Involvement of p32 and Microtubules in Alteration of Mitochondrial Functions by Rubella Virus

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The interaction of the rubella virus (RV) capsid (C) protein and the mitochondrial p32 protein is believed to participate in virus replication. In this study, the physiological significance of the association of RV with mitochondria was investigated by silencing p32 through RNA interference. It was demonstrated that downregulation of p32 interferes with microtubule-directed redistribution of mitochondria in RV-infected cells. However, the association of the viral C protein with mitochondria was not affected. When cell lines either pretreated with respiratory chain inhibitors or cultivated under (mild) hypoxic conditions were infected with RV, viral replication was reduced in a time-dependent fashion. Additionally, RV infection induces increased activity of mitochondrial electron transport chain complex III, which was associated with an increase in the mitochondrial membrane potential. These effects are outstanding among the examples of mitochondrial alterations caused by viruses. In contrast to the preferential localization of p32 to the mitochondrial matrix in most cell lines, RV-permissive cell lines were characterized by an almost exclusive membrane association of p32. Conceivably, this contributes to p32 function(s) during RV replication. The data presented suggest that p32 fulfills an essential function for RV replication in directing trafficking of mitochondria near sites of viral replication to meet the energy demands of the virus.

Rubella virus (RV), a single-stranded RNA virus, is the sole member of the genus Rubivirus in the family Togaviridae and causes a generally mild exanthematous childhood disease. However, severe malformations known as congenital rubella syndrome may result from the infection of seronegative women, especially during the first trimester of pregnancy. The mechanisms contributing to RV teratogenesis remain largely unknown. The 5'-proximal open reading frame (ORF) of the genome encodes the two replicase proteins P150 and P90, while the 3' ORF encodes the structural proteins, the capsid (C) protein and two envelope glycoproteins (E1 and E2). Viral RNA synthesis occurs on replication complexes, which are membrane bound to a structure called the cytopathic vacuole (CPV). CPVs are of endolysosomal origin and surrounded by rough endoplasmic reticulum (RER) cisternae, the Golgi apparatus, and mitochondria (13, 14). CPVs are replication factories and provide a protected environment for virus replication and assembly.

The C protein of RV represents one of the few RNA virus-encoded structural proteins that interact with mitochondria and is so far the only known viral protein that impairs protein transport into mitochondria (17). Additionally, the C protein participates in viral RNA synthesis (42), which is emphasized by its accumulation around CPVs (14). The C protein is also involved in the process of mitochondrial redistribution to a perinuclear region in proximity to CPVs (3, 28) and interacts with the p32 protein (3). Besides its predominant localization to the matrix of mitochondria, p32 is also found in the nucleus and at the plasma membrane. Due to its receptor function for the C1q component of complement, p32 is also known as gC1q-R (21). This evolutionarily conserved protein interacts with several cellular proteins with diverse functions, e.g., splicing factor SF2, the proapoptotic BH3-only protein Hrk, and also viral proteins, such as hepatitis C virus core protein and human immunodeficiency virus Rev protein (20). However, its precise function and, especially, the underlying molecular mechanisms remain to be determined. The importance of p32 for RV replication and for the association of RV with mitochondria has been indubitably shown (2, 13, 14, 33). In addition, the implication of p32 in apoptotic pathways and in oxidative phosphorylation (oxphos) in some human cancer cells (11, 18) suggests an energy-directed interaction between RV, mitochondria, and p32. Moreover, p32 mRNA is increased upon infection with RV (7), and significant amounts of p32 protein were detected in and around CPVs (13). The interaction of RV with mitochondria could result in altered energy metabolism and thus could represent one of the factors contributing to RV teratogenesis.

To address the exact roles and contributions of p32 and mitochondria during RV replication, p32 was downregulated in the present study by RNA interference (RNAi). Furthermore, RV replication was investigated under conditions of impaired respiration. This study indicates that the p32 protein- and microtubule filament-guided interaction of RV with mito-
chondria results in profound alterations of the activity of mitochondria and exerts positive effects on cellular metabolism.

MATERIALS AND METHODS

Cell culture and virus infection. Vero (ATCC CCL-81), BHK21 (ATCC CCL-10), HEK293 (ATCC CRL-1573), and HEK293T/17 (ATCC CRL-11268) cells were cultivated with 10% fetal calf serum (FCS) in humidified 5% CO2-95% air at 37°C. For hypoxic cultivation, the incubator was flushed with a gas mixture of 95% N2, 5% O2, and 1% CO2.

The Therien strain was used at a multiplicity of infection (MOI) of 1 in all experiments. In parallel, control cells were mock infected with sterile medium without virus. After adsorption for 2 h, the virus inoculum was replaced by maintenance medium. Viral titers were determined by standard plaque assay as described previously (8).

Reagents and chemical inhibitors. Cytochalasin D was purchased as a di-methyl sulfoxide (DMSO) stock solution; antimycin A (AMA) was dissolved in ethanol, nocardazole in DMSO, and tetramethylrhodamine ethyl ester (TMRE) in methanol. All reagents except 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich Chemical Company (Taunton, Germany).

Preparation of mitochondria. Vero cell mitochondria were isolated from confluent 75-cm2 cell culture flasks in a medium of 0.2 mM EDTA, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.8) buffer by differential centrifugation. Mitochondria homogenates obtained with a Magna Lyser (Roche, Mannheim, Germany). Differential centrifugation was as described elsewhere (MitochondriaSciences) with the following minor modifications. Before homogenization, 0.01% Triton X-100 and 0.1 mg/ml digitonin were added. Undisrupted cells and nuclei were removed by centrifugation at 1,000 g for 10 min at 4°C. Supernatants were further centrifuged at 12,000 × g for 15 min at 4°C to obtain the crude mitochondrial pellet, which was suspended in 5 ml of Mito buffer supplemented with 10 mM Tris-HCl, pH 7.8, and 1 mM sodium pyruvate (a hydrogen acceptor to regenerate NAD from NADH to support energy generation by glycolysis). The mitochondrial-DNA-depleted derivate 143B.TK was used at a multiplicity of infection (MOI) of 1 in all experiments. In parallel, control cells were mock infected with sterile medium without virus. After adsorption for 2 h, the virus inoculum was replaced by maintenance medium. Viral titers were determined by standard plaque assay as described previously (8).

Application of antimycin A. After incubation of Vero cells with 20 μM AMA for 6 h at 37°C, the cells were infected with RV and cultured in the presence of AMA or the solvent DMSO for 24 h after postinfection (p.i.). The cells were then refed with maintenance medium, which was harvested at 1, 2, and 3 days p.i. and processed for titer determination by plaque assay. The addition of the respective compound at 1 day p.i. was performed directly after collection of the 1-day-p.i. supernatant.

Application of cytoskeletal disrupting agents. Complete disruption of actin and microtubule filament was achieved by incubation of Vero cells with 5 μg/ml cytochalasin D and 10 μM nocardazole, respectively, for 2 h at 37°C. To test their effects on RV infection, either agent or the solvent DMSO was coapplied with RV or added in fresh medium to RV- and mock-infected cells at 2, 6, 24, and 48 h postinfection (p.i.). The cells were then refed with maintenance medium, which was harvested at 1, 2, and 3 days p.i. and processed for titer determination by plaque assay. The addition of the respective compound at 1 day p.i. was performed directly after collection of the 1-day-p.i. supernatant.

Isolation of membrane protein fractions. Hydrophobic membrane and hydrophilic cytoplasmic protein fractions were prepared from 1× 106 to 1× 107 cells using the ProteoJet membrane protein extraction kit (Promega, Mannheim, Germany), according to the manufacturer's instructions. The expression level of p32 mRNA and the amount of genomic viral RNA in RV-infected cell cultures were calculated by qRT-PCR using primers designed by the online BLOCK-iT RNAi Designer tool (Invitrogen). For each p32 shRNA, four short oligonucleotides that covered the antisense, loop, and sense sequence of the shRNA were synthesized. The forward and reverse oligonucleotides were phosphorylated by T4 polynucleotide kinase (NEB, Frankfurt am Main, Germany), annealed, and cloned into HindIII/BamHI-digested pAdshRNA as described previously (10) to yield the plasmid pAdshp32. For a control, a scrambled shRNA was utilized (10). To test the efficacy of the p32 shRNA, cells were cotransfected with the respective shRNA expression plasmid and a p32 expression plasmid. p32 expression was measured by quantitative real-time PCR (qRT-PCR) and immunohistochemistry 72 h later. The shRNA with the target sequence 5'-CCTTCTATGTTCCCTGTAGG-3' (nucleotides [nt] 494 to 514 of the p32 mRNA) had the highest efficiency and was obtained through modifications of a published p32 small interfering RNA (siRNA) sequence (21). For adenoviral vector (AdV) production, plasmids were linearized with SpeI, ligated to the 5'-long arm of the XbaI-digested E1-E3 adenovirus mutant RRS, transfectioned into HEK293 cells, and propagated as described previously (10). This resulted in the AdVs AdVshp32 and AdVsScrambled.

RNA preparation and quantitative real-time PCR. Briefly, cells (grown in 35-mm dishes for 1 to 3 days) were harvested for RNA extraction using TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. The expression level of p32 mRNA and the amount of genomic viral RNA in RV-infected cell cultures were calculated by qRT-PCR using geneNorm version 3.5.4 (43). Gene expression was normalized against HUEL (solute carrier family 30) and TBP (TATA box binding protein).

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GmbH, Aachen, Germany; diluted 1:500). Secondary swine anti-rabbit and rabbit anti-mouse antibodies conjugated with peroxidase (Dako, Hamburg, Germany) were used at a 1:5,000 and a 1:10,000 dilution, respectively. Detection was performed by chemiluminescence using an enhanced chemiluminescence (ECL) solution according to published protocols (15).

Indirect immunofluorescence and staining with fluorescent dyes. Vero cells were cultured on glass coverslips and fixed with 2% (wt/vol) paraformaldehyde–phosphate-buffered saline (PBS) at 48 h to 72 h postinfection. Prior to fixation, mitochondria were stained with 200 nM MitoTracker Red CMXRos or MitoTracker Deep Red 633 in PBS for 45 min before PBS wash and fixation. For intracellular staining, cells were permeabilized in methanol at −20°C. Microtubules were stained with a 1:500 dilution of monoclonal anti-alpha-tubulin antibody, RV C protein with a 1:100 dilution of the mouse monoclonal antibody, p32 with a 1:100 dilution of anti-p32 rabbit polyclonal antibody C1QBP, and RV structural proteins with a 1:5 dilution of a human RV-seropositive serum.

Primary antibodies were applied for 1.5 h at 37°C. Three washes with PBS were followed by incubation with a 1:100 dilution of the following secondary antibodies for 45 min at 37°C: Alexa Fluor 488 goat-anti mouse (Invitrogen, Molecular Probes), DyLight688 goat anti-mouse and anti-rabbit (Dianova GmbH, Hamburg, Germany), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako, Hamburg, Germany). For staining of actin filaments, cells were fixed with 2% (wt/vol) paraformaldehyde and permeabilized with acetone and ethanol (1:1 [vol/vol]) before being incubated with a 1:100 dilution of Alexa Fluor 488-phalloidin (Invitrogen, Molecular Probes) in PBS for 20 min at room temperature. Samples were mounted with entellan (VWR, Darmstadt, Germany) and processed for confocal microscopy (TCS SP2; Leica, Heidelberg, Germany), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako, Hamburg, Germany). By confocal microscopy (TCS SP2; Leica, Heidelberg, Germany) with a 100× objective and oil immersion. Stacks of serial optical slices with a step size of 0.2 μm were recorded for each sample. Postacquisition analysis was performed with Corel Photo-Paint 11 software with minimal alterations to background and contrast. The scale bar presented in each figure is 10 μm.

Statistical analysis. Student’s t test was applied to determine statistical significance. A P value of <0.05 (indicated by an asterisk in the figures) was considered statistically significant. Each bar represents the mean and standard deviation of two or three independent experiments. Each experiment was performed at least two or three times with similar results.

RESULTS

Silencing of p32 in Vero cells. AdVs, as an efficient shRNA delivery and expression system, were applied to assess the effect of p32 knockdown on RV replication. A green fluorescent protein (GFP)-expressing AdV was used to determine the optimal AdV transduction dose for Vero cells. At an MOI of 100, more than 90% of Vero cells were positive for GFP expression without noticeable cytotoxic effects or interference with RV replication (data not shown). Transduction of Vero cells with AdVshp32 at an MOI of 100 was effective only after two rounds of transduction (with an interval of 6 days, including two rounds of subculture). Almost complete downregulation of p32 was observed between 24 and 72 h after plating of two-round-transduced Vero cells (3 to 5 days posttransduction). AdVshp32-transduced Vero cells showed downregulation of p32 mRNA by about 80% in both mock- and RV-infected Vero cells (Fig. 1A). Transduction with AdVshScrambled had only a marginal effect on the p32 mRNA level. As subcellular localization of p32 appears to be variable, downregulation of p32 protein levels were assessed by Western blot analysis for both the hydrophilic (soluble) and hydrophobic (membrane-associated) protein fractions. p32 was almost completely absent in both fractions obtained from AdVshp32-transduced Vero cells (Fig. 1B). For further characterization of p32 knockdown Vero cells, the growth rate and mitochondrial morphology were examined. AdVshp32-transduced Vero cells were characterized by an increase in the number of dead cells over the time of incubation (Fig. 1C) and a reduced growth rate (Fig. 1D) in comparison to the AdVshScrambled-transduced control population. Supplementation of the cultivation medium with 50 μg/ml uridine and 1 mM sodium pyruvate can compensate for a respiratory metabolism deficit, which was described for p32 knockdown in human cancer cells (11). This supplementation reduced the percentage of dead cells in AdVshp32-transduced Vero cells (Fig. 1C) and was therefore applied to all subsequent experiments. To determine the morphology and localization of mitochondria, transduced Vero cells were stained with MitoTracker Red CMXRos. No difference in the mitochondrial staining pattern was detected between pAdVshp32- and pAdVshScrambled-transduced Vero cells (Fig. 1E).

Effect of p32 silencing on RV replication. The effect of p32 downregulation on RV replication and C protein-guided reorganization of mitochondria was examined in p32 knockdown Vero cells. Two days after the second round of transduction, Vero cells were harvested and subcultured for 24 h before infection with RV. A plaque assay was performed to reorganize the amount of newly generated progeny virus 3 days later (6 days posttransduction and 3 days p.i.). Downregulation of p32 reduced the viral titer by about 1 log10 PFU/ml (85%) compared to the AdVshScrambled-infected control population (Fig. 2A). Additionally, total RNA was extracted to quantify the amount of genomic viral RNA by qRT-PCR. Accumulation of intracellular viral RNA was reduced by 80% in comparison to the AdVshScrambled-infected control population (Fig. 2B). The subcellular localization of C protein and its association with mitochondria in RV-infected p32 knockdown Vero cells was comparable to that of the AdVshScrambled-infected control population, but reorganization and perinuclear cluster formation of mitochondria appeared to be absent (Fig. 2C). In summary, RV establishes a productive infection of p32 knockdown Vero cells, albeit with a lower virus production rate, but is not able to induce redistribution of mitochondria.

Disruption of the microtubule network: effect on virus titer and mitochondrial distribution. p32 knockdown reduced RV titers and affected RV-induced reorganization of mitochondria. This led to an investigation of actin and microtubule cytoskeleton involvement in clustering of mitochondria in RV-infected Vero cells. Depolymerization of actin filaments and microtubules was induced with 5 μg/ml cytochalasin D and 10 μM nocodazole, respectively. Figure 3A, b, and B, b, show, respectively, the characteristic and effective disruption of actin and microtubule filaments in Vero cells directly after the washout of the respective compound. Cytochalasin D and nocodazole were applied to RV-infected Vero cells at 6 h p.i., and their effects on mitochondrial distribution were examined by staining of mitochondria with MitoTracker Red CMXRos and MitoTracker Deep Red 633 at 2 days p.i. Cytochalasin D treatment resulted in a clustered distribution of mitochondria (Fig. 3A, f), which was comparable to that of the solvent control population (Fig. 3A, e) and typical of RV infection. However, in contrast to the solvent control population (Fig. 3B, e), most mitochondria remained evenly distributed in the cytoplasm after nocodazole treatment of RV-infected Vero cells (Fig. 3B, f). Moreover, in comparison to the solvent
control, the number of RV-infected Vero cells containing clustered mitochondria was decreased after nocodazole treatment but increased after treatment with cytochalasin D (Fig. 3C).

The next approach to determine the roles of microtubules and actin filaments during RV infection was to apply cytochalasin D and nocodazole between 6 and 24 h p.i. to study their effects on RV titers (Fig. 3D). In comparison to the solvent...
control, the addition of nocodazole at 6 h p.i. caused a noticeable reduction in the infectious titer at 2 days p.i., by more than 90%, from $2 \times 10^7$ to $1.3 \times 10^6$ PFU/ml (Fig. 3D). The lysosomotropic agent sucrose (0.45 M) as a RV entry inhibitor (22) had a strong effect on virus production at all examined time points (Fig. 3D). This indicates that cytochalasin D and nocodazole have only a minor effect on RV entry. A similar experiment was performed with an additional RV strain (M33) and an additional RV-permissive cell line (MCF-7). The reductions in virus titer after application of nocodazole to M33-infected Vero and Therien-infected MCF-7 cells at 6 h p.i. (Fig. 3E) parallel the changes in virus titer observed for Therien-infected Vero cells (Fig. 3D). Hence, the observed effects of nocodazole on RV replication are neither strain nor cell line specific. A cytotoxicity assay was performed to exclude the possibility that titer reduction was due to off-target (cytotoxic) effects of nocodazole. Nocodazole and cytochalasin D did not significantly influence the viability of RV- and mock-infected Vero cells (Fig. 3F).

The results for titer reduction and mitochondrial distribution obtained for coapplication of nocodazole or cytochalasin D together with RV or for an application at 2 h p.i. were similar to those obtained for an application at 6 h p.i. (data not shown). No effect was observed after application of nocodazole or cytochalasin D at time points later than 6 h p.i. Thus, the early hours of RV infection appear to be important for microtubule-directed mitochondrial redistribution.

Infection of 143B.TK<sup>−</sup> rho<sup>0</sup> cells lacking a functional respiratory chain, p32- and microtubule-directed perinuclear clustering of mitochondria in RV-infected Vero cells appear to support efficient progression of RV infection (Fig. 2 and 3). Therefore, the contribution of mitochondrial activity to the RV life cycle was investigated through infection of a cell line that was completely depleted of mitochondrial DNA (26). The so-called rho<sup>0</sup> derivative of the 143B.TK<sup>−</sup> osteosarcoma cell line was generated by a mild enzymatic approach and lacks a functional respiratory chain. It serves as a model for analysis of the importance of oxidative phosphorylation (oxphos) for cellular processes (26, 35). The 143B.TK<sup>−</sup> cell line enables a productive infection with RV and yields virus titers comparable to those of Vero cells (Fig. 4A) but requires a higher initial infectious dose (MOI = 50). The analysis of supernatants.
FIG. 3. Assessment of mitochondrial distribution and viral titer after application of nocodazole and cytochalasin D to RV-infected Vero cells. (A and B) Pharmacological compound was applied at 6 h p.i. Vero cells were analyzed directly after the washout (a and b) and at 2 days p.i. (c to f). (A) Distribution of mitochondria (counterstained with MitoTracker Red [MTX] CMXRos, shown in red) after application of 5 μg/ml cytochalasin D (Cyt D). (B) Distribution of mitochondria (counterstained with MitoTracker Deep Red 633, shown in blue) after application of 10 μM nocodazole (Noc). In contrast to the clustered distribution of mitochondria (single arrows) in RV-infected Vero cells upon treatment with Cyt D or DMSO (solvent control), Noc treatment of RV-infected Vero cells results in even distribution of mitochondria (double arrows). Microtubules (Tub) were stained with anti-alpha-tubulin antibody and IgG conjugated to DyLight 488 (a and b, shown in green) or Alexa Fluor 633 (c to f, shown in blue). SP, structural proteins stained with human RV-seropositive serum and IgG conjugated to fluorescein isothiocyanate (FITC) (shown in green). F-actin (Act) was stained with Alexa Fluor 488-phalloidin (green), and C protein (C) was stained with the respective antibody and IgG conjugated to Alexa Fluor 633 (blue). (C) The percentage of infected cells with clustered mitochondria (accumulation in proximity to the nucleus) was determined by counting 200 infected cells from 10 random fields per coverslip with 2 to 4 coverslips per treatment. The error bars indicate the standard deviation. *, P < 0.05. (D and E) Virus titers on Vero (D) and MCF-7 (E) cells were determined for the indicated time points and treatments by plaque assay. Sucrose (0.45 M) (Suc), as an endocytosis inhibitor, served as a control for possible inhibition of viral entry by Noc and Cyt D. (F) Cytotoxicity was determined after application of Cyt D and Noc by cytotoxicity assay.
collected at 3 days p.i. showed a significant reduction in the virus titer on the 143B.TK-rho0 cell line by about 1.5 log₁₀ PFU/ml in comparison to the parental cell line, 143B.TK/H11002 (Fig. 4A). This supports the hypothesis of a contribution of oxphos to RV replication.

Application of antimycin A as a respiratory chain inhibitor. AMA, as a mitochondrial electron chain blocker, was employed to corroborate the data on RV-infected 143B.TK-rho0 cells and to further evaluate the contribution of a functional oxphos to RV replication. After 72 h of cultivation in the continued presence of AMA, the titer of RV produced by the 143B.TK-rho0 cell line was reduced by about 1.5 log₁₀ PFU/ml in comparison to the solvent control (Fig. 4A). Two separate experimental observations suggest that titer reduction on the 143B.TK-rho0 cell line was not due to different sensitivities of the 143B.TK-rho0 and the parental cell lines to RV infection.

FIG. 4. RV infection of a rho0 cell line and of cell cultures treated with AMA or kept under hypoxia. (A) The effect of the incubation of the indicated cell lines with 20 μM AMA for 72 h on RV infection (Therien and M33 strains) was analyzed in comparison to the mitochondrial-DNA-depleted cell line 143B.TK-rho0. The viral titer was determined by standard plaque assay for the supernatant collected at 3 days p.i. The error bars indicate the standard deviation. *, P < 0.05. (B) Time-dependent effect of the continued incubation of RV-infected Vero cells with 20 μM AMA on viral RNA and new virus progeny production as determined by qRT-PCR and standard plaque assay. (C) Cytotoxicity caused by the continued incubation of Vero cells with 20 μM AMA was determined by cytotoxicity assay. (D) Vero cells were cultured under normoxic and hypoxic conditions for 24, 48, and 72 h. Total RNA was extracted and subjected to qRT-PCR for quantification of RV genomic RNA. The RV titer was determined by plaque assay. (E) Cytotoxicity caused by the continued incubation of Vero cells under hypoxia was determined by cytotoxicity assay.

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First, similar amounts of genomic viral RNA were measured by qRT-PCR in both cell lines at 2 h p.i. and 1 day p.i. Second, the titer on AMA-treated 143B.TK cells was similar to that obtained for 143B.TK cells (Fig. 4A). Further experiments also revealed a reduction in virus titers obtained from AMA-treated vero and MCF-7 cells by about 2 log_{10} PFU/ml, which was similar in RV strains Therien and M33 (Fig. 4A). Additionally, treatment of mock- and RV-infected Vero cells with AMA was characterized by a significant drop in the intracellular ATP content, by about 30%. This indicates that RV does not counteract the effect of AMA on the intracellular ATP level. Moreover, the effect of AMA on RV replication was time dependent. In comparison to the solvent control, AMA-induced reductions in genomic RNA and viral progeny production were highest at 2 days p.i. (Fig. 4B). The effect was more pronounced at the level of intracellular viral genomic RNA than at the level of new progeny virus generation (Fig. 4B). RV- and mock-infected Vero cell viability (assessed by a cytotoxicity assay) was not significantly affected by treatment with AMA (Fig. 4C). Thus, impairment of oxphos by AMA treatment results in a reduction of RV replication and titer, which emphasizes its contribution to the establishment of a successful RV infection.

**Cultivation of RV under hypoxic conditions.** Mitochondrial metabolism is repressed under hypoxic conditions, enabling characterization of RV infection with reduced oxphos that is not induced by a chemical compound. At oxygen levels below 5%, hypoxia-inducible factor 1 (HIF-1) is induced (19). To not induced by a chemical compound. At oxygen levels below

Mitochondrial ATP concentration was reduced in hypoxic mock- and RV-infected Vero cells by 72% and 68%, respectively. Analogous to the application of AMA to RV-infected vero cells, the reduction in viral titer occurred in a time-dependent manner (Fig. 4D) and was highest at 2 days p.i., with almost 2 log_{10} PFU/ml (48 h of cultivation under hypoxia). However, the reduction at the level of viral RNA in hypoxic Vero cells (Fig. 4D) was higher at 2 and 3 days p.i. than in AMA-treated vero cells (Fig. 4B), which could at least in part be due to the higher reduction in intracellular ATP under hypoxia. Additionally, neither treatment with AMA nor cultivation under hypoxia had an effect on RV-induced redistribution of mitochondria (data not shown). There were no obvious differences in cytotoxicity between cells cultivated under normoxia and hypoxia (Fig. 4E).

**Expression level and membrane association of p32.** p32 mainly resides in the mitochondrial matrix but shuttles between the nucleus and mitochondria. The subcellular localization of p32 influences its function and possibly RV replication. Hence, four different cell lines (HEK293T/17, BHK21, vero, and MCF-7) were subjected to extraction of hydrophilic (cytoplasmic) and hydrophobic (membrane) protein fractions to assess the subcellular localization of p32. Calnexin, as a marker protein for subcellular fractions, indicates that little cross-contamination has occurred during protein extraction. Complete disruption of mitochondria was indicated by the mitochondrial matrix protein glutamate dehydrogenase (GDH), which was found only in the hydrophilic fraction of 293T/17, BHK21, and Vero cells (Fig. 5A). Unfortunately, and for unknown reasons, the antibody against GDH did not detect a protein band in MCF-7 cells. However, the suitability of the extraction method for the remaining three cell lines was shown. While p32 mainly found in the hydrophobic protein fraction of RV-permissive BHK21, vero, and MCF-7 cells, HEK293T/17 cells possesses a distinctive portion of p32 in their hydrophilic protein fraction.

Figure 5B suggests fair differences in the band pattern profiles of p32 between mock- and RV-infected vero cells. This indicates that the mitochondrial expression levels of p32 could differ between RV- and mock-infected vero cells. Therefore, isolated mitochondria were directly subjected to Western blot analysis. Figure 5C illustrates that mitochondria isolated from RV-infected vero cells at 3 days p.i. contain a slightly smaller portion of p32 than mitochondria isolated from the mock-infected population. In summary, while the subcellular distribution of p32 appears to vary between different cell lines, RV infection alters the expression and subcellular localization of p32 only to an extent.

**Analysis of mitochondrial membrane potential.** The mitochondrial membrane potential (ΔΨ_{m}) represents an important parameter for cellular metabolism in general and the mitochondrial energy status in particular. Therefore, ΔΨ_{m} was measured in mock- and RV-infected cells with ΔΨ_{m}-sensitive fluorescent dyes. The first approach was to determine ΔΨ_{m} with the dye TMRE. Since this dye does not take changes in

![FIG. 5. Western blot analysis of p32 protein expression levels. RV infection (MOI = 5) was monitored through expression of E2 glycoprotein and C protein. (A and B) Hydrophilic (H) and hydrophobic (M) extracts were obtained with the ProteoJet membrane protein extraction kit. Twenty micrograms of total protein was resolved. Immunoblotting was carried out with antibodies against the indicated proteins. Calnexin and alpha-tubulin were used as internal loading controls. (A) Expression of p32 was characterized for the indicated cell lines (2 days of culture). The presence of the mitochondrial matrix protein GDH in the hydrophilic protein fraction indicates successful preparation of protein extracts. (B) Expression of p32 at 3 days p.i. in mock- and RV-infected Vero cells. (C) Expression levels of the indicated proteins determined by Western blot analysis using isolated mitochondria at 10 μg. Equivalent protein loading was verified using an antibody against GDH.](http://jvi.asm.org/)
mitochondrial mass or volume into account. TMRE uptake was normalized through determination of the fluorescence of MitoTracker Green FM, which accumulates in mitochondria independently of $\Delta W_m$. TMRE loading was finally expressed as a ratio of TMRE fluorescence to MitoTracker Green FM fluorescence. $\Delta W_m$ in RV-infected Vero cells is shown in Fig. 6A and was calculated relative to the corresponding mock-infected control, which was designated 100%. In comparison to the mock-infected population, $\Delta W_m$ in RV-infected Vero cells is significantly increased at 3 days p.i., by up to 30% (Fig. 6A). The second approach involved the dye JC-1, which exists at low fluorescence and at high $\Delta W_m$ as red/orange-fluorescing aggregates (590-nm emission). JC-1 staining was performed for cell lines infected at an MOI of 5 at 3 days p.i., for which staining with TMRE indicated a significant change in $\Delta W_m$. The staining pattern of JC-1 differs between Vero and MCF-7 cells. While MCF-7 cells show both red (Fig. 6B, e) and green (Fig. 6B, f) fluorescence, Vero cells hardly respond to the dye (Fig. 6B, a and b). This is probably attributable to intrinsic and cell line-specific factors affecting $\Delta W_m$ and/or JC-1 uptake. This was not improved by increasing the concentration of JC-1. However, in comparison to the mock-infected population (Fig. 5B, a and e), a remarkable increase in red-emitting, highly energized mitochondria can be detected for both cell lines after infection with RV (Fig. 5B, c and g). Hence, both mitochondrial-selective dyes, TMRE and JC-1, indicate that RV infection increases $\Delta W_m$ over the time of infection. This suggests an influence of RV on mitochondrial functionalities.

Intracellular ATP content of RV-infected Vero cells. $\Delta W_m$, as an important indicator of cellular metabolism, is increased during RV infection. Therefore, the next attempt at characterization of the influence of RV on mitochondrial activity was to determine the intracellular ATP levels of mock- and RV-infected Vero cells. Figure 6C shows an increase in intracellular ATP levels for an MOI of 1 at 1 day p.i. (15%) and for an MOI of 5 at 3 days p.i. (30%). Otherwise, the intracellular ATP level in RV-infected Vero cells was comparable to that obtained for the mock-infected population. Mitochondria use the potential energy that is stored in the $\Delta W_m$ for most of their functions, including ATP generation. Therefore, the observed RV-induced increase in $\Delta W_m$ (Fig. 6A and B) could be associated with the observed increase in intracellular ATP levels.

Mitochondrial respiratory functions in rubella virus-infected cells. The mitochondrial electron transport or respiratory chain generates $\Delta W_m$ and drives the formation of ATP. For further examination of the influence of RV on mitochondrial functions, the enzyme activities of two mitochondrial electron transport chain complexes were determined. There were no obvious differences in the activities of mitochondrial complex I (NADH dehydrogenase complex) between RV- and mock-infected Vero cells (Fig. 6D). On the other hand, at 2 days p.i., a remarkable increase in the activity of respiratory chain complex III (the cytochrome c oxidase complex) was detected (Fig. 6E). This increase was even more profound after treatment of RV-infected Vero cells with cytochalasin D at 6 h p.i. (Fig. 6E, bottom). This could be explained by the increase in mitochondrial cluster formation after cytochalasin D treatment of RV-infected Vero cells (Fig. 3C). Treatment of isolated mitochondria with the complex III inhibitor AMA served as a control and resulted in the expected loss of complex III activity (Fig. 6E, top).

DISCUSSION

Besides the basic function of providing energy, mitochondria are involved in calcium homeostasis, apoptosis, and cellular defense mechanisms. Various viruses target mitochondria to alter some of these functions and/or utilize them for their own purposes. This study was aimed at a better understanding of the contribution of a functional oxphos and the mitochondrial protein p32 to RV replication. The data on RNA interference (RNAi)-mediated knockdown of p32 indicate the involvement of p32 in targeted trafficking of mitochondria to a perinuclear region in RV-infected Vero cells. Moreover, RV infection was associated with remarkable changes of mitochondrial functionalities and thus the energy status of the cell. RV infection results in higher intracellular ATP levels, which could be the result of the observed increase in mitochondrial membrane potential and activity of electron chain complex III.

Besides RV there are two prominent examples of viruses that induce cluster formation of mitochondria. African swine fever virus induces the migration of mitochondria to the site of virus assembly in order to provide energy for this process (39). In hepatitis B virus-infected cells, mitochondria cluster in a perinuclear region, which is induced by the viral X protein (23). However, mitochondria are not always required for the successful establishment of a virus infection. Poliovirus inhibits oxphos (24) and converts almost all cellular organelles except mitochondria into viral replication sites (4). Ethidium bromide treatment of Rous sarcoma virus-infected cells impaired mitochondrial functions, but not virus production or viral RNA synthesis (27). In striking contrast to most other virus infections, RV infection has a positive effect on cellular metabolism.

Despite the amount of data available for p32, the precise function of p32 in mammalian mitochondria remains unknown. A yeast mutant lacking the gene for the p32 homologue is characterized by a low growth rate in glycerol but not in glucose medium. Transfection with the human p32 gene restored the growth rate, suggesting a conserved function of p32 in oxphos (34). Additionally, p32 serves as a marker for metabolically deprived tumor regions, and after knockdown of p32, a shift to glycolysis was detected (11, 12). These results are in accordance with the observed growth retardation upon p32 knockdown in Vero cells. The results of this study regarding RV-infected p32 knockdown Vero cells confirm previous hypotheses on effects mediated by p32: association of C protein with mitochondria occurs independently of p32, and C-p32 complex formation aids in the aggregation of mitochondria (2). Recently, it was demonstrated that C protein impairs p32 import into mitochondria and that RV infection results in higher cytoplasmic levels of p32 than in mock-infected Vero cultures (17). This may result in retention of p32 in the cytoplasm and complex formation with C protein. In turn, complex formation could support trafficking of mitochondria to RV replication sites along microtubules. Besides p32 involvement in the trafficking of the splicing factor U2AF26 and the adenosvirus core protein V to the nucleus (16, 31), the data from this work suggest that p32 directs mitochondrial trafficking, as well. The shift of p32 to the nucleus by the adenosvirus core protein V
FIG. 6. Analysis of mitochondrial activity in RV-infected Vero cells. (A). Changes in mitochondrial membrane potential in RV-infected Vero cells were expressed as the ratio of the fluorescence intensity of TMRE and MitoTracker green and normalized to the mock-infected population, which was set at 100%. The error bars indicate the standard deviation. *P < 0.05. (B) Membrane potential changes in mock- and RV-infected Vero and MCF-7 cells (3 days p.i.) monitored by staining with JC-1 dye. The images were obtained with a fluorescence microscope using FITC and tetramethyl rhodamine isocyanate (TRITC) channels and a 20x objective. (C) The intracellular ATP contents of RV- and mock-infected Vero cells were determined by a CellTiter-Glo luminescent cell viability assay. (D) Assay of complex I activities in mitochondria isolated from mock- and RV-infected Vero cells. (Top) Increase of absorbance at 550 nm after reduction of cytochrome c. (Bottom) Enzyme activity calculated from the initial linear portion of the kinetic curve exemplified at the top.
could explain the Western blot analysis shown in Fig. 1B. There is some controversy in the literature concerning the subcellular localization of p32. Muta and colleagues have reported on an almost exclusive localization of p32 in the mitochondrial matrix of U937 cells (34), whereas the crystal structure of p32 suggests association with the inner mitochondrial membrane through homotrimeric channel formation (20). Western blot analysis of hydrophobic and hydrophilic protein extracts of the four cell lines analyzed in this study suggests that subcellular localization of p32 depends on the cellular context. The p32 protein in the RV-permissive cell lines BHK21, Vero, and MCF-7 appears to be mainly membrane bound, which could account for the reported difficulties in downregulation of p32 in RV-permissive cell lines (17). Knockdown of p32 was successful in other cell lines, e.g., HeLa cells (16, 21) and the 293T cell line (37). The latter showed only partial membrane association of p32 (Fig. 5A). As far as we are aware, this is the first report of cell line-dependent differences in subcellular localization of p32. This could have implications for the ligand-binding properties of p32 and its functions in maintenance of oxphos, which appear to be cell line dependent (11). How this relates to other RV-permissive cell lines and RV infection in general is still unknown. The p32 protein is characterized by three potential N-linked glycosylation sites (38) that could account for the presence of two p32 bands in Western blot analysis of Vero cells (Fig. 5).

The microtubule-directed arrangement of mitochondria in proximity to RV replication complexes apparently supports energy demands of the virus. Our data on depolymerization of microtubules are in agreement with a recent publication on their involvement in RV infection (32). However, we have extended the published time frame to the early hours of infection, which appear to be the only important hours for mitochondrial redistribution. Previously reported increases in the transport rate of mitochondria along microtubules after disassembly of actin filaments (25) and knockdown of myosin V (36) help to explain the data on cytochalasin D-treated infected Vero cells. After application of cytochalasin D, the number of infected cells with clustered mitochondria increases, which in turn is associated with higher activity of respiratory chain complex III than in the RV-infected solvent control. This indicates that p32- and microtubule-directed cluster formation of mitochondria supports the modulation of mitochondrial activity by RV. RV could have evolved a mechanism to efficiently use the energy resources of the infected cell and to provide abundant ATP to the sites of viral replication. On the other hand, other viruses such as vaccinia virus, increase intracellular ATP by up to 80% (6). Three lines of evidence outlined in this study suggest that RV requires a functional oxpho for efficient progression of infection: involvement of p32 as a reported regulator of cellular metabolism (11), microtubule-based trafficking of mitochondria, and modulation of oxpho. According to the literature review, there are only a few examples of increased activity of respiratory chain complexes, such as the HIV-1-induced increase in complexes IV and V (41) and the melatonin-induced increase in activity of complexes I and IV (30). As $\Delta \Psi_m$ and ATP generation are influenced by the transport rate of electrons in the respiratory chain, the hyperactivity of complex III at 2 days p.i. preceded an increase in $\Delta \Psi_m$ (shown in Fig. 6A and B) and intracellular ATP content (Fig. 6C) at 3 days p.i. The effect of altered activity of a respiratory chain complex on $\Delta \Psi_m$ and/or intracellular ATP content can occur with a time delay (45). Besides ATP consumption, RV could require mitochondria to obtain cardiolipin, a constituent of RV particles (1). The mRNA level of cardiolipin synthase (CLS) in yeast varies under different growth conditions and is directly coupled to the protein level (40). However, qRT-PCR analysis revealed that the level of CLS mRNA remained constant during RV infection of Vero cells (data not shown).

The results of this work lead to an interesting question: could the interaction of RV with p32 and mitochondria impair mitochondrial functions and cellular metabolism to such an extent that the pathology of fetal RV infection might be explained? There is evidence that RV spreads through the vascular system of the infected fetus, which is probably associated with extensive focal cytopathic damage to the walls of blood vessels and the lining of the heart (44). Inefficient mitochondrial metabolism can be associated with atherosclerosis and vascular damage (5). Moreover, dysfunction of mitochondria impairs the early development of mammalian embryos (9), highlighting the importance of further efforts to understand the interaction of RV with mitochondria and its influence on cellular metabolism.

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