Physical Interaction and Mutual Transrepression between CCAAT/Enhancer-binding Protein β and the p53 Tumor Suppressor*

The tumor suppressor protein p53 is not only involved in defending cells against genotoxic insults but is also implicated in differentiation processes, a function that it shares with the CCAAT/ enhancer-binding protein β (C/EBPβ). We previously reported an up-regulation of both factors in the cycle-dependent differentiation process of human endometrial stromal cells, termed decidualization. C/EBPβ-mediated activation of a decidualization marker, the decidual prolactin promoter, was antagonized by p53. Here we report that C/EBPβ in turn represses the transcriptional activity of p53. Competition for limiting amounts of coactivator CREB-binding protein/p300 was ruled out as the underlying mechanism of transrepression. Physical interaction between p53 and C/EBPβ was demonstrated in vitro and in vivo and shown to depend on the C-terminal domains of both proteins. In gel shift experiments, C/EBPβ reduced complex formation between p53 and its response element. Conversely, p53 strongly inhibited binding of endogenous C/EBPβ from endometrial stromal cells to the C/EBP-responsive region in the decidual prolactin promoter. The observed negative cross-talk between p53 and C/EBPβ is likely to impact expression of their respective target genes.

The tumor suppressor protein p53 plays a fundamental role in protecting the organism against the emergence of cell populations with an abnormal genotype. The protein is present at low levels in normal cells due to its rapid turnover. In response to various types of genotoxic stress, it is stabilized and regulates the expression of genes that mediate cell cycle arrest and DNA repair or trigger apoptosis. Mice with a null mutation of the TP53 gene show a high incidence of tumor formation, and a large proportion of human cancers have a somatic mutation either in the TP53 gene itself or in genes that encode components of the p53-mediated transcriptional machinery.

The core region of the sequence-specific DNA-binding domain (DBD; residues 102–292) and the C-terminal domain (CTD; aa 300–393) contains the tetramerization domain (T4; residues 323–356) and a basic regulatory region (RR) at the extreme C terminus (aa 363–393) (10). The T4 mediates formation of a p53 tetramer, consisting of two dimers, which binds to a consensus containing two copies of the palindromic sequence 5′-PuPuPuCA(T)/T(A)GPy-PyPy-3′ (11). The RR is involved in nonspecific DNA binding (12).

Among the transcriptional targets of p53 are the genes for the cell cycle regulator p21WAF1 (an inhibitor of cyclin-dependent kinases) and the Mdm2 (mouse double minute-2) oncoprotein (3). Mdm2 and p53 are involved in a negative feedback loop. Whereas p53 up-regulates Mdm2 transcriptionally, Mdm2 initiates the proteasomal degradation of p53 by binding to its TAD and acting as an E3 ubiquitin ligase, adding ubiquitin groups to the C terminus of p53 (13). Mutated p53 fails to induce endogenous Mdm2 and is therefore longer lived than transcriptionally competent wild type (WT) p53.

In addition to being a transcriptional activator, p53 has also been reported to suppress numerous promoters. Among these are the β-fibrinogen, IL-6, insulin receptor, and albumin gene promoters when linked to reporter genes. Whereas the exact mechanism of transcriptional repression by p53 in these cases remained unclear, an interference with C/EBP-dependent transactivation seemed to be involved (14–17).

CCAAT/Enhancer-binding proteins (C/EBPs) belong to the superfamily of transcription factors characterized by the basic region/leucine zipper (bZIP) structure (18). To date, six members of the C/EBP family have been identified: C/EBPα, -β, -δ, -ε, -γ, and -ζ (19). C/EBPs play a fundamental role in differentiation processes and reproductive function. Ablation of the C/EBPβ gene in mice leads to defective mammary gland differentiation and failure to ovulate (20–22) and impairs adipocyte and macrophage differentiation (23, 24). The C/EBPβ mRNA gives rise to two protein isoforms, the so-called liver-enriched activatory protein (LAP) and the liver-enriched inhibitory protein (LIP). The latter is a truncated form lacking the N-terminal transactivation domains of LAP and antagonizes the transcriptional activity of other C/EBP isoforms (25). The most likely explanation for the generation of LAP and LIP is alternative translation initiation, with initiation at codons Met1 or Met24 in the human C/EBPβ mRNA resulting in LAP proteins of 36- and 33.5-kDa molecular mass and initiation at the downstream codon Met199 giving rise to the 16-kDa repressor LIP (26). However, a proteolytic mechanism has also been proposed to explain the presence of LIP in tissue extracts, at least in rodents (27). The leucine zipper of C/EBPs accommodates formation of a multitude of homo- and heterodimers.
between activator and/or repressor isoforms (19). In addition, C/EBPs have been found to interact with members of other families of transcription factors, such as helix-loop-helix (28), zinc finger (29, 30), or Ets proteins (31).

We have previously described up-regulation of C/EBPβ and p53 in the course of decidualization of human endometrial stromal cells (ESC) (32, 33). This differentiation process occurs in the late secretory phase of the menstrual cycle and prepares the uterine lining for implantation of the blastocyst. In primary cultures of human ESC, decidualization can be induced by several days of treatment with cAMP analogues. An exquisite marker of decidualization is activation of the decidual prolactin (dPRL) gene (34). The dPRL promoter is induced by the activating transcription factors, such as helix-loop-helix (28), zinc finger (29, 30), or Ets proteins (31).

Repression of p53 by C/EBPβ

Experimental Procedures

Cell Culture—Soa-2 human osteosarcoma cells (HTB-85; American Type Culture Collection) and COS-7 African Green Monkey kidney cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary cultures of human ESC were prepared as detailed previously (33) from anonymous biopsy samples obtained from premenopausal women at the time of hysterectomy for benign gynecological disorders. Informed consent was obtained, and the study was approved by the local ethics committee. ESC were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12, 10% steroid-depleted fetal calf serum, and antibiotics as above. Stimulations were performed in the same medium supplemented with insulin (1 μg/ml) and 10^{-9} M 17-β-estradiol.

Transient Transfection and Protein Extraction—Transient transfections for luciferase reporter gene assays were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions or by the calcium phosphate precipitation method as described previously in triplicates using 24-well plates (growth area 1.88 cm^2) (35). One μg of reporter construct and amounts of expression vector as indicated in the figure legends were used. Controls received equimolar amounts of empty expression vector, and total DNA was kept constant by the addition of promoterless plasmid DNA. Medium was replaced 16 h later, and cells were harvested 24 h after medium replacement. Luciferase activity was measured with the luciferase reagent kit (Promega). Transfections were repeated at least three times, and the means of three or four independent experiments were analyzed by one-way analysis of variance followed by Bonferroni’s post hoc test.

Cross-linking, Co-immunoprecipitation—Soa-2 cells were transfected with p53 and C/EBPβ expression constructs. Thirty h post-transfection, monolayers were washed twice with phosphate-buffered saline (PBS) and subjected to reversible cross-linking with the cell-permeable, thiol-cleavable reagent diithiobis(succinimidyl)propionate. Cells were exposed to diithiobis(succinimidyl)propionate (5 mM) for 30 min at room temperature, and the reaction was stopped by the addition of Tris, pH 7.5, to 20 mM. After 15 min, monolayers were washed with PBS and extracted with radioimmune precipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Complete Protease Inhibitor Mixture; Roche Applied Science). Whole cell extracts (300 μg) were preabsorbed with anti-hemagglutinin-agarose conjugate (Sigma) for 2 h at 4°C. Supernatants were retrieved and immunoprecipitated with 15 μl of slurry of p53 monoclonal antibody DO-1 cross-linked to agarose beads (Oncogene Research Products) overnight at 4°C. After five washes in radioimmune precipitation buffer, immunocomplexes were disrupted, and disulfide bonds were cleaved by heating the beads in SDS gel loading buffer containing 5% β-mercaptoethanol (10 min, 95°C).

Primary ESC were treated with 0.5 mM 8-bromoadenosine cAMP (8-Br-cAMP; Biolog, Bremen, Germany) for 6 days before harvest. Monolayers were washed once with ice-cold PBS and scraped in PBS, 0.5% Triton X-100. Cells were allowed to swell on ice for 5–15 min and sonicated for 3 × 5 s, and the lysate was incubated on ice for 30 min to solubilize proteins and cleared by microcentrifuging for 15 min at 4°C. Supernatant containing 200 μg of protein was incubated with anti-p53 DO-1 agarose as described above. Immunocomplexes were washed six times with PBS, 0.1% Triton X-100 and disrupted by heating the sediment in SDS gel loading buffer.

SDS-PAGE, Western Blotting, and Immunodetection—Proteins were electrophoresed on 10–12% SDS-polyacrylamide gels (NuPage Bis-Tris; Invitrogen) and transferred onto polyvinylidene difluoride Immobilon membranes (Millipore Corp.). Immunodetection was performed with the enhanced chemiluminescence system (SuperSignal; Pierce). Rabbit antibodies against C/EBPβ (C-19; 100 μg/ml) and p53 (FL-393; 2 mg/ml) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antiserum against the N terminus of C/EBPβ (LAP-specific) was obtained from Cell Signaling Technology. Monoclonal FLAG antibody M2 (2 mg/ml) was from Stratagene, and monoclonal p53 antibodies DO-1 (Ab-6) (0.1 mg/ml) and PAb421 (1 mg/ml) were from Oncogene. The following dilutions were used for Western blot analysis: C/EBPβ antisera, 1:1000; p53 FL-393, 1:5000; all monoclonal antibodies, 1:1000. Secondary antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG; Dianova) were employed at a dilution of 1:2500 and 1:10,000, respectively. For detection of immunoprecipitated proteins with monoclonal antibodies, peroxidase-conjugated goat anti-mouse IgG, F(ab’)_2 fragment-specific, was used (Jackson ImmunoResearch) at 1:5000.

Indirect Immunofluorescence and Confocal Microscopy—ESC were seeded on 4-well chamber slides (BD Falcon) at 1 × 10^5 cells/well and treated with 8-Br-cAMP (0.5 mM) for 6 days. Cells were fixed with MetOH (10 min, −20°C), washed with PBS, and permeabilized with 0.2% Triton X-100 for 10 min. Non-specific binding sites were blocked with normal goat serum. Monoclonal antibody to p53 (PAb421) and polyclonal antibody to C/EBPβ (LAP-specific; Cell Signaling Technology) were diluted in PBS (1:100). Following a 1-h incubation at room temperature and washing, secondary antibodies diluted in PBS, 2% normal goat serum (Cy3-conjugated anti-mouse IgG (1:100) and Cy2-conjugated anti-rabbit IgG (1:50)) (Dianova) were added for 1 h. Nuclei were counterstained with TO-PRO-3 (1:1000; Molecular Probes, Inc. (Eugene, OR)) for 5 min. Slides were mounted with ProLong Antifade (Molecular Probes), and images were captured with an AxioPlan2 confocal microscope (Zeiss).

Reporter Constructs and Expression Vectors—All luciferase gene reporter constructs were generated in the pGL3-Basic plasmid (Promega). The dPRL promoter/luciferase reporter fusion construct dPRL32/luc3, the minimal promoter construct dPRL32/luc3, and the C/EBP-responsive reporter construct CEBP-RE+32/luc3 (previously designated NFL6RE/luc3) have been described elsewhere (32). A reporter construct with the minimal rat pRL promoter element −36/+36 in pGL2-Basic (Promega) was provided by Dr. M. G. Rosenfeld (Howard Hughes Medical Institute, San Diego, CA). The insert was isolated by restriction with BglII and HindIII and cloned into the respective sites in pGL3-Basic to yield −36pRL/luc3. The p53-responsive reporter plasmid p53-RE−36pRL/luc3 was generated based on the
yeast one-hybrid control reporter vector p53BLUE (Clontech), which carries a p53-responsive region in the reporter plasmid pLacZi. The response element was amplified by PCR using primers flanking the response element: LacZ-sense (5′-CTTCTGGGGGAGATTACC-3′) and LacZ-antisense (5′-ATGCCTAACAGGCATTATG-3′). The PCR product was inserted into the Ecl136I site of −36rPRL/luc3 upstream of the rPRL promoter element.

To construct a reporter vector for mammalian one-hybrid experiments, 2×LEX/−36rPRL/luc3, two complementary oligonucleotides (sense sequence, 5′-TACTGCTGTATATAAAAAACAGTGTTATATGTACAGTACTcagg-3′) containing the LexA operon sequence and an overhang (lowercase) to include an XhoI site (underlined) were annealed, cut with XhoI, and ligated head-to-tail. The resulting dimer was inserted into the Smal site of −36rPRL/luc3.

Human p53 expression vectors pcDNA/p53 and pcDNA/p53/FLAG (carrying a C-terminal FLAG epitope) have been described (33). Residues Leu22 and Trp39 of p53 were mutated to Gln and Ser by site-directed mutagenesis with the QuickChange system and Pfu polymerase (Stratagene) on template pcDNA/p53/FLAG to generate pcDNA/p53.M22,23/FLAG. Two complementary oligonucleotides were used, which introduced the two point mutations (lowercase) in codons 22 and 23 (boldface) and destroyed a unique AclI site (underlined) for diagnostic purposes: 5′-CTGAAAACAAtGTTCTGTCCC-3′ (sense), and Arg175 was changed to His by site-directed mutagenesis using complementary oligonucleotides with a point mutation (lowercase) in codon 175 (boldface) and a point mutation (lowercase) to destroy an EcoR47III site (underlined) for diagnostic purposes to yield pcDNA/p53.M175/FLAG: 5′-GAGGCaCTGCCCCCAACCATTGAGGCGTGCTC-3′ (sense sequence). An expression vector with the tetramerization and regulatory domains deleted, pcDNA/p53ΔT4-RR/FLAG, was constructed as follows; the HindIII-Eco1019I fragment of pcDNA/p53/FLAG, extending from the 5′-end of the inserted cDNA to just 5′ of the FLAG sequence, was removed, and the Eco1019I overhang was filled in (pcDNA-FLAG). The HindIII-SspI fragment of pcDNA/p53/FLAG from the 5′-end of the cDNA to the codon for Tyr327 was isolated and inserted into pcDNA-FLAG, resulting in a deletion of amino acids 327–389. For construction of a vector with deletion of the TAD, pcDNA/p53ΔTAD/FLAG, aa 1–65 were removed. A sense primer was designed that spans the codon for Met26 of p53 and creates a Kozak consensus sequence around this triplet (boldface) by three mutations (lowercase) and a 5′-HindIII site (underlined) by one mutation (lowercase): 5′-CAGATGAAGCTtCCAccATGCaGAGGCT-3′ (sense). This primer was paired with an antisense primer anchored in pcDNA3.1(+) downstream of the poly linker for PCR on template pcDNA/p53/FLAG. The PCR product was digested with HindIII and Apal and inserted into the respective sites of pcDNA3.1 (+). p53 expression constructs in pcDNA3.1 were subjected to in vitro transcription/translation using the TNT T7 coupled reticulocyte lysate system (Promega) in a final volume of 25 μl in the presence of [35S]methionine.

For mammalian one-hybrid analysis, the DBD of the LexA repressor was fused to p53, using the vector pLexApolII (kindly provided by Dr. Jean Schneikert, Institute for Genetics, Research Center Karlsruhe, Germany). FLAG-tagged WT p53 cDNA was amplified from pcDNA/p53/FLAG with a 5′-primer mutating the start codon and introducing a BamHI site and a 3′-primer anchored downstream of the poly linker. The BamHI-restricted ampiclon was inserted into the BamHI site of pLexApolII to yield pLex/p53/FLAG.

Expression vector for human p300 (pCMVp-HNA-p300) was kindly provided by Dr. David Livingston (Dana Farber Cancer Institute, Boston, MA) (36).

Expression vectors for human C/EBPs in pSG5 (pSG-C/EBPβ, pSG/LAP, pSG/LIP) have been described previously (32). The LAP insert was excised with EcoRI and BglII and inserted into the EcoRI and BamHI sites of pcDNA3.1(−) (Invitrogen) to yield pcDNA/LAP. For construction of pcDNA/LIP, a restriction fragment was prepared from pSG-C/EBP β extending from the SacII site 49 bp upstream of Met199 to the BglII site in the 3′-polylinker of pSG5. The SacII overhang was polished, and the fragment was inserted into the EcoRV and BamHI sites of pcDNA3.1(−).

On template pSG/LIP, the insert was amplified by PCR with an upstream primer mutating the start ATG (Met199) and adding an EcoRV site and a downstream primer mutating the stop codon and attaching a FLAG epitope followed by a NotI site. After restriction of the ampiclon with EcoRV and NotI, it was inserted into the respective sites of pVP22/Myc-His (Invitrogen). From this construct (pVP22/LIP-FLAG), the Bpu1102I-Pmel fragment was isolated (encompassing the 3′-end of LIP cDNA and the FLAG-, Myc-, and His6, epitopes) and used to replace the Bpu1102I-HindIII (blunted) fragment of pcDNA/LIP. The resultant vector pcDNA/LIP-FLAG carries the LIP cDNA with 3′-FLAG-, Myc-, and His6, epitopes. The vector pcDNA/LAP-FLAG was generated by discarding the 5′ Nhel-Bpu1102I fragment from pcDNA/LIP-FLAG and replacing it with the Nhel-Bpu1102I fragment retrieved from pcDNA/LAP.

**GST Pull-down Assays**—GST pull-down assays were performed essentially as detailed elsewhere using pGEX/LAP and pGEX/LIP for bacterial expression of GST fusion proteins (37). GST, GST-LAP, or GST-LIP, immobilized on glutathione-Sepharose beads (Amersham Biosciences), were incubated with 50 μl of 35S-labeled in vitro translation product generated from p53 expression vectors, and bound material was eluted and electrophoresed in a 10% SDS-polyacrylamide gel. Five μl of the in vitro translation reaction was loaded on the gel as the input of 35S signal. The gel was dried, and proteins were visualized by autoradiography.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear proteins were extracted by the high salt procedure (35, 38) from Saos-2 cells that had been transfected using Lipofectamine from COS-7 cells transfected using the DOTAP liposomal reagent (Roche Applied Science) in 6-well plates (growth area 9.4 cm2) with the indicated amounts of expression plasmid. Double-stranded oligonucleotides used as probes or competitors for EMSA were dPRL promoter fragment dPRL−322/−270, and consensus binding sites for C/EBP (CGBP-RE), p53 (p53-RE), and Ets-1/PEA3 (Ets-RE) (Santa Cruz Biotechnology). Double-stranded oligonucleotides were end-labeled with [γ-32P]ATP. Per binding reaction, 5 μg of nuclear protein, unless indicated otherwise, and 10,000 cpm of probe were used. For probe dPRL−322/−270, the following binding buffer (BP-high salt) was employed: 10 mM HEPES, 5 mM MgCl2, 60 mM KCl, 1,25 mM spermidine, 3.5% Ficoll, and 0.02 units poly(dI-dC) (Roche Applied Science). Preincubations of two different protein preparations were performed overnight at 4°C. For binding to p53-RE and C/EBP-RE, the following buffer (Bf-B) was used: 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl2, 0.1% Nonidet P-40, 10% glycerol, 50 mg/ml bovine serum albumin, 0.02 units of poly(dI-dC). After the addition of probe, incubation was continued for 40 min at 4°C. For supershift analyses, antibody (1–2 μl/reaction) was added for an additional 20 min incubation at room temperature. The following antibodies were employed: monoclonal p53 antibodies clone PAb421 (1 μl) and clone DO-1 (2 μl) (Oncogene); polyclonal antibody to C/EBPβ.
Repression of p53 by C/EBPβ

RESULTS

C/EBPβ Represses p53-dependent Transactivation—We have previously described that p53 represses the C/EBPβ-dependent activation of the dPRL promoter in primary cultures of human ESC (33). To assess whether the inhibition between C/EBPβ and p53 was reciprocal, we transfected the p53-deficient Saos-2 cell line with a p53-responsive reporter construct (p53-RE/-36PRL/luc3; see Fig. 1), which was massively induced by exogenous p53 (Fig. 2). The addition of increasing doses of the activating isofrom of C/EBPβ, LAP, or of the inhibitory isofrom LIP significantly reduced p53-dependent activation, whereas LAP alone even displayed a small activation potential on the p53 reporter construct.

LAP and LIP Do Not Exert Their Inhibitory Function by Impairment of the DNA Binding of p53 in Vivo—To address the question of whether LAP or LIP inhibited p53 transcriptional activity through interference with its DNA binding ability, we performed a mammalian one-hybrid assay. As the activator construct, we utilized a fusion of WT p53 with the DBD of the bacterial repressor LexA. The reporter construct was under the control of the Lex operon. Thus, reporter gene activation would be achieved through the p53 TAD and the LexA DBD but would be independent of the DNA binding function of p53 (Fig. 3A). The massive induction of the reporter gene by LEX/p53 was markedly reduced by co-transfection of either LAP or LIP expression vector. The C/EBPβ isofroms therefore appear to antagonize the transcriptional activity of
Repression of p53 by C/EBPβ

Transrepression of p53 by LAP Is Independent of p300—Both p53 and C/EBPβ are known to interact with the coactivator CBP/p300 (39), rendering competition for limiting amounts of coactivator as a possible explanation for transrepression. This has been put forward as one of the reasons for repression of p53 activity by LAP. The possibility of direct physical interaction between p53 and C/EBPβ and to map the domains involved, we generated a series of mutant p53 constructs (see Fig. 1C). DNA binding ability was destroyed by mutating amino acid 175 (R175H) (construct p53.M175), one of the most frequent naturally occurring mutations (1, 44). The TAD was either deleted (p53ΔTAD) or disabled by mutating residues 22 and 23 (L22Q,W23S) (p53.M22,23) (44). Furthermore, the C terminus with the tetramerization domain and the regulatory region was deleted (p53ΔT4-RR). GST pull-down assays were performed using GST-LAP and GST-LIP as the matrix. In vitro translated radiolabeled WT and mutant p53 constructs (Fig. 4A) were incubated with the immobilized GST fusion proteins, eluted, and analyzed by SDS-PAGE and autoradiography (Fig. 4B). Both LAP and LIP were found to bind all forms of p53, with the exception of p53ΔT4-RR. This demonstrates that p53 and C/EBPβ physically interact in vitro, that a motif within the CTD (beyond aa 326) of p53 is required, and that the C-terminal portion of C/EBPβ (corresponding to LIP) is sufficient for interaction.

Physical association of p53 and C/EBPβ in vitro—To investigate the possibility of direct physical interaction between p53 and C/EBPβ and to map the domains involved, we generated a series of mutant p53 and C/EBPβ constructs (see Fig. 1C). DNA binding ability was destroyed by mutating amino acid 175 (R175H) (construct p53.M175), one of the most frequent naturally occurring mutations (1, 44). The TAD was either deleted (p53ΔTAD) or disabled by mutating residues 22 and 23 (L22Q,W23S) (p53.M22,23) (44). Furthermore, the C terminus with the tetramerization domain and the regulatory region was deleted (p53ΔT4-RR). GST pull-down assays were performed using GST-LAP and GST-LIP as the matrix. In vitro translated radiolabeled WT and mutant p53 constructs (Fig. 4A) were incubated with the immobilized GST fusion proteins, eluted, and analyzed by SDS-PAGE and autoradiography (Fig. 4B). Both LAP and LIP were found to bind all forms of p53, with the exception of p53ΔT4-RR. This demonstrates that p53 and C/EBPβ physically interact in vitro, that a motif within the CTD (beyond aa 326) of p53 is required, and that the C-terminal portion of C/EBPβ (corresponding to LIP) is sufficient for interaction.

Physical association of p53 and C/EBPβ was further investigated by co-immunoprecipitation experiments. Saos-2 cells were transfected with WT p53, p53ΔTAD, p53.M175, and LIP-FLAG alone or in combination. Cellular proteins were reversibly cross-linked in vivo with the
Repression of p53 by C/EBPβ

leading to the detection of WT or mutant p53 multimers in addition to the monomers. Detection of immunoprecipitated samples with C/EBPβ antibody revealed the presence of LIP-FLAG in those cases where WT p53 or p53.M175 had been co-transfected (Fig. 5A, middle panel, lanes 7 and 8) but not when LIP-FLAG had been transfected alone (Fig. 5A, middle panel, lane 5). Appropriate expression of LIP-FLAG in the transfected cells was ascertained by Western blotting the input proteins before immunoprecipitation (Fig. 5A, top panel). The input LIP-FLAG sample loaded in lane 9 was also run on the gels with the precipitated proteins (middle and bottom panels, lane 9) as a size reference and to demonstrate specificity of the antibodies. As seen for p53, cross-linking also led to formation of LIP-FLAG dimers and higher order complexes, which was not fully reversed by the reducing sample treatment.

This experiment clearly shows physical association of p53 and C/EBPβ in vivo and confirms the notion obtained by the GST pull-down assay that the C-terminal portion of the molecule (corresponding to LIP) is sufficient for interaction.

In addition to using overexpressed C/EBPβ and p53 to assess in vivo interaction, we then turned to a cell system in which both proteins are endogenously expressed. Primary cultures of ESC were incubated in the absence or presence of 8-Br-cAMP for 6 days before whole cell extracts were harvested. Western blotting confirmed up-regulation of LAP and p53 in response to the cAMP analogue, as described previously (32, 33) (Fig. 5B, upper panel). Pull-down with anti-p53 DO-1-agarose resulted in precipitation of p53 and co-precipitation of LAP, as shown by immunodetection with the respective antibodies (Fig. 5B, lower panel). It must be noted that the CAMP-induced increase in p53 and LAP is not fully reflected in the immunoprecipitated samples. This might be due to saturation of DO-1-agarose with p53 and/or saturation of the binding capacity of p53 for LAP. Taken together, this experiment demonstrated for the first time physical interaction of endogenous p53 and LAP in vivo.

Subcellular Localization of p53 and C/EBPβ in ESC—The subcellular localization of p53 and C/EBPβ was investigated by indirect immunofluorescence in ESC that had been treated with 8-Br-cAMP for 6 days to up-regulate both factors. Whereas p53 was predominantly in the nucleus.
nucleus, some immunoreactivity was also detected in the cytoplasm. C/EBPβ, visualized with an antibody specific to the activating isoform LAP, was found exclusively nuclear (Fig. 6). An overlay of the confocal images revealed only partial colocalization of p53 and LAP in punctate nuclear structures.

Effect of LAP on p53 DNA Binding in EMSA—Gel shift experiments were performed to more specifically investigate a potential interaction of LAP with p53 on DNA. It is a specific feature of p53 that it usually does not interact with its response element in vitro unless the antibody PAb421, directed against the CTD, is added to activate sequence-specific DNA binding (45). To circumvent this problem, we used p53 extracted from COS-7 cells, because it binds to p53-RE without the requirement for activating antibody. Furthermore, COS-7 cells have very low levels of C/EBPβ, C/EBPδ, or other proteins binding to C/EBP-RE (32, 33). Nuclear extracts from COS-7 cells were incubated with labeled p53 consensus element (p53-RE) as the probe (p53-RE*). As competitors, excess unlabeled p53-RE, C/EBP-RE, or Ets-RE was added. For supershift analysis, monoclonal antibodies to p53 (DO-1 or PAb421), an antibody to C/EBPβ from Santa Cruz Biotechnology (SC; C-19) or antibody to Ets-1/Ets-2 was used. Specific complex; SS, supershifted complexes. B, fluorescently labeled probes p53-RE-Cy5 (upper panel) or CEBP-RE-Cy5 (lower panel) were incubated with nuclear extracts from COS-7 cells that had been transfected with pcDNA/LAP-FLAG (0.5 μg/24-well) or with empty vector. In addition to the antibodies described in A, a C/EBPβ antiserum from a different supplier (Active Motif, AM) and monoclonal C/EBP antibody M2 were employed for supershift analysis. S, specific complex; SS, supershifted complex resulting from the addition of C/EBPβ antibody (Santa Cruz; SC); SS II, supershifted complex containing LAP-FLAG. C, Western blot loading control for the nuclear extracts used in B. Ten μg of the same nuclear extracts as employed for the EMSA experiment depicted in B, from COS-7 cells transfected with empty vector pcDNA (Fig. 7B, EMSA lanes 1–5) or pcDNA/LAP-FLAG (Fig. 7B, EMSA lanes 6–10), were analyzed by SDS-PAGE alongside 10 μg of nuclear extract from COS-7 cells that had been transfected in parallel with untagged expression vector pcDNA/LAP. Four identical blots were generated with these samples and immunodetected with antibodies to p53 (DO-1), FLAG (M2), LAP, and C/EBPβ (C-19). The small arrowheads indicate N- or C-terminal proteolytic cleavage products of LAP and LAP-FLAG recognized by the LAP-specific (N-terminal epitope) or the C/EBPβ (C-terminal epitope) antibody, respectively. The asterisk denotes a nonspecific band seen with the C/EBPβ antiserum. Note the very faint signal for endogenous LAP in the middle lane (mock-transfected cells) of the right panel.

FIGURE 7. C/EBPβ reduces p53 DNA binding in EMSA. A, nuclear extract (NE) from COS-7 cells that had been transfected with empty vector (pcDNA) or with pcDNA/LAP (0.05 or 0.5 μg/24-well plate) was incubated with 32P-labeled p53-RE as the probe (p53-RE*). As competitors, excess unlabeled p53-RE, C/EBP-RE, or Ets-RE was added. For supershift analysis, monoclonal antibodies to p53 (DO-1 or PAb421), an antibody to C/EBPβ from Santa Cruz Biotechnology (SC; C-19) or antibody to Ets-1/Ets-2 was used. Specific complex; SS, supershifted complexes. B, fluorescently labeled probes p53-RE-Cy5 (upper panel) or CEBP-RE-Cy5 (lower panel) were incubated with nuclear extracts from COS-7 cells that had been transfected with pcDNA/LAP-FLAG (0.5 μg/24-well) or with empty vector. In addition to the antibodies described in A, a C/EBPβ antiserum from a different supplier (Active Motif, AM) and monoclonal C/EBP antibody M2 were employed for supershift analysis. Specific complex; SS, supershifted complex resulting from the addition of C/EBPβ antibody (Santa Cruz; SC); SS II, supershifted complex containing LAP-FLAG. C, Western blot loading control for the nuclear extracts used in B. Ten μg of the same nuclear extracts as employed for the EMSA experiment depicted in B, from COS-7 cells transfected with empty vector pcDNA (Fig. 7B, EMSA lanes 1–5) or pcDNA/LAP-FLAG (Fig. 7B, EMSA lanes 6–10), were analyzed by SDS-PAGE alongside 10 μg of nuclear extract from COS-7 cells that had been transfected in parallel with untagged expression vector pcDNA/LAP. Four identical blots were generated with these samples and immunodetected with antibodies to p53 (DO-1), FLAG (M2), LAP, and C/EBPβ (C-19). The small arrowheads indicate N- or C-terminal proteolytic cleavage products of LAP and LAP-FLAG recognized by the LAP-specific (N-terminal epitope) or the C/EBPβ (C-terminal epitope) antibody, respectively. The asterisk denotes a nonspecific band seen with the C/EBPβ antiserum. Note the very faint signal for endogenous LAP in the middle lane (mock-transfected cells) of the right panel.
Repression of p53 by C/EBPβ

observation compatible with the reported physical association of p53 and Ets-1 (46). Whereas an excess of unlabeled p53-RE fully abrogated complex formation, competition with unlabeled CEBP-RE or Ets-RE was without effect. Furthermore, the addition of antibodies against a variety of other transcription factors (i.e. C/EBPβ, C/EBPγ, c-Jun, FOXO1a) or competition with their respective binding sites did not affect p53 binding to its response element (data not shown).

To obtain further evidence for the specificity of the indirect supershift obtained with C/EBPβ antibody, COS-7 cells were transfected with FLAG-tagged LAP, and their nuclear proteins were subjected to gel shift analysis on p53-RE (Fig. 7B, upper panel). Again, p53 binding to its response element was reduced in the presence of overexpressed LAP (lanes 1 and 6), the complex was supershifted by DO-1 (SS II), and the same C/EBPβ antibody as used in Fig. 7A produced a partial supershift (SS I). This antibody (SC) has been raised against a peptide corresponding to residues 258–276 of rat C/EBPβ, which corresponds to aa 306–324 in human C/EBPβ and is 100% conserved. In addition, we employed a C/EBPβ antibody from a different supplier, which had been raised against a peptide comprising residues 228–242 of human C/EBPβ (AM), and monoclonal antibody M2 against the C-terminal FLAG epitope of LAP-FLAG. Neither antibody generated a supershift on the p53-DNA complex. Interestingly, whereas in the presence of overexpressed LAP-FLAG, the p53-DNA complex was reduced, it was restored to the level seen with untransfected cells when C/EBPβ antibody (AM) or FLAG antibody M2 was included (compare lanes 1, 6, 9, and 10).

To confirm the functionality of the antibodies for supershift analysis, we incubated the same COS-7 nuclear proteins with CEBP-RE as the probe (Fig. 7B, lower panel). The antibody (DO-1) has been shown to recognize a specific epitope of LAP-FLAG. Neither antibody detected both transfected LAP and LAP-FLAG but hardly any endogenous LAP. The C/EBPβ antibody (SC), which had produced the indirect supershift in EMSA (Fig. 7A, B), also visualized highly abundant transfected LAP and LAP-FLAG and a very faint band for endogenous LAP in mock-transfected COS-7 cells (Fig. 7B, lower panel). To further substantiate the reduced binding of p53 to its RE in the presence of overexpressed LAP, we quantitated the proteins used in the gel shift experiment (Fig. 7B) by Western blot analysis. Equal amounts of the same nuclear extracts from COS-7 cells, transfected with empty vector pcDNA (loaded in lanes 1–5; Fig. 7B), with pcDNA/LAP-FLAG (loaded in lanes 6–10; Fig. 7B), or with pcDNA/LAP (not shown in Fig. 7B), were immunodetected with antibodies to p53 or C/EBPβ (Fig. 7C). The level of p53 protein was identical in all lanes, ruling out reduced expression of p53 in the presence of overexpressed LAP. The FLAG antibody showed a high level of transfected LAP-FLAG. The LAP-specific antibody detected both transfected LAP and LAP-FLAG but hardly any endogenous LAP. The C/EBPβ antibody (SC), which had produced the indirect supershift in EMSA, produced a supershift in mock-transfected COS-7 cells with two different amounts (0.03 and 0.3 µg/24-well plate) or equimolar amounts of expression vector (−). Means ± S.E. of three independent experiments are shown. *p < 0.01, **p < 0.001. C/EBPβ-induced repression of p53-DNA complex (lanes 1–5; Fig. 7B) was not alleviated by co-expression of p300 (data not shown). We then tested the reverse scenario in the Saos-2 cell line. Reporter constructs contained either the intact C/EBPβ-responsive dPRL promoter (dPRL-332/luc3) or an isolated consensus C/EBPβ-RE adjacent to the minimal dPRL promoter element dPRL-32 (CEBP-RE/-32/luc3) (see Fig. 1A). LAP-induced activation of both reporter constructs was fully suppressed in the presence of p53 (Fig. 8, A and B). Transrepression could not be alleviated by co-expression of p300 (data not shown). We then addressed the question whether p53 interfered with the DNA binding of C/EBPβ. Primary ESC were used as the cell model, since we had dem-
Repression of p53 by C/EBPβ

In this study, we characterize a reciprocal inhibition between the bZIP transcription factor C/EBPβ and the tumor suppressor protein p53. Whereas negative regulation of C/EBPβ-dependent transcription by p53 had been observed previously for the β-fibrinogen, IL-6, insulin receptor, and albumin gene promoters in co-transfection systems, the underlying mechanism had not been resolved (14–17). Our observation of the reverse effect, namely repression of p53 transcriptional activity by C/EBPβ, is, however, novel.

Competition for limiting amounts of endogenous p300/CBP lent itself as an explanation for mutual transrepression. Interaction and functional collaboration does not only operate between p300/CBP and p53 (6, 7, 9) but also between p300 and C/EBPβ (47). The coactivator p300 interacts with the TAD of both p53 and C/EBPβ (6, 8, 9, 39, 47, 48). Two lines of evidence obtained in our study argue against competition for limiting amounts of p300 between p53 and C/EBPβ as the sole explanation for mutual repression. First, p53-mediated activation of a p53-responsive promoter/reporter gene was repressed not only by LAP but also, and even more efficiently, by LAP, which lacks the p300-binding region of full-length C/EBPβ. Second, overexpression of p300 did not alleviate transrepression of LAP by p53 or of p53 by LAP.

As an alternative mechanism for mutual antagonism between p53 and C/EBPβ, we considered the possibility of direct interaction between the two factors, leading to impairment of their DNA binding or transactivation functions, either by blocking relevant epitopes or by sequestration. Association of p53 with androgen, estrogen, glucocorticoid, and thyroid hormone receptors has been reported to interfere with binding of these transcription factors to their respective response elements (49–53). When we added p53 to nuclear extracts of ESC, binding of endogenous C/EBPβ to its response element was abrogated. Conversely, overexpression of LAP in COS-7 cells reduced binding of endogenous p53 to its cognate DNA sequence. However, with an antibody raised against residues 258–276 of rat C/EBPβ, an indirect supershift of the p53/p53-RE adduct was obtained, indicating the presence of C/EBPβ in such adducts. The antigenic peptide sequence of this antibody (SC) is 100% conserved in the human C/EBPβ protein and corresponds to aa 306–324 within the leucine zipper domain (54). This observation, however, could not be reproduced using a different C/EBPβ antibody (AM) directed against residues 228–242 or an antibody against the C-terminal FLAG epitope of co-expressed LAP-FLAG. The possibility cannot be excluded at present that the phenomenon of an indirect supershift is due to cross-reactivity of the C/EBPβ antibody (SC), under nonnaturating conditions, with an unidentified protein present in the COS-7 nuclear extracts that is part of the p53-DNA complex. Taken together, LAP reduces p53 DNA binding when overexpressed. On the other hand, p53 strongly inhibits DNA binding of C/EBPβ. These observations point toward a squelching mechanism.

In GST pull-down assays, we demonstrated a direct physical association of p53 and C/EBPβ, the relevant domains being the C-terminus of p53 (aa 326–390), which includes the T4 and the RR, and the C-terminal portion of C/EBPβ corresponding to LIP (aa 199–345) with the bZIP domain. The bZIP is the interface for interactions of C/EBPβ with other bZIP factors but also with such diverse proteins as nuclear progesterone receptors PR-B and PR-A, forkhead transcription factor FOXO1a, NF-κB, or YY1 (37, 55–58). The CTD of p53 interacts with numerous viral and cellular proteins and carries phosphorylation, acetylation, and ubiquitination sites (10, 13). The physical interaction of p53 and C/EBPβ was verified by co-immunoprecipitation, both with transfected p53 and LIP in Saos-2 cells and with endogenous p53 and LAP in primary cultures of ESC.

To investigate the functional consequences of such an interaction, we employed a mammalian one-hybrid assay. A fusion of the LexA-DBD with full-length p53 was used to activate a reporter construct under the control of LEX operons. Transcriptional activity of the p53 fusion protein was greatly diminished in the presence of co-transfected LAP or LIP, indicating that the latter do not only exert their inhibitory function by perturbing the interaction of the p53-LIP with its cognate response element, as suggested by the EMSA experiments, but possibly also by interference with the transactivating function of p53.

It is an open question whether p53 and C/EBPβ proteins interact which each other in their multimeric forms. It has been reported that p53 exists primarily as a tetramer even in the absence of DNA (59). This makes it likely that in vitro translated p53 was tetramerized when it was found to bind to C/EBPβ in the GST pull-down assay or when it abrogated the DNA binding of endogenous C/EBPβ in ESC nuclear extracts in EMSA studies. C/EBPβ, on the other hand, is also likely to primarily form homodimers or heterodimers with other bZIP proteins even in the absence of DNA (60). Such dimers might be converted to monomers upon binding to p53, leading to disruption of DNA binding ability. Alternatively, interaction of p53 with monomeric or dimeric C/EBPβ might sterically obstruct the DBD of the latter. Co-immunoprecipitation of chemically cross-linked proteins indicated that p53 can associate with multimeric LIP.

The negative cross-talk between C/EBPβ and p53 is likely to influence the transcriptional control of both p53- and C/EBPβ-regulated genes, and it is conceivable that dysregulation of p53-C/EBPβ cross-talk is implicated in tumorigenesis. Enhanced expression of C/EBPβ could antagonize the tumor-suppressive role of WT p53, and in fact, increased levels of C/EBPβ have been reported for ovarian, breast, colorectal, and skin tumors and renal cell carcinomas (61–66). On the other hand, an increased level of p53 wild type protein might keep the tumor-promoting role of C/EBPβ in check and is in fact seen in tissues that undergo pronounced waves of proliferation and differentiation such as the endometrial stroma of the cycling human uterus (33) or the mammary gland during pregnancy and lactation (67).

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