

The nonstructural protein 2c biology essay

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Endoplasmic reticulum (ER)-to-Golgi apparatus transport of proteins is blocked by the FMDV 2BC protein. The observation that 2BC is processed to 2B and 2C during infection and that individual 2B and 2C proteins are unable to block secretion stimulated us to study the effects of 2BC processing on the secretory pathway. Even though 2BC was processed rapidly to 2B and 2C, protein transport to the plasma membrane was still blocked in FMDV-infected cells. The block could be reconstituted by coexpression of 2B and 2C, showing that processing of 2BC did not compromise the ability of FMDV to slow secretion. Under these conditions, 2C was located to the Golgi apparatus, and the block in transport also occurred in the Golgi apparatus. Interestingly, the block in transport could be redirected to the ER when 2B was coexpressed with a 2C protein fused to an ER retention element. Thus, for FMDV a block in secretion is dependent on both 2B and 2C, with the latter determining the site of the block. FMDV 2BC blocks ER-to-Golgi apparatus transport and cell surface expression of a model membrane protein, the TsO45 G-protein of vesicular stomatitis virus (Moffat et al., 2007). This work was stimulated by our observation that the FMDV 2BC precursor protein, but not 2B or 2C, blocks ER-to-Golgi apparatus transport when expressed individually in cells (Moffat et al., 2005). Experiments using the TsO45 G membrane protein of VSV showed that the secretory pathway was indeed blocked in cells infected by FMDV, and the G-protein failed to reach the plasma membrane (Moffat et al., 2007). Picornaviruses may affect the secretory pathway in different ways, because their nonstructural proteins have different amino acid sequences and possibly different functions. The FMDV 3A protein has 50 extra C-terminal amino acids compared with its PV

counterpart and lacks the proline and lysine residues in the N-terminal domain that are critical in PV 3A for blocking secretion (Choe et al., 2005, Wessels et al., 2005). The 2B proteins of PV and FMDV also differ. The FMDV protein is one-third longer at the C terminus, and hydrophobicity plots suggest this may produce a third hydrophobic domain which is absent from PV 2B. A failure to block secretion has also been reported recently for 3A proteins of human rhinovirus 14, enterovirus 71, hepatitis A virus, and Theiler's virus (Choe et al., 2005). These viruses either do not need to block secretion, because inflammatory and immune responses are advantageous to their life cycle, or as we have demonstrated for FMDV, they may use 2BC and its products to block ER-to-Golgi apparatus transport in infected cells. For FMDV the block has the potential to contribute to the development of persistent infections seen in ruminants that recover from acute viral infection, and it will be interesting to see if 2BC and coexpressed 2B and 2C have direct effects on MHC class I trafficking, antigen presentation, and/or cytokine secretion (Moffat et al., 2007). The effects of the 3A, 2B, 2C, and 2BC proteins of FMDV on the function of the secretory pathway were analyzed by following the movement of the TsO45 mutant of the VSV G protein from the ER to the cell surface. Surprisingly, even though FMDV 3A, 2B, and 2C were located in membranes of the early secretory pathway, they did not block transport of the TsO45 GFP protein from the ER to the Golgi apparatus or to the cell surface. It is not possible from our assay to show that 3A, 2B, and 2C did not slow the movement of the G protein from the ER to the Golgi apparatus and to the cell surface. Our study does, however, clearly show that following 30 min at the permissive temperature, 3A, 2B, and 2C

had no obvious effect on movement of the G protein from the ER to the Golgi apparatus. In contrast, in the presence of FMDV 2BC, the TsO45 GYFP protein failed to reach the Golgi apparatus or surfaces of cells but localized with 2BC inside the cell. Triple-staining experiments showed that 2BC causes retention of the G protein in a modified ER compartment and that this retention lasts at least 3 h. The effect of FMDV 2BC on trafficking of the TsO45 GYFP protein was observed in about 200 cells at different levels of expression, and in each case, the expression of 2BC appeared to result in the retention of TsO45 GYFP within an intracellular vesicular compartment. The exact nature of these 2BC-positive structures containing the retained G protein and ER markers is not known but worthy of further investigation (Moffat et al., 2005). Interestingly, in cells infected with PV or transfected with PV 2BC, the 2BC protein localizes to ER exit sites and sec13-positive vesicles (Rust et al., 2001). Sec13 is a component of the COPII complex which is involved in anterograde (ER-to-Golgi) vesicle formation and budding (Barlowe et al., 1994). In our studies, we were unable to colocalize sec13 with 2BC or the structures in which the TsO45 GYFP appears to accumulate (data not shown). The TsO45 GYFP does not therefore appear to accumulate at ER exit sites (Moffat et al., 2005). This study shows that FMDV differs from PV in the ability of nonstructural proteins to modulate the secretory pathway.

Evidence that different picornaviruses may utilize the secretory pathway in different ways has also been provided by previous studies (Gazina et al., 2002, Irurzun et al., 1992) in which it was demonstrated that three genera of the Picornaviridae show different sensitivities to BFA. This agent prevents Arf1-dependent recruitment of COP1 coats onto the Golgi apparatus, and the

differential sensitivity to the drug suggests that different viruses have different requirements for COP1 coats during the formation of membrane vesicles utilized for replication. This is supported by the observation that COP1 is found associated with the replication complexes formed by BFA-sensitive viruses (parechovirus VI, echovirus 11) but not those resistant to the drug (encephalomyocarditis virus [EMCV]) (Gazina et al., 2002). FMDV is also insensitive to BFA (Monaghan et al., 2004), so given the results from the present study, it will be interesting to see if other BFA-resistant viruses use 2BC rather than 3A to block ER-to-Golgi transport (Moffat et al., 2005). The PV 3A protein induces swelling of ER cisternae and a consequent block in the movement of proteins from the ER to the Golgi apparatus. In our study, the FMDV 3A protein also bound to the ER, but from immunofluorescence studies, it did not prevent the trafficking of the TsO45 GYFP protein to the cell surface. An explanation for this may lie in a comparison of the sequences of PV and FMDV 3A proteins. The PV 3A protein is 85 amino acids in length, while the FMDV protein is larger, with an extra 50 amino acids at the C terminus. Both proteins have hydrophobic domains that would explain their membrane association, but the N and C termini are very different (Moffat et al., 2005). Interestingly, mutations near the N terminus of PV 3A reduce its ability to block ER-to-Golgi transport. This is most apparent from the insertion of a single serine residue between threonine 15 and serine 16 which produces a temperature-sensitive mutant unable to block secretion at 37°C (Doedens et al., 1997). This N-terminal region also contains a series of proline residues conserved amongst the rhino- and enteroviruses but absent from the aphthoviruses (FMDV) and cardioviruses (EMCV). The loss of this

region from FMDV may explain the lack of effect of the protein on ER-to-Golgi transport (Moffat et al., 2005). In mammalian cells, the PV 2B and 2BC proteins also slow movement of proteins to the cell surface (Doedens et al., 1995). Since PV 2C does not block secretion when expressed alone, it is generally assumed that 2B is the active component of 2BC. Interestingly, the PV 2B protein does not block movement from the ER to the Golgi apparatus but rather arrests protein traffic in the Golgi apparatus. In our experiments, there was no intracellular arrest of traffic in cells expressing FMDV 2B. In a few cases, in cells expressing high levels of FMDV 2C, the G protein appeared to be retained in the cell, where it colocalized with 2C. However, this was seen in as few as 2% of cells examined. Observations from this study also point to differences in the types of membrane rearrangement induced by nonstructural proteins from different genera of picornaviruses (Moffat et al., 2005). PV 2BC expressed alone in cells produces a punctate cytoplasmic staining which colocalizes with domains of the ER enriched for COP11 proteins, suggesting location to ER exit sites (Rust et al., 2001). In our experiments, FMDV 2BC also produced a punctate staining in the cytoplasm, but we were unable to colocalize these structures with proteins of the COP11 complex (data not shown). We also observed remodeling of the ER into large vesicular structures close to the nuclei in cells expressing FMDV 2BC (Moffat et al., 2005). For PV, it is the 3A protein that remodels the ER by causing swelling of ER cisternae, whereas the PV 2BC protein induces vesicle formation and PV 2C induces extensive tubular structures in the rough ER lumen (Cho et al., 1994, Suhy et al., 2000). The high level of sequence similarity in 3A seen between the rhinoviruses and other enteroviruses

suggests that the 3A protein expressed by each of these viruses will also block membrane traffic, whereas the lower sequence similarity observed for 3A encoded by hepatitis A virus, Theiler's virus, and EMCV raises the possibility that they will behave differently and perhaps more like FMDV (Moffat et al., 2005). Studies of hepatitis A virus-infected cells, using electron microscopy, showed that the 2BC protein modifies the ER into tight crystalloid structures (Teterina et al., 1997). It may be that the hepatitis A 2BC protein also disrupts host membrane protein trafficking. Recent studies have investigated the ability of hepatitis C virus nonstructural proteins to induce membrane rearrangements and block the secretory pathway (Konan et al., 2003). Both the hepatitis C virus NS4B protein and its precursor NS4A/B expressed alone in cells produced a membranous web thought to be derived from the ER (Egger et al., 2002). Interestingly, only the precursor NS4A/B blocked ER-to-Golgi transport (Konan et al., 2003). This is analogous to our results with FMDV where only the precursor 2BC, but not the individual products (2B or 2C), is able to block the secretory pathway (Moffat et al., 2005). The observation that FMDV 2BC inhibits the delivery of membrane proteins to the cell surface raises the possibility that, as seen for PV 3A (Deitz et al., 2000, Dodd et al., 2001, Doedens et al., 1995), the 2BC protein of FMDV may also be able to modulate the recognition of infected cells by the immune system. This could include a block in the secretion of interferons and proinflammatory cytokines or the inhibition of cell surface MHC class 1 expression (Sanz-Parra et al., 1998) and antigen presentation. Such effects could contribute to the development of persistent infections that are frequently seen in ruminants following an acute FMDV infection. This study

should therefore stimulate further investigation of the effects of FMDV 2BC on cytokine secretion and antigen presentation and the role the protein may play during the development of persistent FMDV infections in ruminants (Moffat et al., 2005). The 2C protein encoded by the P2 region of the polyprotein is highly conserved among picornaviruses (Gorbalenya et al., 1989 ↓). During PV infection, 2C and its precursor 2BC migrate to the rough endoplasmic reticulum where they induce the formation of smooth membrane vesicles that bud off and become the site of viral RNA synthesis, the replication complex (Bienz et al., 1987 ↓ , 1992 ↓ , 1990 ↓ ; Cho et al., 1994 ↓ ; Teterina et al., 1997 ↓). 2C is a multifunctional protein and some of these functions include ATPase and GTPase (Rodriguez & Carrasco, 1993 ↓ ; Mirzayan & Wimmer, 1994 ↓ ; Pfister & Wimmer, 1999 ↓), membrane-binding (Kusov et al., 1998 ↓ ; Echeverri & Dasgupta, 1995 ↓ ; Aldabe & Carrasco, 1995 ↓) and RNA-binding activities (Rodriguez & Carrasco, 1995 ↓). In a previous study, we have shown that PV-encoded 2C and the precursor polypeptide 2BC specifically interact with the 3'-terminal sequences of the negative-strand RNA, but not with the corresponding 5'-terminal sequences of the positive-strand RNA (Banerjee et al., 1997 ↓ , 2001 ↓). We have also demonstrated that this interaction requires a stable stem-loop structure to be present at the 3' terminus of the negative-strand RNA. Because PV replication occurs in the cytoplasmic membrane and the 2C protein is capable of interacting with both the membrane and the viral negative-strand RNA, it was hypothesized that the 2C protein anchors the negative-strand RNA to the membrane. This anchoring may be crucial for the synthesis of positive-strand RNA from the negative-strand RNA template. Here, we have

extended our 2C-RNA interaction studies to demonstrate that the purified 2C proteins from both HAV and HRV-14 also interact specifically with the 3'-terminal sequences of the corresponding negative-strand RNAs (Banerjee and Dasgupta, 2001). Viral protein 2B and its precursor 2BC have been suggested to be responsible for membranous alteration in infected cells [van Kuppeveld et al., 1997, van Kuppeveld et al., 1996, Aldabe et al., 1996, Barco et al., 1995]. The cellular proteins of COPII have reportedly been used in the virus-induced production of vesicles [De Jong et al., 2003]. 2B and the precursor 2BC contain two hydrophobic regions, which are α amphipathic α -helix domain, which is important in multimerization, integrating into the membrane of the host Golgi and ER complex, producing virus-induced vesicles, and forming the virporin complex [De Jong et al., 2003, van Kuppeveld et al., 1996]. The accumulation of 2B or 2BC proteins on Golgi changes the permeability of plasma membrane [De Jong et al., 2003] and the disassembly of Golgi complex [Sandoval et al., 1997], causing cell lysis [van Kuppeveld et al., 1997]. The membrane that integrates the 2B/2BC complex also reduces the Ca^{2+} level in ER and Golgi complex by increasing the efflux of Ca^{2+} [van Kuppeveld et al., 1997]. The disruption of Ca^{2+} homeostasis by 2B/2BC is the mechanism why the transport of protein from ER to Golgi is blocked [de Jong et al., 2006, de Jong et al., 2008]. The 2B-induced intracellular Ca^{2+} imbalance is also related to the anti-apoptosis property [Campanella et al., 2004]. Hepatitis A virus 2B protein can reportedly inhibit cellular IFN- β gene transcription by blocking the activation of the interferon regulatory factor 3 (IRF-3), which has been suggested to be crucial to the survival of the virus [Paulmann et al., 2008].

2B

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 Ca^{2+} levels, most likely by forming transmembrane pores, and inhibits protein trafficking through the Golgi complex. 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 Ca^{2+} 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 Ca^{2+} homeostasis and inhibit protein trafficking, demonstrating the importance of Golgi complex localization for its functioning (De Jong et al., 2008). In enterovirus-infected cells, 2B is present both as a mature protein and as part of the 2BC protein, a relatively stable precursor protein that is involved in cytosolic accumulation of the secretory pathway-derived membrane vesicles, where viral replication takes place (Bienz et al., 1987, Rust et al., 2001, Schlegel et al., 1996). Studies of both PV and CBV indicate that 2B plays an important role in the modification of intracellular membrane structures and functions. Increasing evidence indicates that 2B forms homomultimers that build pores in ER and Golgi

complex membranes (Agirre et al., 2002, Cuconati et al., 1998, Van Kuppeveld et al., 2002), thereby reducing the levels of Ca^{2+} and H^{+} in the lumens of these organelles in infected cells (Van Kuppeveld et al., 1997, De Jong et al., 2006). Individual expression of 2B furthermore results in inhibition of protein trafficking through the Golgi complex (De Jong et al., 2006). 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 (De Jong et al., 2006). 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 (Van Kuppeveld et al., 1997). These 2B functions may be required for the activity of the precursor 2BC to accumulate membranous replication vesicles, but other possibilities cannot be excluded (De Jong et al., 2008). CBV3 2B, PV1, HRV14 2B colocalized predominantly with the EGFP-Golgi protein (Fig. 2A and B). The more distantly related HAV, EMCV, and FMDV 2B proteins showed different subcellular localizations. HAV 2B stained in a reticular pattern that included the nuclear envelope and was largely identical to staining of calreticulin (Fig. 2E), indicating that HAV 2B is localized to the ER. Similar to CBV3 2B, the 2B proteins of PV1 and HRV14 efficiently inhibited protein trafficking through the Golgi complex (Fig. 4B to D). In cells expressing these 2B proteins, VSV-G-GFP accumulated in the juxtannuclear Golgi region, where it

colocalized with the respective 2B proteins. This demonstrates that the ability of 2B to inhibit protein trafficking through the Golgi complex is conserved among these three closely related viruses. In contrast, expression of HAV, FMDV, or EMCV 2B did not result in the inhibition of protein trafficking. VSV-G-GFP was predominantly localized on the plasma membrane and showed no colocalization with these 2B proteins (Fig. 4E to G). 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 (Arjan et al., 2008).

2C

The nonstructural protein 2C is highly conserved among picornaviruses and plays an important role in the assembly of mature virions, membrane association, and viral RNA synthesis. The investigation of other potential functions of nonstructural protein 2C from avian encephalomyelitis virus (AEV) resulted in identifying for the first time that the protein 2C is involved in apoptosis. Analysis of the AEV protein 2C showed that the N-terminal domain containing 35 amino acid (aa) residues (between 46 and 80 aa) is associated with apoptotic function. Transfection of the deletion mutant lacking this 35 aa's into CEB and Cos-7 cells failed to induce apoptosis. Furthermore, the AEV protein 2C induced apoptosis in the transfected CEB and Cos-7 cells through activation of caspase-9 rather than caspase-8 followed by activation of caspase-3 pathway. The AEV protein 2C was located in the mitochondria and cytosol of the transfected/infected CEB and transfected Cos-7 cells, but the mutant lost its ability to localize to the

mitochondria. (Liu et al., 2004)The 2C protein encoded by the P2 region of the polyprotein is highly conserved viral protein among picornaviruses (Gorbalenya et al., 1989; Marvil et al., 1999), which has been considered to be directly involved in RNA replication (Cho et al., 1994; Teterina et al., 1997a, 1997b), the assembly of mature virions (Vance et al., 1997), ATPase and GTPase (Mirzayan and Wimmer, 1994; Pfister and Wimmer, 1999; Rodriguez and Carrasco, 1993), membrane-binding (Aldabe and Carrasco, 1995; Echeverri and Dasgupta, 1995; Kusov et al., 1998), and RNA-binding activities (Rodriguez and Carrasco, 1995). Protein 2C is a highly conserved non-structural protein that binds to membranes and RNA, and is crucial in poliovirus replication (reviewed in Goodfellow et al., 2003). It also plays a role in encapsidation (Vance et al., 1997) and also has ATPase/GTPase activity (Rodríguez & Carrasco, 1993). 2C and its precursor 2BC are responsible for poliovirus RNA binding to the cytoplasmic vesicles, whose formation is also induced by this precursor. Its mechanism of action has yet to be determined. FMDV 2C protein is localized in juxtannuclear structures, vesicles that could be derived from Golgi compartments. However, the origin of these vesicles is not clear yet; recent studies argue against the relation with the Golgi (Knox et al., 2005; Moffat et al., 2005). For instance, in FMDV and EMCV infected cells treated with Brefeldin A (BFA), which inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-dependent secretory transport vesicles (Duden et al., 1994), 2C juxtannuclear localization and replication is not inhibited (Gazina et al., 2002). In contrast, replication is inhibited in PV and Rhinoviruses when infected cells are treated with BFA (Cuconati et al., 1998).