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The Phospholipid Composition of Extracellular Herpes Simplex Virions Differs from That of Host Cell Nuclei

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Enveloped viruses of eukaryotes obtain their membrane by budding through a cellular membrane. Therefore, most frequently the lipid composition of the virion envelope reflects that of the membrane where budding took place. In the case of herpes simplex viruses, nucleocapsids assemble in the nucleus and bud through the inner nuclear membrane. The pathway from the perinuclear space to the extracellular medium is as yet poorly understood. Here we demonstrate that the phospholipid composition of extracellular herpes simplex virions differs from that of nuclei isolated from the infected cells. The viral membrane contains threefold higher concentrations of sphingomyelin and phosphatidylserine. These lipids are typically enriched in the Golgi apparatus and plasma membrane. The data are in agreement with a model in which herpes simplex virus, after budding through the inner nuclear membrane, loses its envelope by fusing with the outer nuclear membrane and obtains a new membrane by budding into a compartment late in the exocytotic pathway, very likely the Golgi apparatus or membranes derived from it. Alternatively, because the perinuclear space is continuous with the ER lumen, the virus after its first budding may be transported through the exocytotic pathway without ever leaving the lumen of the subsequent compartments. In that case, either the virions, while budding through the nuclear membrane select for sphingomyelin and phosphatidylserine, or the original lipids of the viral envelope are exchanged for lipids of an exocytotic membrane, most likely by a transient membrane continuity between the virion and the vesicle by which it is surrounded. Light particles, virus-like particles that lack capsid and DNA but contain tegument and envelope proteins, displayed the same lipid composition as complete herpes simplex virions, suggesting that they also acquired their envelope from a Golgi membrane. © 1994 Academic Press, Inc.

Herpes simplex virus (HSV) envelopment, maturation, and transport to the extracellular space have been the subject of numerous investigations in the past 40 years. Two features are firmly established. First, the nucleocapsids acquire the envelope by budding through the inner nuclear membrane into the perinuclear space (*1;* see *2, 3*). The nuclear membranes carry the immature forms of the viral glycoproteins (*4*) and the virion envelope itself acquires immature glycoproteins (*5*). Second, once they have left the perinuclear space, virions interact with the Golgi apparatus. Evidence for this interaction was provided in immunocytochemical and numerous biochemical studies, showing that virion glycoproteins are converted to the mature type (*5–10;* for reviews, see *11–13*) and by the finding that virion egress is reduced in cell mutants defective in Golgi glycosyl transferases (*14, 15*) or in cells treated with Golgi inhibitors, like monensin (*16*) or brefeldin A (*17, 18*). These studies lead to the view that the transport of virions from the perinuclear to the extracellular space follows the vesicular exocytotic pathway (*16;* see *11*). In some cell lines, virions may interact with Golgi-derived membranes, originating from fragmentation of the Golgi apparatus (*19*).

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The details of the interaction between HSV and Golgi apparatus are not yet known. As discussed elsewhere (*2, 20*), electron microscopic observations have produced a uniform set of micrographs, but divergent interpretations. According to one view, virions maintain their structural integrity, occupy a lumenal position (*2*), and move from the perinuclear space to subsequent Golgi cisternae, after which they are finally released into the extracellular space (*11*). Variations on this model envision that virions may travel through the cell enclosed in vesicles that fuse with Golgi-derived transport vesicles (*5*). This model is supported by the electron microscopic findings that herpes simplex virions in transit to the extracellular space are most frequently detected within vesicular or vacuolar structures, which could represent individual transport vesicles, or be part of the endoplasmic reticulum (ER) or Golgi apparatus (*5, 19, 20*). An entirely different view was proposed in studies on cells infected with a herpesvirus of the frog, and, later, on cells infected with HSV and pseudorabies virus (PRV). Naked nucleocapsids in the cytoplasm juxtaposed to the outer nuclear membrane and to cytoplasmic membranes, or partially wrapped by a bilaminar membrane, were interpreted as evidence that virions, after budding into the perinuclear space, lose their envelope by fusing with the outer nuclear

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membrane, and are released into the cytoplasm (*21*). De-enveloped nucleocapsids would then interact with cytoplasmic membranes (*22*), resulting in re-envelopment, probably at the level of the Golgi (*17*), by a process later shown to be exploited by vaccinia virus (*23*). The weakness of the two models is that they depend on subjective interpretations of static images obtained at the electron microscope (for a discussion see *20*). This is particularly true for the de-envelopment–re-envelopment model, where images of envelopment at the Golgi may also be interpreted as de-envelopment from the Golgi into the cytoplasm to be followed by degradation. An indication for degradation of the cytoplasmic nucleocapsids is that their DNA was eccentric and partially extruded (*17*), whereas the DNA of nuclear capsids and of extracellular virions is concentric and contained entirely within the capsids. In addition, detection of capsids in the process of nuclear envelopment is a very rare circumstance, implying that this must be a very rapid phenomenon. By contrast, the relative ease with which the authors detected cytoplasmic nucleocapsids in the process of re-envelopment would require that cytoplasmic re-envelopment is a much slower and hence different process. Finally, in cells infected with the mutant HSV-1(F)U10, which carries a mutation in the viral glycoprotein D, de-enveloped nucleocapsids accumulated in large number in the cytoplasm and concomitantly the release of infectious virus in the medium was reduced. At least in cells infected with this mutant, cytoplasmic de-envelopment is a final event not followed by re-envelopment (*20*).

The lipids of most viral membranes closely resemble those of the cellular membrane through which virions bud (*24*), and viral membranes have been instrumental in elucidating the lipid composition and organization of cellular (plasma) membranes (discussed in *24–27*) and, conversely, to identify the intracellular sites of virion budding (*23*). HSV incorporates preexisting lipids into their envelope (*28, 29*). To investigate if the envelope of the extracellular herpes simplex virions remains the same as that acquired at the inner nuclear membrane or is modified during the transit from the perinuclear to the extracellular space, we compared the phospholipid composition of extracellular virions with that of the nuclear membranes. The rationale of this approach stems from the notion that the lipid composition of the nuclear membranes, being similar or identical to that of ER, differs from that of Golgi and plasma membranes (*30, 31*). Compared to the nuclear membrane, Golgi membranes typically contain two- to threefold higher concentrations of the phospholipids sphingomyelin (SM) and phosphatidylserine (PS) when expressed as percentage of total phospholipids, while this increase is three- to sixfold in the plasma membrane (*30, 31*). It was expected that the lipid composition of extracellular virions should be similar to that of nuclear membranes, if the envelope is not modified relative to that acquired at the inner nuclear membrane. By contrast, the lipid composition of extracellular virions should differ from that of the nuclear membrane, if the virion envelope acquired at the inner nuclear membrane is modified during passage through the Golgi. We report that the latter is indeed the case and discuss the mechanisms by which the modifications in lipid composition may be induced.

The ^{32}P-phospholipid patterns of HSV and of the nuclear fraction from infected African green monkey kidney (Vero) cells are compared in Fig. 1. The quantitative distribution of label over the various phospholipid classes is given in Table 1. HSV is enriched in SM and PS, mostly at the expense of phosphatidylcholine (PC). In view of the original budding of HSV through the inner nuclear membrane it is rather unexpected to see that the clearest difference exists between the virions and the nuclear fraction. If the difference is expressed as the ratio of the relative contribution of each lipid class in the virions over that in the nuclear fraction, we find a 3-fold enrichment in SM and PS. This is accompanied by a more modest 1.4-fold increase in phosphatidylethanolamine (PE) and decreased levels of PC and phosphatidylinositol (PI). It has been noticed that inner and outer nuclear membranes proliferate and fold in HSV-infected cells (see *2*). The inner and outer nuclear membranes do not appear to differ in their glycoprotein and oligosaccharide content (*5*), and because proteins can diffuse between them it is unlikely that they differ in lipid composition. As they cannot be separated without the use of detergents, we have examined a mixture of the two. In addition, the nuclear fraction was contaminated with cytoplasmic membranes, as detergents had purposely been avoided in cellular fractionation. Therefore, the difference in the figures between the nuclear fraction and the virions very likely represents an underestimate of the actual difference.

To test whether these results were cell-type-dependent, HSV was grown in baby hamster kidney (BHK) cells. Extracellular virions were purified on Ficoll gradients (*32*). As shown in Table 2, the distribution of label over the various phospholipid classes in BHK cells is different from that in Vero cells. PC and PE again account for over 85% of the label, but the relative contribution of SM is much higher and that of PI much lower. However, when the lipid composition of the virus is compared to that of the cellular fractions, the same picture emerges as in Vero cells: the lipid composition of virions differs from that of the nuclear fraction. The (virions/nuclei) ratios are remarkably similar to those in the Vero cells. SM and PS were enriched three times in the virions compared to the nuclear fraction. The same enrichments were obtained in a second experiment on BHK cells, although the incorporation of [^{32}P]orthophosphate into the various phospholipid

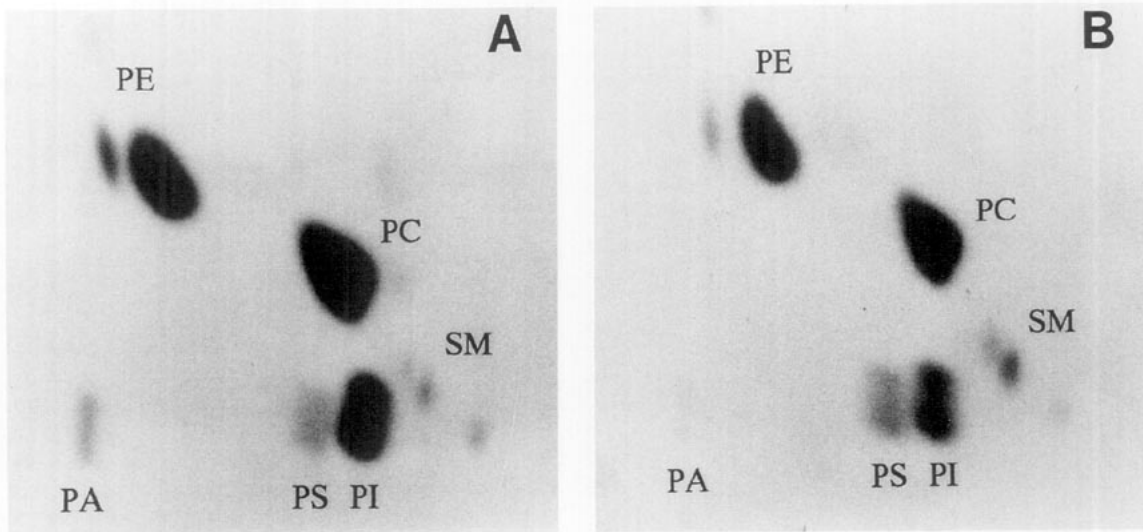

FIG. 1. Thin-layer autoradiogram of ^{32}P-labeled phospholipids of herpes simplex virus (A) and of the nuclear fraction of the infected host cells (B). Vero cells, grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, were labeled for 24 hr prior to infection with 200 MBq [^{32}P]orthophosphate (Radiochemical Center, Amersham)/3 roller bottles, in DMEM with $\frac{1}{10}$ the normal concentration of phosphate. Cells were infected with 3–5 PFU/cell of HSV-1(F) (49) in nonradioactive medium without phosphate. After 1.5 hr of virus adsorption, the inoculum was replaced with the radioactive medium employed for prelabeling; 24 hr after infection, virus was purified from the medium essentially as described (50, 51): medium containing virions was centrifuged at 16,000 *g* for 30 min to remove cell debris. Virions were pelleted by centrifugation for 2 hr at 80,000 *g* in a SW27 rotor, resuspended in phosphate-buffered saline (PBS), layered over a 15–30% Dextran T10 gradient made in 1 m*M* phosphate buffer, pH 7.4, and centrifuged for 2 hr at 65,000 *g*. The virion band was collected by side puncture and diluted in PBS, and virions were pelleted as above. The nuclear fractions were obtained from uninfected and infected cells: 2×10^7 cells were rinsed twice in PBS, allowed to swell for 10 min in 0.5 ml 10 m*M* KCl, 1.5 m*M* $MgCl_2$, buffered with 10 m*M* Tris–HCl, pH 7.4, broken with a Dounce homogenizer, and centrifuged at 1000 *g* for 6 min. The supernatant was subjected to a further centrifugation at 15,500 *g* for 10 min to remove any remaining trapped nuclei. The nuclear fraction was resuspended in 0.25 *M* sucrose in 20 m*M* Tris–HCl, pH 7.8, and centrifuged again over a cushion of 1 *M* sucrose in 20 m*M* Tris–HCl, pH 7.8, for 15 min at 12,500 *g* in an Eppendorf microfuge. Samples of both the nuclear fractions and the postnuclear supernatants were stained with Giemsa and examined under a light microscope. Whereas the nuclear fraction contained some cytoplasmic debris, the postnuclear supernatant was virtually free of nuclei and large nuclear debris. Lipids were extracted, with unlabeled carrier lipid (100 nmol phospholipid from MDCK cells) added before phase separation of the one-phase extraction mixture, separated by 2-D TLC, and located by autoradiography (*26*). Origin: bottom right. First direction, bottom to top: alkaline; second direction, right to left: acidic. SM runs as a double spot due to fatty acid heterogeneity (*52*).

classes was somewhat different (not shown). In both experiments with BHK cells the composition of the postnuclear supernatant was similar to that of the total cells (not shown). In comparison to total cells, the virus contained a 2.5-fold higher concentration of SM and PS. It should be realized that the phospholipid composition of the cells is the mean of membranes with high concentrations of these lipids like the plasma mem-

TABLE 1

^{32}P-PHOSPHOLIPID COMPOSITION OF HERPES SIMPLEX VIRIONS AND INFECTED VERO CELLS

	Infected Vero cells (%)				
	Total cells	PNS	Nuclei	Virions (%)	Ratio virions/nuclei
Sphingomyelin (SM)	1.8 ± 0.2	3.2 ± 0.5	1.1 ± 0.4	3.1 ± 0.4	2.8
Phosphatidylcholine (PC)	58.4 ± 1.1	50.9 ± 0.3	60.5 ± 0.3	51.2 ± 0.7	0.8
Phosphatidylinositol (PI)	10.8 ± 0.1	9.8 ± 0.1	15.6 ± 0.4	11.3 ± 0.6	0.7
Phosphatidylserine (PS)	2.7 ± 0.2	5.0 ± 0.1	1.5 ± 0.2	4.6 ± 0.2	3.1
Phosphatidylethanolamine (PE)	26.3 ± 1.0	31.2 ± 0.6	21.3 ± 0.8	29.8 ± 0.4	1.4

Note. Vero cells were prelabeled with [^{32}P]orthophosphate and infected with HSV, after which extracellular virions, cells, nuclei, and a postnuclear supernatant (PNS) were purified as described in the legend to Fig. 1. The PNS was subjected to a second centrifugation for 6 min at 12,500 *g* in an Eppendorf microfuge before lipid extraction and analysis by TLC. After staining with iodine vapor, the lipid spots were scraped from the plates and radioactivity was counted in a mixture of 0.5 ml Solulyte (J.T. Baker Chemicals, Deventer, NL) and 4.5 ml Ultima Gold (Packard Instrument Co., Downers Grove, IL). Each lipid extract contained over 85 Bq (100%) over a background of 0.3 Bq. Data are the mean of three determinations ± standard deviation.

TABLE 2

COMPARISON OF THE ^{32}P-PHOSPHOLIPID COMPOSITION OF HERPES SIMPLEX VIRIONS, L PARTICLES, AND INFECTED BHK CELLS

	Infected BHK cells					
	Total cells (%)	Nuclei (%)	Virions (%)	Ratio virions/nuclei	L particles (%)	Ratio L particles/nuclei
SM	9.0 ± 0.1	7.1 ± 0.5	24.6 ± 1.4	3.5	23.6 ± 1.5	3.3
PC	65.0 ± 2.6	69.1 ± 2.2	49.3 ± 2.3	0.7	49.8 ± 2.4	0.7
PI	2.7 ± 0.4	2.4 ± 0.6	3.2 ± 0.6	1.3	3.8 ± 0.8	1.6
PS	2.0 ± 0.8	1.8 ± 0.8	4.8 ± 0.8	2.7	5.6 ± 1.3	3.1
PE	21.7 ± 2.0	19.8 ± 1.1	18.2 ± 3.3	0.9	17.1 ± 1.1	0.9

Note. BHK cells, grown in DMEM with 5% fetal calf serum (*5*), were prelabeled with [^{32}P]orthophosphate and infected with HSV, like Vero cells. Virions were purified at 4°C, essentially as described (*29*): medium containing virions was centrifuged at 1000 *g* for 30 min, and virions were pelleted by centrifugation at 80,000 *g* for 2 hr, resuspended in DMEM lacking phenol red, and layered onto a 5 to 15% Ficoll gradient made in the same medium. After centrifugation at 26,000 *g* for 2 hr bands containing virions (heavy) and L (light) particles were collected by side puncture, diluted with the medium, and pelleted by centrifugation (80,000 *g* for 2 hr), gently resuspended in 200 μl PBS, and stored at −80°C. Cells and nuclei were purified, and the lipids analyzed as in Table 1. Data are the mean of three determinations ± standard deviation.

brane, intermediate concentrations like the Golgi apparatus, and membranes that contain very low concentrations like mitochondria and ER including the nuclear membranes (*30, 31*), whereby the relative contributions of these membranes has only been measured for uninfected BHK cells (*33*).

To monitor the extent of purification, a fraction of each radiolabeled virion preparation was processed for electron microscopy prior to lipid analysis. Extracellular virion preparations are frequently contaminated with empty vesicles and what appear to be coreless particles. In the preparations that were analyzed for lipid composition the vesicles and particles together accounted for less than 10% of the virion particles observed. We estimate that under these conditions the contamination will not significantly affect the differences in lipid composition reported in the present study.

L particles are noninfectious structures which resemble normal herpes simplex virions in their tegument and envelope protein content, but do not contain DNA (*32*). To test whether the envelope of L particles differs from that of complete virions, we separated the L particles from the virions by gradient centrifugation and analyzed the lipid compositions. As is shown in Table 2, L particles and complete virions are virtually identical in their phospholipid composition. Both differ in their composition from the nuclear fraction by a threefold increase in SM and PS content.

Changes in lipid metabolism following HSV infection have been reported (*34, 35*). The incorporation of [^{32}P]-orthophosphate and [^{3}H]choline into SM appeared to be especially enhanced. To see whether a change in cellular phospholipid metabolism during infection influenced our compositional data, we compared the phospholipid pattern of infected and uninfected cells. As shown in Table 3, the lipid compositions are fairly similar. A moderate increase in the incorporation of ^{32}P in SM is indeed observed in total cells. However, the lipid composition of the nuclear fraction did not undergo consistent changes.

The analysis reported here shows, both in Vero and in BHK cells, remarkable differences in phospholipid composition between extracellular herpes simplex virions and the nuclear membrane from which they initially obtained their membrane with 3-fold increases in the concentrations of SM and PS, although PC and PE are the major lipids in both membranes (Fig. 1, Tables 1 and 2). The differences are very similar to the differences that have been reported to exist between Golgi membranes and the nuclear membrane/ER. Whereas the composition of the ER has been found to be similar or identical to that of the nuclear membrane, Golgi membranes were reported to possess a 3-fold higher concentration of SM and a 2-fold higher concentration of PS than the nuclear membrane/ER. For the plasma

TABLE 3

LIPID COMPOSITION OF CELLS INFECTED WITH HERPESVIRUS VERSUS CONTROL CELLS

	Ratio infected/control	
	Cells	Nuclei
SM	1.6 ± 0.4	0.9 ± 0.3
PC	1.1 ± 0.2	1.1 ± 0.2
PI	0.6 ± 0.1	0.8 ± 0.2
PS	1.2 ± 0.6	0.8 ± 0.2
PE	1.0 ± 0.2	1.1 ± 0.4

The ^{32}P-phospholipid compositions of infected cells at 24 hr after infection were compared to the ^{32}P-phospholipid compositions of the corresponding uninfected control cells. Uninfected cells were labeled with 14 MBq [^{32}P]orthophosphate per 75-cm^2 flask in DMEM with 1/10 phosphate for 48 hr. Shown are the means of the ratios calculated in three independent experiments, one on Vero cells and two on BHK cells.

membrane, endosomes and lysosomes these increases were 5- and 3-fold, respectively (*30, 31*). As observed for the Golgi, the increase in the concentrations of SM and PS was linked to a reduced concentration of PC (0.8-fold) without consistent changes in the content of PI and PE. It should be realized that the phospholipid distribution may be somewhat different in infected cells, although no increase in the concentrations of SM and PS was observed in the nuclei isolated from infected cells (Table 3) with which the virions were compared (Tables 1 and 2).

Previously, an approach similar to ours was attempted twice, but the techniques employed and the results obtained invalidated the conclusions. In one study (*34*), an inner nuclear membrane fraction was prepared by removing the outer nuclear membrane with Triton X-100. This procedure was later shown to result in the specific extraction of some lipids, notably PC, PI, and PE, and retention of other lipids, notably SM (*36*). Hence, the high SM content of what was defined as "inner nuclear membrane" was very likely a technical artefact. In a later comparison of viral and cellular lipids (*35*), it was reported that nuclei contained more SM and less PC than the postnuclear supernatant in human skin fibroblasts. This is the opposite of what has been consistently found in a variety of cellular systems (reviewed in *31*).

The significance of the current data stems from the implication that the lipid composition of HSV is modified during the transit from the perinuclear to the extracellular space and becomes similar to that of Golgi or of other membranes late in the exocytotic pathway. The evidence available until now indicated that the glycoproteins of herpes simplex virions are processed by interaction with the Golgi apparatus from immature to mature types (*5*; for reviews see *3, 11–13*). This processing may be envisioned as occurring *in trans*, i.e., the glycosidases and glycosyl transferases that reside in the Golgi membrane surrounding the virion act vis-à-vis on the envelope glycoproteins. The remarkable implication of the present findings is that the interaction between the herpes simplex virions and the Golgi-derived membranes is more complex than a simple *in trans* interaction. They impose restrictions onto both models of virion transport discussed above and allow narrowing and better focusing of the possibilities opened by previous findings. First of all, the data would fit the model in which the virus loses its original membrane by fusing with the outer nuclear membrane and obtains a new membrane by a second budding event. The lipid compositions predict that budding would occur into a Golgi-related membrane. When fitting the present data into the model where the virus always remains situated in the lumen of the exocytotic pathway during transit to the extracellular space, the following possibilities can be envisaged.

(i) The striking difference in the concentration of the phospholipids SM and PS between extracellular virion envelopes and nuclear fractions virtually rules out the possibility that extracellular virions carry an unmodified lipid membrane relative to that acquired at the inner nuclear membrane. Although, in principle, the virus could bud through specialized patches of the inner nuclear membrane with a lipid composition different from the bulk of the nuclear membrane, analogous to hypothesized lipid microdomains in the Golgi (*37*), the magnitude of the differences makes this explanation unlikely (cf. *24*).

(ii) The results indicate that the HSV envelope undergoes modifications during the virion transit to the extracellular space. A mechanism that is made unlikely by our results is that the lipid composition of the virus is modified by an exchange of lipid monomers between the virion envelope and the Golgi-derived membranes in the absence of any fusion event. It is hard to imagine how the high PS concentration found in the virion envelope could result from such a mechanism, as PS appears to be selectively concentrated in the cytoplasmic leaflet of the membranes along the secretory pathway (*38*). An interesting comparison can be made with the observation (*39*) that the surface of lipoprotein particles passing through the lumen of the Golgi contained five times less PS than the surrounding Golgi membrane.

(iii) Efficient exchange between the virus and Golgi-derived membranes is likely to occur if the virion envelope becomes transiently continuous with the membrane of the carrier vesicle/vacuole by means of a fusion event: The virus could transiently fuse with the surrounding membrane. "Micro" fusion events between the viral envelope and its carrier vesicle might occur temporarily in very limited areas of the virion–vesicle complex. Such fusion events are plausible inasmuch as the virion as well as the carrier vesicle/vacuole is covered with the viral glycoproteins (*5*), some of which have fusion activity. Thus, the viral glycoproteins D, B, and H are implicated in virus-induced cell fusion and in virus entry (*40–44*), and, in addition, syncytial mutations were mapped to at least four other regions of the HSV-1 genome, namely gK, gL, U_L20, and U_L24 (*45–48*).

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