Delinquency, and Violence in the Dunedin Longitudinal Study (Cambridge Univ. Press, Cambridge, 2001).

39. We thank P. Silva, founder of the Dunedin Multidisciplinary Health and Development Study, Air New Zealand, and the study members, their families, and friends. Supported by the Health Research Council of New Zealand, the University of Wisconsin Graduate School, and by grants from the U.K. Medical Research Council and the U.S. National Institute of Mental Health (MH49414, MH45070). The study protocol was approved by the institutional review boards of the participating universities.

Supporting Online Material
www.sciencemag.org/cgi/content/full/297/5582/851/DC1
Materials and Methods
Tables S1 and S2

27 March 2002; accepted 27 June 2002

Cytomegalovirus Recruitment of Cellular Kinases to Dissolve the Nuclear Lamina

Walter Muranyi,[1]* Jürgen Haas,[1] Markus Wagner,[1] Georg Krohne,[2] Ulrich H. Koszinowski[1]†

The passage of large-sized herpesviral capsids through the nuclear lamina and the inner nuclear membrane to leave the nucleus requires a dissolution of the nuclear lamina. Here, we report on the functions of M50/p35, a β-herpesviral protein of murine cytomegalovirus. M50/p35 inserts into the inner nuclear membrane and is aggregated by a second viral protein, M53/p38, to form the capsid docking site. M50/p35 recruits the cellular protein kinase C for phosphorylation and dissolution of the nuclear lamina, suggesting that herpesviruses target a critical element of nuclear architecture.

Viral genes can target and abuse specific cellular functions for generating a favorable environment for virus maintenance and spread. Well-known examples are viral gene functions for immune evasion from host cell defense or capsid movement within host cells (*1, 2*). To identify new viral functions that target or divert cellular functions, we addressed an important step during morphogenesis: the exit of the newly formed virus capsid from the nucleus. Because the size of herpesvirus capsids (~100 nm) prevents their transport through the nuclear pore complex (NPC), egress requires the penetration of the nuclear envelope (NE) (*3*). Local duplications of the nuclear membrane and patches containing wrapped viral capsids have been observed in cells infected with cytomegalovirus (CMV), which belongs to the subgroup of β-herpesviruses (*4, 5*). However, the inner nuclear membrane (INM) is not easily accessible; it is lined and stabilized by the nuclear lamina (NL) layer, which constitutes an orthogonal filamentous protein meshwork 20 to 80 nm deep. This meshwork is a barrier to capsid budding, but is only dissolved during mitosis by phosphorylation with specific kinases. Here, we present evidence that a virus gene product with homology to resident INM proteins can breach this barrier by recruiting cellular kinases that act to dissolve the obstacle.

In CMV-infected cells, modifications of the NE can be detected by an irregular, sometimes ruffled staining for lamins and a non-uniform lamin-associated polypeptide 2β (LAP2β) staining, as well as by deformation of the nucleus (*4, 5*). To identify the viral gene(s) responsible for this phenotype, we subcloned a series of candidate open reading frames (ORFs) of murine CMV (MCMV) into a eukaryotic expression vector and then screened the ORFs for this phenotype in transient assays. This approach led to the identification of M50, which is predicted to be a type II transmembrane protein 35 kD in size (p35) (Fig. 1A). M50/p35 showed a rim staining, typical for proteins of the INM such as lamins and LAP2β (Fig. 1, B to E, I, J, N, and O). Moreover, in both transfected and CMV-infected cells, M50/p35 was localized in membranous cytosolic structures occasionally containing lamin B. Remarkably, cells expressing only M50/p35 in the absence of any additional viral protein showed substantial alterations of the NE, characterized by exclusion of lamins A, B, and C as well as LAP2β in areas of the INM where M50/p35 accumulated. A similar phenotype was observed after expressing UL50, the homologous protein encoded by human CMV (HCMV) (*6*). Cells were costained for M50/p35 and propidium iodide to test whether the induced phenotype represented a proapoptotic event. In M50/p35-positive cells, no chromatin condensation (Fig. 1, K to M) and no staining for terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) (*6*) were detected 24 hours after transfection, excluding apoptosis. Nonetheless, the M50/p35-induced phenotype is probably incompatible with normal cellular functions, as we failed to isolate stable transfectants. Remarkably, the extent of the M50/p35-induced alterations of the NE differed from those seen in CMV-infected cells (Fig. 1, F to H).

In infected cells, M50/p35 was concentrated in distinct, smaller aggregates associated with the NE. In addition, cytosolic M50/p35 positive structures were less frequent, smaller, and more uniform in size. We therefore tested whether additional viral proteins modulate the M50/p35 distribution to induce the infection phenotype. This led to the identification of M53/p38. Whereas M53/p38 is diffusely distributed within the nucleus if expressed alone (Fig. 2, D to F), in cells cotransfected with M50/p35 and M53/p38 the localization of both viral proteins was changed, resulting in a phenotype indistinguishable from infection (Fig. 2, G to I). The direct interaction of the two proteins was confirmed by coprecipitation (Fig. 2J). Thus, we propose that M50/p35 strongly modifies the NL, whereas M53/p38 qualitatively and quantitatively modulates this effect.

The morphological stability of the NE critically depends on the integrity of the NL. Depolymerization of the NL preceded by a phosphorylation of lamins is required for the major architectural changes of the NE during mitosis (*7*). Phosphorylation and dephosphorylation of lamins and proteins of the INM by different kinases at distinct sites regulate the dynamic properties of the NL during interphase and mitosis (*8–11*). In a cell cycle–dependent manner, NL dissolution during mitosis requires the sequential phosphorylation by protein kinase C (PKC) during the interphase and by $p34^{cdc2}$ during mitosis (*12*). We thus tested whether kinases are also involved in NL dissolution during MCMV infection. By immunofluorescence we examined different kinases for a redistribution to the NL in MCMV-infected cells. Among the kinases tested, only Ca-dependent PKCs were found to be redistributed to the NE by viral proteins (*13*). Because the distribution of Ca-dependent PKC during MCMV infection correlated well with the localization of M50/p35, we hypothesized that M50/p35 recruits Ca-dependent PKCs to the INM. In cells transiently transfected with M50/p35 and M53/p38, Ca-dependent PKCs were recruited to the NE (Fig. 3, A to C). In MCMV-infected cells, M50/p35 colocalized with M53/p38 and Ca-dependent PKC (Fig. 3, G to I) but

[1]Genzentrum and Max-von-Pettenkofer Institut, Ludwig-Maximilians-Universität München, 80336 München, Germany. [2]Division of Electron Microscopy, Biocenter of the University of Würzburg, 97074 Würzburg, Germany.

*Present address: Abteilung Virologie, Hygiene-Institut, Ruprecht-Karls-Universität Heidelberg, 69120 Heidelberg, Germany.

†To whom correspondence should be addressed. E-mail: koszinowski@m3401.mpk.med.uni-muenchen.de

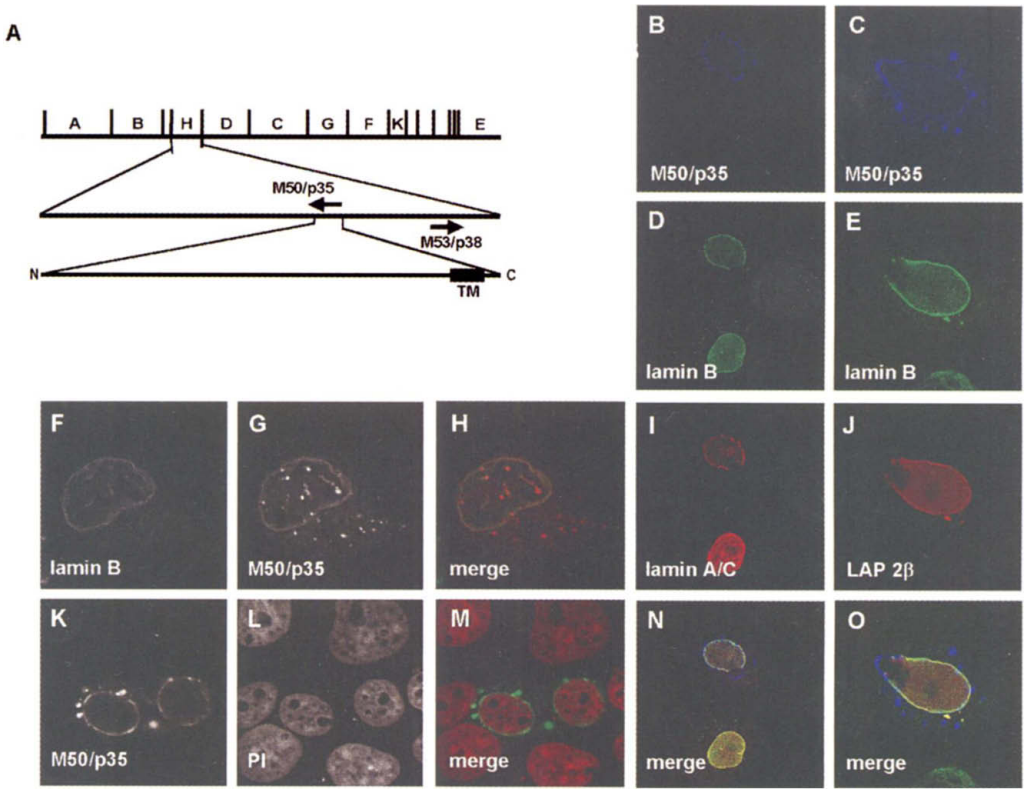


Fig. 1. M50/p35 is responsible for MCMV-induced NL alterations. (**A**) Schematic diagram of the M50/p35 localization within the MCMV genome. Top line, Hind III cleavage map of the 230-kb MCMV genome. Second line, position and transcriptional direction of M50/p35 and M53/p38 within the Hind III fragment H. Third line, M50/p35 with amino- (**N**) and carboxyl terminus (**C**) as well as the transmembrane region (TM) (*28*). (**B** to **E**, **I**, **J**, **N**, and **O**) Isolated M50/p35 expression alters the NL. CV-1 cells transfected with a plasmid expressing immunoglobulin-tagged M50/p35 were subjected to triple staining with antibodies to the Ig-tag of M50/p35, lamin B, lamin A and C, or LAP2β 2 days after transfection (*29*). M50/p35 localized to the NE, and concentrated at sites of herniation from which lamins and LAP2β were largely excluded. (**F** to **H**) M50/p35 is localized at the NL in MCMV-infected NIH 3T3 fibroblasts. NIH 3T3 cells infected with MCMV at a multiplicity of infection (MOI) of 0.5 were stained 24 hours after infection with antibodies to M50/p35 and lamin B. M50/p35 localized at the NE in infected cells. (**K** to **M**) Expression of M50/p35 does not induce apoptosis. CV-1 cells transiently expressing M50/p35 were stained with propidium iodide (PI) to visualize chromatin 2 days after transfection. M50/p35-positive cells show no chromatin condensation as a sign for apoptosis.

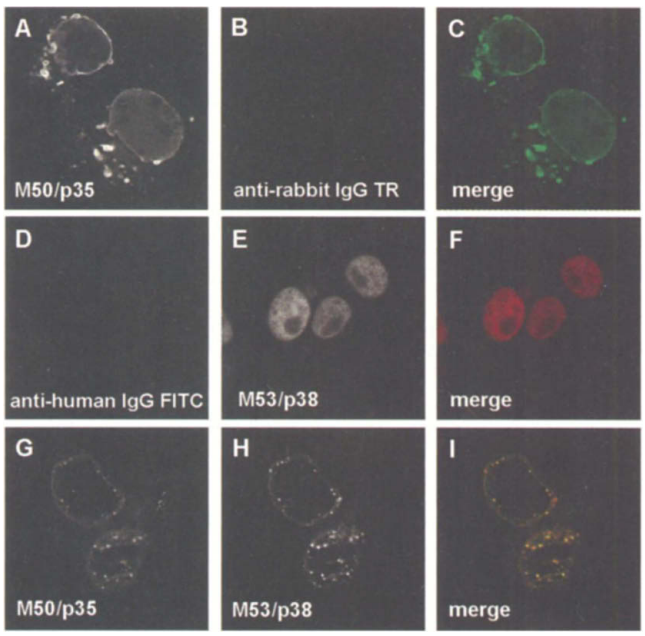
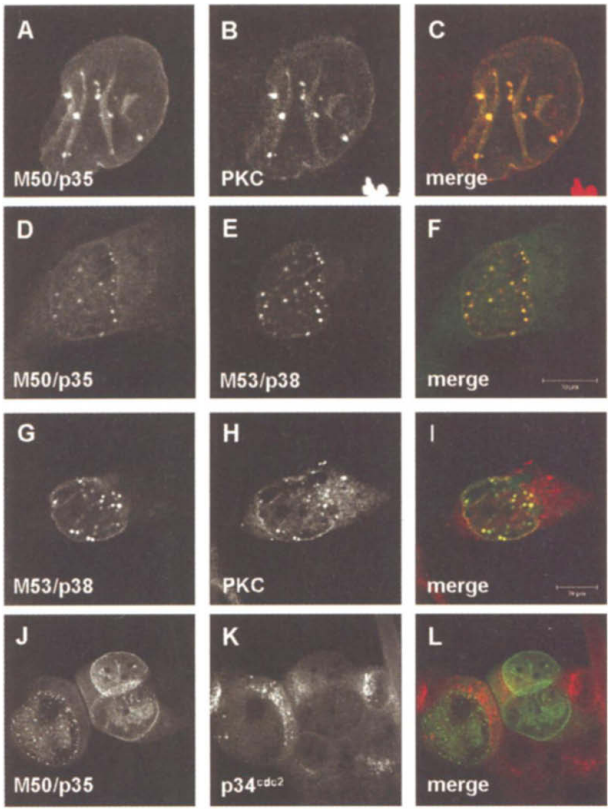


Fig. 2. Coexpression of MCMV M50/p35 and M53/p38 induces NL alterations comparable to infection. 293 cells were transfected with either Ig-tagged M50/p35 (**A** to **C**), FLAG-tagged M53/p38 (**D** to **F**), or Ig-tagged M50/p35 and FLAG-tagged M53/p38 (**G** to **I**) and stained with anti-human Ig and anti-FLAG antibodies. M53/p38 is diffusely distributed in the nucleus in cells expressing M53/p38 alone, but colocalizes with M50/p35 at the NE in small structures of uniform size in cells transfected with both viral genes. (**J**) M50/p35 directly interacts with M53/p38. Coimmunoprecipitation of M50/p35 with M53/p38 in cell lysates from transiently transfected 293 cells. 293 cells were transfected with plasmids expressing either the Ig tag alone, Ig-tagged M53/p38, or FLAG-tagged M50/p35. Lysates of metabolically labeled cells were precipitated either with protein A– sepharose alone (lanes 1, 3, 4) or an anti-Flag antibody and protein A– sepharose (lane 2) and separated by SDS-PAGE on a 10% gel.

not with the mitotic kinase known to phosphorylate lamins, $p34^{cdc2}$ (Fig. 3, J to L). In time course experiments, Ca-dependent PKC was still evenly distributed throughout the cytoplasm after 6 hours of infection (*13*). However, after 16 and 24 hours, Ca-dependent PKC could be detected at the nuclear rim and in perinuclear spherical structures.

Next, we tested whether MCMV-induced kinase recruitment resulted in an augmented phosphorylation of lamins in infected cells. Cells infected with MCMV for 20 hours and mock-infected cells were pulse-labeled with [^{32}P]orthophosphate, lysed, and precipitated with antibodies against lamin A and C (*14*). In MCMV-infected cells, a factor of 2.5 increase of phosphorylated lamin A and C was detected, whereas the total amount of lamin A and C was not altered (Fig. 3M). Lamin B could not be tested in these cells owing to the lack of a precipitating antibody. However, in human fibroblasts infected with HCMV, a factor of 4 increase of phosphorylated lamin B_2 as well as a factor of 2.5 increase of phosphorylated lamin A and C was detected, indicating that the virus-induced increase of lamin phosphorylation is conserved among the β-herpesvirus subgroup (Fig. 3N). This was further supported by an inhibition of CMV-induced lamin phosphorylation by the inhibitor Ro-31-7549 specific for Ca-dependent PKCs (Fig. 3, M and N). During mitosis,



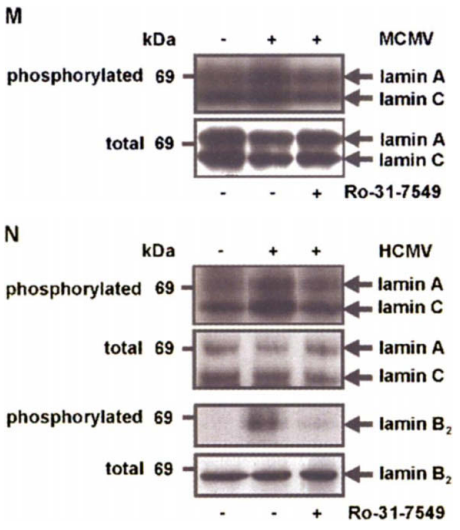


Fig. 3. M50/p35 recruits Ca-dependent PKCs to the INM and induces a lamin phosphorylation. (**A** to **C**) M50/p35 colocalizes with PKC in 293 cells transfected with M50/p35 and M53/p38. Cells were stained with antibodies to the Ig-tag of M50/p35 and Ca-dependent PKC. M53/p38 colocalizes with M50/p35 (**D** to **F**) and PKC (**G** to **I**) in MCMV-infected NIH 3T3 fibroblasts. Because antibodies to both M50/p35 and PKC were generated in rabbit, colocalization of M50/p35 could only be shown indirectly. M53/p38 colocalized with M50/p35 as well as with PKC, indicating that M50/p35 and PKC are similarly distributed. (**J** to **L**) Mitotic kinase $p34^{cdc2}$ is not recruited to the NE by M50/p35. (**M**) MCMV infection induces an increased phosphorylation of lamin A and C in murine NIH 3T3 fibroblasts. Cells infected with MCMV at a MOI of 2 in the presence or absence of 200 nM Ro-31-7549 were labeled with [^{32}P]orthophosphate (phosphorylated) 24 hours after infection, lysed, and precipitated with an antibody to lamin A and C (*30*). (**N**) HCMV infection induces an increased phosphorylation of lamins A, B_2, and C in HCMV-infected human fibroblasts. Cells were infected with HCMV AD169 at a MOI of 2, labeled with [^{32}P]orthophosphate, lysed, and precipitated with antibodies against either lamin A and C or lamin B_2. A fraction of each sample was analyzed by Western blot analysis to prove that the total amounts of lamins were similar.

lamin phosphorylation can be increased up to a factor of 10. However, CMV-induced lamin phosphorylation was only increased by a factor of 2.5 to 4. CMV infection causes a premitotic cell cycle arrest (*15, 16*), and thus kinases involved in lamin phosphorylation at later stages of the cell cycle may not be recruited. Because we could not detect a direct interaction between M50/p35 and Ca-dependent PKCs, binding between these two proteins might be mediated by a PKC-binding protein.

Here, we show that M50/p35 recruits the cellular machinery to dissolve the NL. The recruitment of PKC results in an increased phosphorylation of lamins, which is above that seen in the interphase. Lamin phosphorylation may be below the mitotic level, because the mitotic kinase $p34^{cdc2}$ is not recruited. The M50/p35-induced effect is concentrated in distinct intranuclear areas by a second viral protein, M53/p38. We propose that sites of the NL decorated by the complex of M50/p35 and M53/p38 represent the docking sites for viral capsids. M50/p35, which is homologous to UL34 in α-herpesviruses and ORF67 in γ-herpesviruses (*17*), is essential to viral replication, as MCMV M50/p35 deletion mutants failed to produce progeny unless the gene was reintroduced into the genome (*6*). Alterations of the NL have also been reported for the α-herpesvirus HSV-1 (*18*). However, the homologous viral proteins may not act in an identical fashion. HSV-1 UL34, for example, localizes at the NE but appears to induce a dissociation of the inner and outer nuclear membrane rather than alterations of the NL (*17, 19*). It is unknown which cellular kinases are recruited by α- and γ-herpesviruses (if any), and we thus do not know whether they use a similar or different strategy for capsid egress.

It is possible that M50/p35 shares structural homology with LAP2β and emerin, because M50/p35 is a type II transmembrane protein with a very short COOH-terminal and a long, hydrophilic NH_2-terminal domain. The region between residues 190 and 243 of M50/p35 has limited homology to the LEM (LAP2β, emerin, and MAN1) domains shared by emerin, MAN1, and LAP2β, suggestive of a common evolution (*13*). LEM domains are bound by BAF (barrier to autointegration factor), which is a nuclear protein that colocalizes with chromatin during interphase and mitosis and binds to DNA molecules (*20, 21*). BAF has been shown to support human immunodeficiency virus–type 1 integration into the host cell genome (*22*). Structural analysis of the LAP2β LEM domain has revealed two large parallel α helices, which are connected by a short stretch of seven amino acids (*23*). A sequence comparison between M50/p35 and LEM domains indicates that the highest homology lies between the two α helices and the first half of the second α helix. Because polyproline and polyglycine stretches at either side of this region probably disturb potential α helices, the structure of M50/p35 might differ significantly from true LEM domains, and its potential to bind BAF will be an interesting question for the future.

References and Notes

1. A. Alcami, U. H. Koszinowski, *Immunol. Today* **21**, 447 (2000).
2. S. Cudmore, P. Cossart, G. Griffiths, M. Way, *Nature* **378**, 636 (1995).
3. T. C. Mettenleiter, *J. Virol.* **76**, 1537 (2002).
4. E. S. Mocarski Jr., in *Fields Virology*, B. N. Fields, D. M. Knipe, P. M. Howley, Eds. (Lippincott-Raven, Philadelphia, ed. 3, 1996), pp. 2447–2492.
5. K. D. Radsak, K. H. Brucher, S. D. Georgatos, *Eur. J. Cell Biol.* **54**, 299 (1991).
6. W. Muranyi, J. Haas, M. Wagner, G. Krohne, U. H. Koszinowski, data not shown.
7. L. Gerace, G. Blobel, *Cell* **19**, 277 (1980).
8. Y. Ottaviano, L. Gerace, *J. Biol. Chem.* 260, 624 (1985).
9. H. Otto, M. Dreger, L. Bengtsson, F. Hucho, *Eur. J. Biochem.* **268**, 420 (2001).
10. M. Peter, J. Nakagawa, M. Doree, J. C. Labbe, E. A. Nigg, *Cell* **61**, 591 (1990).
11. G. Dessev, C. Iovcheva, B. Tasheva, R. Goldman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2994 (1988).
12. B. Buendia, J.-C. Courvalin, P. Collas, *Cell. Mol. Life Sci.* **58**, 1781 (2001).
13. See supporting data on *Science* Online.
14. A. P. Fields, G. R. Pettit, W. S. May, *J. Biol. Chem.* **263**, 8253 (1988).
15. F. M. Jault *et al.*, *J. Virol.* **69**, 6697 (1995).
16. M. Lu, T. Shenk, *J. Virol.* **70**, 8850 (1996).
17. G. J. Ye, K. T. Vaughan, R. B. Vallee, B. Roizman, *J. Virol.* **74**, 1355 (2000).
18. E. S. Scott, P. O'Hare, *J. Virol.* **75**, 8818 (2001).
19. R. J. Roller, Y. Zhou, R. Schnetzer, J. Ferguson, D. DeSalvo, *J. Virol.* **74**, 117 (2000).
20. M. S. Lee, R. Craigie, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1528 (1998).
21. K. Furukawa, *J. Cell Sci.* **112**, 2485 (1999).
22. H. Chen, A. Engelman, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15270 (1998).
23. C. Laguri *et al.*, *Structure* **9**, 503 (2001).

24. W. D. Rawlinson, H. E. Farrell, B. G. Barell, *J. Virol.* **70**, 8833 (1996).
25. W. Kolanus *et al.*, *Cell* **86**, 233 (1996).
26. M. Del Val, K. Munch, M. J. Reddehase, U. H. Koszinowski, *Cell* **58**, 305 (1989).
27. T. H. Hoger, C. Grund, W. W. Franke, G. Krohne, *Eur. J. Cell Biol.* **54**, 150 (1991).

28. ORFs M50 to M53 were amplified by the polymerase chain reaction (PCR) from the subcloned Hind III H fragment of the MCMV strain Smith genome [American Type Culture Collection (ATCC) VR-194] and subsequently cloned into the pCR3 (Invitrogen) plasmid by Eco RI and Xba I restriction sites. Primers for PCR were selected from the published MCMV sequence (GenBank accession number U68299) (*24*). All viral genes were expressed as immunoglobulin-tagged (cIg) (*25*) or FLAG-tagged fusion proteins. CV-1 cells (ATCC CCL-70), 293 cells (ATCC 1573), NIH 3T3 cells (ATCC CRL 1658), or human foreskin fibroblasts were seeded on cover slips before infection or transfection (SuperFect, Qiagen), incubated 24 hours in 5% CO_2 at 37°C, and then subjected to immunofluorescence.
29. Cells were incubated with the following antibodies: anti-lamin B (Santa Cruz Biotechnology), anti-Ca-dependent PKC polyclonal rabbit serum (M. Ueffing, GSF Munich, Germany), anti-pp89 (*26*), anti-LAP2β (R. Foisner, Biocenter Vienna, Austria). For the detection of M50/p35, an affinity-purified polyclonal rabbit antiserum raised against a synthetic M50/p35 peptide with the amino acid sequence PPGQRWGSL-RKHG (G, Gly; H, His; K, Lys; L, Leu; P, Pro; Q, Gln; R, Arg; S, Ser; W, Trp) was used (Eurogentec, Herstal, Belgium). Cells were washed and incubated with fluorescein isothiocyanate– or Texas Red–conjugated secondary antibodies (Dianova, Hamburg, Germany). Cells subjected to DNA staining were incubated with 1 μg/ml propidium iodide in the presence of ribonuclease A and 1 mM EDTA. Before imaging on a confocal laser scanning microscope in a sequential scan mode (Leica SP2, Bensheim, Germany), cover slips were mounted with Histogel (Linaris, Wertheim, Germany).
30. Mock-infected and MCMV-infected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). For orthophosphate labeling, cells were washed in phosphate-free DMEM (Life Technologies) and incubated with [^{32}P]orthophosphate (100 μCi/ml; Amersham, Braunschweig, Germany) for 3 hours (*14*). Cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM tris (pH 7.5), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 1 mM sodium vanadate, and 10 μg/ml of each of the protease inhibitors leupeptin, aprotinin, and soybean trypsin inhibitor. Protein concentration of lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL), and identical amounts of protein were subjected to immunoprecipitation. An aliquot of the lysate was used for a Western blot control. For precipitation, the following antibodies to lamins were used: anti-lamin A/C (clone X67), anti-lamin B_2 (clone R29), and anti-lamin clone PKB8 (*27*). Samples were analyzed by 10% SDS–polyacrylamide gel electrophoresis (PAGE). The PKC inhibitor Ro-31-7549 (Calbiochem, San Diego, CA) was used at a final concentration of 200 nM.

31. Supported by Deutsche Forschungsgemeinschaft grants SFB 413 and SFB 455. We thank C. Atzler for technical help and M. Ueffing, R. Foisner, G. Gill, and K. Wilson for reagents.

Supporting Online Material

www.sciencemag.org/cgi/content/full/297/5582/854/DC1

Materials and Methods

Figs. S1 to S3

5 March 2002; accepted 20 May 2002