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Hydroxyproline in the Major Capsid Protein VP1 of Polyomavirus†

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Amino acid analysis of [^{3}H]proline-labeled polyomavirus major capsid protein VP1 by two-dimensional paper chromatography of the acid-hydrolyzed protein revealed the presence of ^{3}H-labeled hydroxyproline. Addition of the proline analog L-azetidine-2-carboxylic acid to infected mouse kidney cell cultures prevented or greatly reduced hydroxylation of proline in VP1. Immunofluorescence analysis performed on infected cells over a time course of analog addition revealed that virus proteins were synthesized but that transport from the cytoplasm to the nucleus was impeded. A reduction in the assembly of progeny virions demonstrated by CsCl gradient purification of virus from [^{35}S]methionine-labeled infected cell cultures was found to correlate with the time of analog addition. These results suggest that incorporation of this proline analog into VP1, accompanied by reduction of the hydroxyproline content of the protein, influences the amount of virus progeny produced by affecting transport of VP1 to the cell nucleus for assembly into virus particles.

The DNA genome of polyomavirus codes for three structural proteins: VP1, VP2, and VP3 (8, 11, 14, 16, 25, 28). Major capsid protein VP1 can be separated into six species, designated A to F, by isoelectric focusing (2, 5, 13, 23, 26, 27). This charge heterogeneity is due at least in part to phosphorylation (1, 2, 5, 13, 19, 26, 27) and sulfation (20). Since the polyomavirus genome does not code for any enzymes capable of modifying its own protein structure, posttranslational modifications, such as the previously reported phosphorylation and sulfation, must therefore be the result of host cell enzymatic activity (19). In addition to contributing to the charge heterogeneity of VP1, these modifications, by imparting negative charges, may also influence the conformation of this protein, thereby playing a role in maintaining the intact virion structure. The divalent cation calcium also appears to be involved in stabilizing the intact virus, as well as being crucial for in vitro reassembly of infectious polyomavirus particles (6–8, 32) and stabilization of purified VP1 (29). Recently, we have localized the site of this calcium association to the carboxyl-terminal portion of VP1 (21). In keeping with our ongoing investigation into the modification of polyomavirus capsid proteins and their function(s) in the virus life cycle, the present report demonstrates that VP1 is also modified by hydroxylation of the amino acid proline. In addition, the use of the proline analog L-azetidine-2-carboxylic acid (LACA), which has been shown to inhibit hydroxylation of proline residues (4, 15, 31), appears to affect polyomavirus progeny assembly following incorporation and subsequent reduction in the hydroxyproline content of VP1.

The primary mouse kidney cells (MKC) used for these experiments were prepared as described previously (24, 30). Wild-type polyomavirus was used to infect cells at a multiplicity of 10, and infected cell cultures were maintained in serum-free Eagle medium. Preparation of radioisotopically labeled virus was accomplished in the following manner. Infected cells were maintained in serum-free Eagle medium containing all essential and nonessential amino acids minus proline and supplemented with L-[2,3,4,5-^{3}H]proline (ICN Radiochemicals, Irvine, Calif.) at a concentration of 10 μCi/ml of medium. Virus was also labeled with [^{35}S]methionine by maintaining infected MKC in serum-free, methionine-free Eagle medium supplemented with [^{35}S]methionine Translabel (ICN) at a concentration of 10 μCi/ml of medium. Infected cells and medium were harvested 3 to 5 days postinfection, and virus was purified as previously described (22). The CsCl gradients used to purify the virus were prepared as described by Brunck and Leick (9) and were described in greater detail previously (7, 8, 33).

The proteins of purified [^{3}H]proline-labeled polyoma virions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 15% acrylamide gel and a 0.2% bisacrylamide cross-linker (8). [^{3}H]proline-labeled VP1 was electroeluted from the gel after localization by autoradiography of a parallel lane containing VP1 labeled with ^{125}I by the chloramine-T method (12). [^{3}H]proline-labeled VP1 was then acid hydrolyzed in 6 N HCl at 105°C for 2 h. Following evaporation of the HCl, the remainder was lyophilized for analysis by ascending two-dimensional paper chromatography with phenol-distilled water (100 g of phenol, 39 ml of distilled water) in dimension 1 and *N*-butanol–acetic acid–distilled water (100:22:50) in dimension 2. In addition to hydrolyzed VP1, the sample contained 10 μg each of proline and hydroxyproline amino acid standards. The results of this analysis are shown in Fig. 1. The autoradiogram of the [^{3}H]proline-labeled VP1 hydrolysate (Fig. 1B) shows ^{3}H radioactivity migrating identically to that of the proline and hydroxyproline standards identified by spraying the filter paper with a solution of 0.2% isatin in acetone and developing the color at 65°C in a humid chamber (Fig. 1A). A densitometric tracing of this autoradiogram was made, and by comparing the individual peak areas with their sum, 90% of the ^{3}H radioactivity was found with the proline standard and 10% was found with the hydroxyproline standard. Similar quantities were obtained in parallel experiments when these amino acid standards were excised, eluted from the paper in distilled water, and scintillation counted (88 and 12%, respectively).

The amino acids proline and hydroxyproline impose conformational restrictions in structural proteins, enzymes, and hormones because of the pyrrolidine ring (3). Hydroxyproline formation has been shown to be a critical step in the biosynthesis of collagen. Procollagen chains that are low in hydroxyproline content cannot form a stable triple helix, and

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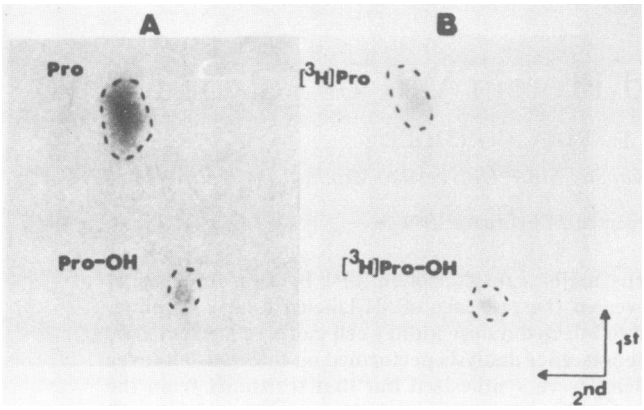

FIG. 1. Two-dimensional paper chromatography of acid-hydrolyzed polyomavirus VP1. (A) Visualization of proline and hydroxyproline amino acid standards (10 μg each) by isatin staining. (B) Autoradiography of acid-hydrolyzed [^{3}H]proline-labeled VP1 (5×10^4 cpm).

as a consequence, transport and eventual secretion may be inhibited (4, 10, 15). To investigate the possible roles of proline and hydroxyproline in the biosynthesis of polyomavirus structural proteins and in progeny virus production, we supplemented the proline-free culture medium used to maintain infected cells with the proline analog LACA. This analog has been shown to substitute for proline in procollagen at substitution ratios ranging from 2 to 40% when added to cell culture medium without detriment to normal cell biochemical processes (17, 18, 31). LACA, which is incapable of being hydroxylated by prolyl hydroxylases, also inhibits hydroxylation of the remaining prolines by altering the structure of the polypeptide backbone (4, 15, 31). In pilot experiments, various concentrations of LACA were tested to determine the amount which would not affect MKC total protein synthesis. When compared with cells that did not receive this analog over a time course of 24 h, a maximum concentration of 25 μg of LACA per ml of culture medium was found to reduce total MKC protein synthesis minimally (15%) as measured by trichloroacetic acid-precipitable counts of [^{35}S]methionine-labeled cells (data not shown). LACA (25 μg/ml) was then added to infected MKC cultures at various times postinfection. Following fixation, these cells were analyzed by indirect immunofluorescence (25) with both rabbit anti-polyomavirus immunoglobulin G (IgG) directed against dissociated purified virions and rat anti-polyomavirus tumor antigen IgG following harvest at 36 h postinfection. This experiment was performed to determine what effect, if any, LACA exerts on the overall synthesis of two polyomavirus-coded proteins, large T antigen and the structural protein VP1, as shown in Fig. 2. Uninfected control cells did not exhibit fluorescence when reacted with anti-polyomavirus IgG (Fig. 2A) or anti-tumor antigen IgG (data not shown). Infected cells demonstrated the same pattern of positive nuclear fluorescence following reaction with anti-polyomavirus tumor antigen IgG regardless of whether LACA was added at 12, 24, or 30 h postinfection (Fig. 2B). An identical fluorescence pattern was observed with infected control cells which did not receive this proline analog (data not shown). The time of LACA addition did, however, appear to affect the pattern of capsid protein fluorescence in infected cells following reaction with anti-

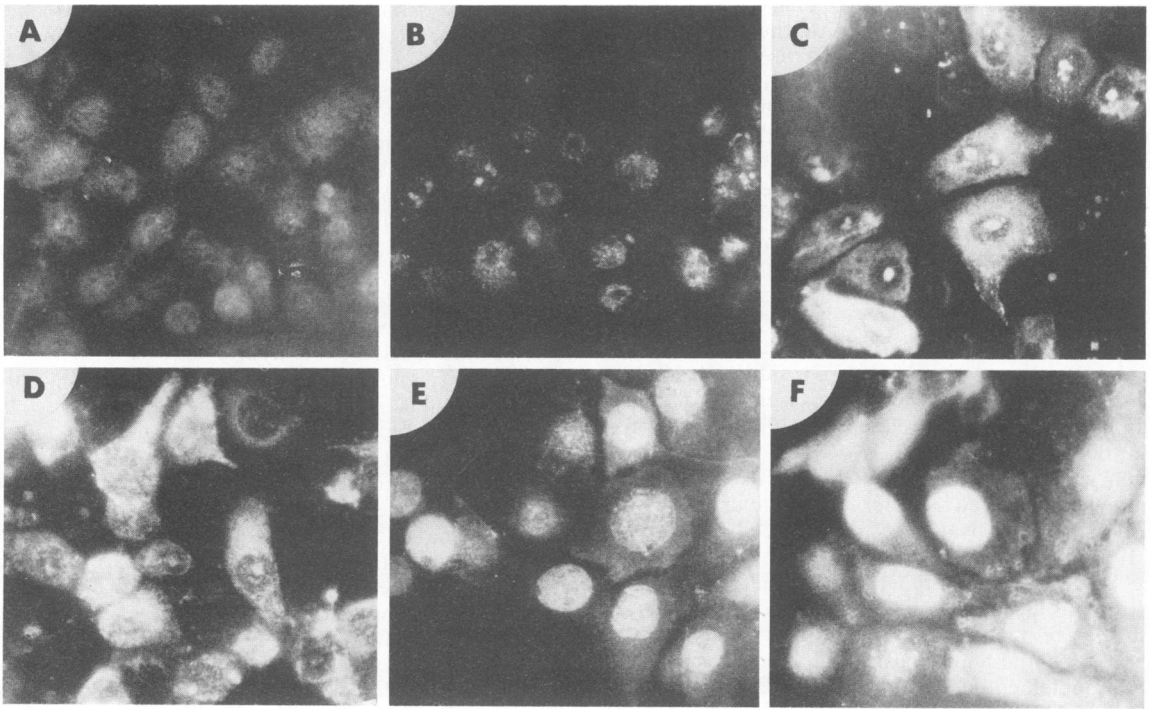

FIG. 2. Immunofluorescence of LACA-treated infected cells. Cells were harvested at 36 h postinfection. The final concentration of LACA was 25 μg/ml of culture medium. Uninfected cells were reacted with rabbit anti-polyomavirus IgG (A). Infected cells were reacted with rat anti-polyomavirus tumor antigen IgG, and LACA was added at 12 h postinfection (B). Infected cells were reacted with rabbit anti-polyomavirus IgG, and LACA was added at 12 (C), 24 (D), or 30 (E) h postinfection. Infected control cells reacted with rabbit anti-polyomavirus IgG with no LACA addition are shown in panel F.

polyomavirus IgG. Cells to which LACA (25 μg/ml) was added at 12 h postinfection (Fig. 2C) showed nucleolar and cytoplasmic fluorescence. When LACA was added at 24 h postinfection, immunofluorescence was observed predominantly in the cell cytoplasm (Fig. 2D). LACA addition to infected MKC at 30 h postinfection revealed nuclear immunofluorescence (Fig. 2E) identical to that seen in untreated infected cells (Fig. 2F).

While the cellular location of structural protein immunofluorescence differed between control infected and early LACA-treated cells as a function of the time of analog addition, the overall positive numbers and fluorescence intensities of LACA-treated and untreated cells were the same. Since LACA slightly reduced overall MKC protein synthesis (15%), we analyzed the infected cells to determine whether VP1 synthesis was affected by the drug. VP1 was immunoprecipitated from both LACA-treated and untreated cells and quantitated by densitometry of autoradiograms resulting from ^{125}I-labeled pansorbin-probed immunoblots. LACA addition to cells at either 12 or 24 h postinfection reduced the amount of detectable VP1 by 12% compared with that in untreated infected cells (data not shown). In addition, experiments were also performed on infected cells that were maintained in medium containing [^{3}H]proline, and these cultures received LACA at either 12 or 24 h postinfection and were harvested at 36 h postinfection. VP1 was isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid analysis was then performed on the isolated VP1 as described for Fig. 1. Scintillation counting of the proline and hydroxyproline spots excised from the chromatograms of two separate experiments revealed only background levels of radioactivity migrating with the hydroxyproline spots; proline radioactivity was reduced 14 and 37%, respecively, compared with untreated infected cell preparations. These data indicate that addition of the proline analog LACA to infected cell culture medium at either 12 or 24 h does not prevent VP1 synthesis as determined by quantitative immunoblots but does prevent or greatly reduce hydroxylation of proline in VP1 as determined by amino acid analysis. Incorporation of the proline analog LACA and subsequent prevention or reduction of proline hydroxylation likely affects proper protein folding and, in turn, influences cellular transport of VP1 to the nucleus.

It was also of interest to determine what effect LACA addition to infected MKC cultures would have on virion assembly. In vivo [^{35}S]methionine-labeled virus was partially purified from infected cell lysate through a sucrose gradient. The 240S peak containing virions was then isolated and further purified through a CsCl gradient (33). A correlation was observed between the amount of virus assembled and the time of LACA addition (Fig. 3). On the basis of the total amount of [^{35}S]methionine contained within the virion peak (fractions 10 to 13) for each of the LACA-treated preparations compared with the untreated infected preparation (Fig. 3D), only 1% of the radioactivity was found after LACA addition at 12 h postinfection (Fig. 3A). When LACA was added at 24 h postinfection, 26% of the radioactivity was present (Fig. 3B), and addition at 30 h revealed 70% of the level of incorporation attained by untreated, infected MKC (Fig. 3D). These data indicate that polyoma virion assembly can be affected by addition of the proline analog LACA to infected MKC culture medium at various times postinfection.

In this report, evidence has been presented that major capsid protein VP1 of polyomavirus is modified by hydroxylation of the amino acid proline (Fig. 1). Perturbation of proline hydroxylation was facilitated by the use of LACA, a proline analog which can substitute for proline in nascent protein chain synthesis. While incapable of being hydroxylated by prolyl hydroxylases, LACA, when present in protein chains, can also inhibit hydroxylation of incorporated proline residues by altering the structure of the polypeptide backbone (4, 15, 31). After addition of LACA to infected MKC culture medium, immunofluorescence (Fig. 2) demonstrated that polyomavirus structural protein synthesis occurred at a level comparable to that of untreated control cells. Addition of this analog to infected MKC culture medium did, however, appear to affect the transport of polyomavirus structural protein VP1 from the cytoplasm, the site of synthesis, to the nucleus, where virus assembly occurs. Incorporation of LACA and subsequent reduction of proline hydroxylation did not drastically interfere with the VP1 synthesis observed by immunofluorescence (Fig. 2) or by immunoprecipitation of infected cells but may have affected the proper folding of VP1 in preparation for transport to the nucleus. The importance of proline hydroxylation for proper protein folding and cellular transport of proteins has been reported for collagen (4, 10, 15). This phenomenon was most evident in our experiments when LACA was

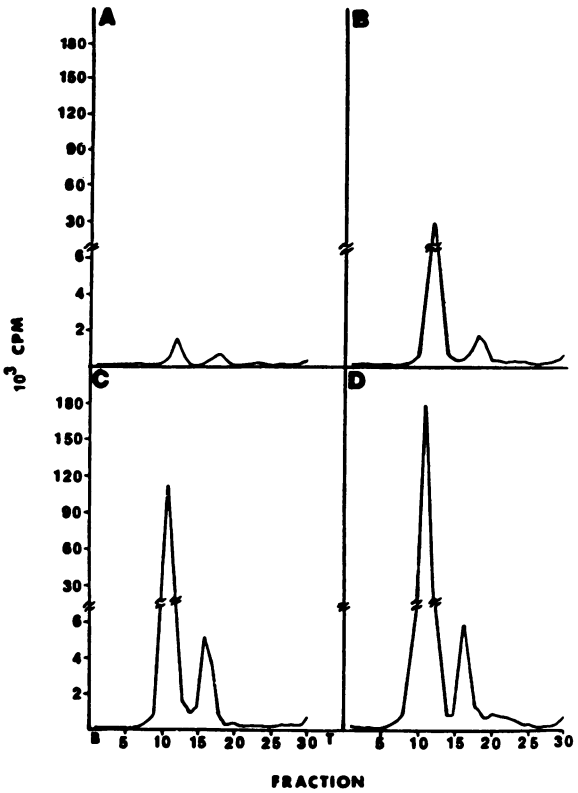

FIG. 3. The effect of LACA on polyomavirus progeny as determined by CsCl gradient ultracentrifugation. Cells were infected in the presence of ^{35}S-labeled methionine, and LACA (25 μg/ml) was added at various times postinfection. [^{35}S]methionine-labeled virus was partially purified through a 10 to 30% sucrose gradient to isolate the peak of 240S virions, which was then subjected to CsCl gradient analysis. The CsCl gradient profiles show virions (fractions 10 to 13) and empty capsids (fractions 15 to 18). LACA was added to infected cells at 12 (A), 24 (B), or 30 (C) h postinfection. Infected control cells (D) which did not receive LACA were used for comparison. B, Bottom of gradient; T, top of gradient.

added at up to 24 h postinfection. With less of the viral structural proteins being transported to the nucleus, one would expect less progeny to be produced. Fewer virus particles were isolated when the analog was added at up to 24 h postinfection (Fig. 3). After 24 h postinfection, addition of LACA resulted in virus protein transport and assembly comparable to those of untreated, infected control cells. This seems reasonable, since the maximum level of virus protein synthesis occurs between 24 and 30 h postinfection. Thus, the proline analog LACA must be present before the onset of maximum viral structural protein synthesis to result in the greatest possible perturbation of VP1 proline hydroxylation. The effect of LACA on the hydroxylation of proline contained within the amino acid sequences of specific cellular proteins was not investigated in this study. Therefore, the possibility that this proline analog perturbs proline hydroxylation in a cellular protein(s) which facilitates transport of polyomavirus structural proteins from the cytoplasm to the nucleus cannot be ruled out. Thus, although VP1 is modified by hydroxylation of proline, incorporation of the proline analog LACA and subsequent reduction of the hydroxyproline content of this protein appear to play a role in the transport of VP1 from the cytoplasm to the nucleus. Studies in which perturbation of a protein modification has an observable biological consequence, as presented here, should provide impetus for further investigation into virus protein transport within host cells.

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