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Functional Analysis of Picornavirus 2B Proteins: Effects on Calcium Homeostasis and Intracellular Protein Trafficking

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Received 19 September 2007/Accepted 26 December 2007

The family Picornaviridae consists of a large group of plus-strand RNA viruses that share a similar genome organization. The nomenclature of the picornavirus proteins is based on their position in the viral RNA genome but does not necessarily imply a conserved function of proteins of different genera. The enterovirus 2B protein is a small hydrophobic protein that, upon individual expression, is localized to the endoplasmic reticulum (ER) and the Golgi complex, reduces ER and Golgi complex Ca2+ levels, most likely by forming transmembrane pores, and inhibits protein trafficking through the Golgi complex. At present, little is known about the function of the other picornavirus 2B proteins. Here we show that rhinovirus 2B, which is phylogenetically closely related to enterovirus 2B, shows a similar subcellular localization and function to those of enterovirus 2B. In contrast, 2B proteins of hepatitis A virus, foot-and-mouth disease virus, and encephalomyocarditis virus, all of which are more distantly related to enteroviruses, show a different localization and have little, if any, effects on Ca2+ homeostasis and intracellular protein trafficking. Our data suggest that the 2B proteins of enterovirus and rhinovirus share the same function in virus replication, while the other picornavirus 2B proteins support the viral life cycle in a different manner. Moreover, we show that an enterovirus 2B protein that is retained in the ER is unable to modify Ca2+ homeostasis and inhibit protein trafficking, demonstrating the importance of Golgi complex localization for its functioning.
thereby reducing the levels of Ca²⁺ and H⁺ in the lumens of these organelles in infected cells (8, 14, 39). Individual expression of 2B furthermore results in inhibition of protein trafficking through the Golgi complex (14, 17). It is unknown whether these activities represent different functions of 2B or whether the one activity is the consequence of the other. The observation that 2B mutants that are impaired in increasing the efflux of ions from the ER and Golgi complex are also impaired in inhibiting protein trafficking suggests that these activities are somehow connected (8, 14). The relevance of these 2B activities for the viral life cycle is still poorly understood. Mutations that interfere with the ability of 2B to disturb ER and Golgi complex ion homeostasis and/or to inhibit membrane trafficking cause early defects in viral RNA replication (9, 40). These 2B functions may be required for the activity of the precursor 2BC to accumulate membranous replication vesicles, but other possibilities cannot be excluded.

In this study, we investigated whether the structural and functional aspects of the enterovirus 2B protein are conserved among other members of the picornavirus family. To this end, the phylogenetic relationships and degree of conservation between the 2B proteins of CBV, PV, rhinovirus, hepatovirus, aphthovirus, and cardiovirus were defined. In addition, subcellular localization and possible effects on organelle Ca²⁺ homeostasis and intracellular protein trafficking were investigated. Finally, the importance of the subcellular localization of enterovirus 2B for its functions was studied.

MATERIALS AND METHODS

Cells and medium. Buffalo green monkey (BGM) cells were grown in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum, 100 units penicillin per ml, and 25 µg streptomycin per ml. Cells were grown at 37°C in a 5% CO₂ incubator.

Antiserum. Rabbit polyclonal anti-enhanced green fluorescent protein (anti-EGFP) was described previously (15). Mouse monoclonal anti-c-emc (clone 9E10) and anti-Flag M2 antiserum were obtained from Sigma-Aldrich. Rabbit polyclonal anti-calreticulin was obtained from Affinity Bioreagents, Inc. Fluorescein isothiocyanate-conjugated goat anti-rabbit polyclonal antibody, Texas Red-conjugated goat anti-mouse polyclonal antibody, and Texas Red-conjugated goat anti-rabbit polyclonal antibody were obtained from Jackson ImmunoResearch Laboratories.

Plasmids. pSVSV-G(tos65)-EGFP (37) was a kind gift from P. Keller and T. Simons, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. The expression constructs pER-AEQ (30), pGolgi-AEQ (27), p2B-EGFP, 2B-EGFP-AAAA, 2B-EGFP-KKAA, and p2B-myc p2B-I64S/V66S were obtained from Simons, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. The expression constructs pER-AEQ (30), pGolgi-AEQ (27), p2B-EGFP, 2B-EGFP-AAAA, 2B-EGFP-KKAA, and p2B-myc p2B-I64S/V66S were obtained from Simons, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany.

Immunofluorescence. Cells were transfected with EGFP-tagged and myc-tagged or Flag-tagged 2B proteins. At 24 h posttransfection, aequorins were reconstituted with coelenterazine-N. To efficiently renature the aequorin chimeras and to measure [Ca²⁺] response curves of mutant aequorin (2). Inhibition of protein trafficking. Immunofluorescence was performed as described above (see “Subcellular localization”). BGM cells were transfected with 0.5 µg p2B-myc and 0.5 µg pVSV-G-GFP(ts045), incubated at 40°C for 18 h, and additionally incubated at 32°C for 2 h. Cells were fixed, permeabilized, and stained. Cells were analyzed by CSLM (Leica TCS NT; Leica Lasertech GmbH, Heidelberg, Germany).

Comunoprecipitation. BGM cells were cotransfected with EGFP-tagged and myc-tagged or Flag-tagged 2B proteins. At 24 h posttransfection, cells were lysed and EGFP-tagged proteins were precipitated using an anti-EGFP antibody, as described previously (15). Samples were run in sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and immunodetected with antisera against both EGFP and either c-myc or Flag. Proteins were visualized using a chemiluminescence detection system (Amersham Pharmacia Biotech).

Statistical analysis. Data are presented as mean values ± standard deviations. Differences were tested for significance by means of Student’s t test.

RESULTS

Phylogenetic analysis of picornavirus 2B proteins. A phylogenetic analysis of the 2B proteins of enterovirus, rhinovirus, cardiovirus, aphthovirus, and hepatovirus was performed. The enterovirus genus can be divided into two subgroups (on the basis of differences in amino acid sequence), the CBV-like subgroup and the PV-like subgroup. Both CBV3 2B and PV 2B were included for a direct comparison of their effects in the same cell type, using the same expression constructs. The rhinovirus genus is divided into groups A and B (34). Sequence comparison of the 2B proteins of HRV group A and HRV group B showed that they were very similar (data not shown), and therefore only HRV14 (HRV group B) was used as a representative of the rhinovirus genus. EMCV was used as a representative of the cardiovirus genus, FMDV was used as a repre-
sentative of the aphthovirus genus, and HAV was used as a representative of the hepatovirus genus.

Phylogenetic analysis showed that the 2B proteins of CBV3, PV1, and HRV14 are closely related (Fig. 1A). The 2B proteins of both PV1 and HRV14 share 51% and 55% identity (71% and 74% similarity), respectively, with CBV3 2B and 49% identity (71% similarity) with each other (Fig. 1B). Like CBV3 2B, both PV1 2B and HRV14 2B contain two hydrophobic regions (referred to as HR1 and HR2) (Fig. 1C), of which the first is predicted to form a cationic amphipathic α-helix, typical of membrane-lytic polypeptides (Fig. 1D), separated by a short hydrophilic sequence (38).

The 2B proteins of EMCV, FMDV, and HAV share little, if any, relationship (<20% sequence identity) to the enterovirus and rhinovirus 2B proteins (Fig. 1A and B). They also share little sequence identity with each other or with the 2B proteins of CBV3, PV1, and HRV14.
of aichivirus (kobuvirus), human parechovirus 2 (parechovirus), porcine teschovirus (teschovirus), and equine rhinitis B virus (erbovirus) (Fig. 1A and B). The EMCV, FMDV, and HAV 2B proteins are larger than the enterovirus and rhinovirus 2B proteins (EMCV 2B, 151 aa; FMDV 2B, 155 aa; and HAV 2B, 251 aa). They contain one or more hydrophobic regions, but none of them contains a cationic amphipathic α-helix typical of the group of membrane-lytic polypeptides, as can be found in all enterovirus and rhinovirus 2B proteins.

Subcellular localization of picornavirus 2B proteins. CBV3 2B is present predominantly in the Golgi complex upon individual expression (15). Here we determined the subcellular localization of the other picornavirus 2B proteins. To this end, the coding regions of the picornavirus 2B proteins were amplified and cloned into a eukaryotic expression vector that drives the expression of proteins carrying a c-myc tag at the C terminus. The experiments were performed with BGM kidney cells because all viruses used in this study are able to replicate in kidney cell lines, indicating that the 2B proteins have the ability to fulfill their function(s) in these cells (5, 7, 25, 26). BGM cells were transfected with the expression constructs, and the subcellular distribution of the 2B proteins was compared with that of markers for the Golgi complex (EGFP-Golgi) and the ER (calreticulin), as described previously (15).

Like CBV3 2B, PV1 2B colocalized predominantly with the Golgi marker (D) but was colocalized predominantly with the ER marker (E). FMDV 2B (F and G) and EMCV 2B (H and I) did not colocalize with the Golgi and ER markers. Bars = 10 μm.

FIG. 2. Subcellular localization of picornavirus 2B proteins. (A to D, F, and H) BGM cells were cotransfected with EGFP-Golgi and the indicated 2B-myc constructs, fixed at 24 h posttransfection, and stained with anti-c-myc antiserum. (E, G, and I) BGM cells were transfected with the indicated 2B-myc constructs, fixed at 24 h posttransfection, and stained with anti-c-myc and anti-calreticulin antisera. Cells were analyzed by CLSM. The 2B-myc proteins of CBV3 (A), PV1 (B), and HRV14 (C) colocalized with the Golgi marker. HAV 2B did not colocalize with the Golgi marker (D) but was colocalized predominantly with the ER marker (E). FMDV 2B (F and G) and EMCV 2B (H and I) did not colocalize with the Golgi and ER markers. Bars = 10 μm.
In lowly and moderately expressing cells, FMDV and EMCV 2B proteins showed no colocalization with either calreticulin or EGFP-Golgi (Fig. 2F to I), indicating that these proteins are not present in the ER or the Golgi complex. In cells expressing high levels of FMDV 2B, staining and morphology of both the ER and the Golgi complex were often affected, yet there was still no obvious colocalization of 2B and the ER or Golgi marker (not shown).

Intraorganelle calcium concentrations in 2B-expressing cells. We previously demonstrated that expression of CBV3 2B reduced ER and Golgi complex Ca^{2+} levels (8, 14). Here we analyzed whether the other picornavirus 2B proteins also have the ability to modify ER and Golgi complex Ca^{2+} homeostasis, using the Ca^{2+}-sensitive photoprotein aequorin as described previously (8). To this end, BGM cells were cotransfected with the different 2B-myc constructs and either pCI-ER-AEQ, pCI-Golgi-AEQ, or the empty pCI expression vector. Ca^{2+} measurements were performed at 24 h posttransfection. Offline calibration of the aequorin bioluminescence data into [Ca^{2+}] values. Representative traces are shown for CBV3 2B. (A) ER Ca^{2+} levels were reduced in cells expressing CBV3, PV1, HRV14, or EMCV 2B but not in cells expressing HAV 2B. (B) Golgi complex Ca^{2+} levels were reduced in cells expressing CBV3, PV1, or HRV14 2B but not in cells expressing HAV or EMCV 2B.

Effects of picornavirus 2B proteins on protein trafficking through the secretory pathway. Next, we investigated whether the other picornavirus 2B proteins are able to interfere with protein trafficking through the secretory pathway. We used a temperature-sensitive mutant of the envelope glycoprotein of vesicular stomatitis virus (VSV-G-ts045; hereafter referred to as VSV-G) fused to fluorescent EGFP (VSV-G–GFP) to study protein trafficking. We used a temperature-sensitive mutant of the envelope glycoprotein of vesicular stomatitis virus (VSV-G-ts045; hereafter referred to as VSV-G) fused to fluorescent EGFP (VSV-G–GFP) to study protein trafficking. The temperature-sensitive VSV-G–GFP mutant is misfolded at 40°C, resulting in its accumulation in the ER. Upon shifting of the temperature to 32°C, the protein
is refolded correctly and transported via the secretory pathway, resulting in its exposure on the plasma membrane (16).

BGM cells were cotransfected with expression constructs for VSV-G–GFP and the picornavirus 2B-myc proteins. Cells were incubated at 40°C for 18 h to accumulate VSV-G–GFP in the ER and subsequently incubated at 32°C for 2 h (B to G). Cells were fixed, stained with the anti-c-myc antiserum, and processed for CLSM analysis. (A) Accumulation of VSV-G–GFP in the ER upon incubation at 40°C (left) and on the plasma membrane upon additional incubation at 32°C (right). VSV-G–GFP accumulated in the Golgi complex upon incubation with CBV3 2B (B), PV1 2B (C), and HRV14 2B (D). VSV-G–GFP was exposed on the plasma membrane in cells expressing HAV 2B (E), FMDV 2B (F), or EMCV 2B (G). Bars = 10 μm.

FIG. 4. Analysis of protein trafficking in cells expressing picornavirus 2B proteins. BGM cells were transfected with the VSV-G–GFP construct (A) or cotransfected with VSV-G–GFP and the picornavirus 2B-myc constructs (B to G). Cells were incubated at 40°C for 18 h to accumulate VSV-G–GFP in the ER and subsequently incubated at 32°C for 2 h (B to G). Cells were fixed, stained with the anti-c-myc antiserum, and processed for CLSM analysis. (A) Accumulation of VSV-G–GFP in the ER upon incubation at 40°C (left) and on the plasma membrane upon additional incubation at 32°C (right). VSV-G–GFP accumulated in the Golgi complex upon incubation with CBV3 2B (B), PV1 2B (C), and HRV14 2B (D). VSV-G–GFP was exposed on the plasma membrane in cells expressing HAV 2B (E), FMDV 2B (F), or EMCV 2B (G). Bars = 10 μm.

Is Golgi complex localization of 2B required for alterations in calcium homeostasis and protein trafficking? Thus far, we have demonstrated that the 2B proteins of PV1, CBV3, and HRV14 are closely related and that these proteins localize predominantly to the Golgi complex, reduce ER and Golgi complex Ca²⁺ levels, and inhibit protein trafficking. We asked ourselves whether the Golgi complex localization of enterovirus and rhinovirus 2B proteins is required for their ability to modify Ca²⁺ homeostasis and to inhibit protein trafficking or if 2B can also exert these functions from the ER. To this end, we made use of wild-type CBV3 2B-EGFP carrying a C-terminal KKAA dilysin motif (2B-EGFP-KKAA), which acts as an ER retention signal in mammalian cells (15). Cells were cotransfected with expression constructs for ER-targeted or Golgi complex-targeted aequorins and either 2B-EGFP-KKAA or the control plasmid EGFP or 2B-EGFP-AAAA, and the ER and Golgi complex Ca²⁺ concentrations were determined as described above. Figure 5A and B show that expression of 2B-EGFP-KKAA had no effect on the filling state of the ER and Golgi complex Ca²⁺ stores. The finding that 2B-EGFP-AAAA expression reduced both ER and Golgi complex Ca²⁺ levels demonstrates that the presence of a C-terminal tag does not inhibit 2B-EGFP function. Consistently, the ER-retained Flag-2B-KKAA did not alter VSV-G–GFP trafficking, while Flag-2B-AAAA expression resulted in the accumulation of VSV-G–GFP in the juxtanuclear Golgi region, where it colocalized with 2B-AAAA (Fig. 5C). The possibility that the functions of ER-retained 2B-EGFP-KKAA are impaired because
the KKAA tag somehow interferes with 2B homomultimerization, which is a prerequisite for 2B to exert its functions, was considered (8, 12, 14, 15). To investigate multimerization, coimmunoprecipitation experiments were set up. First, multimerization of wild-type 2B was tested using this method. BGM cells coexpressing wild-type 2B-EGFP and 2B-myc were lysed at 24 h posttransfection, and the EGFP-tagged proteins were precipitated using an anti-EGFP antibody as described previously (15). Western blot analysis showed that 2B-myc was efficiently coimmunoprecipitated with 2B-EGFP but not with EGFP alone or the multimerization-deficient mutant 2B-I64S/V66S-EGFP (13) (Fig. 5D). To investigate homomultimerization of 2B-KKAA, BGM cells coexpressing Flag-2B-KKAA and 2B-EGFP-KKAA expression constructs. Figure 5D shows that Flag-2B-KKAA was coimmunoprecipitated with 2B-EGFP-KKAA but not with EGFP. Analysis of the lysates that were used as input for the coimmunoprecipitation experiments demonstrated that all proteins were properly expressed.

the presence of the KKAA tag. All proteins were efficiently expressed, excluding the possibility that differences in coprecipitation were due to different expression levels.

Together, these results further emphasize the importance of the Golgi complex localization of enterovirus 2B, and most likely also of rhinovirus 2B, for its activities in the host cell.

**DISCUSSION**

This study was performed to gain more insight into the phylogenetic conservation of the structure and function of the picornavirus 2B proteins. Our results demonstrate that the structural and functional properties of enterovirus 2B (represented in this study by CBV3 and PV1 2B) and rhinovirus 2B (represented by HRV14 2B) are strongly conserved. Phylogenetic analysis revealed that CBV3, PV1, and HRV14 2B proteins are closely related and share approximately 50% identity.
and >70% similarity. Despite differences in amino acid sequence, CBV3, PV1, and HRV14 2B proteins share remarkable structural similarities. These proteins are similar in length (97 to 99 aa) and contain two hydrophobic regions, the first of which is predicted to form a cationic amphipathic α-helix typical for the group of lytic polypeptides. The amphipathic α-helices of these 2B proteins all contain three lysine residues in the (small) hydrophilic face of the helix. CBV3 2B is present at ER and Golgi complex membranes (15) and is responsible for the release of Ca^{2+} and H⁺ from these organelles, most likely by forming membrane-integral pores (8, 14). Moreover, CBV3 2B has been shown to inhibit protein trafficking through the Golgi complex and to increase hygromycin B entry (14, 40). Here we demonstrate that PV1 and HRV14 2B proteins also localize to the Golgi complex, reduce ER and Golgi complex Ca^{2+} levels, and inhibit VSV-G trafficking through the Golgi complex, whereas the more distantly related HAV, FMDV, and EMCV 2B proteins behave differently.

The 2B protein of HAV (a member of the hepatoviruses) shares little or no sequence and structural homology with the other picornavirus 2B proteins. HAV 2B had little, if any, effect on ER and Golgi complex Ca^{2+} homeostasis. Moreover, it did not inhibit protein trafficking through the secretory pathway. The latter finding is consistent with the hypothesis that HAV uses the secretory pathway to leave the cell, as virus release of the cell culture-adapted HAV strain from polarized epithelial cells was shown to be sensitive to trafficking inhibiting drugs (6). Analysis of the subcellular localization of HAV 2B demonstrated that it localized predominantly in the ER and was absent from the Golgi complex. Immunoelectron microscopy analysis of the subcellular localization of HAV 2B in transfected cells (FRhK-4 and HeLa) demonstrated that the protein was present almost exclusively in a tubular-vesicular network, whose origin (i.e., the presence of organelle-specific markers) was not investigated (19). Our finding that HAV 2B largely colocalizes with a marker for the ER suggests that the tubular-vesicular structures may be derived, at least partly, from the ER.

The 2B proteins of FMDV and EMCV (members of the aphthoviruses and cardioviruses, respectively) are closely related to any of the other picornavirus 2B proteins. FMDV 2B and EMCV 2B appeared to be absent from the ER and the Golgi complex. Instead, they showed a punctate localization pattern in the cytosol, whose exact nature remains to be established. The localization of these proteins in highly expressing cells is less clear, and we cannot exclude the possibility that in these cells there is some degree of colocalization with ER markers, as seen for FMDV 2B (23, 24). Although EMCV 2B does not show a clear ER localization and lacks an amphipathic helix that is typical for the group of lytic polypeptides, expression of this protein was found to reduce the ER Ca^{2+} level (but not the Golgi complex Ca^{2+} level). The mechanism underlying this reduction remains to be established. Unfortunately, possible effects of FMDV 2B on ER and Golgi complex Ca^{2+} levels could not be investigated reliably because overexpression of this protein interfered with the correct localization of the calcium-sensitive aequorin probes. Neither FMDV 2B nor EMCV 2B interfered with protein trafficking through the secretory pathway. We are not aware of studies addressing protein trafficking in EMCV-infected cells, but early secretory pathway transport in FMDV-infected cells is inhibited (23). In agreement with our results, Moffat and coworkers recently showed that FMDV 2B alone does not inhibit transport but that coexpression of 2B and 2C or expression of the 2BC precursor protein is required for this effect (24).

Expression of the 2B proteins of both PV1 and CBV3 was previously shown to result in increased entry of hygromycin B, a drug that under normal conditions enters cells poorly (12, 15, 17, 40). The mechanism by which these enterovirus 2B proteins increase hygromycin B entry is still unknown. A possible explanation was recently provided by Cornell et al., who showed that endocytosis is upregulated upon individual expression of CBV3 2B and 2BC (10). There are strong indications that this effect is directly linked to the abilities of 2B to reduce ER and Golgi complex Ca^{2+} levels and to inhibit protein transport because mutations that disrupt these abilities also impair the ability of 2B to increase hygromycin B entry (8, 12, 14, 15, 40). Consistently, we found that expression of HRV 2B caused a similar strong increase in hygromycin B entry to that of CBV 2B, whereas expression of HAV, FMDV, and EMCV 2B proteins had little effect (data not shown).

To investigate the importance of the Golgi complex localization of the enterovirus 2B protein for its function(s), we tested a (wild-type) CBV3 2B protein that is retained in the ER by virtue of an ER retention signal. Through this approach, evidence was obtained that the ability of 2B to reduce ER and Golgi complex Ca^{2+} levels as well as to inhibit protein trafficking critically depends on its Golgi complex localization. The observation that this ER-retained protein was unable to reduce the ER Ca^{2+} level was surprising. Immunoprecipitation experiments showed that this was not due to impaired formation of multimers in the ER. The finding that formation of 2B multimers in the ER is not sufficient to trigger the efflux of Ca^{2+} from this store indicates that 2B multimerization is not sufficient for pore formation. A possible explanation is that the mode of membrane interaction of 2B in the ER differs from that in the Golgi complex, possibly due to differences in lipid composition of these organelles that may affect the architecture of 2B multimers. Previously, we showed that the ER-retained CBV 2B also failed to increase the entry of hygromycin B (15). Although its exact function in the viral life cycle remains to be elucidated, the observations reported here point to the Golgi complex as the major target organelle of the enterovirus/rhinovirus 2B proteins, from where they exert (part of) their function(s).

Taken together, our results have demonstrated that the abilities of the enterovirus 2B protein to reduce ER and Golgi complex stored Ca^{2+} and to inhibit protein trafficking through the Golgi complex are conserved in the closely related rhinovirus 2B protein but not in the more distantly related 2B proteins of HAV, FMDV, and EMCV. We conclude that the 2B proteins of enterovirus and rhinovirus are closely related and most likely exhibit the same function in the viral life cycle.

ACKNOWLEDGMENTS

We thank P. Keller and K. Simons for the kind gift of the pSVS-G(td045)-GFP construct, R. Andino for the kind gift of the pXpA plasmid, R. Rueckert for the pWR3.26 plasmid, S. Emerson for the