Regulation of Marburg virus (MARV) budding by Nedd4.1: a different WW domain of Nedd4.1 is critical for binding to MARV and Ebola virus VP40

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The VP40 matrix protein of Marburg virus (MARV) has been shown to be the driving force behind MARV budding, a process in which the PPPY L-domain motif of VP40 plays a critical role. Here, we report that Vps4B and Nedd4.1 play critical roles in MARV VP40-mediated budding. We showed that unidentified activities of the Nedd4.1 HECT domain, along with its E3 ubiquitin ligase activity, may be required for MARV budding. Moreover, we showed that the first WW domain of Nedd4.1, WW1, is critical for binding to MARV VP40, indicating that MARV VP40 and Ebola virus VP40 are recognized by a different WW domain of Nedd4.1. This is the first report showing that the viral L-domains containing PPxY have specificities for binding to WW domains. Our findings provide new insights into MARV budding, which may contribute to the development of novel anti-MARV therapeutic strategies.

INTRODUCTION

Marburg virus (MARV) is a member of the family Filoviridae in the order Mononegavirales (MNV), and causes a haemorrhagic fever that is associated with high morbidity and mortality in humans (Peters, 2005). At present, no licensed vaccines or antiviral drugs are available to prevent or treat filoviral diseases.

The RNA genome of MARV encodes seven genes: glycoprotein (GP), nucleoprotein (NP), RNA-dependent RNA polymerase (L), VP35, VP30, VP40 and VP24. VP40, the most abundant protein in the virion, is the counterpart of the matrix (M) protein found in most MNVs and plays a key role in virus assembly and budding (Kolesnikova et al., 2002, 2004; Swenson et al., 2004; Urata et al., 2007a). The filovirus surface glycoprotein (GP) mediates virus binding to cellular receptors and cell entry, including a pH-dependent fusion event between viral and cellular membranes required for release of the virus ribonucleoprotein into the cytoplasm of infected cells (Becker et al., 1995).

Previous reports indicated that M proteins of many enveloped MNVs play critical roles during the late stage of virus budding. Many of these M proteins are, in the absence of any other viral polypeptides, competent in budding and formation of virus-like particles (VLPs). Budding of M proteins is often directed by late (L)-domain motifs, which are most frequently PT/SAP, PPxY, YxxL or FPIV (Bouamr et al., 2003; Burleigh et al., 2005; Ciancanelli et al., 2006; Gottlinger et al., 1991; Harty et al., 1999, 2000; Huang et al., 1995; Irie et al., 2007; Jasenosky et al., 2001; Jayakar et al., 2000; Parent et al., 1995; Schmitt et al., 2005; Strecker et al., 2003; Urata et al., 2007a; Wills et al., 1994; Yasuda & Hunter, 1998). Efficient budding requires interaction between viral L-domains and host factors, many of which are involved in the cellular multivesicular body (MVB) sorting pathway (Chen & Lamb, 2008). These findings suggested that budding into the lumen of MVBs in late endosomes and virus budding at the plasma membrane or late endosomal surface are topologically identical and share a common mechanism.

MARV VP40 protein is a bona fide virus budding protein containing the PPxY motif as an L-domain (Kolesnikova et al., 2002, 2004; Swenson et al., 2004; Urata et al., 2007a). Previously, we reported a PPxY-dependent Tsg101–MARV VP40 interaction that plays an important role in VLP budding (Urataha et al., 2007a). Nedd4.1 and Nedd4-like ubiquitin ligase have been shown to interact with viral L-domain PPxY, found in vesicular stomatitis virus (Harty et al., 1999), Ebola virus (EBOV) (Harty et al., 2000; Yasuda et al., 2003), Roux sarcoma virus (Kikonyogo et al., 2001), human T-cell leukemia virus (HTLV-1) (Blot et al., 2004; Bouamr et al., 2003; Heidecker et al., 2004, 2007; Sakurai et al., 2004) and Mason–Pfizer monkey virus (MPMV) (Yasuda et al., 2002), and this interaction regulates virus budding. Similarly, Vps4 has been reported to participate in the assembly and budding of several viruses (Dong et al., 2005; Garrus et al., 2001; Gosselin-Grenet et al., 2007; Gottwein et al., 2003; Licata et al., 2003; Martin-Serrano et al., 2003; Schmitt et al., 2005; Urata et al., 2004, 2007; Strecker et al., 2003; Wills et al., 1994; Yasuda, 2000; Yasuda & Hunter, 1998). Efficient budding requires interaction between viral L-domains and host factors, many of which are involved in the cellular multivesicular body (MVB) sorting pathway (Chen & Lamb, 2008). These findings suggested that budding into the lumen of MVBs in late endosomes and virus budding at the plasma membrane or late endosomal surface are topologically identical and share a common mechanism.

The VP40 matrix protein of Marburg virus (MARV) has been shown to be the driving force behind MARV budding, a process in which the PPPY L-domain motif of VP40 plays a critical role. Here, we report that Vps4B and Nedd4.1 play critical roles in MARV VP40-mediated budding. We showed that unidentified activities of the Nedd4.1 HECT domain, along with its E3 ubiquitin ligase activity, may be required for MARV budding. Moreover, we showed that the first WW domain of Nedd4.1, WW1, is critical for binding to MARV VP40, indicating that MARV VP40 and Ebola virus VP40 are recognized by a different WW domain of Nedd4.1. This is the first report showing that the viral L-domains containing PPxY have specificities for binding to WW domains. Our findings provide new insights into MARV budding, which may contribute to the development of novel anti-MARV therapeutic strategies.
These findings prompted us to examine the roles of Nedd4.1 and Vps4 in VP40-induced budding of MARV VLPs.

RESULTS

Functional involvement of Nedd4.1 and Vps4 in MARV VLP budding

To examine the contributions of Nedd4.1 and Vps4 to MARV VP40-induced VLP budding, we used a small interfering RNA (siRNA)-based approach. Specific depletion of Nedd4.1 or Vps4B by siRNA reduced the production of VP40-induced VLPs significantly (Fig. 1), indicating that Nedd4.1 and Vps4B are involved in MARV VLP budding.

Domains of Nedd4.1 required for budding of MARV VLPs

Nedd4.1 contains an N-terminal C2 domain, four WW domains and a C-terminal HECT domain. The C2 domain is thought to be responsible for Ca\(^{2+}\)-dependent binding of Nedd4.1 to membrane phospholipids. WW domains are 35–40 aa in length and contain two conserved tryptophan (W) residues spaced 21 aa apart. The WW domains usually interact with the PPxY motif. The HECT domain comprises approximately 350 residues and is responsible for ubiquitin transfer from a conserved cysteine residue within the HECT domain to a lysine residue in the substrate protein. To examine the contribution of each functional domain of Nedd4.1 to MARV budding, we generated a variety of Nedd4.1 mutants (Fig. 2) and examined the effects of their overexpression on MARV VP40-induced VLP production (Fig. 3). Overexpression of wild-type (WT) Nedd4.1 enhanced VLP release markedly (Fig. 3a, c). The ΔC2 and ΔQ mutants with deletion of the N-terminal region also enhanced VLP production to similar levels to WT Nedd4.1, suggesting that the N-terminal region containing the C2 domain is dispensable for the role of Nedd4.1 in MARV budding (Fig. 3b, c). On the other hand, the WW mutant containing all four WW domains and the ΔHECT mutant reduced the production of VLPs to 29 and 28 % of the control, respectively (Fig. 3b, c). The C894A mutant, which has a cysteine-to-alanine substitution, showed a complete loss of function (Fig. 3b, c).

(a) Nedd4.1

(b) Vps4B

Fig. 1. Roles of Nedd4.1 and Vps4B in MARV VLP release. 293T cells were pretreated with siRNA specific for Nedd4.1 (a) or Vps4B (b) or with control RNA 1 day before plasmid transfection. The following day, these cells were co-transfected with pMV-VP40 and siRNA or control RNA. At 48 h after transfection, cell lysates and VLPs were collected and analysed by Western blotting (WB). Cell-associated and VLP-associated VP40 were detected by using rabbit anti-VP40 antibody. Intracellular depletion of endogenous Nedd4.1 by siRNA was confirmed by WB using rabbit anti-Nedd4.1 antibody (a). Depletion of Vps4B was examined by monitoring Vps4B expressed from pFL-Vps4B co-transfected with siRNA using mouse anti-FLAG monoclonal antibody (b). The intensities of the bands for cell- and VLP-associated VP40 were quantified by using a LAS3000 imaging system (Fuji Film). The efficiency of VP40-induced VLP budding in cells co-transfected with pMV-VP40 and control RNA (VLP/cellular) was set to 1.0. The data are shown as means ± SD of three independent experiments.

Fig. 2. Schematic representation of Nedd4.1 and its mutants. Nedd4.1 contains an N-terminal C2 domain, four WW domains and a C-terminal HECT domain. All constructs were expressed as proteins containing a Myc tag at their N termini.
substitution at the active-site cysteine in the HECT domain, reduced MARV VLP production slightly (Fig. 3b, c). As predicted, the WG1234 mutant, with a tryptophan (W)-to-glycine (G) mutation of the first W residue in each of the four WW domains, W224, W381, W454 and W506, had no effect on VLP production, which was probably due to the loss of binding ability to the PPPY motif within VP40 (Figs 3b, c, 5b). These results suggested that Nedd4.1 interacts with the PPPY motif of VP40 via WW domains.

**Contribution of the PPPY motif of MARV VP40 to enhancement of budding by Nedd4.1**

Next, we examined whether Nedd4.1 enhances VP40-ΔPPPY VLP production. Previously, we showed that the PPPY motif of MARV VP40 is critical for VLP production (Urata et al., 2007a). Although VP40-ΔPPPY was expressed efficiently in culture cells, only low numbers of VLPs were released from cells. VLP production by MARV VP40-ΔPPPY was not enhanced by Nedd4.1 expression (Fig. 3d), probably due to the loss of binding ability to Nedd4.1 (Fig. 5a).

**Intracellular localization of VP40 and Nedd4.1**

We next examined the intracellular localization of MARV VP40 and Nedd4.1 by immunofluorescence confocal microscopy (Fig. 4). VP40 and VP40-ΔPPPY were detected mainly in clusters in the cytoplasm and the plasma membrane, corresponding to previous reports (Kolesnikova et al., 2007a, b, 2009). Nedd4.1 was also

![Fig. 3. Effects of Nedd4.1 mutants and the PPPY motif within VP40 on MARV VLP production. COS-7 cells were co-transfected with pMV-VP40 and an expression plasmid for Nedd4.1 (a) or its mutants (b). At 48 h after transfection, cell lysates and VLPs were collected and analysed by WB. Cell- and VLP-associated VP40 were detected by using a rabbit anti-VP40 antibody. Mouse anti-Myc monoclonal antibody was used to confirm the expression of Nedd4.1-WT (a) and mutants (b) by WB. (c) The intensities of the bands for cell- and VLP-associated VP40 in (a) and (b) were quantified as described in the legend to Fig. 1. The efficiency of VLP budding in cells co-transfected with pMV-VP40 and the empty vector (control) (VLP/cellular) was set to 1.0. The data are shown as means±SD of three independent experiments. (d) At 48 h after transfection using pMV-VP40-ΔPPPY and expression plasmid for Nedd4.1 or empty plasmid, cell lysates and VLPs were collected and analysed by WB. Cell- and VLP-associated VP40 were detected by using a rabbit anti-VP40 antibody. Mouse anti-Myc monoclonal antibody was used to confirm the expression of Nedd4.1-WT.

![Fig. 4. Intracellular localization of VP40 and Nedd4.1. 293T cells expressing MARV VP40-WT or -ΔPPPY, along with Nedd4.1-WT or mutants (-ΔC2 or -WG1234), were fixed and processed for immunofluorescence analysis as described in Methods. Staining: VP40-WT and VP40-ΔPPPY, green; Nedd4.1-WT and mutants, red; 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining, blue. Magnification ×600 (upper three rows); ×350 (lower row).]
detected in clusters, but somewhat diffused in cytoplasm, as reported previously (Anan et al., 1998; Plant et al., 1997). VP40 clearly co-localized with Nedd4.1 in the cytoplasm and plasma membrane, whereas VP40-ΔPPPY did not show any co-localization with Nedd4.1, despite the fact that VP40-ΔPPPY showed similar intracellular localization to WT. Nedd4.1-WG1234 did not also co-localize with VP40-WT. These results suggest strongly that the PPPY motif within VP40 interacts with the WW domain(s) of Nedd4.1. On the other hand, Nedd4.1-ΔC2 co-localized with VP40-WT, suggesting that Nedd4.1-ΔC2 can still interact with VP40 in cells.

### Interaction between MARV VP40 and Nedd4.1

To investigate further the interaction between VP40 and Nedd4.1, we performed glutathione S-transferase (GST) pull-down (PD) assays. Recombinant GST, GST–VP40 or GST–VP40-ΔPPPY proteins purified from *Escherichia coli* were incubated with 293T cell lysates overexpressing Nedd4.1-WT, Nedd4.1-WW or Nedd4.1-ΔC2 mutant, followed by the GST PD assay, as described in Methods. As shown in Fig. 5(a), GST–VP40 interacted with WT, WW mutant and ΔC2 mutant of Nedd4.1, whereas GST–VP40-ΔPPPY failed to bind to Nedd4.1, indicating that the PPPY motif within VP40 is critical for binding of VP40 to Nedd4.1. The WW mutant and ΔC2 mutant of Nedd4.1 were able to bind to VP40, indicating that the WW domains of Nedd4.1 are sufficient for binding to VP40. Nedd4.1 has four WW domains within its central region. To determine which WW domain is responsible for recognition of the PPPY motif of MARV VP40, we performed GST PD assays using the WW mutants with W-to-G mutations in each WW domain, WG1, WG2, WG3 and WG4 (Figs 2b, 5b). As shown in Fig. 5(b), the expression levels in cells varied among WW mutants. Therefore, to compare the binding abilities of WW mutants to VP40, we quantified the intensities of the band for Nedd4.1 WT and mutants in input and pull-down samples, and the binding efficiency of each WW mutant to VP40 was calculated as pull-down/input. The WG4 mutant interacted with VP40 with a similar efficiency to Nedd4.1-WT, whereas the WG1 mutant showed a significantly reduced ability to interact with VP40, similar to that of mutant WG1234. The WG2 and WG3 mutants showed modest binding to VP40. These results indicated that the first WW domain, WW1, within Nedd4.1 is the major binding site of Nedd4.1 binding to MARV VP40.

### DISCUSSION

Previously, we reported that Tsg101 interacts with MARV VP40 and plays an important role in MARV VLP budding (Urata et al., 2007a). We also showed that the PPPY motif within VP40 is critical for VP40- and VP40/GP/NP-induced VLP budding. Nedd4.1 and Nedd4-like ubiquitin ligase interact with the PPPY L-domain motif present in a variety of viral budding proteins, and this interaction contributes to the regulation of virus budding (Blot et al., 2004; Bouamr et al., 2003; Harty et al., 1999, 2000; Heidecker et al., 2004, 2007; Kikonyogo et al., 2001; Sakurai et al., 2004; Yasuda et al., 2002, 2003). In the present study, we have provided

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**Fig. 5.** Identification of the WW domain(s) critical for interaction with MARV VP40. (a) Recombinant GST, GST–VP40 and GST–VP40-ΔPPPY immobilized on glutathione–Sepharose beads were incubated with cell lysates overexpressing Nedd4.1-WT, Nedd4.1-WW or Nedd4.1-ΔC2. (b) Recombinant GST and GST–VP40 immobilized on glutathione–Sepharose beads were incubated with cell lysates overexpressing WW mutants of Nedd4.1. The binding of Nedd4.1 WT and mutants to the immobilized GST-fusion proteins was detected by WB using an anti-Myc antibody (middle panels). Input (upper panels): Nedd4.1 WT and mutants in 1/30 vol. of the input cell lysates were detected by WB using anti-Myc antibody. Lower panels: Coomassie brilliant blue (CBB) staining of GST, GST–VP40 and GST–VP40-ΔPPPY proteins in the samples after pull-down. The intensities of the bands for Nedd4.1 proteins in the input and pull-down fractions were quantified by using LAS3000. The binding efficiencies of Nedd4.1 proteins to VP40 were calculated as pull-down/input and that in Nedd4.1-WT was set to 1.00. The binding abilities of WW mutants are indicated below the figure as a ratio relative to that of Nedd4.1-WT.
evidence for the involvement of Nedd4.1 in MARV VP40-mediated VLP budding.

Mutation–function studies showed that the C2 domain of Nedd4.1 was dispensable for the function of Nedd4.1 in MARV VLP budding, a finding consistent with our previous observations on EBOV and M-PMV budding (Yasuda et al., 2002, 2003).

Mutation C894A within Nedd4.1 showed only a weak effect on MARV VLP budding, whilst deletion of the HECT domain greatly reduced VLP production. As both ΔHECT and C894A have been shown to be inactive forms of Nedd4.1 (Anan et al., 1998; Sakurai et al., 2004; Yasuda et al., 2003), our results suggest that an as-yet-undetermined activity of the HECT domain, distinct from its E3 ubiquitin ligase activity, is required for the budding of MARV, and probably also murine leukemia virus (MLV) and HTLV-1 (Heidecker et al., 2007; Martin-Serrano et al., 2005). However, this additional function of the HECT domain that greatly affects MARV VLP budding has yet to be characterized.

The WW domains interact with proline-rich motifs (Sudol, 1996). Therefore, it is possible that one or more of the four WW domains present in Nedd4.1 interacted with the PPPY motif within MARV VP40. Consistent with this hypothesis, the WGI234 mutant showed complete loss of binding ability to MARV VP40 (Figs 4, 5b) and did not regulate MARV VLP budding (Fig. 3b, c). This finding, together with our observations that mutant VP40-ΔPPPY did not bind to Nedd4.1 (Figs 4 and 5a) and Nedd4.1 did not enhance VP40-ΔPPPY-mediated production of VLPs (Fig. 3d), indicate that Nedd4.1 interacts with the PPPY motif of MARV VP40 via its WW domains.

The WW domains are present in many cellular proteins, and mediate specific protein–protein interactions (Sudol, 1996). The results of GST PD assays indicated that the first WW domain in Nedd4.1, WW1, was the most critical for binding of Nedd4.1 to MARV VP40, whereas the second and third WW domains, WW2 and WW3, interacted more weakly than WW1 (Fig. 5b). In contrast, the fourth WW domain, WW4, was not required for binding of Nedd4.1 to MARV VP40. Previously, we reported that multiple WW domains of Nedd4.1 function as sites of interaction with the EBOV VP40 L-domain, although WW3 appeared to be the most important (Yasuda et al., 2003). In addition, another group showed that WW3 of Nedd4.1 is critical for binding to EBOV VP40, although they did not examine WW1 (Timmins et al., 2003). These results suggest strongly that the four WW domains of Nedd4.1 are involved differently in recognizing EBOV or MARV VP40 (Fig. 6). EBOV VP40 has an overlapping L-domain motif, PTAPPEY, whereas MARV VP40 has only PPPY as an L-domain motif. The flanking sequences around the L-domain are not conserved between EBOV and MARV VP40, and may affect the specificity of binding to the different Nedd4.1 WW domains.

Previously, we reported that Tsg101 interacts with MARV VP40, and that this interaction depends on the VP40 PPPY motif and plays a critical role in MARV VLP budding (Urata et al., 2007a). Nedd4.1 may facilitate the recruitment of Tsg101 to VP40, as Tsg101 does not appear to bind directly to VP40. However, we were unable to find a direct interaction between Nedd4.1 and Tsg101 by using both a mammalian two-hybrid assay and co-immunoprecipitation assays, suggesting that Tsg101 may require another cellular factor(s) to interact with Nedd4.1.

In this study, we also showed that Vps4 is involved in MARV VLP budding. Vps4 is an AAA-type ATPase that has a key function in the endocytic pathway for degradation of membrane proteins. The function of Vps4 is linked closely to ESCRTs, which are multiprotein complexes required for the sorting of endocytosed transmembrane proteins into intraluminal vesicles of morphologically distinctive endosomes known as MVBs. Mutant forms of Vps4 defective for ATPase activity are potent inhibitors of budding of many viruses, including HIV-1, HTLV-1, MLV, equine infectious anemia virus, M-PMV, EBOV, Lassa virus, simian virus 5 and Sendai virus (Garrus et al., 2001; Gosselin-Grenet et al., 2007; Gottwein et al., 2003; Licata et al., 2003; Martin-Serrano et al., 2003; Schmitt et al., 2005; Urata et al., 2006, 2007b). A recent study by Kolesnikova et al. (2009) also showed that a Vps4A mutant defective for ATPase activity inhibited MARV production. These findings suggest that all of these very divergent viruses utilize the cellular MVB sorting pathway to release their infectious progeny.

Our results provide valuable novel information to gain a detailed understanding of the molecular interaction underlying MARV VP40-mediated budding. Moreover, our data contribute to the body of knowledge showing that budding of many different viruses involves the contributing components of the MVB pathway of the host cell. Therefore, drugs capable of disrupting the interactions...
between viral and host factors involved in budding may exert antiviral activity against different viruses.

**METHODS**

**Plasmids and antibodies.** Plasmids for expression of MARV VP40, GST–MARV VP40, GST–MARV VP40-APPPY and FLAG-tagged Vps4B (pMV-VP40, pGEX-MVVP40-WT, pGEX-MVVP40-APPPY and pFL-Vps4B) were described previously (Urata et al., 2006, 2007a). The Myc-tagged Nedd4.1 expression plasmid, pBj-Myc-hNedd4, and the plasmids for Nedd4.1 mutants other than QA and AHECT were also reported previously (Anan et al., 1998; Yasuda et al., 2003). For Nedd4.1-AQ, Bmi1-H sites were introduced by standard PCR into Nedd4.1-AQ at both the 5′ and 3′ ends and subcloned into pBj-Myc. Nedd4.1-AHECT was constructed by introducing a stop codon at K591 in pBj-Myc-hNedd4, using a KOD Plus mutagenesis kit (Toyobo). The plasmid for expression of MARV VP40-APPPY, pMV-VP40-APPPY, was constructed by deleting the PPPPY motif using a KOD Plus mutagenesis kit (Toyobo). Polyclonal antibodies against MARV VP40 and Nedd4.1 were described previously (Urata et al., 2007a; Yasuda et al., 2003). Monoclonal antibodies against Myc and FLAG were purchased from Cell Signaling Technology and Sigma, respectively.

**VLP production assay.** COS-7 and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS). Aliquots of 293T cells (2 x 10^5) were pretreated with 100 pmol siRNA specific for Vps4B (siVps4B) or Nedd4.1 (siNedd4.1) or control siRNA (Urata et al., 2006, 2007a), 1 day before plasmid transfection. The following day, these cells were co-transfected with 50 pmol siVps4B, siNedd4.1 or control siRNA, and 0.03 μg pMV-VP40. At 48 h after transfection, VLPS released from cells and cell lysates were collected and analysed as described previously (Urata et al., 2007a). siVps4B and siNedd4.1 were purchased from Ambion. For analysis of Nedd4.1 mutants, COS-7 cells (1 x 10^6) were co-transfected with 0.1 μg pMV-VP40 and 2.0 μg expression plasmid for Nedd4.1 or its mutants using Trans IT-LT1 (Mirus). At 48 h after transfection, VLPS released from cells and cell lysates were collected and analysed as described previously (Urata et al., 2007a).

**GST PD assay.** Recombinant GST, GST–VP40 and GST–VP40–APPPY proteins were expressed in E. coli BL21–Gold and purified by using glutathione–Sepharose 4B beads (Amersham Biosciences). The immobilized GST–fusion proteins were incubated for 2 h at 4 °C with 293T-cell lysates overexpressing Nedd4.1-WT or mutants, washed extensively and eluted, followed by detection by Western blotting (WB) as described previously (Urata et al., 2007a).

**Immunofluorescence microscopy.** At 48 h post-transfection, 293T cells were fixed in a 1:1 ratio of methanol and acetone for 15 min at –20 °C. The fixed cells were treated with 1% FBS/PBS (−) for 30 min. Mouse anti-Myc monoclonal antibody (9E10; Sigma) or rabbit anti-VP40 antibody (Urata et al., 2007a) was used to stain Myc–Nedd4.1 or MARV VP40, followed by Alexa Fluor 568–goat anti-mouse IgG or goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Invitrogen), respectively. Cells were observed under a confocal microscope (LSM 710; Carl Zeiss).

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