Kin17 is an evolutionarily conserved DNA-binding protein, which forms intranuclear foci in proliferating cells. Recent data have suggested that human kin17 protein is associated with cell proliferation and unrepaired DNA lesions. Herein, we show that human fibroblasts (MRC5-V2 and CHSV4) immortalized with SV40 overexpress endogenous kin17 protein, as compared with normal diploid human fibroblasts. We observed that certain carcinoma cell lines also up-regulated kin17 protein, suggesting that increased kin17 protein levels may be a consequence of the immortalized phenotype. We report here that the endogenous kin17 protein is located in nucleoplasmic foci and colocalizes with SV40 large T antigen. Purification of human kin17 protein allowed analysis of the physical interaction with T antigen by several in vitro and in vivo assays. Large T antigen and human kin17 protein are part of the same high molecular weight multiprotein complex in human cells. Furthermore, human kin17 protein interacts with T antigen by several in vitro and the introduction of increased amounts of human kin17 protein in an in vitro assay reduced T-antigen-dependent DNA replication, suggesting that kin17 protein may be involved in the DNA replication process in human cells.

INTRODUCTION

SV40, a small DNA virus belonging to the polyomavirus family, has been extensively studied as a model for understanding the regulation of mammalian cell growth and proliferation. The SV40 large T antigen, a 90-kDa nuclear multifunctional phosphoprotein, is the only viral protein essential for SV40 DNA replication and host cell transformation. T antigen possesses several biochemical activities, such as ATPase, DNA binding, ATP-dependent oligomerization, and DNA helicase. These activities map to discrete domains that can act independently of the rest of the polypeptide (1). After infection, T antigen alters host gene expression and growth control by binding cellular retinoblastoma family proteins, such as pRb and p107 (7), DNA transcription factors (2–4), components of the replication machinery (5–7), and ionizing radiations (17–21). We have reported recently that in vivo kin17 protein is directly associated with chromosomal DNA as judged by cross-linking experiments on living human cells. The amount of DNA-bound kin17 protein increases 24 h after γ-irradiation of RKO cells. At this time, kin17 protein concentrated into large nuclear foci with RPA, which has been suggested to localize at sites of unrepaired DNA damage (22). RKO clones expressing a human KIN17 antisense transcript elicited major disruptions in the S phase progression, as well as a significant decrease in clonogenic cell growth and cell proliferation (21). Taken together, our observations indicate that kin17 protein is a DNA maintenance protein involved in DNA replication and in the cellular response to DNA damage.

Because mouse kin17 protein and T antigen colocalize in intranuclear foci of transfected HeLa cells (23), we assumed that these proteins may participate in a common pathway of DNA metabolism. Herein, we show that SV40-immortalized human cell lines present higher levels of kin17 protein as compared with human diploid fibroblasts. kin17 protein associates directly with T antigen as evidenced by coimmunoprecipitation, fusion protein binding assay, ELISA, and affinity chromatography. In vivo, kin17 protein and T antigen belong to the same high molecular weight complex. Moreover, we show that the interaction between these two proteins is able to reduce DNA synthesis, suggesting that kin17 protein may inhibit DNA replication.

MATERIALS AND METHODS

Materials. Restriction endonucleases were from New England Biolabs. T4 polynucleotide kinase, [α-32P]CTP (3000 Ci/mmol), ribonucleoside, deoxyribonucleoside triphosphates, DNase I-activated calf thymus DNA, and enhanced chemiluminescence system were from Amersham Pharmacia Biotech. T antigen and DNA replication assay kit were from Molecular Biology Resources (Milwaukee, WI). PAGE-purified oligonucleotides were synthesized by Sigma-Genosys. Create phosph ate, create phosphokinase, and proteinase K were from Boehringer Mannheim.

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3 The abbreviations used are: RPA, replication protein A; PVDF, polyvinylidene difluoride; GST, glutathione S-transferase; MRC, multiprotein DNA replication complex; ADH, alcohol dehydrogenase; mAb, monoclonal antibody; PBS-T, PBS-0.1% Tween 20; T/BST, Tris-buffered saline with 0.1% Tween 20; PCNA, proliferating cell nuclear antigen.

45-kDa nuclear protein remarkably conserved during evolution and ubiquitously expressed in mammals (11). The kin17 protein displays common epitopes with the RecA protein and shares 47% homology over a 40-residue stretch in the RecA COOH-terminal region (12). This region is involved in the regulation of DNA binding and in the SOS response in Escherichia coli (13). To date, major features of the kin17 protein are its ability to: (a) bind to curved DNA found at the hot spots of illegitimate recombination of human cells (14, 15); (b) complement the functions in deficient bacterial strains of a bacterial transcriptional factor called H-NS, which binds to curved DNA and controls gene expression (16); and (c) be up-regulated after UV and ionizing radiations (17–21). We have reported recently that in vivo kin17 protein is directly associated with chromosomal DNA as judged by cross-linking experiments on living human cells. The amount of DNA-bound kin17 protein increases 24 h after γ-irradiation of RKO cells. At this time, kin17 protein concentrated into large nuclear foci with RPA, which has been suggested to localize at sites of unrepaired DNA damage (22). RKO clones expressing a human KIN17 antisense transcript elicited major disruptions in the S phase progression, as well as a significant decrease in clonogenic cell growth and cell proliferation (21). Taken together, our observations indicate that kin17 protein is a DNA maintenance protein involved in DNA replication and in the cellular response to DNA damage.
core origin of replication (24). To test the specificity of T antigen for SV40 origin of replication, 5′ GGAGCGAACACCAATTTGATGCTTTAAGA and reverse: 5′ GAGATGCATGCTTTGCATACTTCTGCCTGCTGGGGAGGGAATTCT- was described elsewhere (29). Recombinant baculovirus Bac-Kin17 was constructed by cotransfection of BacPAK6 Bsu 36I-digested DNA with pBacPAK-His3-KIN17 into Sf9 insect cells by the same buffer. Fractions (1.5 ml) were collected, analyzed by 10% SDS-PAGE, and stained for protein using the zinc staining kit according to the manufacturer’s protocol (Bio-Rad). Peak fractions of (His)6-kin17 were pooled and diluted five times in 20 mM phosphate buffer (pH 7.4), 1% Igepal CA-630, and a protease inhibitor. The diluted protein was then applied as described above to a 5-ml Hi-Trap heparin column (Amersham Pharmacia Biotech) equilibrated with 20 mM phosphate buffer (pH 7.4), 150 mM NaCl, 1% Igepal CA-630, and protease inhibitors as above. After extensive washing, (His)6-kin17 protein was eluted with a linear gradient over 10-column volume from 150 mM to 1 M NaCl in the same buffer without Igepal CA-630. Peak fractions of (His)6-kin17 were pooled and concentrated using a Concentrator-10 spin column (Vivascience) to achieve a protein concentration of 1–2 mg/ml.

Purified protein was analyzed by gel filtration and sucrose density gradient sedimentation. (His)6-kin17 protein was fractionated by gel filtration through a Superdex 200 HR 10/30 column (Smart System; Amersham Pharmacia Biotech) equilibrated in PBS. The columns were run at 40 μl/min at room temperature. Fractions (40–50 μl) were collected and analyzed by SDS-PAGE, followed by Western blotting. (His)6-kin17 protein (500 ng) was then fractionated at room temperature as described above. Aprotinin (a multimer of 440 kDa), β-amylace, ADH, BSA, and cytochrome c (a monomer of 12.7 kDa) were analyzed as protein standards under the same conditions. The molecular mass of (His)6-kin17 protein was determined from the calibration curve of molecular masses of protein standards. For sucrose density gradient sedimentation, a mixture (50 μl in PBS) of purified (His)6-kin17 protein (500 ng) and the molecular mass markers (Sigma) β-amylace (30 μg; tetramer, 200 kDa), ADH (50 μg; tetramer, 150 kDa), and BSA (25 μg; monomer, 68 kDa) was layered onto a 10-ml linear 5–20% sucrose density gradient in PBS and 2 mM β-mercaptoethanol. Fractions (500 μl) were collected after centrifugation at 27,000 rpm for 24 h in a Beckman SW27 rotor at 4°C and analyzed by 10% SDS-PAGE. Fractions containing (His)6-kin17 protein were detected by Western blotting with anti-His antibody and revealed by chemiluminescence. Molecular mass markers were detected by zinc staining.

Production of Polyclonal and mAbs. Purified human His-tagged k17 protein (500 μg) was mixed with complete Freund’s adjuvant (volume/volume) and injected intradermally into two rabbits. Three boosts (equivalent amounts of k17 protein in complete Freund’s adjuvant) were given to the rabbits at 1-month intervals. The serum was collected before the first injection and 10 days after each injection, and its specificity determined by Western blot and immunoprecipitation analyses. The best titer was obtained 77 days after the first injection and this fraction named pAb77.2 was additionally used in this report.

mAbs against the k17 protein were raised in mice (Biozzi’s High Responder strain) as described previously (28). The presence of murine anti-(His)6-kin17 antibodies in the corresponding antisera was monitored as described elsewhere (29–31).

ELISA Assays. Two different ELISA assays were used: (a) a conventional two-site immunometric assay (sandwich immunassay) to quantify the amount of k17 protein; the used mAbs (mAb K3 and mAb K31 conjugated to a reporter enzyme) recognize nonoverlapping epitopes of k17 protein and was developed essentially as described previously (29); and (b) a simple ELISA test was used to investigate the direct interaction between (His)6-kin17 protein and T antigen. A 96-well ELISA plate was coated with 1 μg of (His)6-kin17 protein in 100 μl PBS per well for 1 h. Incubations were done at room temperature, and wells were washed three times between each incubation step with PBS-T unless otherwise indicated. The wells were then saturated by incubating them with 1% BSA in PBS for 1 h. Increasing amounts of T antigen in PBS containing 1% BSA and 0.1% Triton-X 100 were incubated in the (His)6-kin17-coated wells for 1 h. After washing the plates, anti-T-antigen antibody (mAb 416) directed against diluted 100-fold in PBS-T containing 5% nonfat dry milk was added to the wells and incubated overnight at 4°C followed by antimouse IgG peroxidase-conjugated secondary antibody, diluted 2,000-fold in PBS-T-milk for 1 h. After three final washes in PBS-T, the interactions were revealed by adding to each well 100 μl of 0.4 mg/ml of ortho-phenylenediamine in a buffer containing 0.012% hydrogen peroxide, 0.1 M citric acid and 0.1% H2O2 (pH 5.0). The reaction was stopped with 0.1 M H2SO4 and the intensity of the yellow color measured at A492nm. The values were used as a measure of the interaction between both proteins after correcting for the background values of (His)6-kin17-uncoated wells.

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Coimmunoprecipitation and Immunoblotting. For each immunoprecipitation experiment, 4 x 10^6 CHSV4 cells and 10^7 S9-infected cells were collected by centrifugation and the pellet washed once with ice-cold PBS. All of the subsequent manipulations were performed at 4°C. The cells were resuspended in 300 μl of lysis buffer [PBS (pH 7.4), 1% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride] and incubated for 20 min. The lysate was then centrifuged at 20,000 × g for 30 min. The supernatant was transferred to a fresh tube containing the antibody used for immunoprecipitation and incubated for 2–4 h. One-hundred μl of 10% (volume/volume) protein A-agarose beads were then added, and the tube was gently rotated overnight. Three μl of anti-His mAb, 20 μl of anti-kin17 antibody (mAb K36) hybridoma cell supernatant, 100 μl of anti-T-antigen antibody (mAb 416) hybridoma supernatant, and 5 μl of ascites fluid of an anti-thyroglobulin mAb (Sigma; isotype control) were used in these experiments. The beads were then washed three times with 1 ml of lysis buffer, resuspended in Laemmli sample buffer, and boiled for 10 min (32). Proteins were separated by 10% SDS-PAGE, transferred to a PVDF membrane, incubated with the indicated antibodies, and revealed by chemiluminescence.

Soluble and Insoluble Protein Extraction. For each cell line, 0.5 × 10^6 cells were seeded in 100 mm dishes 3 days before trypsinization. Cells (5 × 10^6) of each cell line were lysed in 500 μl of buffer A [10 mM Tris-HCl (pH 7.9), 10 mM NaCl, 1.5 mM MgCl₂, 1% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride] and incubated for 20 min on ice. After 20 min on ice, lysed cells were spun at 20,000 × g for 30 min. The supernatant extracted from 5 × 10^6 of HEK 293 cells expressing either T antigen or both (His)₆-kin17 protein and T antigen was then applied to a nickel-affinity column (Qiagen) and analyzed by SDS-PAGE and Western blotting using purified IgG from anti-kin17 antibodies K36 and K58 at a concentration of 25 ng/ml. Other antibodies used were anti-p53 and anti-kin17 antibody (mAb K36) hybridoma supernatant (1:1,000) for 90 min at room temperature. After three washes with TBST for 10 min, the blots were incubated with antimouse horseradish peroxidase antibody (1:1,000) for 90 min at room temperature. The blots were then washed three times with TBST for 20 min each and developed by chemiluminescence.

Generation, Expression, Purification, and Binding Assay of GST Fusion Proteins. E. coli Y1090 transformed with pGEX fusion plasmids expressing GST fusions of T antigen, and regions T1 to T5 of T antigen, protein expression, and purification were described previously (4). The same amount of GST proteins (20 μg) was used in the binding reactions, as determined by the method of Bradford. GST protein binding assays were performed at 4°C with gentle agitation. The kin17 protein was prepared using the TnT-coupled in vitro transcription-translation system in accordance with the recommendations of the manufacturer (Promega). Briefly, (His)₅/kin17 cDNA was amplified by PCR with specific primers. Sequences necessary for T7 polymerase transactivation and three methionine codons were added upstream of the unique polymerase radioactive labeling. PCR product was used as template for in vitro transcription-translation. Full-length kin17 protein was labeled with [35S]methionine. The radiolabeled proteins (one-third of the total reaction) were incubated for 1 h with the GST fusion proteins attached to glutathione beads in NETN” buffer plus 3% BSA, which served as a blocking agent. The beads were then washed four times with NETN” buffer and bound proteins eluted by boiling the beads in Laemmli sample buffer. The eluted proteins were separated by electrophoresis on an SDS-10% polyacrylamide gel and visualized by autoradiography.

DNA Coimmunoprecipitation. Single-stranded DNA was 32P-labeled with T4 polynucleotide kinase. Double-stranded oligonucleotides were formed by incubating complementary pairs of oligonucleotides in hybridization buffer [20 mM Tris-HCl (pH 7.4) and 7 mM MgCl₂] at 90°C for 4 min. Annealed double-stranded labeled oligonucleotides containing the SV40 origin of replication or not (64-bp, 5 x 10⁵ cpm, 500 fmol) and T antigen (500 ng) were preincubated for 5 min at 30°C in 200 μl of hybridization buffer (7 mM MgCl₂, 0.5 mM DTT, 4 μM ATP, 40 μM creatine phosphate (pH 7.6), 24 μM of creatine phosphate kinase, 50 μg of BSA, and 50 pmol of a nonspecific oligonucleotide competitor). Five-hundred ng of kin17 protein were then added to the reaction and incubated at 30°C for 1 h. Protein A-Sepharose beads (100 μl, 10% slurry) were incubated for 2 h at room temperature with 600 μl of either the hybridoma supernatant anti-T-antigen antibody (mAb 416) or a mixture of the four hybridoma supernatants anti-kin17 antibodies (mAb K19, K36, K44, and K58; 150 μl each). After two washes in binding buffer, the antibody-bound protein A-Sepharose beads (100 μl) were then incubated with the protein-DNA mixture for 1 h at room temperature with gentle agitation. Beads were washed twice in binding buffer and incubated with 0.5 mg/ml of proteinase K and 0.1% SDS for 15 min at 37°C. After phenol-chloroform extraction and overnight precipitation at −20°C, the DNA was analyzed by electrophoresis in a 6% polyacrylamide-Tris-borate-EDTA gel containing 7 M urea. The gel was dried and placed in a PhosphorImager cassette, scanned, and the pattern of radioactivity digitalized. Quantitation of the immunoprecipitated DNA bands was performed with a PhosphorImager device (Molecular Dynamics).

SV40 in Vitro DNA Replication Assay. Cytoplasmic extracts were prepared from HEK 293 and B399.3 cells as described (34). In vitro SV40 DNA replication assay (34) was performed as described by the manufacturer instructions (CHIMERX, Milwaukee, WI). Briefly, extracts from HEK293, B399.3, and HeLa cells (350 μg) in replication buffer were supplemented with 50 ng of SV40 origin-containing plasmid pUC.HSO or a control plasmid devoid of SV40 origin pUC.S-8,4, with or without 1 μg of purified T antigen, and 1 μCi of [α-32P]dCTP. The mixtures (final volume 25 μl) were incubated at 37°C for 4 h in the presence of the indicated amounts of purified (His)₅/kin17 protein or with the same protein buffer as control. The reaction was then stopped and DNA was electrophoresed after phenol-chloroform extraction on an 1% agarose gel in 1 x TAE at 55 V. Gels were dried and DNA replication products were visualized by exposure to a PhosphorImager screen (Molecular Dynamics). To quantify DNA replication activity, one-fifth of the reaction was mixed with 50 μl of yeast RNA and coprecipitated with 1 ml of 10% trichloroacetic acid for 10 min. The precipitate was recovered in a Whatman GF/C filter disk, which was thoroughly washed with 10% trichloroacetic acid followed by 95% ethanol, Incorporated radioactivity was determined by scintillation counting.

DNA Polymerase Activity Assay. DNA polymerase activity was tested as described previously (35) in 60 μl of buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT, 50 μg/ml BSA, 0.5 μg DNAse I-activated calf thymus DNA, 25 μM each dATP, dCTP, and dGTP, and 5 μCi [α-32P]dCTP (3,000 Ci/nmol). The DNA polymerase activity of 10 μg of the HSP-4...
fraction was tested in the presence of increasing amounts of purified (His)_6-kin17 protein or in the presence of 10 μM aphidicolin (Sigma), an inhibitor of DNA polymerase α activity. Incorporation of radiolabeled nucleotides was determined by trichloroacetic acid precipitation.

Isolation of High Molecular Protein Complex. SP9 cells were fractionated as described by Wu et al. (36). Briefly, 2 g of SP9 frozen cells [confected previously with baculoviruses encoding either T antigen or (His)_6-kin17 proteins] were thawed and homogenized. The homogenate then was subjected to a series of centrifugation steps to obtain postmicrosomal and nuclear fractions. The crude nuclear extract was resuspended and gently stirred for 2 h at 4°C. The extracted nuclei were centrifuged at 100,000 × g for 1 h, and the supernatant was collected. The latter supernatant and the postmicrosomal supernatant were pooled and brought to 2 M in KCl. Polyethylene glycol was added to a final concentration of 5% and the mixture stirred gently for 1 h at 4°C. Polyethylene glycol-precipitated material was pelleted by centrifugation, and the supernatant was dialyzed for 3 h. The clarified dialysate was layered over a 2 M sucrose cushion and subjected to centrifugation at 100,000 × g for 18 h at 4°C. The material above the sucrose interface was collected and designated the HS-4 fraction (8/10 of the total). The sucrose interphase fraction was collected and designated HSP-4 (2/10 of the total). The HS-4 fraction was dialyzed and was layered onto a 9-ml 5–20% sucrose gradient formed over a 1-ml cushion of 2 M sucrose in polyallomer tubes or subjected to size-exclusion chromatography in the same buffer. A 0.5-ml aliquot of protein fraction was loaded onto the preformed gradient and centrifuged at 100,000 × g for 18 h at 4°C. Marker proteins (thyroglobulin, 195; apoferritin, 175; ADH, 75) were sedimented on parallel gradients. After centrifugation, the gradient was collected from the top of the centrifuge tube, and proteins were loaded on 10% SDS-PAGE and analyzed by Western blot.

Indirect Immunofluorescence Staining. Cells were plated at 5,000 cells per cm² on glass coverslips and treated as described previously (21). Representative fields for each cell line are presented.

RESULTS

Chromatin-bound Proteins Are Unequally Distributed in the Nucleus of Cultured Human Tumor Cells. Because cultured cells present important variations in the levels of KIN17 RNA as judged by Northern blots, and considering its short half-life (11, 19), we asked here for the first time whether human kin17 and other DNA-binding proteins involved in DNA metabolism present these variations and whether they may be correlated with the status of normal, SV40-immortalized or tumor cells. We separated cytoplasmic and soluble nuclear proteins (called here detergent-soluble fraction) from nuclear proteins anchored to DNA (DNA-bound fraction) by using a hypotonic buffer containing 0.05% Triton X-100. The presence of proteins bound to DNA was detected by Western blot using mAbs directed against p53, PCNA, SV40 T antigen, and kin17 protein. In the particular case of kin17 protein, we used two mAbs (mAb K36 or mAb K58), which recognize different regions of kin17 protein (data not shown). We observed that all of the cell lines displayed kin17 protein mainly localized in the nuclear fraction as a protein anchored to DNA (Fig. 1A). Only a weak signal was detected in the detergent-soluble fraction, particularly with the mAb K38 antibody (Fig. 1A). We conclude that endogenous kin17 protein is associated with the DNA, whatever the cell line used. Our results also revealed many kin17 protein levels depending on the cell type used, as mentioned below.

Under the same conditions, we assessed the levels of PCNA, p53, and SV40 T antigen. These three proteins were distributed in equivalent amounts between both the detergent-soluble fraction and the DNA-bound fraction (Fig. 1A). This result suggested that the association of endogenous kin17 protein with nuclear structures is stronger than that observed for PCNA, p53, and T antigen. CHSV4 and MRC5-V2, the two human fibroblast cell lines immortalized with SV40, presented high expression levels of p53 and T antigen. Because these cell lines express high levels of T antigen, it could be suggested that saturating amounts of T antigen are responsible for the distribution of this nuclear protein in both detergent-soluble and nuclear-bound fractions.

To quantify accurately the amount of kin17 protein in each cell line, we used a two-site immunometric assay (sandwich immunoassay) using two antibodies directed against kin17 protein (mAb K3 and mAb K31) and pure (His)_6-kin17 protein for calibration. Within the range of 0–400 pg of (His)_6-kin17 protein, the assay was linear (data not shown). The amount of kin17 protein in the samples analyzed in Fig. 1A was determined twice by ELISA. Similar results were obtained when the amount of kin17 protein was expressed as the number of kin17 molecules per cell (Fig. 1B) or as the number of pg of kin17 protein per µg of total protein (data not shown). Normal human fibroblasts presented the lowest amount of kin17 protein, with 18,000 kin17 molecules per cell (Fig. 1B). Immortalized cells presented increased numbers of kin17 molecules with ~26,000 and 41,000 molecules per cell for CHSV4 and MRC5-V2, respectively. H1299 and RKO carcinoma cells exhibited the greatest number of kin17 molecules, with 97,000 and 90,000 molecules/cell, respectively (Fig. 1B). We ruled out the possibility that this difference between cell lines arose from their embryonic origin because the epithelial HeLa cells elicited a moderate level of kin17 protein (30,000 molecules/cell). These data were confirmed by immunohistochemical staining using the mAb K36 (Fig. 1C). All of the cell lines were seeded and analyzed together under the same conditions. We detected the endogenous human kin17 protein in discrete nucleoplasmic foci in these types of cells, confirming the pattern reported previously for mouse kin17 proteins using antibodies raised in rabbits (23). Fibroblastic cells (normal human fibroblasts, CHSV4 and MRC5-V2) presented a punctate staining inside the nuclei and a slight fluorescence in the cytoplasm (Fig. 1C). In contrast, RKO and H1299 cells presented strong staining inside nuclei (Fig. 1C) in agreement with the pattern described previously using mAb directed against the human kin17 protein (21). We conclude that CHSV4 and MRC5-V2, SV40-immortalized cells, and certain tumor cells like H1299 and RKO cells display increased levels of kin17 protein.

Physical Interaction of T Antigen and Kin17 Protein. We sought to determine whether kin17 protein directly interacts with the T antigen in SV40-immortalized human fibroblasts. We first overexpressed the kin17 protein, tagged at the amino terminus by the addition of six histidine residues, in the baculoviral translation system and purified it from infected-cell extracts in two steps by metal-affinity and heparin-based chromatography. (His)_6-kin17 protein has an apparent molecular weight of M_r 49,000 (Fig. 2A). The determination of the molecular mass of (His)_6-kin17 protein by gel filtration using a Superdex 200 HR 10/30 column with an exclusion volume of 6 × 10^5 Da indicated a molecular weight lower than M_r 67,000 corresponding to BSA (data not shown). The absence of (His)_6-kin17 protein in the exclusion volume indicated that the majority of the protein was soluble and was not aggregated under our experimental conditions (data not shown). This molecular mass was confirmed by velocity sedimentation in a sucrose gradient (data not shown). These results indicate that the purified (His)_6-kin17 has a monomeric structure and behaves as a globular protein. This protein preparation was used to additionally characterize the interaction of kin17 protein with T antigen.

Immunoprecipitation was performed to analyze this interaction molecularly. Cell extracts from infected SF9 overexpressing both T antigen and (His)_6-kin17 proteins, and an SV40 immortalized fibroblastic cell line CHSV4 cells (with endogenous T antigen and kin17 proteins) were used in immunoprecipitation experiments using anti-T antigen (mAb 416), anti-kin17 (mAb K36), anti-His tag, and anti-thyroglobulin mAbs (used as isotype control). The
Fig. 1. Increased levels of kin17 protein in SV40-immortalized human fibroblasts and carcinoma cell lines. Cells (0.5 × 10^6) were seeded in 10 cm Ø dishes 3 days before trypsinization. Cells (5 × 10^6) of each line were lysed in buffer A for exactly 3 min on ice as described in “Materials and Methods.” Proteins anchored to nuclear structure and soluble proteins (cytoplasmic proteins as well as soluble nuclear proteins) were recovered separately. A, Western blot analysis of both fractions in the different cell lines used. The equivalent of 2 × 10^5 cells/slot were loaded and proteins separated onto a 10% SDS-polyacrylamide gel. Proteins transferred onto a membrane were incubated with the indicated antibody and revealed by chemiluminescence with a light-sensitive film. B, quantitation by ELISA of kin17 protein levels of the same samples analyzed in Fig. 1A. Results are expressed as the number of kin17 molecules per cell. The mean of two determinations is shown. C, immunocytochemical detection of kin17 in different human cells. Proliferating cells were fixed with acetone/methanol and stained with the anti-kin17 mAb K36 and a Cy2-conjugated affinity-purified goat antimouse IgG. DNA was visualized with 4',6-diamidino-2-phenylindole (small panels). Magnification: ×500. The determinations were performed more than three times; bars, ±SD.
precipitated proteins were analyzed by Western blotting. Antipolyhistidine tag and anti-kin17 protein (mAb K36) antibodies immunoprecipitated T antigen together with kin17 protein from Sf9 (Fig. 2B, top panel, Lane 1) and CHSV4 cell extracts (Fig. 2B, bottom panel, Lane 1) as revealed by Western blotting of the immunoprecipitated proteins using mAbs against both T antigen and kin17 protein. Conversely, both proteins also coimmunoprecipitated with anti-T-antigen antibody followed by immunoblotting with polyclonal or mAbs against kin17 protein and anti-T antigen mAb (Fig. 2B, Lanes 3). Western blotting of the immunoprecipitated proteins with and without antithyroglobulin (isotype control) did not reveal the presence of either T antigen or kin17 protein (Fig. 2B, Lanes 2).

Because both T antigen and kin17 are DNA-binding proteins, ethidium bromide (up to 100 μg/ml) was added in some immunoprecipitation experiments to test whether the interaction between both proteins was dependent on DNA (37). T antigen and kin17 protein association was not selectively inhibited by ethidium bromide in the immunoprecipitation reaction, suggesting that the interaction between both proteins was DNA-independent (data not shown). To exclude the possibility of cross-reaction of the antibodies, we performed immunoprecipitation experiments with anti-T-antigen antibody (mAb 416) in extracts not expressing T antigen. Immunoblotting with anti-kin17 antibody K36 did not reveal the presence of (His)₆-kin17 protein. We also performed
immunoprecipitation controls with anti-His and anti-kin17 antibody K36 in cell extracts of uninfected Sf9, which do not express (His)6-kin17 protein. As expected, immunoblotting with anti-T-antigen antibody did not reveal the presence of (His)6-kin17 protein (data not shown).

We next asked whether the interaction between (His)6-kin17 protein and T antigen was detectable in human cell extracts. Metal chelate chromatography experiments were performed with extracts from HEK 293 human cell line after ectopic overexpression of either T antigen or (His)6-kin17 protein together with T antigen. This affinity chromatography is based on the selective binding of (His)6-kin17 protein to a nickel column. Cell lysates from HEK 293 cells were loaded onto a chelating column as described in “Materials and Methods.” After extensive washing of the column, a single-step elution procedure with imidazole released the bound proteins. The eluted proteins were analyzed by Western blot with specific antibodies. T antigen coeluted with kin17 protein bound to the metal-chelating column by its hexahistidine tag (Fig. 2C, left panel). Note that the majority of the kin17 protein was bound to the column. In the absence of (His)6-kin17 protein, only a very small amount of T antigen was retained by the nickel column representing the nonspecific binding (Fig. 2C, right panel). These results indicate that (His)6-kin17 protein and T antigen may exist as a complex in transfected human cells.

Interaction between T Antigen and (His)6-kin17 Protein. To test whether the structure of kin17 protein allows this putative interaction, we used the overlay procedure in which pure (His)6-kin17 protein was immobilized on PVDF membranes. After a partial renaturation of the immobilized protein, the membranes were incubated with lysates of baculovirus-infected Sf9 cells expressing recombinant T antigen as described in “Materials and Methods.” The presence of T antigen was revealed using anti-T-antigen mAb and visualized by chemiluminescence. After T-antigen overlay, anti-T-antigen antibodies detected a 49-kDa band at the position where (His)6-kin17 protein migrates (Fig. 3A). The intensity of the revealed bands increased with the amount of immobilized (His)6-kin17. It was noteworthy that neither BSA (500 ng) nor molecular weight markers were revealed in the T-antigen overlay assay. No bands were detected on a control blot treated in exactly the same manner apart from incubation of the membrane with extracts of uninfected Sf9 cells (data not shown). This approach confirmed the association of (His)6-kin17 protein with T antigen. Nevertheless, we cannot exclude the involvement of a third accessory factor in this interaction.

To additionally analyze this interaction, we used a classical ELISA. Basically, constant amounts of purified (His)6-kin17 protein were coated on the bottom of a 96-well ELISA plate, and increasing amounts of purified recombinant T antigen were added to the wells. Fig. 3B shows the detection of T antigen bound to the (His)6-kin17 protein-coated wells. These results suggest that, in vitro, recombinant (His)6-kin17 protein and T antigen may interact directly with each other, although we cannot exclude the possibility of another factor that could be bound to the purified recombinant T antigen.

Molecular Basis of the Interaction between (His)6-kin17 Protein and T Antigen. We sought to determine whether the interaction between T antigen and kin17 protein involves a specific domain of T antigen. We performed pull-down experiments using the recombinant GST-T-antigen fusion proteins bound to glutathione agarose. T antigen is a 707-amino acid protein with protein-binding domains that are functionally distinct. The major domains of T antigen are: the retinoblastoma protein-binding domain (amino acids 102–115), the DNA-binding domain (amino acids 131–259), the putative zinc finger (amino acids 302–320), the p53- and DNA polymerase α protein-binding domains (amino acids 259–517), the ATPase domain (amino acids 418–627), and the host range region (amino acids 682–708; Ref. 1). To determine the kin17-binding domain in T antigen, full-length T antigen and 5 regions of T antigen were fused to the glutathione-binding domain of GST (full-length T antigen, amino acids 1–707; T1, amino acids 5–172; T2, amino acids 168–383; T3, amino acids 379–561; T4, amino acids 557–707; and T5, amino acids 5–383; Ref. 4). Equivalent amounts of these fusion proteins plus the whole GST protein were bound to glutathione-Sepharose beads, and were then used in a binding assay with in vitro-transcribed/translated 35S-labeled kin17 protein. The full-length T antigen (T-ag), the T2 and the T5 (which includes the T1 and T2 peptides) fusion proteins produced the strongest signal and a weak nonspecific band was observed in the negative controls (GST protein bound to glutathione-Sepharose beads, lane GST, and glutathione-Sepharose beads, lane reduced glutathione, in Fig. 4A). These results indicate that T antigen and human kin17 protein can bind directly to each other preferentially through the amino acids 168–383 region (T2) of T antigen.

We asked whether (His)6-kin17 protein could interact with T antigen when bound to its SV40 DNA core origin of replication by immunoprecipitating protein-DNA complexes. In the presence of ATP, T antigen specifically interacts with the core of the replication origin and assembles into a double-hexamer structure that covers the entire 64-bp DNA core. In this assay, a T antigen-DNA core ratio of 10 was used to minimize the binding of the kin17 protein to free DNA core. A 100-fold excess of unlabeled nonspecific oligonucleotide competitor was present in each sample. The specificity of T-antigen binding to its own origin was compared under the same conditions to the binding of a 64-bp labeled DNA without SV40 origin of replication. T-antigen-DNA coimmunoprecipitation with this control DNA produced a weak signal corresponding to the background of the technique (data not shown). T antigen alone was incubated with the labeled 64-bp DNA core and immunoprecipitated with anti-T-antigen antibody (mAb 416). As expected, the target sequence was efficiently bound.
recovered (Fig. 4B, Lane 1). Conversely, mAbs specific for kin17 protein were unable to immunoprecipitate T antigen-DNA complex (Fig. 4B, Lane 4). However, when T antigen was incubated with (His)_6-kin17 protein and the core origin DNA and thereafter immunoprecipitated with a mixture of anti-kin17 mAbs, we observed a signal because of the radiolabeled core DNA that corresponded to 25% of the DNA precipitated with anti-T-antigen antibody (Fig. 4B, Lane 2; 4-fold weaker than signal of Fig. 4B, Lane 1). In the absence of T antigen, only a weak binding of the purified kin17 to the labeled DNA was detected (Fig. 4B, Lane 3; 20-fold weaker than the signal of Fig. 4B, Lane 2). We conclude that kin17 protein interacts with T antigen bound to its SV40 DNA core origin of replication.

**Kin17 Protein Inhibits DNA Replication.** In mammalian cells, overexpression of mouse kin17 protein inhibits *in vivo* DNA replication (23, 33). Furthermore, endogenous kin17 and RPA colocalize in nucleoplasmic foci of human cells after ionizing irradiation (21). To explore this point additionally, we prepared extracts from cells presenting a basal level of endogenous kin17 protein (HEK 293) and from a derived clone overexpressing kin17 protein (B399.3 cells) and presenting reduced proliferation rates (data not shown). We compared their effect on DNA replication activity promoted by T antigen from a SV40 origin by using the *in vitro* assay described by Li et al. (34). A 40% decrease in T-antigen-dependent DNA synthesis was observed when comparing the activity of extracts from B399.3 cells (Fig. 5A, Lane 3) to that of HEK 293 cells (Fig. 5A, Lane 2), as evidenced by scintillation counting and autoradiography (Fig. 5A). To determine whether the binding of human kin17 protein to T antigen-DNA complexes accounts for the inhibition of DNA replication, we examined the ability of pure kin17 protein to interfere with DNA replication using HeLa cell extracts and the replication assay. The introduction of kin17 protein in the reaction reduced the replication activity (Fig. 5B). This reduction was dependent on the amount of kin17 protein present in the reaction. A 2-fold reduction in the incorporation of radiolabeled nucleotide was obtained when 1 μg of purified protein was added in the replication assay, as shown by scintillation counting. As control experiments, we tested the effect of (His)_6-kin17 protein on SV40 DNA replication activity. Increasing amounts of human (His)_6-kin17 protein were incubated with pUC.HSO DNA in replication buffer supplemented with HeLa cell extract at 37°C for 4 h. At the end of the incubation period, an aliquot was removed from each reaction mixture, and the incorporation of labeled nucleotides into DNA was assessed by precipitation with 10% trichloroacetic acid and scintillation counting. Results are expressed as percentage of control. The remainder of each reaction mixture was run on a 1% agarose gel and visualized by PhosphorImager. A, superhelical form I DNA, B, relaxed and open circular form II DNA, RI, replicative intermediate DNA; C, detection of T antigen and (His)_6-kin17 in a multiprotein complex. Extracts of infected S9 cells expressing T antigen and (His)_6-kin17 proteins were fractionated to obtain fraction HSP-4 containing a multiprotein complex of high molecular weight according to Wa et al. (36). Sucrose gradient sedimentation of this fraction was then performed, and the presence of T antigen and (His)_6-kin17 proteins in 10 collected fractions was determined by Western blot as described in Fig. 1A. The pattern obtained by chemiluminescence is shown.

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**Fig. 4.** Identification of the T antigen domain interacting with kin17 protein. A, binding of *in vitro*-translated kin17 protein to fragments of T antigen fused to the GST protein. *In vitro*-translated [35]S-labeled kin17 protein was incubated with reduced glutathione-Sepharose beads, or with the GST-Sepharose beads (GST), or fusion proteins GST-T1, T2, T3, T4, or T5 (20 fmol). Bound proteins were eluted and analyzed by SDS-PAGE as described in “Materials and Methods.” The autoradiographical detection pattern of 35S-labeled kin17 protein is shown. B, (His)_6-kin17 protein binding to T antigen-DNA complex. T antigen (500 ng) was incubated with the labeled 64-bp DNA core origin of replication in the presence or absence of (His)_6-kin17 protein (500 ng). T antigen was added to reaction mixtures (containing labeled DNA) in Lanes 1, 2, and 4. (His)_6-kin17 protein was added to reaction mixtures in Lanes 2 and 3. T antigen was immunoprecipitated with anti-T-antigen antibody (Lane 1), and (His)_6-kin17 protein was immunoprecipitated with a mixture of anti-kin17 mAbs (Lanes 2 and 3). In parallel T antigen was immunoprecipitated with a mixture of anti-kin17 mAbs (Lane 4). Immunocomplex precipitated DNA was purified, resolved by PAGE, and visualized by PhosphorImager. A digital image of the separated radioactive DNA is shown.
human kin17 protein inhibits T antigen and SV40 origin-dependent DNA replication in vitro.

**T Antigen and (His)_6-kin17 Protein Copurify with a Multiprotein Complex in S9 Cells.** Because kin17 protein inhibits T-antigen-dependent DNA replication in vitro and considering that T antigen is an essential protein for viral DNA replication, we wondered whether these proteins are part of the replication complex. We partially purified the MRC of S9 cells coexpressing both T antigen and (His)_6-kin17 proteins by using the protocol described by Wu et al. (36) with minor modifications. The extracted proteins were separated onto a 2 m sucrose cushion to produce a fraction called HSP-4 containing the MRC and other high molecular weight proteins, and a supernatant fraction consisting of soluble molecules (fraction HS-4). The HSP-4 fraction was subjected to velocity sedimentation in a 5–20% sucrose gradient containing 0.5 M KCl. T antigen and (His)_6-kin17 proteins were detected in the fractions collected from the gradient. Fractions 1–4 presented the highest level of T antigen, and fraction 2 contained the majority of (His)_6-kin17 protein, as shown by Western blot analysis of the sucrose gradient fractions (Fig. 5C). We repeatedly observed (His)_6-kin17 and T antigen as part of a protein complex, which sedimented in the range 17S–19S as judged by the protein markers [apoferritin (17S) and thyroglobulin (19S)] run on a parallel gradient. It should be noted that in insect cells, T antigen and (His)_6-kin17 proteins were detected in both HSP-4 and HS-4 fractions (data not shown). We confirmed that the MRC purified migrated in the 17S–21S range reported previously for numerous different cell lines and tissues (36). These results show that in vivo (His)_6-kin17 and T antigen are components of the same multiprotein complex.

**DISCUSSION**

Whereas the precise role of the DNA-binding kin17 protein remains unknown, much evidence suggests its involvement in DNA replication. In proliferating cells, kin17 protein is mainly localized in nucleoplasmic foci with a staining pattern resembling those of proteins involved in DNA metabolism (18, 21). Elevated ectopic expression of kin17 protein in human cultured cells leads either to cell death in most cell lines tested or to a drastic drop in clonogenic growth (33). Transient kin17 protein overexpression also induces deformations of nuclear morphology and an inhibition of DNA synthesis, as demonstrated by the lack of bromodeoxyuridine uptake in transfected HeLa cells (23). The overexpression of both kin17 protein and T antigen leads to the colocalization of these proteins in nuclei of transfected cells and to the in vivo abrogation of the T-antigen-dependent replication (23). The hypothesis of an involvement of human kin17 protein in DNA replication has been reinforced by the characterization of RKO cells expressing a KIN17 antisense transcript (RKO antisense kin17 cells). These cells display major disruptions in the S phase progression, as well as a significant decrease in clonogenic cell growth and cell proliferation (21). RKO antisense KIN17 cells accumulate in early and mid S phase, but only a few cells were detected in late S phase, as evidenced by flow cytometry analysis of pulse bromodeoxyuridine-labeled cells. Compelling evidence also emphasizes the recruitment of both mouse and human kin17 proteins in the presence of unrepaired DNA damage (11, 17, 19). In particular, DNA-bound human kin17 protein concentrates in nucleoplasmic foci 24 h after ionizing irradiation and colocalizes with RPA (21). Because it has been suggested that RPA foci concentrates after the completion of DNA repair at sites of unrepairable DNA damage (22), our results highlight the involvement of kin17 protein in DNA replication in the presence or absence of DNA damage.

To additionally detail the relationship between kin17 protein and DNA replication, we used the well-characterized SV40 DNA replication model. The characterization of the mechanism by which SV40 perturbs viral control processes allowed the identification of important regulatory proteins involved in DNA metabolism, such as p53 and pRB tumor suppressor proteins (1). SV40 T antigen disrupts cell growth control mechanisms, primarily by binding to and abolishing the normal functions of both p53 and pRb. The overproduction of human kin17 protein in insect cells and its purification affords us the opportunity to perform for the first time biochemical analysis of human kin17 protein, and to develop polyclonal and mAbs. Accurate quantification of endogenous human kin17 protein content in different cell lines revealed increased levels in two SV40-immortalized fibroblast cell lines, as compared with normal diploid fibroblasts. Because the KIN17 gene has been mapped on human chromosome 10p (11), we rather expected reduced expression levels of KIN17 gene in SV40-transformed cells, which display characteristic patterns of chromosomal imbalances including multiple deficiencies for the 8p, 11p, 6q, 10p, 13q, 17p, and 18p arms (38, 39). Therefore, increased kin17 protein levels observed in immortalized cells would be a consequence of uncontrolled proliferation or genomic instability.

The preferential binding of kin17 protein to curved DNA could lead to a general poisoning of DNA metabolism (14–16). This preferential binding could slow down the propagation of replication forks, thus explaining the inhibition of DNA replication observed after transient overexpression of mouse kin17 protein in vivo (23) and in vitro using pure human kin17 protein and cell extracts overexpressing it (this report). The physical interaction between human kin17 protein and T antigen described here, which is independent of the cellular type used, offers an alternative interpretation. In fact, T antigen binds to specific sequences within the core origin of the replication region to melt the DNA partially, thus allowing replication. During these steps, T antigen associates with components of the replication machinery, such as DNA polymerase α and RPA, resulting in the formation of the initiation complex (1). Notwithstanding that the SV40 core origin of replication contains curved DNA sequences, the binding of kin17 protein was greater when T antigen was already bound to its own core origin. Our results support the idea that high amounts of kin17 protein may inhibit T-antigen-dependent DNA replication through a direct interaction with T antigen instead of a general poisoning mechanism because of the binding to curved DNA.

We show here that T antigen directly interacts with kin17 protein through a relatively short stretch of amino acids (168–383, known as region T2) located in the NH2-terminal portion of T antigen. These amino acids form part of a major domain of the T antigen responsible for DNA binding, and for the interaction with p53 and DNA polymerase α protein. Three regions of T antigen are essential for cellular transformation. The NH2 terminus (amino acids 1–82) contains a J domain, which binds the hsc70 molecular chaperone protein and is presumably involved in assembly and disassembly of protein complexes. A separate domain (amino acids 102–115) proximal to the NH2-terminal J domain is required for binding to pRb-related tumor suppressor proteins (pRb, p107, and p130/pRb2). The third region contains the p53-binding sites (amino acids 350–450 and 533–626). T antigen sequesters p53 abolishing its function and allowing cells with genetic damage to survive and enter the S phase. This leads to accumulation of T-antigen-expressing cells with genomic mutations that may promote tumorigenic growth (1, 40).

The relatively stable interaction of the kin17-T antigen complex supports the idea that these proteins form part of a nuclear multiprotein complex in vivo. Most of the endogenous kin17 protein was associated with nuclear structures (Fig. 1A) of high molecular weight (bottom fractions 1 and 2, Fig. 5C), whereas T antigen was distributed...
between both detergent-soluble and nuclear-bound fractions (Fig. 1A; Fractions 1–9, Fig. 5C). This could indicate that kin17-T-antigen interaction occurs in vivo in a multiprotein complex associated with nuclear structures. Furthermore, the functional interaction between human kin17 protein and T antigen takes place in a nuclear multiprotein complex of high molecular weight devoid of nucleic acid (Fig. 5C). Replication proteins are easily isolated under native conditions with members of the well-characterized MRC such as PCNA, RPA, DNA polymerase, and ATPase activities. These results additionally support the idea that the kin17-T-antigen complex is important for the essential function of DNA replication.

SV40 can transform a variety of human cultured cells and is a potent tumor inducer in experimental animals. The types of tumors induced by SV40 in laboratory animals are similar to those seen in human cancers found to contain SV40 DNA (46, 47). Increasing evidence indicates that SV40 infects humans. Its association with certain types of human tumors may have important implications in the future for cancer etiology and treatment (48). Our results confirm that the SV40 model is a useful tool in characterizing the consequences of the interaction between kin17 protein and T antigen. Annu. Rev. Biochem., 65: 3553–3558, 1991.


Human Kin17 Protein Directly Interacts with the Simian Virus 40 Large T Antigen and Inhibits DNA Replication

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