JOURNAL OF VIROLOGY, Nov. 1993, p. 6551–6557
0022-538X/93/116551-07$02.00/0

Vol. 67, No. 11

Simian Virus 40 Large T Antigen Associates with Cyclin A and $p33^{cdk2}$

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Received 10 June 1993/Accepted 23 July 1993

In this paper we provide evidence that a fraction of large T antigen of simian virus 40 (SV40) interacts with cyclin A and $p33^{cdk2}$ in both virus-infected and stably transformed cells. Immunoprecipitates of SV40 large T antigen from SV40-infected or SV40 large-T-antigen-transformed cells contain cyclin A, $p33^{cdk2}$, and histone H1 kinase activity. Conversely, immunoprecipitates of cyclin A from these cells contain SV40 large T antigen. In this respect, SV40 large T antigen has properties similar to those of the E1A oncogene of adenoviruses and the E7 oncogene of human papillomaviruses.

Transforming genes encoded by DNA tumor viruses bind to a series of host cellular proteins which are implicated in tumorigenesis. Simian virus 40 (SV40) large T antigen binds p53 and the product of the retinoblastoma susceptibility gene (pRb) (for a review, see reference 27). Similarly, the adenovirus E1A protein and the human papillomavirus E7 gene product bind pRb (6, 12, 46) while the adenovirus E1B protein and the human papillomavirus E6 gene product bind p53. A very similar set of interactions between the pRb-related protein p107 and adenovirus E1A and between human papillomavirus E7 and SV40 large T antigen has been identified (11, 12, 47). The binding site for pRb has been localized to two small regions in the amino terminus of E1A and T antigen. Deletions and point mutations in this common region (32) compromise the transforming efficiency of T antigen and E1A, suggesting that binding to pRb is a prerequisite stage in transformation (3, 47).

Adenovirus E1A also interacts with cyclin A (16, 39). Cyclins are essential activating and targeting subunits for the cdc2 family of protein kinases (2, 7, 34, 37). The levels of cyclins A, B, and E oscillate in phase with the cell cycle (8, 13, 38), with cyclin E showing a peak in late G_1, cyclin A showing a peak in S phase, and cyclin B showing a peak in G_2. Cyclin A is usually found in the nucleus of fibroblasts (40) and can bind to at least two different protein kinase subunits, $p34^{cdc2}$ and $p33^{cdk2}$ (24, 39, 43). The complex between adenovirus E1A and cyclin A appears to contain $p33^{cdk2}$ but not $p34^{cdc2}$ (22, 43). A similar set of interactions has been identified for the human papillomavirus 16 E7 oncogene (11, 42). The sequences conserved between E1A, E7, and SV40 large T antigen have been shown to be responsible for the interaction of pRb and cyclin A with E1A and E7, respectively (11).

Although the specific roles of these kinases are not yet completely clear, there is evidence that cyclin A plays a part in controlling both S phase (17, 35) and the onset of mitosis (26, 44). A possible role in transcriptional control has also been proposed, since cyclin A and cdk2 are found associated with the cellular transcription factors E2F and DRTF1 (1, 33).

In view of the association between adenovirus E1A protein and cyclin A, it was of some interest to determine whether cyclin A was also associated with SV40 large T antigen. In this paper we confirm the previous observations that T antigen is associated with a protein kinase activity (18, 41) and present evidence that cyclin A-$p33^{cdk2}$ is responsible, at least in part, for this activity, for we find cyclin A and $p33^{cdk2}$ associated with SV40 large T antigen in both virus-infected and large-T-antigen-transformed cells.

MATERIALS AND METHODS

Cell culture and virus infection. Tissue culture cells were grown in E4 medium supplemented with 10% fetal calf serum. SVK cells are an SV40-transformed derivative of the human neonatal epithelial keratinocyte cell line K14.

Subconfluent monkey CV1 cells were lytically infected by incubation with SV40 strain 830 at a multiplicity of infection of 5 for 2 h. The medium was then replenished. After 24 h, the efficiency of infection was determined by cell staining with antibodies against large T antigen, and the cells were harvested.

Cell labelling, immunoprecipitation, and antibodies. Cells were labelled for 1 or 2 h with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans-Label; ICN) in E4 medium lacking methionine and cysteine and containing 10% dialyzed fetal calf serum. For immunoprecipitation, 2.5×10^7 subconfluent and rapidly dividing cells from a 15-cm-diameter dish were rinsed in phosphate-buffered saline, lysed in 1.5 ml of buffer A (20 mM Tris-Cl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), spun at 50,000 rpm in a Beckman TL100 centrifuge for 10 min at 4°C, and precleared with 25 μl of protein G-Sepharose beads. Monoclonal antibodies (MAbs) (2.5 μg) or antiserum (5 μl) was bound to 25 μl of protein G beads for 15 min and washed in buffer A, and the pelleted beads were incubated with the cell lysate for 2 h. The beads were washed three times in buffer A, resuspended in 50 μl of sample buffer containing 3% sodium dodecyl sulfate (SDS), and boiled for 2 min, and 10 μl of the supernatant was applied to SDS-polyacrylamide gels. For the immunoprecipitations subsequently probed with antibodies against cyclin-dependent kinases (see Fig. 6), the antibodies were cross-linked onto the protein A beads (20) before incubation with the cell lysate in order to minimize interference from the antibody heavy and light chains used for the immunoprecipitations in the subsequent immunoblotting detection system.

MAbs against SV40 T antigen were PAb203, PAb419, and PAb423 (19, 31). MAbs against p53 were PAb421, which reacts with both murine and human p53, and PAb246, which is specific for murine p53 and does not recognize the simian or human protein (49). MAb against $p34^{cdc2}$ was A17 (24), and

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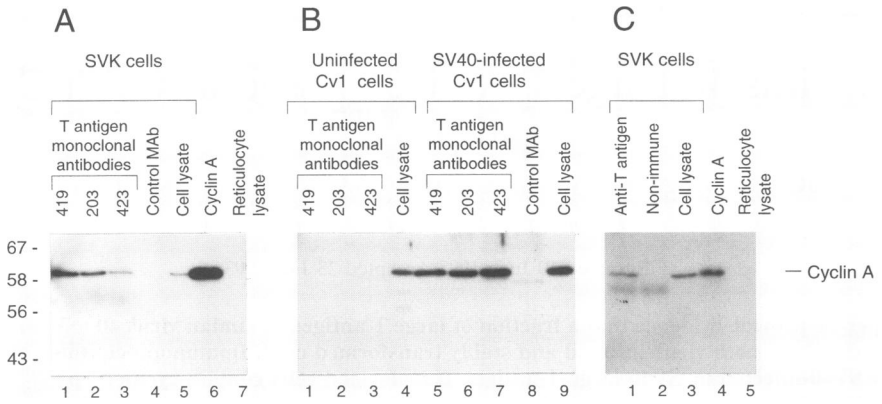

FIG. 1. SV40 large T antigen is associated with cyclin A. (A) Extracts from SVK cells were immunoprecipitated with anti-T-antigen MAbs. Lane 1, PAb419; lane 2, PAb203; lane 3, PAb423; lane 4, PAb246 (a control MAb specific for murine p53). Immune complexes were collected on protein G-Sepharose beads, resolved on SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with rabbit anti-bovine cyclin A. To check the specificity of the antiserum and the mobility of cyclin A on the gel, lanes containing total SVK extract equivalent to 5×10^5 cells (lane 5) and reticulocyte lysate programmed with either human cyclin A mRNA (lane 6) or no added mRNA (lane 7) are also included. Molecular masses (in kilodaltons) are on the left. (B) Extracts from uninfected CV1 cells (lanes 1 to 4) or CV1 cells infected 24 h earlier with SV40 strain 830 at a multiplicity of 5 (lanes 5 to 9) were immunoprecipitated with anti-T-antigen MAbs: lanes 1 and 5, PAb419; lanes 2 and 6, PAb203; lanes 3 and 7, PAb423; lane 8, PAb246 (a control MAb specific for murine p53). The immunoprecipitates were processed and immunoblotted with rabbit anti-cyclin A antiserum as described for panel A. Total cell extract from uninfected CV1 (lane 4) and SV40-infected CV1 cells (lane 9) are also shown to provide markers. (C) Extracts from SVK cells were immunoprecipitated with rabbit anti-T antigen (lane 1) or nonimmune rabbit serum (lane 2). Immunocomplexes were collected on protein G-Sepharose beads, resolved by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with mouse monoclonal E68, which specifically recognizes mammalian cyclin A. SVK extract (lane 3) and reticulocyte lysate programmed with either human cyclin A mRNA (lane 4) or no mRNA (lane 5) are also included. The position of cyclin A is indicated on the right.

MAb against PSTAIRE peptide was from M. Yamashita (48). The polyclonal antiserum against $p33^{cdk2}$ has previously been described (25). Antiserum against human cyclin A was from J. Pines (39), and bacterially expressed bovine cyclin A was used to raise antibodies in rabbits (1) and in mice to produce MAbs E23 and E68, which can recognize human, monkey, and mouse cyclin A (15a).

Immunoblotting. Samples were analyzed on SDS–15% polyacrylamide gels (17.5% for the visualization of cyclin-dependent kinases—see Fig. 6) and transferred with a Hoefer Semi-dry blotting apparatus onto nitrocellulose filters. The filters were probed with antibodies in 100 mM Tris-Cl [pH 8.0], 300 mM NaCl, 0.5% Tween 20, and 2% nonfat milk powder. Antisera were used at a dilution of 1:1,000, and hybridoma supernatant was used at a dilution of 1:5. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse immunoglobulin G as appropriate, and the proteins were visualized by using the Amersham ECL system.

Partial proteolytic mapping. Partial proteolysis with *Staphylococcus aureus* V8 protease (4) was performed as described by Harlow and Lane (20). Proteins were labelled with [^{35}S]methionine metabolically or by in vitro translation, immunoprecipitated, and separated on a 15% polyacrylamide gel. The bands were localized by autoradiography, excised, and loaded on a second SDS–15% polyacrylamide gel together with various amount of protease. The digestion patterns were visualized by autoradiography.

Protein kinase assays. Histone kinase assays were performed as previously described by Minshull et al. (30). Briefly, immunoprecipitations were collected as described above, washed with kinase buffer [80 mM sodium β-glycerophosphate, 20 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 15 mM $MgCl_2$], and incubated in 10 μl of this buffer containing 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 1 μg of histone H1 (Boehringer) for 10 min at 25°C. The reactions were stopped with 25 μl of sample buffer, applied to SDS-polyacrylamide gels, and exposed overnight on X-ray film.

Antibody depletion procedure. Extracts from infected or noninfected CV1 cells were precleared by rotating at 4°C for 2 h with a first 25-μl batch of beads and then for a further 14 h with a second 25-μl batch of beads. The beads used were protein G-Sepharose loaded with 5 μl each of rabbit serum against human cyclin A or normal rabbit serum, $p13^{suc1}$ or bovine serum albumin (BSA) coupled to cyanogen bromide-activated Sepharose, or protein G-Sepharose loaded with 2.5 μg of PAb421 or PAb246.

RESULTS

Cyclin A and SV40 large T coimmunoprecipitate. To investigate the possible interactions between SV40 large T antigen and cyclin A, cell extracts were prepared from subconfluent rapidly dividing SVK cells and immunoprecipitated with three different anti-T-antigen MAbs directed against separate epitopes on T antigen (31). All three antibodies precipitated a protein that was recognized in an immunoblot by a rabbit polyclonal antiserum against bovine cyclin A (Fig. 1A, lanes 1 to 3). No such band was present when a control MAb that recognized murine (but not human) p53 was used (Fig. 1A, lane 4). The immunoreactive polypeptide comigrated with the only protein recognized by this anti-cyclin A antiserum in total cell extract (Fig. 1A, lane 5), which had the same electrophoretic mobility as the product of human cyclin A mRNA translated in reticulocyte lysate (Fig. 1A, lane 6). Identical results were obtained by probing a similar immunoblot with an antiserum raised against human cyclin A by Pines and Hunter (39) (data not shown). In order to investigate whether cyclin A could be found associated with large T antigen in cells lytically infected by SV40, the same set of anti-T-antigen and control MAbs was used to immunoprecipitate extracts prepared from subconfluent monkey CV1 cells infected with SV40 24 h

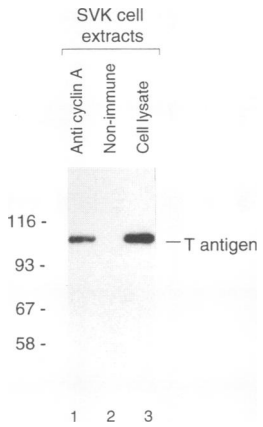

FIG. 2. Immunoprecipitates of cyclin A contain SV40 large T antigen. Extracts of SVK cells were immunoprecipitated with rabbit anti-bovine cyclin A antiserum (lane 1) or nonimmune rabbit serum (lane 2), resolved by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with the MAb PAb419 against T antigen. Lane 3, total SVK cell extract equivalent to 5×10^5 cells. Molecular masses (in kilodaltons) are on the left.

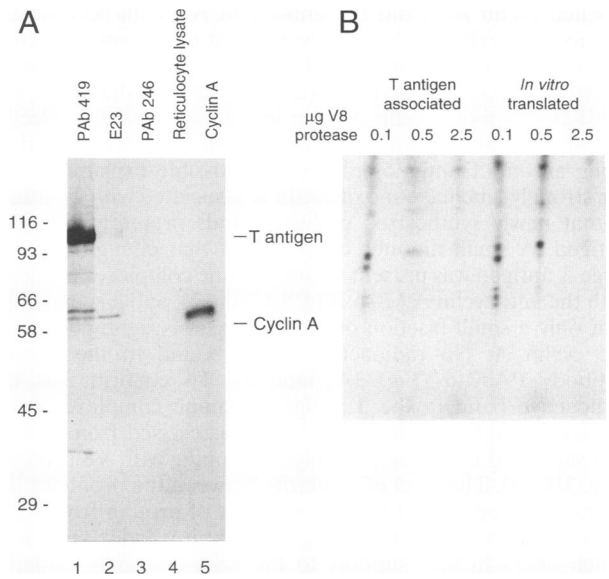

FIG. 3. Only a fraction of SV40 large T antigen binds to cyclin A, and only a fraction of cyclin A binds SV40 large T antigen. (A) Extracts from SVK cells which had been labelled for 1 h with a mixture of [^{35}S]methionine and [^{35}S]cysteine in methionine- and cysteine-free medium were immunoprecipitated with anti-T-antigen MAb PAb419 (lane 1), anti-cyclin A MAb E23 (lane 2), or control MAb PAb246 (lane 3). To locate the position of cyclin A on the gel, [^{35}S]methionine-labelled reticulocyte lysate programmed with human cyclin A mRNA (lane 5) or no mRNA (lane 4) was included. Gels were exposed to X-ray film (Hyperfilm β-max; Amersham) for 10 days. Molecular masses (in kilodaltons) are on the left. (B) Proteolytic mapping of cyclin A. SVK cells were labelled with [^{35}S]methionine for 2 h and immunoprecipitated with a mixture of three anti-T-antigen MAbs, PAb203, PAb419, and PAb423. Human cyclin A was labelled with [^{35}S]methionine by translating human cyclin A mRNA in reticulocyte lysate and immunoprecipitated with rabbit anti-cyclin A. The immune complexes were resolved by SDS-PAGE, and the cyclin A bands were excised after autoradiography and subjected to partial proteolytic mapping as described elsewhere (20), by using 2.5, 0.5, and 0.1 µg of V8 protease.

previously. All three MAbs immunoprecipitated a protein recognized by the rabbit anti-bovine cyclin A (Fig. 1B, lanes 5 to 7) in the virus-infected cells but failed to do so in uninfected cells (Fig. 1B, lanes 1 to 3). The control immunoprecipitate (Fig. 1B, lane 8) did not contain an immunoreactive band with the mobility of cyclin A.

To confirm the identity of the polypeptide that reacted with the rabbit anti-bovine cyclin A antiserum, extracts from subconfluent SVK cells were immunoprecipitated with a rabbit polyclonal antiserum raised against T antigen, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with the anti-cyclin A MAb E68. The substitution of a rabbit polyclonal antiserum against T antigen for the mouse anti-T-antigen MAbs allowed detection of the anti-cyclin A antibody without too much interference from immunostaining of the heavy and light immunoglobulin chains by the second antibody. The anti-cyclin A MAb identified a protein present in the rabbit anti-T-antigen immunocomplexes (Fig. 1C, lane 1) with a molecular weight similar to that of the product of a human cyclin A mRNA translated in reticulocyte lysate (Fig. 1C, lane 4). Thus, both polyclonal and monoclonal anti-cyclin A antibodies recognize a polypeptide with the gel mobility of cyclin A in immune complexes of large T antigen.

Roughly equal amounts of cyclin A were detected in the total lysate (Fig. 1A, lane 5) and in the anti-T-antigen immunoprecipitates (Fig. 1A, lanes 1 to 3) as judged from the immunoblot, even though far more cell equivalents were used for immunoprecipitation and loaded on the gel (5×10^6 cells) than were used when the total cell extract was loaded onto the gel (5×10^5 cells). These results suggest that only a fraction of cyclin A was present in the T-antigen complex.

If immunoprecipitates of T antigen contain cyclin A, and if this reflects a meaningful association, the converse ought to be true: immunoprecipitates of cyclin A should contain T antigen. To test this, immunoprecipitations were performed on extracts from subconfluent SVK cells by using the polyclonal rabbit anti-bovine cyclin A antiserum. The immune complexes were resolved by SDS-PAGE and immunoblotted with PAb419 against large T antigen. A band of 98 kDa corresponding to T antigen could be seen in the immune complexes obtained with anti-bovine cyclin A antiserum and was absent in the control nonimmune immunoprecipitate (Fig. 2, lanes 1 and 2). The immunoreactive band in the immunoprecipitate had the same mobility as T antigen present in the cell lysate (Fig. 2, lane 3).

Cyclin A is one of the major T-antigen-associated bands. In order to confirm the association between SV40 large T antigen and cyclin A and to obtain an estimate of its quantitative relevance, subconfluent SVK cells were metabolically labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 1 h, at which time lysates were prepared as described in Materials and Methods. These extracts were immunoprecipitated with MAbs directed against T antigen (PAb419) (Fig. 3A, lane 1) or cyclin A (E 23) (Fig. 3A, lane 2). Several radioactive bands coimmunoprecipitated with T antigen, one of which comigrated with cyclin A (Fig. 3A, lane 1; compare mobilities with those in lanes 2 and 5). The strongly labelled band above cyclin A is probably p53, a protein which is found associated with T antigen in SV40-transformed cells. The band around 35 kDa is likely to be p35, a cellular protein known to cross-react with PAb419 (5).

The band comigrating with cyclin A is one of the major proteins coimmunoprecipitating with T antigen, confirming the specificity of the association. We were surprised to find that the anti-T-antigen MAb was able to immunoprecipitate as much

labelled cyclin A as did the anti-cyclin A MAb, because the previous experiments had suggested that only a small fraction of cyclin A was present in the T-antigen complex, as discussed above. Immunoblots revealed (data not shown) that there was substantially more cyclin A in an immunoprecipitate collected by using an anti-cyclin A antibody than in one collected by using an anti-T-antigen antibody. A possible explanation for the strongly labelled band of cyclin A associated with T antigen is that newly synthesized cyclin A binds preferentially to T antigen. A small amount of a protein that comigrated with large T antigen was present in the immune complexes collected with the anti-cyclin A MAb (Fig. 3A, lane 2), and it seems clear that only a small fraction of the T antigen is tightly bound to the cyclin A. No radioactive bands bound to the control antibody PAb246 (Fig. 3A, lane 3). To confirm that the radioactive band in the T-antigen immune complexes really corresponded to cyclin A, the band was excised from the gel and subjected to partial proteolytic mapping with V8 protease (Fig. 3B) (4). The level of similarity between the profile of this coimmunoprecipitated protein and that of protein from an in vitro translation of cyclin A suggests that it is indeed cyclin A, which offers further support to the immunological evidence presented above.

T antigen is associated with a cyclin A-dependent H1 kinase activity. More than 10 years ago, some excitement was generated by the finding that SV40 large T antigen was associated with a protein kinase activity (18, 41). It emerged that this was not an intrinsic property of large T antigen itself but was due to a cellular serine/threonine protein kinase that associated with the viral protein through many steps of purification (41, 45). At the time, the identity of this protein kinase was not established.

In the light of the evidence for the association between T antigen and cyclin A presented above, it seemed plausible that the protein kinase activity associated with T antigen was due to a cdc2-like kinase associated with the cyclin. To assess this possibility, we first checked whether immunoprecipitates from SVK cells collected either with anti-T-antigen MAbs or with rabbit polyclonal anti-T antigen were able to phosphorylate histone H1, a commonly used model substrate for cyclin-dependent kinases. Figure 4A shows that the immunoprecipitates indeed had a histone H1 protein kinase activity (Fig. 4A, lanes 1 to 3 and 5) while much less activity was detected in immunoprecipitates collected by using a control MAb (Fig. 4A, lane 4; see also Fig. 4B, lane 7) or preimmune rabbit serum (Fig. 4A, lane 6). Similar results were obtained with immunoprecipitates collected from extracts prepared from subconfluent CV1 cells infected with SV40 24 h previously (Fig. 4B, lanes 4 to 5) but not with those from uninfected control cells (Fig. 4B, lanes 1 to 3). It was noteworthy that T antigen itself became labelled with $^{32}PO_4$ in these assays.

In order to test whether the T-antigen-associated protein kinase activity corresponded to cyclin A-associated protein kinase, a lysate of SV40-infected subconfluent CV1 cells was immunodepleted either with an antiserum against human cyclin A or with normal rabbit serum. Immunoblots of the depleted extracts confirmed that more than 90% of detectable cyclin A was removed from the extracts (data not shown). The T antigen was subsequently immunoprecipitated from the precleared supernatant with a mixture of the anti-T-antigen MAbs PAb423 and PAb419, and the immune complexes were assayed for their histone H1 kinase activity. Removal of the cyclin A component resulted in a considerable loss of histone H1 kinase activity (Fig. 5A; compare lanes 1 and 2), although the majority of the T antigen from the original cell extract was still present in the immunoprecipitate (data not shown). We

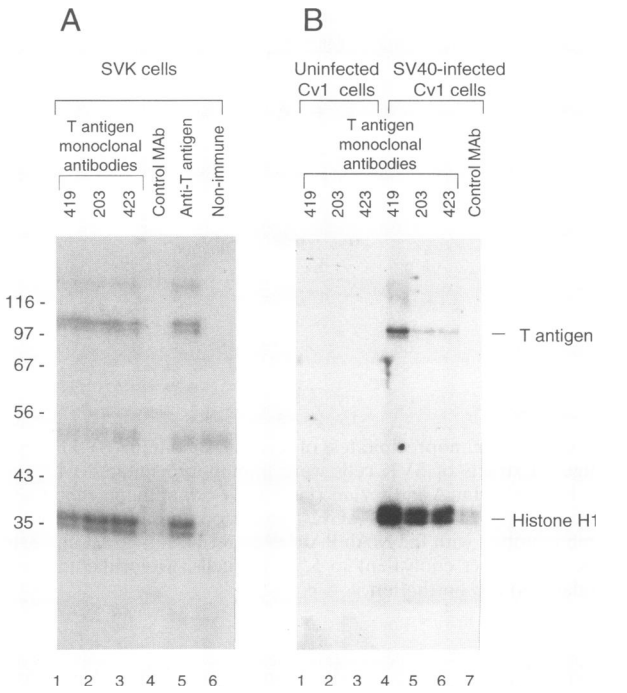

FIG. 4. T-antigen immunoprecipitates display histone H1 kinase activity. (A) Extracts from SVK cells were immunoprecipitated with anti-T-antigen MAbs. Lane 1, PAb419; lane 2, PAb203; lane 3, PAb423; lane 4, PAb246 (a control MAb specific for murine p53); lane 5, rabbit anti-T antigen; lane 6, normal rabbit serum. Immune complexes were collected on protein G beads and assayed for histone H1 kinase activity (30). Molecular masses (in kilodaltons) are on the left. (B) Extracts from uninfected CV1 cells (lanes 1 to 3) or CV1 cells infected 24 h earlier with SV40 (lanes 4 to 7) were immunoprecipitated with anti-T-antigen MAbs. Lanes 1 and 4, PAb419; lanes 2 and 5, PAb203; lanes 3 and 6, PAb423; lane 7, PAb246. Immune complexes were collected on protein G beads and assayed for histone H1 kinase activity (30). The positions of T antigen and histone H1 are indicated on the right.

interpret this to mean that a large fraction of the H1 protein kinase activity of T-antigen immunoprecipitates can be ascribed to a kinase associated with cyclin A.

An alternative approach to this question used Sepharose beads coupled to the 13-kDa polypeptide product of the *Schizosaccharomyces pombe* $p13^{suc1}$ gene, which is a reasonably specific affinity matrix for cyclin-dependent kinases. When $p13^{suc1}$-Sepharose beads were used to deplete cell extracts of cyclin-dependent H1 protein kinases, the supernatant of the $p13^{suc1}$-Sepharose-depleted extracts showed much less T-antigen-associated H1 kinase activity (Fig. 5B; compare lanes 1 and 2) than did mock-depleted extracts. Immunoblotting confirmed that all of the detectable cyclin A was depleted by the $p13^{suc1}$-Sepharose (data not shown). This indicates that the majority of the H1 protein kinase activity associated with the T-antigen immunoprecipitates is capable of binding to $p13^{suc1}$-Sepharose. Although the specificity of $p13^{suc1}$ is not precisely understood and this matrix certainly binds cellular proteins other than members of the cdc2 family, the most likely interpretation is that a cyclin-dependent protein kinase is associated with T antigen.

T antigen is associated with $p33^{cdk2}$ but not with $p34^{cdc2}$. The immunogenic amino acid sequence EGVPSTAIREIS LLKE is a characteristic feature of the cdc2 family of protein

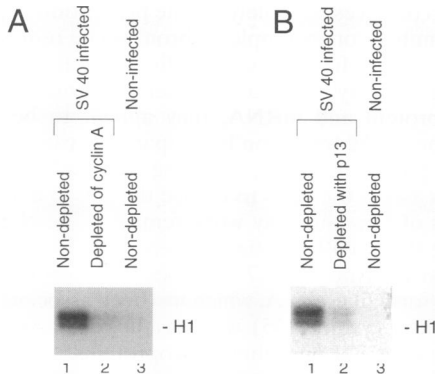

FIG. 5. The kinase activity of anti-SV40 large T antigen is depleted by anti-cyclin A antibodies and by $p13^{suc1}$-Sepharose. (A) Extracts from CV1 cells infected 24 h earlier with SV40 (lanes 1 and 2) or uninfected CV1 cells (lane 3) were immunodepleted with rabbit anti-human cyclin A (lane 2) or nonimmune rabbit serum (lanes 1 and 3). The supernatant was then immunoprecipitated with a mixture of the anti-T-antigen MAbs PAb419 and PAb423 and assayed for histone H1 kinase activity. (B) The protocol was identical to that described for panel A, except that the extracts from SV40-infected CV1 cells (lanes 1 and 2) or uninfected CV1 cells (lane 3) were preincubated with BSA-Sepharose beads (lanes 1 and 3) or with $p13^{suc1}$-Sepharose beads (lane 2).

kinases and can be detected by an antipeptide MAb (48). To see whether a protein containing this motif was bound to T antigen, extracts from subconfluent SVK cells were immunoprecipitated with anti-T-antigen MAbs, and these immune complexes were subsequently probed with the anti-PSTAIRE MAb. Figure 6A (lanes 11 to 13) shows that this antibody identified a polypeptide in the anti-T-antigen immune complexes that had a mobility similar to those of bacterially synthesized $p34^{cdc2}$ and $p33^{cdk2}$ standards. Cyclin A can bind equally well to both $p34^{cdc2}$ and $p33^{cdk2}$, and when extracts from the SVK cells were immunoprecipitated with rabbit anti-cyclin A and immunoblotted with the anti-PSTAIRE MAb, a series of bands were detected; the upper bands probably correspond to phosphorylated forms of $p34^{cdc2}$ (Fig. 6A, lanes 15 and 16). To identify which cyclin-dependent kinase was associated with the T antigen-cyclin A complexes, the immunoprecipitates were immunoblotted with the specific anti-$p34^{cdc2}$ MAb A17 (Fig. 6B) or a rabbit antiserum raised against human $p33^{cdk2}$ (Fig. 6C). The specificities of these two antibodies are clearly shown in Fig. 6B and C. The anti-$p34^{cdc2}$ antibody recognized bacterially synthesized $p34^{cdc2}$ (Fig. 6B, lanes 1 to 10) but did not react with bacterially synthesized $p33^{cdk2}$ (Fig. 6B, lanes 17 to 23). The anti-$p33^{cdk2}$ antibody recognized bacterially synthesized $p33^{cdk2}$ (Fig. 6C, lanes 17 to 23) but did not react with the $p34^{cdc2}$ standards (Fig. 6C, lanes 1 to 10). The antibody against $p33^{cdk2}$ recognized a band associated with T antigen (Fig. 6C, lanes 11 to 13), whereas the anti-$p34^{cdc2}$ antibody did not react with the immunoprecipitates of T antigen, although it was at least as sensitive as the anti-$p33^{cdk2}$ antiserum. Similar results were obtained with a different antiserum against $p33^{cdk2}$ obtained from John Pines (data not shown). We conclude that the most likely identity of the T-antigen-associated histone H1 kinase is cyclin A-$p33^{cdk2}$.

The association of cyclin A and large T antigen is independent of p53. T antigen associates with a number of cellular proteins, including p53, pRb and p107, all of which have been implicated in the ability of T antigen to transform cells. All three of these T-antigen-associated proteins have been reported to bind to cyclin-dependent kinases (14, 23, 29). It was of therefore of interest to investigate a possible role for these proteins in the interaction between large T antigen and cyclin A. To test whether p53 was involved in the T antigen-cyclin A complex, we immunodepleted extracts from SVK cells of p53. We then asked if the remaining T antigen, presumably not bound to p53, was still associated with cyclin A and possessed histone H1 kinase activity. When the supernatants from these p53-depleted extracts were immunoprecipitated with anti-T-antigen MAbs, these immunoprecipitates indeed contained no detectable p53 (Fig. 7A). However, cyclin A (Fig. 7B, lanes 1 and 2) and histone H1 kinase activity (Fig. 7C, lanes 1 and 2) were readily detected at similar levels in both p53-containing

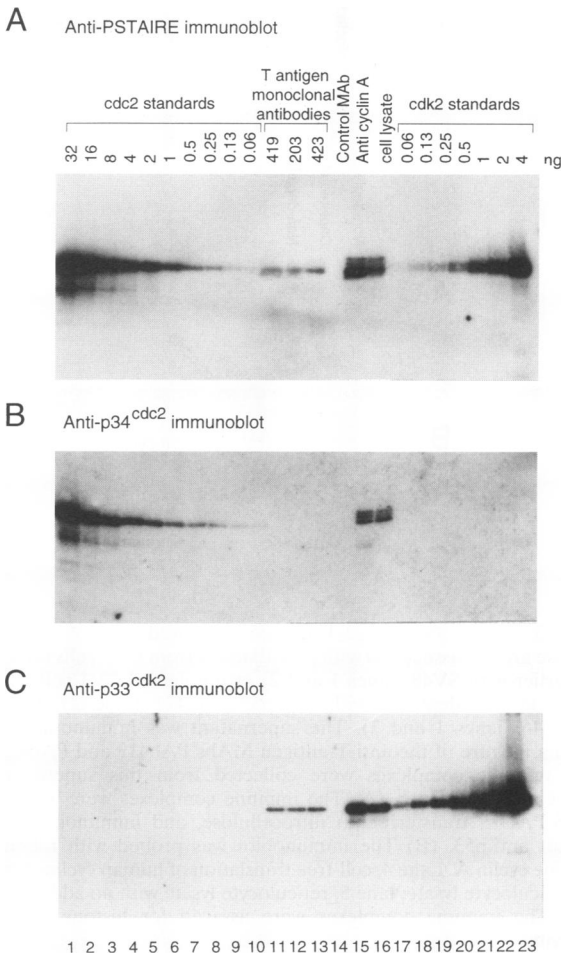

FIG. 6. Immunoprecipitates of SV40 large T antigen contain $p33^{cdk2}$. Extracts from SVK cells were immunoprecipitated with anti-T-antigen MAbs. Lane 11, PAb419; lane 12, PAb203; lane 13, PAb423; lane 14, PAb246 (control MAb against murine p53); and lane 15, rabbit anti-cyclin A cross-linked onto protein G-Sepharose (20). The immunoprecipitates were resolved by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with the antibodies described below. To demonstrate the specificity and sensitivity of the antibodies, lanes 1 to 10 contained serial twofold dilutions of human $p34^{cdc2}$ (32 to 0.0625 ng), and lanes 17 to 23 contained serial twofold dilutions of $p33^{cdk2}$ (0.0625 to 4 ng). Lane 16 shows 1 μl of crude SVK cell extract. (A) The immunoblot was probed with the MAb against the PSTAIRE peptide of Yamashita et al. (48); (B) the immunoblot was probed with MAb A17 raised against $p34^{cdc2}$ (24); (C) the immunoblot was probed with an antibody against $p33^{cdk2}$ (25).

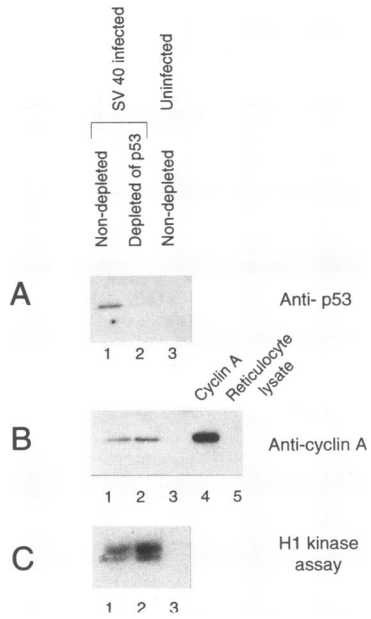

FIG. 7. The SV40 large-T-antigen-associated cyclin A and histone kinase are not associated with p53. Extracts from CV1 cells infected 24 h earlier with SV40 (lanes 1 and 2) or uninfected CV1 cells (lane 3) were immunodepleted with anti-p53 PAb421 (lane 2) or control PAb246 (lanes 1 and 3). The supernatant was immunoprecipitated with a mixture of the anti-T-antigen MAbs PAb419 and PAb423, and the immune complexes were collected from this supernatant on protein G-Sepharose. (A) The immune complexes were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit anti-p53. (B) The immunoblot was probed with rabbit anti-bovine cyclin A. Lane 4, cell-free translation of human cyclin A mRNA in reticulocyte lysate; lane 5, reticulocyte lysate with no added mRNA. (C) The immune complexes were assayed for histone H1 kinase activity.

and p53-depleted extracts. The available antibodies against pRb or p107 were unable to deplete extracts of these proteins sufficiently completely to make the equivalent tests possible in these cases. It is thus possible that what we detect are quaternary complexes containing the viral oncoprotein, the cyclin-dependent kinase, and either pRb or p107, but we have not investigated this point further.

DISCUSSION

In order to use the host cell replication machinery for their own propagation, small DNA tumor viruses presumably need to "drive" the cells they infect into the S phase of the cell cycle, if they infect quiescent cells. Permissive cells normally enter S phase about 12 h after infection with SV40, and they remain in S phase for another 24 h until the cells die and viral progeny are released (21). In this report, we show that a fraction of SV40 large T antigen forms a complex with a portion of cellular cyclin A and $p33^{cdk2}$. Adenovirus E1A protein and the product of the human papillomavirus E7 gene also associate with cyclin A and $p33^{cdk2}$ (11, 16, 22, 39, 42, 43). This suggests that the cyclin A-cdk2 kinase may fulfil an important function for these viruses.

A possible clue to this function is suggested by studies in which microinjection of anti-cyclin A or anti-$p33^{cdk2}$ antibodies inhibits or delays S phase in somatic cells (17, 35, 36). The role of cyclin A cannot be considered unambiguously settled, however, because in extracts of activated frog eggs, cyclin A is reported to be necessary solely for the prevention of premature entry into mitosis on incomplete chromosome replication (44). Moreover, when homozygous cyclin A mutant *Drosophila melanogaster* embryos run out of their maternal stockpiles of cyclin A protein and mRNA, they appear to be unable to undergo the G_2-M transition but apparently can complete the previous S phase (26). Thus, assuming that the T antigen-cyclin A complex assists the virus to induce the cell to enter S phase, the details of how this may work remain to be clarified. It is interesting that SV40 T antigen, adenovirus E1A, and human papillomavirus type 16 E7 all specifically bind the cdk2-associated form of cyclin A, which has been associated with the S-phase role of cyclin A (35), whereas the cdc2-associated form apparently does not bind these oncoproteins.

Addition of cdc2 kinases to cell extracts prepared from G_1 cells strongly stimulates SV40 DNA replication; such extracts are otherwise unable to support this activity (9). The phosphorylation both of T antigen and of the replication factor RPA by cyclin-dependent kinases is believed to be crucial for SV40 viral replication (10, 28). Some of these phosphorylation events might be taking place on the replication origin, especially as cyclin A has also been found associated with replicating SV40 minichromosomes (15). It may be important to viral origins of DNA replication for T antigen to recruit cyclin A, either to promote viral DNA synthesis or to aid in abrogating the block to rereplication for the viral DNA. In nonpermissive cells, it is possible that the interaction of large T antigen with cyclin A-$p33^{cdk2}$ contributes to the transforming potential of this oncogene.

Although the association we observe between T antigen, cyclin A, and $p33^{cdk2}$ is stable against the repeated washings involved in immunoprecipitation, it seems that only a minor fraction of either participant is involved in these complexes, which may mean that the association between them is indirect and requires a third or even a fourth party. The most likely candidates for such a linker are the recessive oncoproteins pRb and p107, both of which can bind cyclin A and $p33^{cdk2}$ independently of viral oncoproteins (14, 23), but it is not clear whether it is possible to form complexes containing pRb, cyclin A, $p33^{cdk2}$, and SV40 large T antigen all at the same time or whether the presence of the active cyclin A-$p33^{cdk2}$ kinase in these complexes plays a significant physiological role.

ACKNOWLEDGMENTS

We are grateful to John Pines for human cyclin A constructs and antibodies, Li-Huei Tsai and Ed Harlow for human cdk2 constructs, Katsumi Yamashita for the rabbit serum against cdk2, and Masakane Yamashita for the PSTAIRE MAb. We thank Massimo Tommasino, Lionel Crawford, Lan Bandara, and Nick LaThangue for helpful discussions and Thomas Lindahl for reading the manuscript.

J.P.A. was supported by a graduate scholarship from the Stiftung Stipendienfond des VCI e.V. during the early stages of this research.

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