Two Phosphorylated Subclasses of Polyomavirus Large T Antigen That Differ in Their Modes of Association with the Cell Nucleus†

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Two classes of polyomavirus large T antigen were distinguished, differing in their modes of association with the cell nucleus. A weakly associated class, the nucleoplasmic T antigen, representing 30 to 40% of the total, was solubilized when cells were lysed in isotonic buffer at pH 7.2. A more tightly bound class retained in isolated nuclei, the retained T antigen, was extractable either at pH 9.0 or in 2 M NaCl. The retained T antigen contained an additional mole of phosphate, 4 mol of PO₄ per mol of T antigen, compared with the nucleoplasmic T antigen (3 mol of PO₄ per mol of T antigen). Limit digestion with staphylococcal V8 protease yielded equivalent amounts of five peptides ranging in size from 7.5 to 20 kilodaltons. Additional phosphorylation within a 12-kilodalton peptide accounted for most of the difference in phosphate content between retained and nucleoplasmic T-antigen classes.

The polyomavirus large T antigen is an 88-kilodalton (kDa) phosphoprotein which accumulates in nuclei of polyomavirus-infected cells from 12 to 27 h after infection (1, 9). During the viral lytic cycle, the large T antigen both regulates viral transcription and is required to initiate each round of viral replication (for reviews, see references 4 and 8). How this single protein can mediate these different functions is not clear, but efforts have focused on resolving the role(s) of structural subclasses of the T antigen (1, 3, 4, 7, 11, 12; G. W. Humphrey and V. Pigiet, *Exp. Cell Res.*, in press). Both the polyomavirus large T antigen and the homologous simian virus 40 large T antigen contain seven or eight phosphorylation sites (5, 10). Moreover, there is evidence for different phosphorylated classes of T antigen distinguished by their modes of association with the cell nucleus. Jones and Su (7) found that a tightly associated class of simian virus 40 large T antigen retained in isolated nuclei (radiolabeled *in vivo* with ³²P_i) contained more phosphate than a loosely associated nucleoplasmic class which appears in the cytosolic fraction. In contrast, Scheidtmann et al. (11) found that simian virus 40 large T antigen retained in isolated nuclei contained half as much phosphate as the nucleoplasmic large T antigen. This discrepancy might be due to the relatively short radiolabeling periods, 2 h (7) or 4 h (11); uniform radiolabeling requires 24 h (13). Though this discrepancy exists in the simian virus 40 literature, it is agreed that phosphorylation of T antigen differs between the nucleoplasmic and retained T-antigen classes. Here we compared the phosphate content of the homologous polyomavirus large T antigen in the nucleoplasmic class with that retained in isolated nuclei by using methodology that is free from the ambiguity of radiolabeling kinetics.

To measure the phosphate content of polyomavirus large T antigen, polyomavirus-infected mouse 3T6 cells were radiolabeled with ³²P_i *in vivo* for 24 h before harvest. At 27 h after infection, cells were lysed in isotonic buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic

acid] [pH 7.2], 222 mM sucrose, 1 mM MgCl₂, 0.5 mM CaCl₂, 0.2% Nonidet P-40), yielding a cytosolic fraction and isolated nuclei (1; Humphrey and Pigiet, *in press*). Nuclei were subsequently extracted in STN buffer (20 mM Tris hydrochloride [pH 9.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) (9). T antigen was quantified in the cytosolic fraction (nucleoplasmic T antigen), and the STN nuclear extract (retained T antigen) was quantified by densitometry of anti-T immunoprecipitates (1, 9; Humphrey and Pigiet, *in press*) resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie R250 dye as previously described (14). The large T antigen, migrating with an apparent molecular mass of 100 kDa (Fig. 1), was quantified by comparison with equivalent samples of protein standards, either bovine serum albumin (Fig. 1) or phosphorylase *b* (data not shown), loaded on the same gel. Approximately one-third of the T antigen appeared in the nucleoplasmic class (Table 1). The remaining two-thirds of the T antigen was retained in isolated nuclei and was extractable at pH 9.0 (9), consistent with our previous results obtained by immu-

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TABLE 1. Phosphate contents of polyomavirus large-T-antigen subclasses

Subcellular fraction ^a	T antigen (picomoles) ^b	% Total T antigen	PO ₄ (picomoles) ^c	Moles of PO ₄ /moles of T antigen
Cytosol	26	37	76	2.9
Nuclear extract (pH 9)	43	56	172	4.0
High salt extract (2 M NaCl)	40	56	160	4.0
Nuclear matrix	5	7	22	4.4

^a Subcellular fractions prepared from polyomavirus-infected 3T6 cells radiolabeled for 24 h before harvest (at 27 h postinfection) with ³²P_i (50 μ Ci/ml) as described in the legend to Fig. 1.

^b Picomoles of large T antigen per 2×10^7 cells were determined by quantitative densitometry of anti-T immunoprecipitates resolved by using SDS-polyacrylamide gels stained with Coomassie R250 dye as in Fig. 1. The data represent the mean of two independent experiments.

^c Picomoles of PO₄ bound to large T antigen per 2×10^7 cells were calculated from ³²P disintegrations per minute incorporated into 100-kDa bands (as in footnote b) and the specific radioactivity of total phosphate in the culture medium, 106 dpm/pmol. The data represent the mean of two independent experiments.

noblotting (Humphrey and Pigiet, in press). Radiolabeled T antigen (in ³²P disintegrations per minute) was determined by liquid scintillation counting of the 100-kDa bands excised from gels (1; Humphrey and Pigiet, in press). The molar amount of phosphate bound covalently to T antigen was calculated by using the specific radioactivity of total ³²P in the culture medium, 106 dpm/pmol (14). The retained T antigen extracted from isolated nuclei at pH 9.0 contained an additional mole of phosphate, 4 mol of PO₄ per mol, compared with the nucleoplasmic T antigen, 3 mol of PO₄ per mol (Table 1).

When isolated nuclei are sequentially extracted with 1% Triton X-100, DNase I, and 2 M NaCl, a small fraction of the polyomavirus large T antigen, 5 to 10%, remains bound to the resulting nuclear matrices, with most of the retained T antigen class extracted in 2 M NaCl (1; Humphrey and Pigiet, in press). The nuclear matrix has been identified as a site of viral and cellular DNA replication and transcription (1, 3, 7, 8, 12; Humphrey and Pigiet, in press), providing a functional rationale for the retained T antigen class. The

FIG. 1. Detection and quantitation of polyomavirus large T antigen in subcellular fractions. Polyomavirus-infected mouse 3T6 cells were scraped from 150-mm (diameter) plates (approximately 2×10^7 cells per plate) at 27 h after infection and lysed by vortex mixing in isotonic buffer, yielding a cytosol fraction (C) and isolated nuclei; all procedures were performed at 4°C. Nuclei were extracted with STN buffer to yield nuclear extract (NE) or sequentially extracted with 1% Triton X-100, DNase I, and 2 M NaCl (as described by Humphrey and Pigiet [in press]) to prepare nuclear matrices (NM). The cytosol fraction was prepared for immunoprecipitation by addition of STN buffer components; nuclear matrices were solubilized with 1% SDS and then diluted 1:10 in STN buffer. Anti-T (T) or preimmune (N) immunoprecipitates of the cytosol (0.4 plate), nuclear extract (0.2 plate), and nuclear matrix (0.55 plate) fractions were resolved by using SDS-polyacrylamide gels (1; Humphrey and Pigiet, in press). Gels were stained with 0.1% Coomassie R250 dye in 10% methanol–10% acetic acid. T antigen, migrating at 100 kDa, was quantified by densitometry with standards amounts of bovine serum albumin (BSA; 68 kDa) or phosphorylase b (94 kDa; not shown) electrophoresed in the same gel. Gels were scanned at 600 nm with a Quick Scan R & D scanning densitometer.

phosphate content of the matrix-bound T antigen was determined by using nuclear matrices solubilized with 1% SDS by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as described above (Fig. 1). T antigen bound to the nuclear matrix had the same phosphate content, 4 mol of PO₄ per mol, as the bulk of the T antigen which was retained in isolated nuclei and subsequently extracted in 2 M NaCl (Table 1). Thus, all species of polyomavirus large T antigen retained in isolated nuclei contained an additional mole of bound phosphate compared with the nucleoplasmic species (Table 1).

Further evidence that this difference in phosphate content between retained and nucleoplasmic classes of T antigen was due to covalently bound phosphate was obtained by analyzing the protein digestion products of ³²P-labeled T antigen in 100-kDa bands excised from acrylamide gels (see above). A limit digest with staphylococcal V8 protease (2) was obtained by using a molar ratio of V8 protease to T antigen of 20:1. Six V8 phosphopeptides were resolved by using an 18% SDS-urea polyacrylamide gel (6) with apparent molecular masses ranging from 20 to 7.5 kDa (Fig. 2). By visual inspection, the 12-kDa phosphopeptide was more heavily phosphorylated in either of the retained T-antigen classes, both STN nuclear extract and nuclear matrix, compared with that in the nucleoplasmic class (Fig. 2).

To determine quantitatively whether this 12-kDa V8 phosphopeptide could account for the overall difference in phosphate content between the retained and nucleoplasmic T-

FIG. 2. Phosphopeptide analysis of polyomavirus large T antigen. Polyomavirus large T antigen, radiolabeled in vivo with ³²P_i (50 μ Ci/ml) as in Table 1, was isolated from cytosol (C), nuclear extract (NE), and nuclear matrix (NM) fractions by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as for Fig. 1. The 100-kDa bands, containing approximately 1 μ g of T antigen, were excised and digested in situ with a 20-fold molar excess (20 μ g) of staphylococcal V8 protease (2), and the resulting peptides were resolved by electrophoresis in an 18% SDS-urea slab gel (6). Six phosphopeptides visualized by autoradiography (cytosol and nuclear extract exposed for 5 days; nuclear matrix exposed for 14 days) had apparent molecular masses of 20, 18, 12, 10, 9, and 7.5 kDa as estimated with prestained molecular mass markers (Bethesda Research Laboratories).

TABLE 2. Phosphopeptide analysis of polyomavirus large T antigen^a

Peptide molecular mass (kDa)	Nucleoplasmic T antigen ^b			Retained T antigen ^b		
	³⁵ S cpm	³² P cpm	³² P/ ³⁵ S ratio	³⁵ S cpm	³² P cpm	³² P/ ³⁵ S ratio
20	130	170	1.3	156	420	2.7
18	208	536	2.6	258	730	2.8
12	306	100	0.3	340	592	1.7
10 + 9	554	568	1.0	798	718	0.9
7.5	400	364	0.9	546	544	1.0

^a Acrylamide gel slices containing approximately 1 µg of large T antigen (100 kDa) radiolabeled for 24 h with [³⁵S]methionine (5 µCi/ml) and ³²P_i (50 µCi/ml) as for Fig. 1 and Table 1 were digested in situ with 20 µg of staphylococcal V8 protease, and the resulting peptides were resolved by electrophoresis in an 18% SDS-urea gel as for Fig. 2. The Radiolabel incorporated in individual V8 peptides was quantified by liquid scintillation counting of excised gel bands.

^b cpm, Counts per minute. Nucleoplasmic T antigen contained a total of 1,686 cpm of ³⁵S and 1,770 cpm of ³²P, with a ³²P/³⁵S ratio of 1.0. Retained T antigen contained 2,098 cpm of ³⁵S and 3,004 cpm of ³²P, with a ³²P/³⁵S ratio of 1.4.

antigen classes, T antigen was isolated by immunoprecipitation from cells radiolabeled in vivo with both [³⁵S]methionine and ³²P_i (see above) and digested with V8 protease. Radiolabel incorporated into individual phosphopeptides from digests of retained and nucleoplasmic T antigens was determined by liquid scintillation counting (see above). The 12-kDa phosphopeptide in the retained class contained six-fold more ³²P radiolabel than did that in the nucleoplasmic class (Table 2). A smaller (twofold) difference in phosphate content was also observed in the 20-kDa phosphopeptide, whereas only minor differences were observed between the other phosphopeptides in either class (Table 2). When total ³²P and ³⁵S counts per minute were summed over all the phosphopeptides in each digest, the retained T-antigen class contained 40% more phosphate than the nucleoplasmic class (Table 2, footnote b), consistent with the data for undigested T antigen (Table 1). The yields of ³⁵S-labeled peptides were equivalent for both classes of T antigen, demonstrating that the 12- and 20-kDa peptides were more heavily phosphorylated in the retained T-antigen class.

This study demonstrated that the polyomavirus large T antigen is differentially phosphorylated. Moreover, the additional phosphorylation associated with the retained T antigen resides within a discrete region of the polypeptide (Table 1; Fig. 2). T antigen bound to the nuclear matrix was phosphorylated to the same extent as T antigen extractable from nuclei, suggesting that all T-antigen species retained in isolated nuclei constitute a single phosphorylated class, as distinct from the loosely associated nucleoplasmic T antigen.

This observation is consistent with our previous finding that all of the T antigen retained in isolated nuclei exists in an association with the nuclear matrix which is stabilized through intermolecular disulfide cross-linking induced in vitro (Humphrey and Pigiet, in press).

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