Association of Simian Virus 40 Small-t Antigen with the 61-Kilodalton Component of a Cellular Protein Complex

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Two cellular proteins, 61 and 37 kDa, are found in association with the simian virus 40 (SV40) small-t antigen. Fractionation in standard chromatography systems showed that these proteins were associated with one another in uninfected cells, suggesting that the small-t antigen may bind the complex as a whole and not each individual protein independently. In the presence of *N*-ethylmaleimide, the 37-kDa protein was selectively released from immune complexes, leaving the small-t antigen and 61-kDa protein in association. This result suggests that the small-t antigen may bind only the 61-kDa protein and that the 37-kDa protein may be associated with immune complexes by virtue of its association with the 61-kDa cellular protein.

Several cellular proteins have been identified in association with the small-t antigen of simian virus 40 (SV40) (14) and the middle- and small-t antigens of polyomavirus (4, 10). Two of these proteins, proteins with molecular masses of 61 and 37 kDa, have been shown to associate both with the middle-T antigen of polyomavirus and with the small-t antigens of polyomavirus and SV40 (5, 9, 11). Recently, it has been shown that these proteins are regulatory (A) and catalytic (C) subunits of the protein phosphatase 2A. The initial breakthrough in this identification came from comparisons of tryptic peptides of the 37-kDa protein associated with polyomavirus middle-T antigen with known sequences present in the GenBank data base (6). This finding led to direct comparisons of the 37-kDa protein with the C subunit and of the 61-kDa protein with the A subunit, using specific antisera raised to the phosphatase. The pairs of proteins were also compared by using two-dimensional gel electrophoresis and chymotryptic mapping. Subsequently, these observations were confirmed and extended (13) by using an antiserum raised to the 61-kDa protein found associated with the middle-T antigen. The 61-kDa protein had been cloned by using oligonucleotides predicted from partial amino acid sequencing, and a TrpE fusion protein of the cloned protein allowed antibody production (12).

The consistent and stoichiometric recovery of the 61- and 37-kDa proteins with the viral antigens had been observed for many years. It is now apparent that this finding reflected the interaction of the viral proteins with cellular proteins which were already associated with one another in cell extracts. One indication that this was the case was obtained by finding that the two proteins which could complex with polyomavirus middle-T antigen comigrated in glycerol gradients (3). In our studies of proteins associated with SV40 small-t antigen, similar observations have been made by G200 column chromatography (Fig. 1).

We have shown previously (8) that the 37-kDa protein can be efficiently labeled *in vitro* by [*methyl*-³H]S-adenosylmethionine ([³H]SAM). The 37-kDa protein is one of the major labeled products under these conditions and can be detected directly in sodium dodecyl sulfate (SDS)-polyacrylamide gels without the need for interaction with the small-t antigen

and immunoprecipitation. In the experiment shown in Fig. 1, an extract of uninfected monkey kidney CV-1 cells was labeled *in vitro* with [³H]SAM and then applied to a G200 column. The proteins present in every third fraction of the column, beginning with fraction 32, were concentrated by trichloroacetic acid and separated by SDS-gel electrophoresis (lanes 2 to 13). The void volume (*v* in Fig. 1) was at fraction 35 (lane 3). The 37-kDa protein was found in a peak that eluted shortly after the void volume, in a region of the column consistent with a molecular mass of about 100 kDa. For comparison, the elution position of bovine serum albumin (68 kDa) is indicated (*b* in Fig. 1). In a separate fractionation using the same column, monomeric small-t antigen (17 kDa) eluted in fractions 78 to 80. Proteins present in fractions 38 to 48 were concentrated by using DEAE-cellulose, from which the 37-kDa protein was eluted with 400 mM NaCl (Fig. 1A, lane 1, labeled D).

The fractionation of the 37-kDa protein suggests that none of the SAM-labeled protein is found in monomeric form and that it behaves like a complex in this column. It has not been possible to show conclusively that all molecules of the 37-kDa protein have been labeled with SAM under these conditions, but estimates of the amount of the total protein and the molar quantities of methyl groups obtained from SAM suggest that a large fraction of the total 37-kDa protein is modified in the *in vitro* labeling. With the new understanding that the 37-kDa protein is the catalytic subunit of protein phosphatase 2A and the availability of antibodies to this enzyme, it should be possible to determine whether monomeric 37-kDa protein is present but not labeled with SAM.

Similar fractionations were performed by using uninfected cell extracts labeled with [³⁵S]methionine (Fig. 1B). After application of total extract proteins to G200, fractions 38 to 48 were pooled and applied to a DEAE-cellulose column, which was eluted with a 100 to 500 mM NaCl gradient. Radioactive protein eluted as a broad peak between 170 and 250 mM NaCl. Two pools were collected, one from 170 to 210 mM NaCl (pool 1) and the second from 210 to 250 mM NaCl (pool 2), either side of the peak of protein. Proteins present in these pools were concentrated by using phenyl-Sepharose and eluted with 1% Nonidet P-40. These proteins were then incubated with unlabeled extracts that contained small-t antigen and were immunoprecipitated by using anti-tumor serum that recognizes the SV40 viral antigens but

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FIG. 1. Fractionation of 61- and 37-kDa proteins. Extracts of uninfected monkey kidney CV-1 cells were used for fractionation. (A) Three 10-cm-diameter dishes of confluent cells were extracted and labeled with [3 H]SAM as described previously (8). The extract was then pumped onto an upward-flow G200 column (2.5 by 40 cm) and fractionated in 20 mM Tris hydrochloride (pH 8) containing 100 mM NaCl and 1 mM dithiothreitol. Fractions of 2.5 ml were collected, and a sample of every third fraction, beginning with fraction 32, was precipitated with trichloroacetic acid for SDS-gel electrophoresis (lanes 2 to 13). Peak fractions were concentrated on DEAE-cellulose and are shown in lane 1 (labeled D). v, Void volume; b, elution position of bovine serum albumin (68 kDa). (B) One 10-cm-diameter dish of confluent CV-1 cells was labeled overnight with 500 μ Ci of [35 S]methionine and extracted as previously described (8). The extract was applied to a G200 column as described above. Fractions 38 to 48 were pooled and applied to a DEAE column (2 by 5 cm), and then proteins were separated by using a 100 to 500 mM NaCl gradient. Fifty 2-ml fractions were collected in two pools, which were concentrated and used for immunoprecipitation. Patterns shown are immunoprecipitates of pool 1 after incubation with wild-type SV40-infected cell extract (lane 1), pool 2 after incubation with wild-type SV40-infected cell extract (lane 2), and pool 2 after incubation with d1888-infected cell extract, which lacks the small-t antigen (lane 3). Positions of the 61-kDa (61K) and 37-kDa (37K) proteins are shown.

does not directly recognize the cellular 61- and 37-kDa proteins. This *in vitro* association assay has been described previously (8, 14).

As expected from the behavior of the SAM-labeled 37-kDa protein, both the 37- and 61-kDa proteins were found in fractions of the G200 column that eluted just after the void volume. Several similar experiments have been performed in which fractions from other regions of the G200 column were analyzed, the two proteins have been found together only with an elution in the 100-kDa region of the column (data not shown). Free 61- or 37-kDa subunits have never been found after G200 chromatography, but these subunits may not be detectable by using interaction with small-t antigen as an assay.

The 61- and 37-kDa proteins also co-fractionated on DEAE-cellulose (Fig. 1B), and similar results have been obtained with hydroxylapatite (data not shown). However, ratios of the 61-kDa to 37-kDa proteins detected in different regions of DEAE-cellulose columns varied (Fig. 1B). The 61-kDa protein often appeared to be found preferentially, although it seems unlikely that monomeric 61-kDa protein would have been present in the region of the G200 column used for further fractionation on DEAE-cellulose. It is possible, however, that the 61-kDa protein might interact with proteins other than the 37-kDa protein, resulting in elution from G200 region indicative of higher-molecular-mass proteins. Other proteins that interact with the A and C subunits of protein phosphatase 2A have been described (1).

The apparently preferential recovery of the 61-kDa protein has been a consistent observation in steps of purification beyond G200 chromatography as protein concentrations

become more dilute. This suggested the possibility that the two proteins dissociate and that the small-t antigen is able to recognize the 61-kDa protein directly in the absence of the smaller protein.

Evidence that the SV40 small-t antigen may interact directly with the 61-kDa subunit and only indirectly with the 37-kDa protein was obtained using *N*-ethylmaleimide (NEM) treatment of immunoprecipitates. Immunoprecipitates of SV40-infected CV-1 cells contained large-T antigen, small-t antigen, and the 61- and 37-kDa cellular proteins (Fig. 1A, lane 1). Immunoprecipitates were isolated on *Staphylococcus aureus* and then incubated with 0.1 M NEM for 1 h on ice. After this incubation, proteins released from immune complexes were separated from the bacteria by centrifugation, concentrated by acid precipitation, and analyzed on SDS-gels. Two separate experiments are shown in Fig. 2A and B, analyzing proteins that remained associated with immune complexes (Fig. 2A, lane 2; Fig. 2B, lane 1) and proteins released from the immune complexes (Fig. 2A, lane 3; Fig. 2B, lane 2). A second incubation with NEM did not result in further protein release (Fig. 2B, lane 3). The NEM treatment did modify the small-t antigen, as indicated by its altered migration on SDS-gels (2, 7), but did not result in the release of the small-t antigen from immune complexes. In addition, the 61-kDa protein remained associated with the small-t antigen, whereas the 37-kDa protein was quantitatively released by NEM treatment.

This result might occur if the small-t antigen formed two individual complexes, one with the 61-kDa protein and a second with the 37-kDa protein. This seems unlikely because the two cellular proteins are normally associated with one

FIG. 2. Release of the 37-kDa protein from immune complexes with NEM. Wild-type SV40-infected CV-1 cells were labeled overnight with [35 S]methionine, extracted, and immunoprecipitated as described previously (8). Washed immunoprecipitates were incubated with 0.1 M NEM for 60 min on ice. Two separate experiments are shown in panels A and B. The patterns shown in panel A are the entire immunoprecipitate (lane 1), proteins left in association with immune complexes after NEM treatment (lane 2), and proteins released by NEM (lane 3). The patterns shown in panel B are the proteins left in association with immune complexes after NEM treatment (lane 1) and proteins released by NEM (lane 2). A second treatment with NEM did not result in further release of any proteins (lane 3). Positions of large-T antigen (T), small-t antigen (t), 61-kDa (61K) protein, and 37-kDa (37K) protein are shown.

another. It is more likely that the small-t antigen is associated directly with the 61-kDa protein and that the 37-kDa protein is present in immunoprecipitates because of its association with the 61-kDa protein. Additional support for this hypothesis comes from the observation that the interaction between the 61- and 37-kDa proteins is sensitive to NEM in the absence of the small-t antigen. It is known that the protein phosphatase 2A A and C subunits can be dissociated by NEM (M. Mumby, personal communication).

One implication of this observation is that the small-t antigen associates with a regulatory subunit of protein phosphatase 2A and does not directly interact with the catalytic subunit. This information may be important in future studies to examine the effect of association of viral proteins with the phosphatase on the activity of this enzyme. Phosphatase 2A activity has been detected in immunoprecipitates of polyomavirus-infected cells (6), suggesting that interaction with at least this viral antigen does not inhibit enzymatic activity. Alterations in the interaction of regulatory subunits with the catalytic 37-kDa protein may, however, be of importance in

determining the activity of the phosphatase on different substrates. In this regard, it is interesting to note that varying forms of an additional regulatory subunit, termed the B subunit, are found naturally in cell extracts (1). It has been hypothesized that the third B subunit may play a role in modifying catalytic activity or substrate preference. Replacement of the B subunits by the small-t antigen could play a similar role, a suggestion that has been made by others as well (6, 13). Our evidence that the small-t antigen may interact with the A subunit (61 kilodaltons) lends support to this possibility.

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