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Complexes of polyoma virus medium T antigen and cellular proteins

(anti-peptide antibodies/transformation/affinity chromatography)

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ABSTRACT **Antibodies against synthetic peptides corresponding to the carboxyl-terminal six amino acids, Lys-Arg-Ser-Arg-His-Phe (KF), and an internal region, Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu (EE), of polyoma virus medium T antigen were used successively to purify medium T antigen by affinity chromatography. Medium T antigen from cell extracts was first bound to anti-KF antibodies and released from the immune complex with excess KF peptide; then it was bound to anti-EE antibodies and released with excess EE peptide. Two proteins, pp60$^{c\text{-}src}$ and a new protein of ≈61,000 Da (61-kDa protein), were copurified because they formed complexes with medium T antigen. The 61-kDa protein–medium T antigen complex was detected in extracts from wild-type-infected and transformed cells but not from cells infected with NG59 virus, which has a mutation in the medium T gene and is transformation defective. Instead, NG59 medium T antigen formed a complex with another cellular protein of ≈72,000 Da.**

The transforming protein of polyoma virus, medium T antigen, is a membrane protein (1–3) associated with a tyrosine-specific protein kinase activity (4–6). Biochemical studies have indicated that this activity is not intrinsic to medium T antigen (7, 8) but instead resides in a minor fraction sedimenting with a size of 200,000 Da, whereas the bulk of medium T antigen is monomeric and kinase inactive (9). It was shown subsequently that the kinase activity is due to an association between medium T antigen and pp60$^{c\text{-}src}$, the cellular homolog of the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (10). The interaction between medium T antigen and pp60$^{c\text{-}src}$ may be facilitated by the fact that both proteins are membrane associated and, therefore, come into close contact. In a similar fashion, medium T antigen might interact with other neighboring membrane proteins. We asked how one might identify such proteins without having a functional assay or a specific antiserum available. In the present paper, we demonstrate that in addition to pp60$^{c\text{-}src}$, medium T antigen also forms a complex with a cellular protein of ≈61,000 Da. This was achieved by affinity chromatography with two anti-peptide antibodies directed against different regions of medium T antigen.

MATERIALS AND METHODS

Cell Culture, Virus Infection, and Radiolabeling. Mouse 3T6 cells were maintained in Dulbecco's modified Eagle's medium suppplemented with 10% calf serum. For infection by polyoma viruses, the cells were seeded on 10-cm tissue culture dishes at 1×10^6 cells per dish. The cultures were infected on the following day and radiolabeled 24–27 hr after infection for 3 hr with [^{35}S]methionine at 250 μCi/ml (1 Ci = 37 GBq) in medium lacking methionine but supplemented with 5% dialyzed calf serum. Cell extracts were prepared in 10 mM sodium phosphate buffer, pH 8/140 mM NaCl/3 mM $MgCl_2$/0.5% Nonidet P-40/1 mM dithiothreitol/50 μM leupeptin (lysis buffer).

Preparation of Antisera and Immunoabsorbents. The production and characterization of the antiserum against the carboxyl-terminal peptide Lys-Arg-Ser-Arg-His-Phe (KF) has been published (9, 11). A description of monoclonal antibodies against the peptide Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu (EE) will be published elsewhere. The latter peptide has been used by Schaffhausen *et al.* to produce antisera against medium T antigen in rabbits (12). IgG from antiserum and hybridoma cell supernatants was purified on protein A Sepharose according to Ey *et al.* (13). Peptide-specific IgG was obtained by affinity chromatography on peptide-Sepharose as described (9) and was coupled covalently to protein A Sepharose with dimethyl pimelimidate according to Schneider *et al.* (14). An amount of 8 mg of purified IgG was coupled per ml of protein A Sepharose.

Purification of Medium T Antigen. Infected or uninfected cell extract (500 μl) was first incubated for 1 hr at 4°C with 50 μl of anti-KF Sepharose plus 50 μl of plain Sepharose 4B. After the incubation, the extract was removed and the Sepharose was washed three times with lysis buffer. The resin was then incubated for 30 min at 4°C with 100 μl of Nonidet P-40 release buffer (2% Nonidet P-40/140 mM NaCl/10 mM Tris·HCl, pH 7.2/1 mM dithiothreitol) containing 10 μg of KF peptide. In some experiments, release was carried out in RIPA buffer containing 1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% $NaDodSO_4$ instead of 2% Nonidet P-40. The released protein was incubated with 25 μl of EE antibody Sepharose and 75 μl of plain Sepharose 4B, and the bound material was released with EE peptide as described for KF peptide. During all incubations, the reaction tube was slowly rotated. Aliquots for analysis were removed from the Sepharose pellet and from the supernatant at each step. Tyrosine-specific protein kinase activity of purified medium T antigen complexes using enolase as a substrate was determined in small aliquots (10 μl) from the material released with peptide (15). Phosphorylation of tyrosine was verified by phosphoamino acid analysis.

RESULTS

Identification of the Complex Between Medium T Antigen and 61-kDa Protein. The identification of the medium T antigen–pp60$^{c\text{-}src}$ complex was possible because of the availability of an assay for pp60$^{c\text{-}src}$ (the tyrosine-specific kinase activity) and because of the availability of specific antisera against both medium T antigen and pp60$^{c\text{-}src}$ (10). The identification of specific complexes between medium T antigen and other cellular proteins of unknown function, against which antibodies

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were not available, seemed feasible by rigorous purification. If a highly purified medium T antigen preparation contains one or more additional proteins, then such proteins may be specifically associated with medium T antigen. In the past, we have purified medium T antigen by affinity chromatography with antibodies against a synthetic peptide, KF peptide, corresponding to the carboxyl terminus of medium T antigen (9). Extracts from polyoma virus-infected mouse cells containing medium T antigen were incubated with anti-KF Sepharose, and bound medium T antigen was released from the antibody by a large excess of KF peptide. Although a considerable purification was achieved, the best preparation still contained many proteins, as visualized by polyacrylamide gel electrophoresis. Therefore, it was not possible to distinguish between proteins associated with medium T antigen in a specific manner and background bands. To further purify medium T antigen, two different anti-peptide antibodies were used in sequence. First, medium T antigen from cell extracts was bound to antibodies against the carboxyl-terminal peptide (KF) and was released with excess KF peptide. In the second step, the released prepurified medium T antigen was bound to antibodies against a different peptide, EE, corresponding to an internal region of medium T antigen (amino acids 311–319), and was released with excess EE peptide. As shown in Fig. 1, this approach yielded highly purified medium T antigen. In addition, we observed a protein of 61,000 Da (61-kDa protein) (lane c) that was copurified under these conditions. Medium T antigen purified with only one antibody is shown in lane a. Although in the latter case, medium T antigen and the 61-kDa protein are major components, additional proteins that were subsequently removed in the second step of purification are still present in this preparation. In a control experiment with extract from uninfected cells (lanes b and d), neither medium T antigen nor the 61-kDa protein was detected.

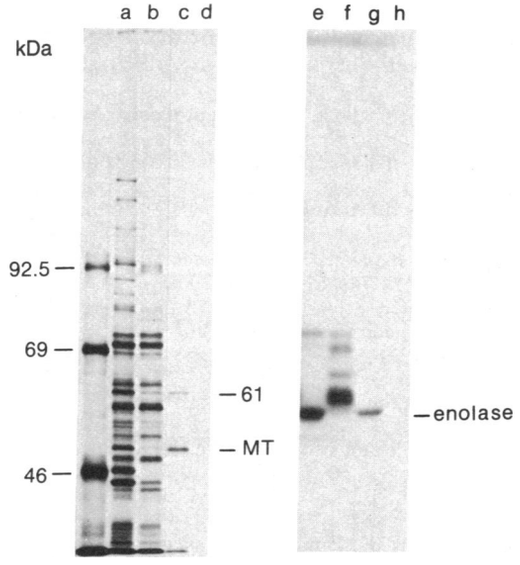

FIG. 1. Purification of the medium T antigen–61-kDa protein complex. [^{35}S]Methionine-labeled extracts from polyoma virus dl8-infected (lanes a and c) and uninfected cells (lanes b and d) were incubated with anti-KF Sepharose (100 μl of Sepharose per 500 μl of extract). After washing to remove unbound material, the resin was incubated with 100 μl of Nonidet P-40 release buffer containing KF peptide. The released protein was incubated with anti-EE Sepharose and bound material was released with EE peptide in Nonidet P-40 release buffer. Released proteins were analyzed on a 7.5% polyacrylamide gel as described (11). Lanes a and b show proteins released with KF peptide; lanes c and d show proteins released with EE peptide. Exposure times of the fluorogram were 3 days for lanes a and b and 14 days for lanes c and d. The recovery of medium T antigen released with EE peptide (lane c) was 10% as compared to the first release with KF peptide (lane a). Lanes e–h show the result of protein kinase reactions carried out with the material shown in lanes a–d. Enolase was used as a substrate. Lanes e (infected) and f (uninfected) show kinase reactions with proteins released with KF peptide; lanes g (infected) and f (uninfected) show kinase reactions with proteins released with EE peptide. MT, medium T antigen; 61, 61-kDa protein.

To demonstrate directly that the 61-kDa protein and medium T antigen form a complex, polyoma virus-infected cell extracts were fractionated on a 5–20% glycerol gradient. Subsequently, medium T antigen was purified from each fraction by single-step affinity purification with anti-EE antibody. Fig. 2 shows that medium T antigen and the 61-kDa protein comigrated on the gradient, forming a complex of ≈200,000 Da. The medium T antigen-associated kinase activity migrated ahead of the 200-kDa complex. Thus, medium T antigen forms complexes with at least two cellular proteins. Fig. 2 shows very little monomeric medium T antigen, in contrast to previous experiments in which the bulk of medium T antigen was monomeric and the medium T antigen-associated kinase migrated as a 200-kDa complex (9). The difference is that the previous experiments were carried out with extracts prepared in RIPA buffer containing $NaDodSO_4$ and deoxycholate in addition to Nonidet P-40. We also observed that both complexes, the medium T antigen–61-kDa protein complex and the medium T antigen–pp60$^{c\text{-}src}$ complex, sedimented faster on sucrose gradients when prepared from extracts containing Nonidet P-40 buffer than from RIPA buffer extracts. It seems that $NaDodSO_4$ and/or deoxycholate partially disrupted the larger structures observed in Nonidet P-40 buffer. The 61-kDa protein is not a phosphoprotein and is not precipitable with antibodies against pp60$^{c\text{-}src}$ (unpublished data). Therefore, the 61-kDa protein and pp60$^{c\text{-}src}$ are different proteins. It is noteworthy that, in contrast to the 61-kDa protein, pp60$^{c\text{-}src}$ is not detectable by [^{35}S]methionine labeling in purified preparations of medium T antigen (Figs. 1 and 2), although it can be easily identified in a protein kinase assay (Fig. 1, lanes e–h) and by immunoprecipitation with anti-pp60$^{c\text{-}src}$ antibodies followed by a protein kinase assay of the precipitate (data not

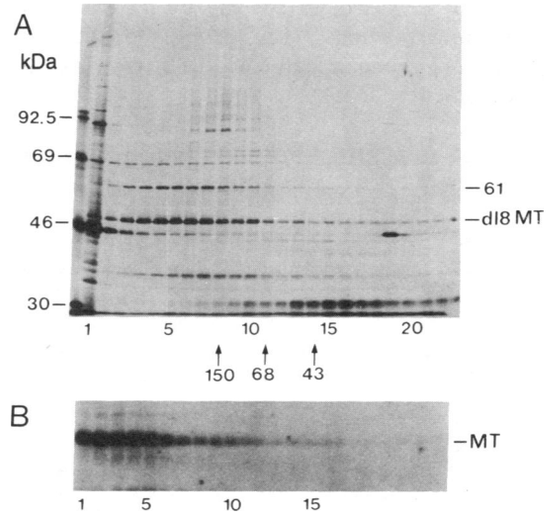

FIG. 2. Sedimentation analysis of the medium T antigen–61-kDa protein complex. Freshly prepared extract (230 μl) from dl8-infected 3T6 cells was loaded on a 5–20% glycerol gradient in lysis buffer and spun at 40,000 rpm in a Beckman SW50.1 rotor at 4°C. Medium T antigen from each fraction was purified by a one-step immunoaffinity purification using anti EE (*A*). An aliquot from each fraction was used to assay medium T antigen-associated kinase activity as described (9) (*B*). Proteins were analyzed on a 7.5% $NaDodSO_4$/polyacrylamide gel. Only that portion of the gel with autophosphorylated medium T antigen is shown in *B*. Migration of molecular size marker proteins was determined in a parallel gradient. MT, medium T antigen; 61, 61-kDa protein.

shown). This emphasizes the idea that only a very small fraction of pp60$^{c\text{-}src}$ becomes associated with medium T antigen. We have not studied the relative stabilities of the medium T antigen–61-kDa protein and medium T antigen–pp60$^{c\text{-}src}$ complexes under various conditions of salt, pH, or heat.

Fig. 3 shows double-affinity-purified medium T antigen from 3T6 cells infected with polyoma wild-type (lane e), dl8, a deletion mutant encoding a smaller medium T antigen than wild type (lane c), and NG59, a transformation-defective mutant of medium T antigen (lane d). The mutation in NG59 involves addition of an isoleucine residue as well as a change of an aspartic acid to asparagine at amino acid 179 (16). Lanes f and g show purified medium T antigen from two polyoma virus-transformed cell lines. It is apparent that all medium T antigen preparations, except that from NG59-infected cells, contain the 61-kDa protein. This suggests that the 61-kDa protein, like pp60$^{c\text{-}src}$, might play a role in transformation. It is interesting that purified medium T antigen from NG59-infected cells shows an additional protein of 72 kDa (lane d). A double-purification experiment using extract from mock-infected cells is shown in lane b (control). Medium T antigen purified with only one antibody (against the carboxyl terminus) is shown in lane a. Some of the small proteins in lanes d and e presumably represent breakdown products of medium T antigen. In addition to the 61-kDa protein, we consistently observed three minor [^{35}S]methionine-labeled proteins of ≈88,000, ≈37,000, and ≈31,000 Da in highly purified medium T antigen preparations. These proteins have not been further characterized.

DISCUSSION

Polyoma virus medium T antigen was purified from infected cell extracts with a combination of antibodies against the carboxyl terminus and an internal region. Two other proteins, pp60$^{c\text{-}src}$ and the 61-kDa protein, were purified concomitantly because they formed stable complexes with medium T antigen. pp60$^{c\text{-}src}$ was detected only by its protein kinase activity as shown previously (10), whereas the 61-kDa protein was much more abundant and could be easily visualized by [^{35}S]methionine labeling. On glycerol gradients, the medium T antigen–61-kDa protein complex sedimented differently from the medium T antigen–pp60$^{c\text{-}src}$ complex. The biological significance of the medium T antigen–61-kDa protein interaction is suggested by the fact that medium T antigen of NG59, a transformation-negative mutant, failed to form a complex with the 61-kDa protein and associated with another protein (72-kDa protein) instead. Silver *et al.* (17) described a protein of 63,000 Da in immunoprecipitates from polyoma virus-infected cells. Like the 61-kDa protein, it was also not found in extracts from cells infected with NG59 or other hrt mutants. This protein might be the same as the 61-kDa protein described here. On the other hand, the 63-kDa protein described by Silver *et al.* was apparently related to large T antigen, whereas the 61-kDa protein is not because extracts from cells transformed by medium T antigen only also contained the 61-kDa protein bound to medium T antigen (unpublished results).

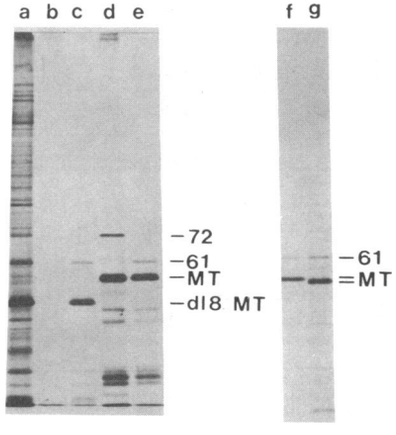

FIG. 3. Purification of medium T antigen from infected and transformed cells. Two antibodies were used in lanes b–g as described in the legend to Fig. 1. Only one antibody (KF) was used in lane a. Extracts were prepared from cells infected with dl8 (lanes a and c), NG59 (lane d), wild type (lane e), transformed mouse cells, line A31 (lane f), and transformed rat cells, line B4 (lane g). A control preparation was assayed with extract from uninfected cells (lane b). The peptide release was carried out in RIPA buffer. MT, medium T antigen; 61 and 72, 61- and 72-kDa proteins.

Protein purification by affinity chromatography with anti-peptide antibodies in combination with the corresponding peptides is a fast, gentle, and efficient method that can be carried out on a small scale with as few as 5×10^4 cells (9). In the present paper, we demonstrated the use of anti-peptide antibodies against two different regions of the same protein for protein purification. This procedure considerably improves the previous method in which only one anti-peptide antibody was used and lends itself to the study of protein–protein interactions of other oncogenic proteins. For example, one can envisage that the proteins encoded by the *ras* gene family, presumably involved in signal transduction through the plasma membrane (18), also form functional complexes with neighboring proteins, which could be isolated and studied with the procedure described here.

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