JOURNAL OF VIROLOGY, May 2007, p. 4429–4437 Vol. 81, No. 9
0022-538X/07/$08.00+0 doi:10.1128/JVI.02354-06

Herpes Simplex Virus Infection Induces Phosphorylation and Delocalization of Emerin, a Key Inner Nuclear Membrane Protein[▽]

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Received 27 October 2006/Accepted 2 February 2007

The inner nuclear membrane (INM) contains specialized membrane proteins that selectively interact with nuclear components including the lamina, chromatin, and DNA. Alterations in the organization of and interactions with INM and lamina components are likely to play important roles in herpesvirus replication and, in particular, exit from the nucleus. Emerin, a member of the LEM domain class of INM proteins, binds a number of nuclear components including lamins, the DNA-bridging protein BAF, and F-actin and is thought to be involved in maintaining nuclear integrity. Here we report that emerin is quantitatively modified during herpes simplex virus (HSV) infection. Modification begins early in infection, involves multiple steps, and is reversed by phosphatase treatment. Emerin phosphorylation during infection involves one or more cellular kinases but can also be influenced by the US3 viral kinase, a protein whose function is known to be involved in HSV nuclear egress. The results from biochemical extraction analyses and from immunofluorescence of the detergent-resistant population demonstrate that emerin association with the INM significantly reduced during infection. We propose that the induction of emerin phosphorylation in infected cells may be involved in nuclear egress and uncoupling interactions with targets such as the lamina, chromatin, or cytoskeletal components.

The nuclear envelope is composed of a double lipid bilayer, the inner and outer nuclear membranes (INM and ONM), and is underpinned on the nucleoplasmic side by the nuclear lamina, a dense meshwork of intermediate filaments formed from interlaced dimers of the lamins A/C and B. Herpes simplex virus (HSV), like all herpesviruses, replicates and packages its genome into newly formed capsids inside the nucleus of infected cells. Progeny nucleocapsids, with a size of 100 nm, are too large to pass through nuclear pores, which have a gating mechanism for soluble proteins and assemblies through an aqueous channel with a diameter of about 9 nm (reviewed in references 1, 42, 55, and 56). The mechanism by which HSV exits the nucleus remains a matter of controversy, but it has been generally accepted that a primary pathway of exit is via nucleocapsid attachment to the INM and subsequent budding into the lumenal space, thereby acquiring a primary lipid envelope (reviewed in references 13, 32, and 51). However, we currently have limited understanding of the modifications to the INM and lamina, the interactions between these components, and the mechanism involved in different stages during exit from the nucleus.

The ONM is a continuation of the endoplasmic reticulum, whereas the INM has a unique composition and contains specific resident proteins, including the lamin B receptor, the LEM-domain proteins emerin, MAN1, and lamin-associated polypeptides (LAPs), and nurim (6, 10, 11). Many more INM proteins have recently been identified (45, 46). Although the precise routes of localization are not known in all cases, one mechanism by which these proteins are thought to localize is by a diffusion and retention mechanism at the INM via interactions between their exposed nucleoplasmic domains and the underlying lamina and/or chromatin components (15, 23, 39, 44, 52).

We have previously reported changes at the INM after HSV infection, exemplified by altered diffusional mobility of the lamin B receptor and dissociation of a population of lamin A/C from the lamina (47). Alterations in nuclear structure, the nuclear lamina, and the INM were also reported in subsequent studies (4, 43, 49, 50) and have also been reported during cytomegalovirus (CMV) replication (36). Analysis of the localization of green fluorescent protein-lamin fusion proteins during infection (47, 50) demonstrated that such alterations in the lamin components were not the result of epitope masking as had been suggested earlier (43) but resulted from loss of a population of lamins. Although the function of many of the INM proteins remains to be fully described, emerin is emerging as an important factor in maintaining nuclear membrane integrity (2, 61).

Emerin is a 254-residue type II transmembrane protein with its single transmembrane domain located 11 amino acids from its carboxy-terminal luminal tail (3, 30, 39). Emerin belongs to a family of nuclear proteins defined by the conservation of a ca. 40 residue motif termed the LEM domain (28). Emerin binds directly to the lamina proteins lamin A/C, an interaction thought to be important in its recruitment to the INM (7, 14, 53, 57). Emerin also binds via the LEM domain to the essential nuclear protein BAF (barrier to autointegration factor), a major structural protein, essential for coordinating chromatin structure and regulating gene expression (16, 25, 31, 48, 58). The interaction with BAF is also thought to be involved in emerin recruitment during nuclear envelope reformation after mitosis (18). Several other partners have recently been described, including transcription regulators, actin, and the spec-

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▽ Published ahead of print on 14 February 2007.

trin repeat domain proteins, nesprins (2, 21, 22, 24, 27, 35, 60). Emerin interaction with actin has also been reported to enhance actin polymerization and has led to the proposal that emerin could be involved in stabilizing actin at the nuclear membrane (2, 61). Emerin is also known to be phosphorylated in a cell cycle-dependent fashion (12, 19) and, while the relevant kinases remain to be identified, such modification is critical in the mitotic compartmentalization and restructuring of the nuclear envelope and may regulate its interaction with BAF and/or lamins and other partners at the INM.

Here we continue our analysis of alterations to the INM and nuclear envelope components during HSV replication and show that emerin is quantitatively modified during infection. HSV infection induces multiple novel phosphorylated emerin species, and this is mainly due to the activity of one or more cellular kinases, although US3 may influence modification directly or indirectly. Further results from biochemical extraction analysis and from immunofluorescence after detergent treatment demonstrate that emerin association with the INM is altered during infection. This is the first demonstration of a specific posttranslational modification to a key INM protein, and the results are discussed in the light of a possible role of emerin in nuclear membrane integrity, alteration of which may be involved in virus association with or transit through the INM.

MATERIALS AND METHODS

Tissue culture. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% newborn calf serum at 37°C in a 5% CO_2 environment. Infections were routinely performed at a multiplicity of infection (MOI) of 10 PFU/cell in DMEM containing 2% newborn calf serum. Cells were incubated for 1 h in a depleted volume of medium to allow adsorption of virions, after which the medium was topped up to 3 ml. At this stage, phosphonoacetic acid (400 μg/ml), and kinase inhibitors were added if required. For incorporation of [^{32}P], cells were infected as indicated in 1 ml of phosphate-free DMEM containing 5% whole DMEM. After adsorption, the medium was topped up to 3 ml with phosphate-free DMEM containing 5% whole DMEM and including 150 μCi of [^{32}P]orthophosphate, and infection continued for the times indicated. The strains of virus used were HSV-1 (strain 17) and a variant (8) of strain 17 lacking UL13 (ΔUL13) and wild-type (wt) HSV-2 (strain 186), a US3 deletion mutant of strain 186 (ΔU_S3), or a rescue mutant of ΔU_S3 (U_S3-R) as previously described (38).

Cell lysate preparation. Cells were washed in ice-cold phosphate-buffered saline (PBS) (containing 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and Complete EDTA-free protease inhibitor cocktail tablets [Roche Diagnostics]), lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT], 10 nM okadaic acid, and Complete EDTA-free protease inhibitor cocktail tablets [Roche Diagnostics]), and incubated on ice for 5 min. Samples were vortexed, the cell debris was pelleted in a refrigerated microfuge, and the supernatant was retained.

Immunoprecipitation and Western blotting. Immunoprecipitations were carried out at 4°C. Cell lysates were diluted 1:4 in RIPA buffer, and 200 μl of the diluted lysates was precleared with 10 μl of protein G-Sepharose beads for 1 h at 4°C. Precleared soluble extracts were incubated with antibody overnight, and immunocomplexes bound to 20 μl of protein G-Sepharose beads for 2 h. The beads were then washed four times with 1 ml of RIPA buffer, and bound proteins were lysed in 200 μl of sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 7.0], 2% SDS, 5% 2-mercaptoethanol, 3% glycerol, and bromophenol blue). Equal quantities of samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and either Western blotted onto nitrocellulose membranes or stained and dried for autoradiography. Anti-emerin mouse immunoglobulin G1 (IgG1) from Novocastra was used at a 1:1,000 dilution.

Phosphatase assays. Cells lysate was prepared as before, but we omitted the phosphatase inhibitors. A total of 100 μl of phosphatase buffer (Tris 50 mM [pH 8.0], 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 μM Zn_2SO_4, 1 mM DTT, and Complete EDTA-free protease inhibitor cocktail tablets [Roche Diagnostics]) was added to 20 μl of lysate, followed by incubation with 1 U of PP1 (Upstate, Ltd.) at 37°C. The reactions were quenched by the addition of an appropriate amount of 4× SDS sample buffer.

Immunolocalization. For immunolocalization studies, cells were plated and transfected on 16-mm borosilicate glass coverslips placed in 35-mm dishes. Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min, rinsed, and permeabilized where indicated in PBS containing 0.5% Triton X-100 (TX-100). The coverslips were then blocked in PBS containing 10% fetal calf serum for 10 min, followed by incubation for 20 min with the different primary antibodies diluted in blocking buffer as follows: anti-emerin antibody, 1:200 (Novocastra); anti-lamin B1 antibody, 1:100 (Oncogene Sciences); and anti-LAP2 1:200 (BD). After three 5-min washes in PBS, bound antibodies were detected by using TRITC (tetramethyl rhodamine isothiocyanate)-conjugated secondary antibodies. Coverslips were mounted in Mowiol (Sigma).

For analysis of retention at the nuclear rim, cells were extracted prior to fixation. For this process, after being washed with ice-cold PBS, the cells were incubated on ice for 5 min in extraction buffer (HEPES 10 mM [pH 7.9], 80 mM KCl, 16 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 30% glycerin, 0.5% TX-100, and Complete protease inhibitor tablets). Cells were then further processed and analyzed for immunofluorescence as described above. Images were routinely acquired by using a Zeiss LSM 410 confocal microscope with a Plan-Apochromat ×63 oil immersion objective lens, NA 1.4, and zoom factors ranging from 1 to 8 of the LSM 410 acquisition software.

Cell Fractionation. Mock-infected or HSV-1-infected cells (plated in 60-mm dishes) were harvested at 8 h or 16 h postinfection (p.i.), washed in ice-cold PBS, and scraped into 1 ml of PBS, and the cells were pelleted at 250 × *g* for 2 min at 4°C. Cell pellets were then fractionated as described previously (29). Briefly, the cells were lysed on ice in 200 μl of a high-salt detergent-containing buffer (HB; 2% Triton X-100, 40 mM HEPES [pH 7.5], 300 mM NaCl, 5 mM DTT, 10% sucrose, 1 mM EGTA, Complete protease inhibitor, and 50 mM β-glycerophosphate). The lysates were centrifuged at 12,000 × *g* for 15 min at 4°C to separate the detergent-soluble and pellet fractions. Soluble supernatants were transferred into fresh tubes, and pellet fractions were resuspended in 200 μl of HB. Samples were denatured with 1 volume of 2× SDS loading buffer, boiled for 3 min, and sonicated prior to separation by SDS-PAGE and immunoblotting as described above.

RESULTS

Emerin modification during HSV-1 infection. Emerin is a 254-amino-acid protein of the LEM domain class (i.e., conserved in LAP2, emerin, and MAN1, see Fig. 1) of INM proteins that may play a key role in nuclear membrane integrity and the proper maintenance of the INM structure and function (for reviews, see references 2 and 63). Here we extend our analysis of the INM during HSV infection, examining the effect of infection on emerin modification and localization.

In the first series of experiments, cells were infected with HSV-1 (MOI = 10), total cell extracts were prepared at various intervals, and equivalent amounts were then examined for the presence of emerin by SDS-PAGE and Western blot analysis with a monoclonal anti-emerin antibody (Fig. 1). In uninfected cells emerin migrated as a major band of ca. 34 kDa (Fig. 1b, lane M), which on shorter exposures or by running on optimal conditions on different percentage gels (Fig. 1e, lane M) could be resolved into two species, labeled U_0 and U_1, of approximately equal abundance. These species are denoted by solid arrows in the figures. In longer exposures, a third species, U_2, could also be seen in uninfected cells.

Infection induced the appearance of several novel emerin species, the most obvious being a species migrating at about 39 kDa and labeled I_2, observed at between 4 and 8 h and accumulating thereafter. (For ease of reference, species observed in uninfected cells are given the prefix Ux, and those observed in infected cells are denoted by the prefix Ix.) At approximately

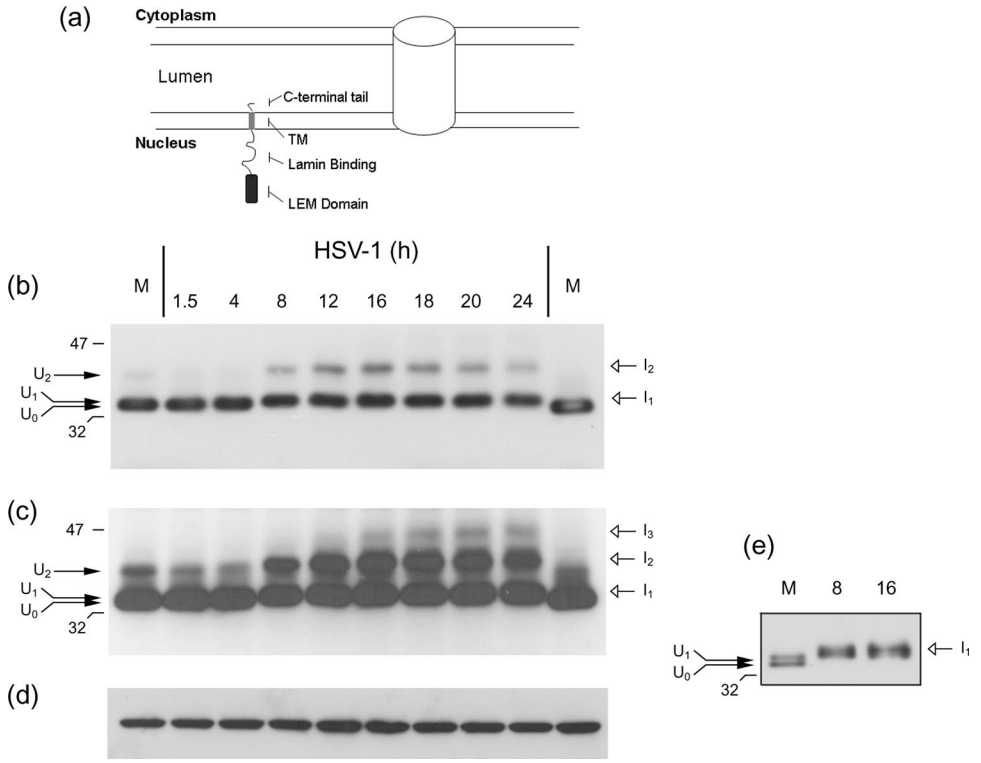

FIG. 1. Emerin modification during HSV1 infection. (a) Schematic summary of emerin features illustrating the N-terminal region, including the Lem domain disposed toward the interior nucleoplasmic face, the single transmembrane domain, and the short C-terminal tail orientated within the intralumenal space. (b) HeLa cells were infected with HSV-1 (MOI = 10), lysed at the times indicated and proteins resolved by SDS-PAGE. Emerin isoforms were analyzed by Western blotting with a monoclonal anti-emerin antibody. Isoforms present in uninfected cells, denoted U_x, are indicated by solid arrows. Novel isoforms induced in HSV-infected cells are denoted I_x and indicated by open arrows. A longer exposure of the same Western blot is shown in panel c. (d) The same lysates were probed with an anti-lamin B1 antibody. (e) Separation of emerin isoforms after electrophoresis in gradient (4 to 10%) SDS-PAGE gels. Identical experimental conditions were used except that infections were performed in Hep2 cells, and shorter exposures were used to optimize separation of the novel forms. Emerin could be readily observed as a doublet (U_0/U_1) in mock-infected cells that was shifted completely to a higher molecular weight (I_1) after infection.

the same time, it was also evident that the migration of the main species had also shifted in infected cells to a form we labeled I_1. The shift to I_1 could be readily seen by loading an uninfected sample adjacent to the later time points (Fig. 1b). This novel form was particularly easy to demonstrate and resolve by using lower loadings of samples after infection of Hep2 cells (Fig. 1e). Finally, on longer exposures, we also observed a further very-low-abundance species in infected cells (Fig. 1c, I_3). Virtually all of the emerin population in infected cells, the majority isoform being I_1, was shifted by approximately 1 kDa (to 35 kDa), with none of the initial doublet U_0/U_1 remaining (Fig. 1e). Both virus-induced isoforms I_1 and I_2 were detectable from relatively early time points in virus infection and were still induced under conditions in which virus DNA replication was inhibited (in the presence of phosphonoacetic acid [data not shown]), indicating that emerin modification is an early event during replication.

Although the exact precursor-product relationship between these forms is not established by these observations, it is tempting to speculate that a modification resulting in a 1- to 2-kDa migration shift converts the uninfected isoforms U_0/U_1 to I_1 and probably isoform U_2 to I_2.

HSV infection induces emerin phosphorylation. Emerin is known to be phosphorylated during nuclear disassembly and reassembly in mitosis (12), and we next sought to examine whether the modifications induced during HSV infection were, as we suspected, due to emerin phosphorylation. We first examined whether the modifications were reversible by phosphatase treatment, and then we sought to determine whether we could demonstrate that the novel forms were indeed phosphospecies by in vivo labeling studies with radiolabeled orthophosphate. We note that although during immediate solubilization in denaturing buffers for SDS-PAGE the emerin isoforms were reasonably stable, solubilization in nondenaturing buffers for further analyses frequently resulted in the loss of modified forms. Although this was suggestive of labile modifications such as phosphorylation, it also made certain analyses difficult.

Nevertheless, to investigate this aspect, infected and mock-infected cells were solubilized in RIPA buffer (lacking any phosphatase inhibitors) and then treated with protein phosphatase 1 (PP1) at 37°C. PP1 was chosen since previous studies have demonstrated emerin dephosphorylation by this phosphatase (12). As indicated above, this type of analysis was hindered by the lability of the emerin modifications such that in control experiments, incubation at 37°C without any phosphatase inhibitors, but without any additional phosphatase added, was sufficient to result in significant loss, particularly of

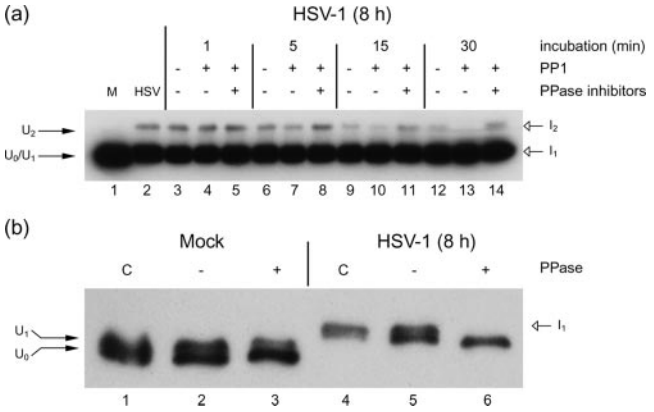

FIG. 2. Emerin modification is reversed by phosphatase treatment. Soluble lysates were prepared from mock-infected (M) or infected cells (8 h p.i.) as described in Materials and Methods. (a) Aliquots of the soluble lysates were incubated without (−) or with (+) PP1 in the absence or presence of phosphatase inhibitors as indicated at 37°C for either 1, 5, 15, or 30 min as indicated. (b) Soluble extracts made in the absence of phosphatase inhibitors were incubated without or with a mixture of PP1 and alkaline phosphatase (PPase) at 37°C for 30 min. After phosphatase treatments, samples were analyzed by Western blotting and immunoprobing for emerin. Control lysates (C) were denatured directly in SDS sample buffer. Short exposures demonstrate the shift of U_0/U_1 to the I_1 form and the shift by phosphatase treatment to higher mobility forms. Note that despite the combined use of PP1 and alkaline phosphatase, the I_1 form was not completely returned to the isoform seen in mock-infected cells.

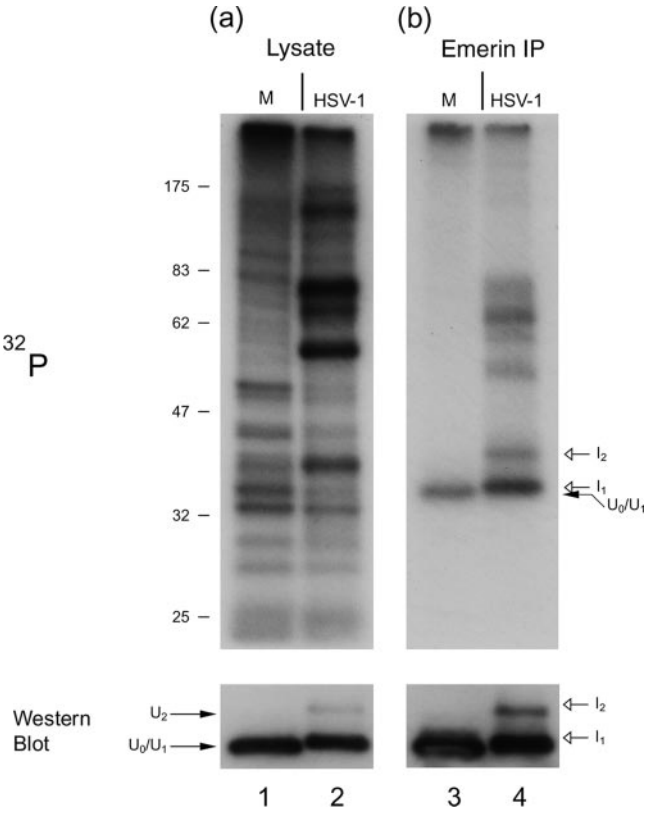

FIG. 3. In vivo [^{32}P]orthophosphate radiolabeling of emerin isoforms. Mock-infected or HSV-1-infected cells were radiolabeled with [^{32}P]orthophosphate beginning at 1 h p.i and harvested at 16 h p.i. Samples were prepared as described in Materials and Methods. Total samples from mock-infected and infected lysates (a) or immunoprecipitates from soluble extracts using anti-emerin antibody (b) were then analyzed by SDS-PAGE and autoradiography. The upper panels are autoradiographs, whereas the lower panels are Western blots from identical gels with probed with anti-emerin antibody. The results confirm the presence of the U_0/U_1 isoforms and the novel I_1 and I_2 forms as phosphorylated species.

I_2 (Fig. 2a, c, and f, lanes 2 and 12). This notwithstanding, we examined the effect of added PP1 on the emerin isoforms in the presence or absence of PP1 inhibitors. The results indicate that incubation with PP1 caused greater loss of I_2 than control incubations (cf. lanes 6, 7, 9, 10, 12, and 13) and that inclusion of phosphatase inhibitors during the incubation protected against the PP1-induced loss and to some degree against the intrinsic loss of the modified forms (cf. lanes 10 and 11 and lanes 13 and 14).

We also examined the effect of phosphatase treatment on the migration of the additional isoforms (using higher-resolving-gradient gels), in this case with a combination of PP1 and alkaline phosphatase. Again, incubation alone of the infected soluble extract caused a loss of some of the I_2 form, resulting in the appearance of a doublet, with the lower form comigrating with cellular U_1 isoform (Fig. 2b, cf. lanes 4 and 5). Inclusion of the phosphatases resulted in complete conversion to the lower form of this doublet. Interestingly, however, the phosphatase treatment did not result in complete conversion of I_1 back to U_0, indicating that this novel HSV-induced isoform is resistant to these phosphatases (or formally that another modification could be involved).

These results indicate that the most likely explanation for the novel isoforms induced during HSV infection was from the induction of novel emerin phosphospecies. To confirm this, we next examined in vivo phosphorylation by radiolabeling with [^{32}P]orthophosphate. HeLa cells were mock infected or infected with HSV-1 (MOI = 10) and incubated in medium supplemented with 10 μCi of [^{32}P]orthophosphate/ml. Extracts were then prepared and analyzed by immunoprecipitation and autoradiography and by Western blotting (Fig. 3). The total profiles of ^{32}P-labeled proteins from mock-infected and infected cells are shown in panel a, and the anti-emerin immunoprecipitates are shown in panel b. In mock-infected cells, the anti-emerin antibody immunoprecipitated a band which migrated at the position of the U_0/U_1 doublet (Fig. 3, lane 3). Although, due to limits in resolution of ^{32}P-labeled bands, we could not readily assign the phosphorylated band specifically to one or the other of the U_0/U_1 species (even with better resolving conditions), the result indicates that emerin is constitutively phosphorylated. In HSV-infected cells the shift in migration to the I_1 species was observed, and this species was phosphorylated as expected (Fig. 3b, cf. lanes 3 and 4). Qualitative comparison of the total amount of emerin in uninfected and infected cells from the immunoprecipitates (Fig. 3, bottom panel) with the abundance of the phosphospecies (Fig. 3, top panel) indicates that I_1 was relatively more highly labeled than U_0/U_1. The I_2 species was clearly also a phosphoprotein (Fig. 3b, lane 4). Moreover, whereas I_2 was significantly less abundant, as judged by Western blotting, than the U_0/U_1 band (cf. bottom panel, lanes 1 and 2), it was present at about equal levels as judged by ^{32}P incorporation. This result indicates also

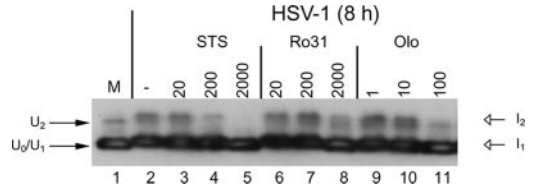

FIG. 4. Examination of the effect of inhibitors on HSV-induced emerin phosphorylation. Replicate cultures of HSV-1-infected cells were supplemented with different kinase inhibitors at various concentrations at 4 h p.i. Cells were harvested 8 h p.i., lysed, and analyzed by Western blotting with anti-emerin antibody. The inhibitors shown here were STS and the PKC inhibitor Ro31-7549 (Ro-31), with concentrations indicated in nM, and the cdc2 inhibitor olomoucine (Olo) concentrations, with concentrations indicated in μM.

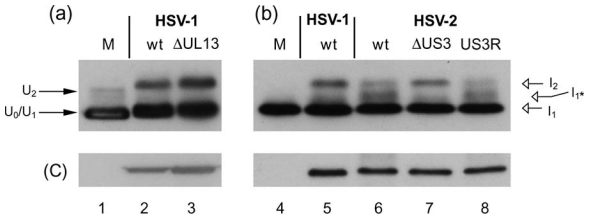

FIG. 5. Examination of the involvement of HSV kinases UL13 and US3 in emerin modification. (a) Emerin modification was compared in cells mock infected (M) or infected with wt HSV-1 (strain 17) or a strain 17 deletion mutant lacking UL13 (ΔUL13). Cells were harvested at 16 h p.i. and processed as described in the text. (b) Cells were infected with wt HSV-2 (strain 186), a US3 deletion mutant of strain 186 (ΔUS3), or a rescue mutant of ΔUS3 (US3-R) harvested at 16 h p.i., and emerin modification was examined as described in the text. (c) As controls for the relative progress of infection, lysates were probed with anti-VP5 antibody.

that although I_2 is a less-abundant species, it is more highly phosphorylated, again consistent with its slower migration.

Taken together, all of these results provide strong evidence that HSV infection induces modification of the majority of emerin and that this modification is due, at least in part, to phosphorylation at multiple sites in the protein.

Effect of kinase inhibitors on HSV-induced emerin phosphorylation. Emerin is known to be phosphorylated by cellular kinases in a cell cycle-dependent manner (12). The phosphorylation of emerin in infected cells could be due to endogenous cellular kinase(s) or a virus-encoded kinase or both. Although the cellular kinases(s) involved in emerin phosphorylation have not been identified, as a first approach we examined the effect of various kinase inhibitors on emerin phosphorylation in HSV-infected cells. The amino acid sequence of emerin revealed reasonable consensus sequence matches for protein kinase A (PKA), PKC, and cdc2 kinase. We therefore initially examined the broad spectrum inhibitor staurosporine (STS), the classical/novel PKC inhibitor Ro-31-7549 (Ro-31), the cdc2 inhibitor olomoucine, and the PKA inhibitor KT5720. Cells were infected and treated with a range of concentrations of each inhibitor at 4 h p.i., before the onset of significant emerin phosphorylation, and harvested at 8 h p.i. Each compound was used at approximately 0.1×, 1×, and 10× the respective 50% inhibitory concentrations (IC_{50}) of the most sensitive target kinase. STS reduced emerin phosphorylation at its IC_{50} (Fig. 4, lane 4) and had a pronounced inhibitory effect at the 10× concentration. Although this is consistent with a kinase activity being involved in emerin modifications the broad-spectrum action of STS does not aid target identification. Of the other more specific inhibitors, Ro-31 had only a minor effect (Fig. 4, lanes 6 to 8), whereas KT5720 had no detectable affect, even at the 10× IC_{50} (data not shown). A modest but consistent effect was observed with olomoucine, wherein the appearance of the infected cell form I_2 was selectively inhibited at concentrations between 10 and 100 μM (Fig. 4, lanes 9 and 11). This effect of olomoucine was explored further in the context of virus mutants. Although there appeared to be a nonspecific effect of inhibitors on the I_1 form, this was not observed in other assays.

Emerin phosphorylation is mainly due to a cellular kinase. We next investigated whether emerin phosphorylation was due to cellular kinase activity and whether either of the two viral encoded kinases UL13 and US3 might play a role. To confirm emerin phosphorylation in HSV-2-infected cells and examine the possible involvement of US3, we examined a US3-ve virus in the background of HSV-2 (38). Infection with an HSV-1 mutant virus (8) lacking UL13 kinase activity (ΔUL13) resulted in an emerin phosphorylation profile virtually identical to that of wt HSV-1 (Fig. 5a). In the case of the US3-ve mutant in HSV-2, we first noticed that the parental HSV-2, while clearly also inducing emerin modification, resulted in a profile that was subtly different from that of HSV-1. In HSV-2, the slower-migrating I_2 form was less pronounced, and instead we observed the accumulation of an intermediate form labeled I_1* (Fig. 5, cf. lanes 5 and 6). Although we have no precise explanation, this subtle difference was highly reproducible and has a bearing on a potential involvement of US3. Thus, in the US3-ve mutant, the profile of emerin species was altered and, if anything, now resembled that seen in HSV-1, with relatively more abundant I_2 species and the I_1* disappearing. (Fig. 5, cf. lanes 6 and 7). The differences between HSV-1 and HSV-2 and between HSV-2 and its US3-ve mutant were not quantitative ones due, for example, to a difference in the relative progression of infection, and indeed the pattern seen in the US3-ve virus was seen to revert to that of the parent in the separate analysis of the US3 rescuant (Fig. 5b, cf. lanes 6 to 8). These results indicate that US3 is not required for the appearance of the I_2 form of emerin in infected cells, but they indicate that US3 could play some modulatory role directly or indirectly on I_2 modification by cellular kinases (see below).

One possibility to explain these results was that the presence of US3 modulated the spectrum of one or more kinases that may be involved in emerin phosphorylation. Although olomoucine had a modest effect on emerin phosphorylation, we compared its effects on the emerin profile seen with wt HSV-2 versus the US3-ve mutant (Fig. 6). In untreated samples, the shift in ratio of the I_2 and I_1* forms in wt virus versus US3-ve-infected cells was again clearly visible (Fig. 6, cf. lanes 2 and 6). For wt HSV-2, olomoucine progressively inhibited the appearance of the I_2 form (Fig. 6, lanes 2 to 6). Interestingly, the I_1* form was relatively resistant and, if anything, there was a relative increase in the production of this form in the presence of olomoucine. (This difference in relative sensitivity was confirmed in additional experiments and scanning densitometry of the best-resolved gels [data not shown].) Although speculative, it is possible that there is a relationship between I_2 and I_1* in

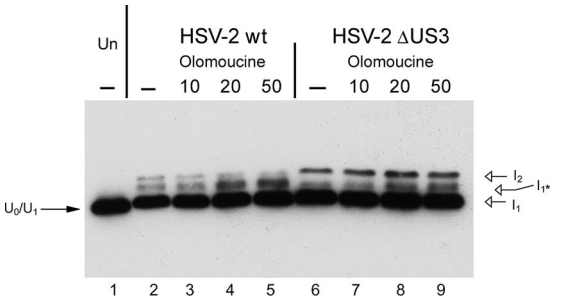

FIG. 6. Comparison of the effect of olomoucine on emerin phosphorylation in the presence or absence of US3-ve cells. Cells were infected (MOI = 10) with HSV-2 (lanes 2 to 5) or HSV2 US3-ve (lanes 6 to 9) strains and incubated with various concentrations (10, 20, or 50 μM) from 4 to 8 h p.i. Cells were then harvested, lysed, and analyzed by Western blotting with anti-emerin antibody.

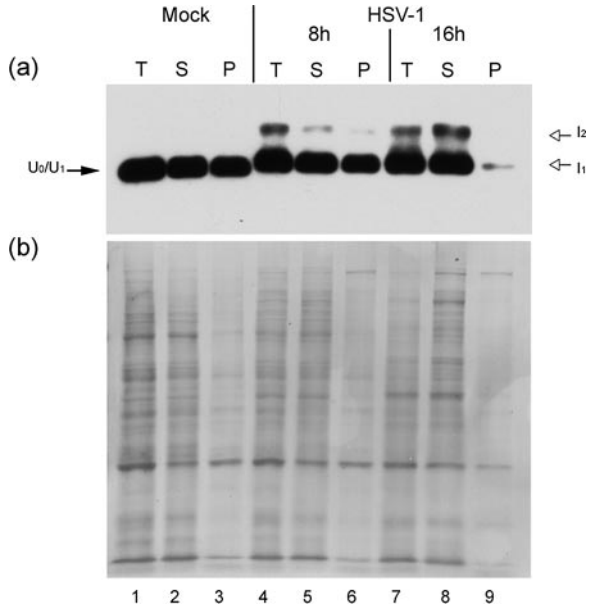

FIG. 7. Effect of infection on nuclear association of emerin. Cells were mock infected (Mock) or infected (HSV-1), and cells were harvested at 8 or 16 h and lysed in an extraction buffer containing 0.3 M NaCl and 0.5% Triton X-100. Aliquots were retained as total lysates (T), and the samples were separated by centrifugation into a supernatant (S) and nuclear pellet (P). (a) Equal cell equivalents from each sample were then separated by SDS-PAGE and probed for emerin. (b) As a loading control, the same membrane was stained for total protein. With this fractionation, most of the total protein partitioned in the soluble fraction, whereas emerin partitioned equally between the soluble and pellet fractions. Between 8 and 16 h p.i., there was a clear difference in fractionation, with little emerin remaining in the pelleted form at the later time.

that if the phosphorylation event yielding I_2 was antagonistic to the event yielding I_1*, inhibition of I_2 might yield more of the I_1* form (see Discussion).

Somewhat surprisingly, however, we observed a difference in the olomoucine sensitivity of the I_1 and I_2 forms in wt HSV-2 versus the US3-ve mutant. As indicated above, in the absence of drug the I_2 form was more prominent in the US3-ve mutant but, in contrast to this form in wt virus-infected cells, this species in the US3-ve virus-infected cells was relatively resistant to olomoucine (Fig. 6, cf. lanes 2 to 5 and lanes 6 to 9). Possible explanations for these results are given in the Discussion.

HSV induces alterations in emerin localization at the INM. Emerin is known to be tightly bound in the INM, anchored by multiple interactions with the lamina and other nuclear components (2, 18, 25). Thus, a significant population of emerin is highly resistant to detergent extraction in biochemical fractionation analysis (12). We next examined the effect of infection on emerin nuclear association first by fractionation into detergent-resistant and detergent-soluble populations and then by immunolocalization either with or without prior detergent extraction of the cells in situ.

Mock-infected and infected cells (MOI = 10 PFU/cell), harvested at 8 or 16 h p.i., were extracted as described in Materials and Methods. Equal cell equivalents of the soluble or pelleted material were separated by SDS-PAGE and immunoblotted for emerin or stained for the total protein profile (Fig. 7). About 50% of the emerin remained bound in the nuclear-detergent-resistant population (Fig. 7a). This did not significantly change at the 8-h time point. However, by 16 h p.i. we found that most of the emerin species in infected cells were now in the detergent-soluble form (Fig. 7a). We observed no obvious difference between isoforms in this increased extraction into a soluble form. Controls showed approximately equal protein loading within each of the pellet fractions (Fig, 7, lanes 3, 6, and 9).

To reveal possible differences in INM anchoring or localization in infected cells, we also examined emerin by immunofluorescence in cells that were treated with or without detergent before fixation. (In this case, the detergent treatment was less harsh to preserve cells attached to the coverslips. Nevertheless, distinct differences in emerin localization could be observed in infected cells compared to uninfected cells.) Thus, in the absence of detergent extraction (Fig. 8a) the levels of emerin were modestly reduced by 8 h p.i., and by 16 h p.i. increased granularity and aggregates or punctae were observed. Loss of emerin and alteration in distribution were more readily detected after detergent extraction (Fig. 8b, low and high magnifications). There was a reproducible reduction in the nuclear-detergent-resistant emerin by 8 h p.i., which was very obvious by 16 h (arrows), accompanied by recruitment of emerin into numerous punctate foci (Fig. 8b, cf. mock and HSV at 8 and 16 h).

DISCUSSION

A feature common to all proposed models of exit of HSV nucleocapsids from the nucleus is budding through the INM (5, 13, 26, 33, 34, 59). Nevertheless, we currently have limited information on the details of alterations to the INM components and underlying lamina during infection, and the possible involvement of cellular proteins in the exit of nucleocapsids from the nucleus.

Emerin is a ubiquitously expressed, single transmembrane protein, which is anchored in the INM with its N terminus projecting into the nucleoplasmic side and its short C terminus projecting into the lumen. Emerin interacts with a number of partners, including the A-type and B-type lamins, the chroma-

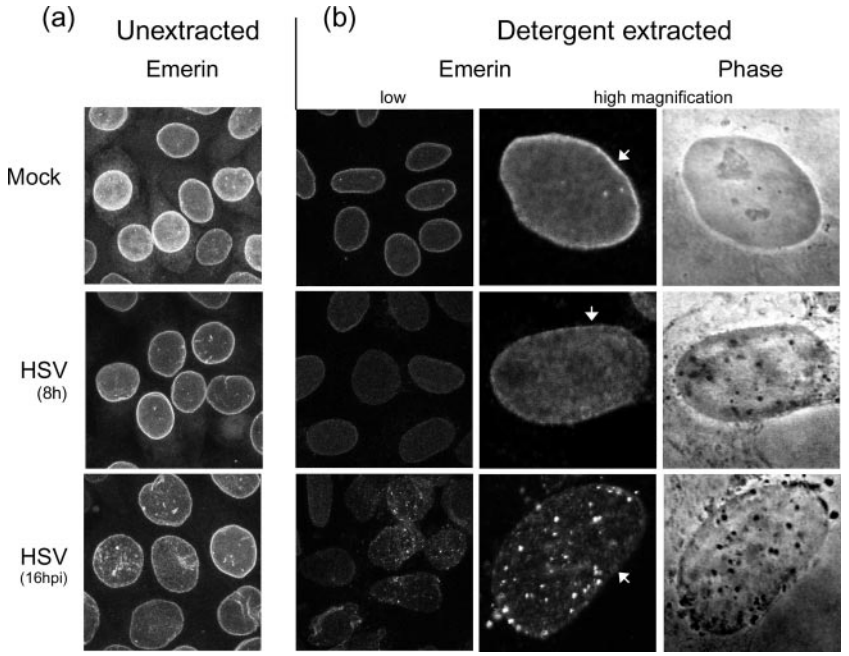

FIG. 8. Altered localization of emerin in HSV-infected cells. Cells plated on coverslips were mock infected (Mock) or infected with HSV-1 (MOI = 10) and at 8 or 16 h p.i., washed in PBS, and fixed directly in 4% PFA (left-hand panels, unextracted) or first extracted with 0.5% TX-100 in the cold prior to fixation with PFA as described in Materials and Methods (right-hand panels, detergent extracted). Endogenous emerin was detected with emerin antibody, and the location was analyzed by laser scanning confocal microscopy using a Zeiss LSM410. Images were obtained by using identical microscope settings in all cases except that, for higher-magnification images of typical cells with corresponding phase-contrast images, an increase in the zoom factor was used in the LSM software.

tin component BAF (for barrier to autointegration factor), several nuclear envelope proteins including nesprin-1α, actin, and a number of transcriptional or splicing regulators (2, 21, 61). It has been suggested that an emerin-nucleoprotein complex stabilizes the nuclear membrane against mechanical stress (14) and that via these multiple interactions emerin plays an important role in nuclear membrane integrity (for a review, see reference 2).

Here we demonstrate that HSV infection induces the quantitative modification of emerin resulting in the appearance of several novel emerin phosphospecies. We also show that the tightly anchored, detergent-resistant population of emerin is significantly altered during infection.

Our results indicate that HSV induced emerin phosphorylation is carried out by one or more cellular kinases but that US3 might have a modulatory role in the process. This conclusion stems from the observation that when phosphorylation during HSV-1 and HSV-2 infection are compared, the ratio of specific emerin phosphospecies differed and that for HSV-2 the altered pattern depended upon the presence of US3. A second line of evidence for an involvement of US3 comes from the observation of altered patterns of sensitivity to the inhibitor olomoucine that are dependent upon the presence of US3.

Although the precise nature of the involvement of US3 requires further clarification, what is clear is that infection very efficiently induces emerin phosphorylation, that this is mainly by cellular kinases, that the profile of specific species differs in ratio between HSV-1 and HSV-2, and that US3 influences phosphorylation. We do not know why the profile of the emerin phosphospecies appears to be different for parental HSV-1 and HSV-2 viruses. We note though that, while sequence conservation of US3 would make it unlikely that the gene has fundamentally different roles, nevertheless, we have found with an anti-US3 antibody that US3 appears to be significantly more abundant during HSV-2 infection compared to HSV-1. This could affect the relative contributions of US3 in HSV-1 versus HSV-2 emerin phosphorylation.

Although it is known that emerin is phosphorylated in a cell cycle-dependent manner in vivo (12), an event that is implicated in efficient dismantling of the nuclear envelope during mitosis, neither the target sites nor the identity of the kinase(s) responsible have been identified. Olomoucine is thought to be a selective inhibitor of cdc2 kinase, and so partial inhibition by olomoucine could reflect a contribution by cdc2 in emerin phosphorylation in infected cells. Previous work has indicated that cdc2 is involved in phosphorylation of another INM protein, the lamin B receptor, in a cell cycle-dependent fashion (37, 54). However, a potential cdc2 site within emerin (S49) is rather weak, is not conserved in (for example) murine emerin, and overlaps with the consensus PKA site. Moreover, from our current work, it appears that any potential contribution from cdc2 would be a minor one. The identity of the relevant kinases in infected cells, the mutation of candidate sites, and how phosphorylation relates to the cell cycle-dependent emerin phosphorylation will be the subject of future analysis.

Previous work examining CMV replication has implicated a cellular kinase in nuclear exit, in this case playing a role in the disruption of the lamina, by lamin phosphorylation (36). The authors of that study indicated that PKC may be involved and reported that PKC was recruited to the INM and that lamins B

and A/C were phosphorylated by 24 h after infection. Furthermore, a recent report indicates that HSV-1 infection induces the recruitment of PKCδ and, to a lesser extent, PKCα to the nuclear rim and that both PKC and lamin B are phosphorylated in infected cells (41). We have examined PKC location using similar methods but find little evidence for significant PKC recruitment to the INM, although we note that the previous report indicates that PKC staining was in general relatively light and recruitment could not be observed in a significant subpopulation of cells. We also found that unlike the case reported for lamin B phosphorylation, the PKC specific inhibitor Ro-31 had little effect on emerin phosphorylation, even at doses 10-fold greater than its IC_{50}. Obtaining definitive evidence for the identity of a specific kinase from broad classes can be difficult. Future work using both in vitro and in vivo analyses will be directed at identifying the kinase(s) involved.

What is clear is that emerin is phosphorylated very efficiently during infection from relatively early time points. In addition to lamin interactions, recent results have indicated possible roles of emerin phosphorylation in the interaction with nuclear actin (21, 24) and with the chromatin-associated protein BAF, where in vitro phosphorylation on S175 appears to affect binding interaction (19), and these interactions could therefore be the subject of phosphorylation-dependent modulation in infected cells. Moreover, one of the strongest emerin binding partners is the large INM protein nesprin-1α (35, 63), which binds to both the nucleoplasmic partners emerin and the lamins (27, 40) and also interacts with the Sun domain class of proteins (20). This interaction is thought to bridge the inner and outer nuclear membranes (9, 17, 62). Intriguingly, depletion of these factors results in alterations in the luminal anchoring of the INM, causing expansion and convolution of the lumenal space, alterations which also occur during HSV replication.

In summary, we provide the first evidence for a direct virus-induced modification of an INM protein. Our results indicate that most of the population of emerin is phosphorylated during infection, and emerin binding at the INM-lamina complex is disrupted. We believe that modification of emerin, which coordinates multiple interactions between the lamina, INM, lumen, and ONM could be directly involved in the alterations observed by microscopy analyses and contribute to access and egress at the stage of nuclear exit.

ACKNOWLEDGMENTS

We thank Yukihiro Nishiyama for provision of the HSV-2 viruses and advice and Duncan McGeoch for supplying the HSV-1 UL13-ve virus. We thank Kathy Wilson for the kind of provision of reagents and advice.

This study was funded by Marie Curie Cancer Care and by a Wellcome Trust International Fellowship to H.H.

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