transcription factors or irrelevant antibody (mouse anti-human IgG, Transduction Laboratories, Lexington, KY) was added to nuclear extract at ice temperature for 1 h prior to incorporation of labeled probe. In all experiments, after incubation, DNA−protein complexes were resolved from free DNA probe on 5% (w/v) polyacrylamide gel in 0.1 M Tris, 0.09 M boric acid, and 0.001 M EDTA (pH 8.4). Gels were dried and labeled complexes were visualized by autoradiography and quantitated by computer-assisted scanning densitometry, as previously described (*11*).

Protamine−Agarose Precipitation Assays. A DNA binding protein (protamine) covalently linked to agarose was used to purify specific DNA−nucleoprotein complexes. Nuclear extract (25−50 μg of protein) was preincubated with unlabeled oligonucleotide probe (4.0 pmol) for 30 min at room temperature, followed by 30 min on ice. Protamine−agarose gel (20 μL) was then added to bind DNA−nucleoprotein. After centrifugation, the complex was washed and then extracted with 2× sample buffer (*11*). Following SDS−polyacrylamide gel electrophoresis and transfer to nitrocellulose, the blots obtained were incubated with antibodies to transcription factors and visualized with Supersignal CL-HRP peroxidase substrate (Pierce, Rockford, IL) (*27*). Levels of transcription factors within nuclear extracts prepared from arrested and dividing cells (0−8 h) were also assayed.

Assays of FC Efflux and Growth. Arrested cells transfected with E2F, SREBP, or wild-type or mutant p53 plasmids, or with empty vector, were equilibrated 24 h before use with

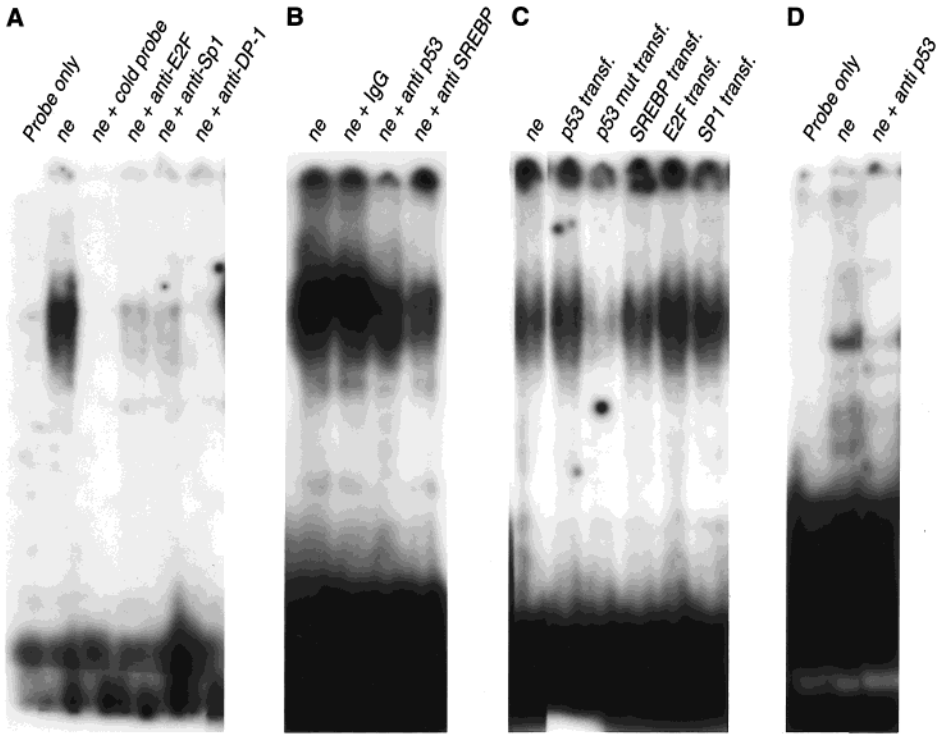

FIGURE 1: (A) EMSA with ^{32}P-labeled oligonucleotide (−157 to −133 bp) of the caveolin gene promoter sequence. Nuclear extract (ne) from arrested human skin fibroblasts was preincubated with anti-E2F, anti-Sp1, or anti-DP-1 antibodies. The treated extract was then fractionated on polyacylamide gels as described under Experimental Procedures. (B) EMSA under the same conditions with antibody to p53 or SREBP or with IgG. (C) EMSA under the same conditions with nuclear extracts from cells transfected with plasmids coding for E2F, Sp1, wild-type or mutant p53, or SREBP. (D) EMSA with ^{32}P-labeled probe encompassing −297 to −259 bp of the caveolin gene promoter sequence. Nuclear extract from arrested fibroblasts was incubated in the presence or absence of anti-p53 antibody.

DME−10% FBS labeled with ^{3}H-FC to a final specific activity of (0.8−1.2) × 10^5 cpm μg^{-1}. FC efflux was assayed from the rate of transfer of isotopic FC from labeled cells to unlabeled DME−10% FBS medium (*11*). Additionally, following removal of aphidicolin, the cells were allowed to divide for 36 h and then counted as previously described.

RESULTS

EMSA of Wild-Type and Mutant Promoter Sequences. Nuclear extract from aphidicolin-arrested cells was incubated with a 25 bp ^{32}P-labeled probe which encompassed a potential E2F/Sp1 consensus site (−151 to −138 bp) (5′-TTTGGCGGGCGGCC-3′). A single major nucleoprotein complex was observed (Figure 1, panel A). When nuclear extract was preincubated with antibodies to either E2F or Sp1 this band was almost completely lost. Antibody to DP-1, which forms a stable heterodimer with E2F, reduced complex formation, as did antibody to p53 or SREBP (Figure 1, panel B). There was no effect of irrelevant antibody (IgG). When TT in this sequence was modified by mutation to AA to inhibit E2F binding, complex formation was significantly reduced. The same result was obtained if GCGG (−143 to −140 bp) was modified to ATAT to reduce Sp1 binding (data not shown). No evidence of a complex with a modified migration rate was observed in the presence of any of these antibodies. This suggests that E2F/DP-1 and Sp1 bound stably to the oligonucleotide only in the presence of each other and of p53.

Nuclear extract from cells transfected with expression plasmids coding for human E2F-1 or wild-type p53 produced an increased level of complex, compared to extract from control (sham-transfected) cells (Figure 1, panel C). Nuclear extract from cells transfected with Sp1 and SREBP showed smaller changes. In contrast, nuclear extract from cells transfected with the mutant p53 plasmid gave a much reduced band. These data suggest p53 promoted complex formation at the E2F binding site, while mutant p53 destabilized it. These results also indicate that E2F and p53 are limiting for complex formation within the nuclei of arrested human skin fibroblasts.

The question whether p53 also bound to DNA via consensus half-sites within the −297 to −259 bp sequence was addressed by incubating nuclear extract with this oligonucleotide in the presence or absence of anti-p53 antibody. A labeled complex was observed, whose formation was blocked by anti-p53 antibody (Figure 1, panel D). These data suggest that p53 binds directly to caveolin promoter DNA, as well as to the E2F/Sp1 complex first identified.

Protamine−Agarose Precipitation. To confirm the presence of E2F and p53 proteins in the complex with the −157 to −133 bp oligonucleotide, unlabeled DNA encompassing this sequence was incubated with nuclear extract and then purified with agarose−protamine as described under Experimental Procedures. Adsorbed proteins bound to DNA were solubilized in SDS buffer, fractionated by electrophoresis, and identified by Western blotting. Coprecipitation of E2F and p53 was observed (Figure 2). To confirm whether p53 bound directly to caveolin promoter DNA, double-stranded DNA encompassing the −292 to −283 and −273 to −264 bp sites was incubated with nuclear extract and purified with protamine−agarose. As shown in Figure 2, p53 protein was identified in the purified complex.

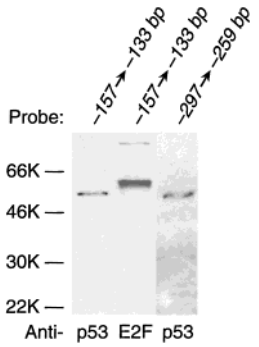

FIGURE 2: Protamine−agarose precipitation of protein−DNA complexes formed between oligonucleotide including −157 to −133 bp or −297 to −259 bp and nuclear extract from arrested cells. Following precipitation, the complex was extracted with SDS buffer and fractionated by electrophoresis. Western blotting with the antibodies shown was carried out as described under Experimental Procedures.

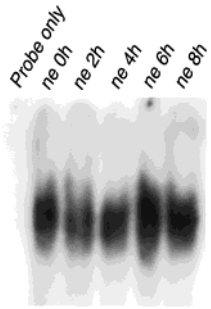

FIGURE 3: Nucleoprotein binding to ^{32}P-labeled oligonucleotide (−157 to −133 bp) during the cell cycle. Cells arrested in aphidicolin were released into inhibitor-free DME−10% FBS medium. At the intervals shown, nuclei were isolated, nuclear extracts were prepared, and EMSA was carried out as described in the legend to Figure 1.

Transcriptional Complex Formation during the Cell Cycle. Transcription of the caveolin gene was shown previously to decrease significantly at S-phase but to recover by 8 h following release from aphidicolin arrest (*11*). In this study, complex formation between nucleoproteins and oligonucleotide encompassing the E2F/Sp1 site was compared in nuclear extract from arrested cells and in extract from cells collected at intervals during the cell cycle after removal of aphidicolin. Complex formation, initially high, had decreased by half at 2 h (−51% ± 5%), coincident with the peak of S-phase. This was restored to its original value in a reversal complete by 8 h (Figure 3). As a result, caveolin gene transcription and complex formation changed in parallel during the cell cycle.

The activity of many transcription factors involved in cell cycle regulation, including each of those identified in the transcription complex of caveolin, is modulated during the cell cycle via phosphorylation (*28*−*30*). The protein phosphatase inhibitor OKA reduced the expression of cell surface caveolae (*31*) and FC efflux (*3*). In the present research, under similar conditions, OKA significantly inhibited formation of the complex formed between nucleoproteins and oligonucleotide encompassing the −151 to −138 bp site (Figure 4, left) while the protein kinase inhibitor STA increased it. Consistent with the finding described above, when the probe was modified by the substitution of AA for TT to inhibit E2F binding, complex formation was almost completely abolished (Figure 4, center).

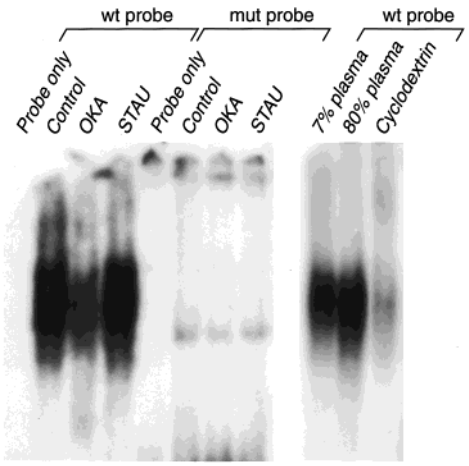

FIGURE 4: (Left) EMSA of nucleoprotein complexes formed between wild-type oligonucleotide encompassing the E2F/Sp1 site and nuclear extract from arrested cells incubated in 10% FBS−DME with okadaic acid (OKA) (1.0 μM) or the same concentration of staurosporine (STA). (Center) The same nuclear extracts incubated with probe in which TT was mutated to AA. (Right) EMSA of complexes formed between the same oligonucleotide and nuclear extract from cells preincubated (6 h, 37 °C) with either human plasma (80% v/v) or hydroxymethylcyclodextrin (0.05 M).

Caveolin mRNA levels and expression are also modified in response to changes in cell FC (*6*, *7*). To determine if nucleoprotein complex formation was modified by changes in FC, caveolin was upregulated by FC in 80% plasma medium (*3*) or FC was depleted with cyclodextrin (*32*). EMSA complex formation was increased by 80% plasma and significantly reduced by cyclodextrin (Figure 4, right). These data suggest factors other than those regulating cell cycle progression mediate their effects via the same central mechanism.

Effects on Caveolin Gene Transcription of Overexpression of E2F, Sp1, SREBP, and p53. The roles and relationship of p53, E2F, and Sp1 in caveolin transcription were further defined with human skin fibroblasts transfected with pGL3-cav carrying wild-type or mutant caveolin promoter sequences, and cotransfected with plasmids expressing E2F, SREBP, or wild-type or mutant p53 nucleoproteins. Luciferase yield was increased ∼4-fold (relative to control) in cells overexpressing E2F (Figure 5). When wild-type caveolin promoter DNA was replaced with the mutant promoter sequence in which E2F binding was deleted, luciferase activity was greatly diminished. Sp1 overexpression was without effect on luciferase production, confirming that Sp1 was not rate-limiting in nuclear extract from arrested cells (data not shown).

Transfection with wild-type p53 was associated with a ∼6-fold increase in caveolin promoter transcription above control levels. In contrast, the reaction in cells cotransfected with mutant p53 was at the level of control cells. Transfection with SREBP increased caveolin transcription 4-fold, while deletion of the G/C-rich box at −395 bp from the caveolin promoter, which is required for SREBP binding (*11*), decreased baseline luciferase yield by 10−20-fold (Figure 5).

FC Efflux and Caveolin Gene Expression. If E2F/DP-1, Sp-1, and p53 formed a complex required for effective caveolin transcription, then cells transfected with E2F or wild-type p53 would show the same response of slowed

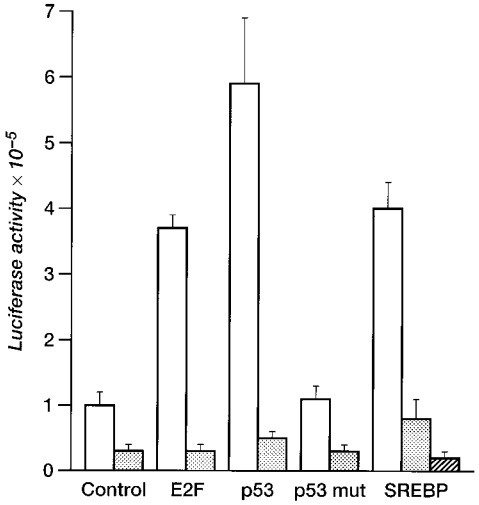

FIGURE 5: Luciferase expression from wild-type or modified caveolin gene promoter fragments ligated into the pGL3 expression vector. Human skin fibroblasts were cotransfected with empty pcDNA3vector or with plasmids expressing E2F, mutant or wild-type p53, or SREBP. Open bars, wild-type caveolin promoter; stippled bars, mutant caveolin promoter in which the sequence between −139 and −150 bp was deleted to inhibit E2F binding (*11*); hatched bar, mutant caveolin promoter in which the G/C-rich box at −395 bp had been deleted (*8*). In all experiments, the expression from pSV-*β*-galactosidase cotransfected with pGL3 was used to correct for transfection efficiency between dishes.

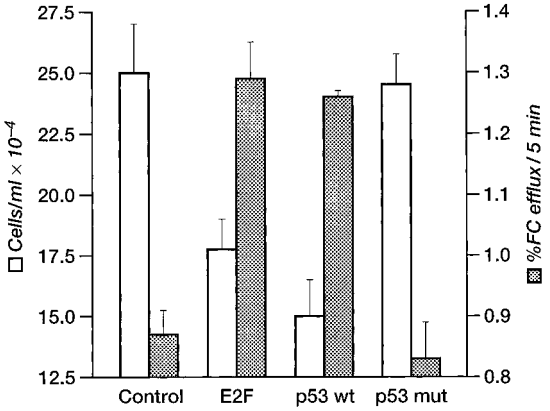

FIGURE 6: FC efflux and growth in human skin fibroblasts transfected with empty pcDNA3 vector or with plasmids coding for E2F or for wild-type or mutant p53. The cells were labeled to equilibrium with ^{3}H-FC prior to arrest. FC efflux (stippled bars) was assayed in the arrested cells as described under Experimental Procedures and has been expressed as a percent of total cell label to correct for differences in cell number between dishes. Growth (open bars) was assayed as cell number per 3.5 cm 36 h following removal of aphidicolin. FC efflux from these cells was 0.8%/5 min. The bars shown represent increases above these initial values.

growth and activated FC efflux as cells transfected with caveolin itself. Cells transfected with mutant p53 would be expected to show little or no change. To test these possibilities, dishes of cells transfected with plasmids expressing one of these proteins and then labeled with ^{3}H-FC were synchronized with aphidicolin. FC efflux from arrested cells, and increase in cell number over the subsequent 36 h period after removal of aphidicolin, were determined as described under Experimental Procedures.

Cells transfected with either wild-type p53 or E2F showed increased FC efflux and a significant reduction in growth rate (Figure 6). This result was not seen in cells transfected with the plasmid coding for mutant p53. These changes parallel the effects of transfection with sense or antisense caveolin cDNA, respectively (*6*, *11*).

DISCUSSION

E2F and Sp1 transcriptionally regulate many of the genes that determine progression through the cell cycle. Interaction of these proteins with DNA is most commonly regulated by phosphorylation, directly or via effects on accessory proteins (*12*). Other genes with key roles in growth and apoptosis are regulated by p53. SREBP-1 binds to G/C-rich elements within the promoters of FC-responsive genes, including caveolin (*8*), usually in association with one or more Sp1 sites (*15*). Regulation of the activity of each gene is mediated by a unique combination of multiple proteins binding directly or via other proteins to its promoter DNA sequence (*33*). Regulation of the transcriptional activity of the caveolin promoter complex appears to differ, in a number of ways, from that of other genes regulating cell cholesterol levels (*34*). Caveolin expression in FC-upregulated cells is inhibited, not activated, by the cysteine protease inhibitor ALLN (*8*, *35*). Caveolin, also unlike the genes coding for proteins of FC synthesis and LDL receptor protein (*34*), does not require cholesterol-deficient medium for expression (*11*). Caveolin is downregulated under these conditions (*7*). Finally, in the presence of increased levels of lipoprotein cholesterol, caveolin expression in upregulated (*6*, *8*, *35*) while that of the other genes is suppressed. Nevertheless, the data in this study clearly indicate that caveolin is a SREBP-regulated gene.

Caveolin transcription was inhibited, not activated, at S-phase (*11*), unlike the case of most other cell cycle genes regulated by E2F-1 (*12*). These effects appear to be mediated via the same short promoter DNA sequence (−151 to −138 bp) identified earlier via deletional mutagenesis (*11*). The mechanism of the E2F-mediated effect could not be determined in the earlier study, because of low luciferase yields, although transcription was consistently reduced at S-phase. More extensive data obtained here by use of cells cotransfected with E2F cDNA show that E2F is an activator of caveolin transcription.

It is a major conclusion of this study that the expression of caveolin by a variety of different stimuli is mediated by a transcriptional complex of E2F/DP1 and Sp1 stabilized by p53. A second conclusion is that p53 binds to both E2F and Sp1 and also to a DNA binding site within the sequence −297 and −259 bp, probably to potential half-binding sites at −292 to −283 and −273 to −264 bp.

Evidence for E2F binding is as follows. EMSA of nucleoprotein binding to the site spanning −151 to −138 bp identified a complex that was depleted by prior incubation of nuclear extract with anti-E2F antibody. Incubation of the nucleoprotein−DNA complex with protamine−agarose precipitated E2F as identified by Western blotting. Formation of this complex was increased in nuclear extract from cells that had been transfected with a plasmid containing the coding sequence for E2F-1. Complex formation was greatly reduced when the E2F consensus site was mutated to an inactive sequence. Within the intact cell, cotransfection with the E2F plasmid increased luciferase product from a construct containing the wild-type caveolin promoter, but not one containing a mutant promoter in which E2F binding had been

abolished. DP-1 was detected by antibody depletion in EMSA assays. This indicates that E2F is present, as usual, as a heterodimer with DP-1 (*12*). E2F binding was inhibited by anti-Sp1 antibody or when the Sp1 consensus site was mutated. These data indicate that simultaneous binding of Sp1 and E2F is required for effective complex formation. While several other promoters of cell cycle genes, such as thymidine kinase and dihydrofolate reductase, bind both E2F and Sp1 (*13*, *14*), the caveolin promoter is unusual for the proximity of the two binding sites. This may explain the finding that the complex is stable only in the presence of a third nucleoprotein, p53.

Although interaction between p53 and E2F or Sp1 has been previously reported (*19*, *36*), the presence of p53 as part of a caveolin transcriptional complex with both factors was unexpected because the caveolin gene promoter, unlike the promoters of other p53-dependent genes with DNA binding sites for this protein, lacks a TATA box. Evidence that caveolin transcription is p53-dependent is as follows. p53 bound to the promoter sequence between −297 and −259 bp. This conclusion is based on the loss of labeled complex after incubation of nuclear extract with anti-p53 antibody. p53 was detected in Western blots of the complex between nuclear proteins and −297 to −259 bp oligonucleotide purified with protamine−agarose. It was also part of the complex binding to the −151 to −138 bp site. Anti-p53 antibody prevented formation of this complex, where p53 protein was also identified following protamine−agarose precipitation. Complex formation was increased in nuclear extract from cells transfected with plasmid coding for wild-type p53 and decreased under the same conditions with the p53 mutant ($143_{V\rightarrow A}$) when analyzed by EMSA. Luciferase production from pGL3-cav was increased by cotransfection with wild-type p53 and strongly inhibited by the mutant plasmid. Thus, by accepted criteria (*16*, *17*), caveolin appears to be a strong candidate for the limited roster of p53-dependent genes, even though its mechanism of activation appears to be atypical.

An earlier study from this laboratory (*11*) showed that the decrease in caveolin gene transcription evidenced at S-phase in synchronized cells was mediated by the −151 to −138 bp promoter sequence. There was a contemporaneous decrease in formation of the E2F−Sp1 complex. Significant changes in p53 and E2F protein levels were not seen over this period in human skin fibroblasts (data not shown). A possible regulatory mechanism is suggested by the data with the metabolic inhibitors OKA and STA. OKA, an inhibitor of protein serine and threonine phosphatase activities (*37*), markedly reduced DNA−nucleoprotein complex formation at the −151 to −138 bp site. STA, a protein kinase inhibitor (*38*), increased it. These data make it likely that phosphorylation of one or more of the proteins in this complex (E2F/DP-1, Sp1, and p53) inhibits complex formation at S-phase. Since each protein is variably phosphorylated (*19*, *26*, *36*), multiple sites may contribute to the inhibition of transcription seen.

Deletion of the SREBP-binding site at −395 bp of the caveolin promoter was shown earlier to greatly reduce caveolin transcription rates assayed with the promoter−luciferase gene construct (*8*). However, ALLN inhibited caveolin expression, in contrast to its effect on other SREBP-dependent genes. The new studies here provide a more detailed view of the mechanism of SREBP-mediated activation and demonstrate that its effect, like that of p53, is mediated by the E2F/DP-1−SP1 site. Antibody to SREBP-1 reduced formation of the complex between E2F/DP1, Sp1, and p53. Cotransfection of cells with a plasmid coding for the functional moiety (codons 1−440) of SREBP-1 strongly stimulated transcription rates assayed with pGL3-cav. These findings provide additional support for the concept (*11*) that caveolin is a SREBP-dependent gene. Details of the mechanism of activation remain to be determined. Stable association between SREBP-1 and Sp-1 has been described (*15*), while interaction between SREBP and either p53 or E2F have not. Levels of SREBP in the nuclei of arrested human fibroblasts were evidently sufficient to partly (∼20%) activate maximal transcription rates in basal (10% FBS) medium. Consistent with this, conditions (80% plasma) that promoted SREBP-dependent upregulation of caveolin expression increased formation of the E2F/Sp1/p53 complex, while cyclodextrin, which depleted cellular FC pools, decreased it. ALLN produced opposite effects on the expression of caveolin, compared to that of LDL receptor protein and HMGCoA synthase. In the case of the latter two genes, it has been shown that the effect of ALLN on SREBP is expressed at the proteosomal level (*34*), but ALLN has other, distinct effects, including an inhibition of plasma membrane endocytosis by the coated pit pathway (*39*). This mechanism mediates the uptake of LDL free cholesterol to which caveolin expression responds (*6*, *27*). We suggest that ALLN may affect caveolin expression at the level of substrate delivery, rather than by inhibition of proteasomal protease activity.

Finally, if an effective response of caveolin to either cell cycle events or FC depended on the same transcriptional complex, changes in the levels of its rate-limiting components (E2F and p53) would have the same effects as caveolin overexpression. This hypothesis was tested by measuring FC efflux and growth rate. As predicted, E2F and p53 both increased FC efflux and inhibited cell division.

Identification of a common mechanism of transcriptional activation for cell-cycle and FC-mediated effects integrates for the first time both functions of caveolin and identifies it as a significant suppressor of cell division via a checkpoint possibly mediated by FC directly. The identification of caveolin as a gene regulated by tumor suppressor p53, the high natural frequency of p53 and E2F mutations, and the loss of caveolin expression in many human cancer cells is consistent with such a role. Additionally, mutations within the coding regions of transcription factors required for caveolin activation, or of their binding sites within promoter DNA as identified here, could contribute to the suppression of caveolin. Direct studies of the regulation of caveolin expression in well-defined cancer cell lines will now be needed to measure the relative importance of these different mechanisms.

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NOTE ADDED IN PROOF

An additional potential p53 half-site between −317 and −308 bp may contribute to the biological function of the caveolin transcription complex.

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