

## Multimerization reactions of coxsackievirus proteins 2B, 2C and 2BC: a mammalian two-hybrid analysis

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Recently, homomultimerization and heteromultimerization reactions of the poliovirus P2 region proteins were investigated using a yeast two-hybrid approach (Cuconati *et al.*, *Journal of Virology* **72**, 1297–1307, 1998). In this study, we investigated multimerization reactions of the 2B, 2C and 2BC proteins of the closely related coxsackie B3 virus (CBV3) using a mammalian two-hybrid system. This system allows the characterization of protein:protein interactions within a cellular environment that more closely mimics the native protein environment. Homomultimerization reactions were observed with the 2BC protein and, albeit weakly, with the 2B protein, but not with the 2C protein. To identify the determinants involved in the 2BC and 2B homomultimerization reactions, several mutants containing deletions or point mutations in the 2B region were tested. Disruption of the hydrophobic character of either the cationic amphipathic  $\alpha$ -helix or the second hydrophobic domain of the 2B protein disturbed both the 2BC:2BC and the 2B:2B homomultimerization reactions. Disruption of either the cationic or the amphipathic character of the  $\alpha$ -helix or deletion of the N-terminal 30 amino acids of the 2B protein, however, had no effect on the 2BC and 2B homomultimerization reactions. Heteromultimerization reactions were observed between proteins 2BC and 2B, and also between proteins 2BC and 2C, but not between the 2B and 2C proteins. The 2BC:2B and 2BC:2C heteromultimerization reactions were also mediated by hydrophobic determinants located in the amphipathic  $\alpha$ -helix and the second hydrophobic domain. The nature of the interactions and their implications for the virus life-cycle are discussed.

### Introduction

Enteroviruses (poliovirus, coxsackievirus, echovirus and several unnamed enteroviruses) are admirably efficient viruses. These cytolitic viruses possess a relatively small plus-strand RNA genome (7.5 kb) that harbours the genetic information for only a limited number of proteins. The nonstructural replication proteins are required both for the physical process of vRNA replication, which takes place in the cytoplasm, as well as for the induction of a variety of host cell modifications (e.g. inhibition of host cell transcription and translation, remodelling of the vesicular membrane system, permeabilization of host cell membranes, inhibition of protein secretion, inhibition of nucleocytoplasmic transport) in order to create the microenvironment required for vRNA replication,

to circumvent antiviral host cell responses and to induce cell lysis (Porter, 1993; Wimmer *et al.*, 1993). To execute all of these complex functions, enteroviruses have developed elegant strategies to make optimal use of the limited size of the vRNA genome. Processing of the viral polyprotein not only yields the P1 capsid proteins and the mature nonstructural P2 and P3 region proteins (2A<sup>pro</sup>, 2B, 2C, 3A, 3B, 3C<sup>pro</sup>, 3D<sup>pol</sup>) but also a number of stable cleavage intermediates (2BC, 3AB and 3CD<sup>pro</sup>) with functions distinct from their cleavage products. Furthermore, most of the nonstructural proteins are equipped with multiple functions, and the nonstructural proteins may exhibit different activities upon interaction with different binding partners (Agol *et al.*, 1999).

Recently, the yeast two-hybrid system has been successfully applied to catalogue interactions between the P2 and P3 region nonstructural proteins of poliovirus (PV) (Cuconati *et al.*, 1998; Hope *et al.*, 1997; Xiang *et al.*, 1998). Using this approach, several homomultimerization and heteromulti-

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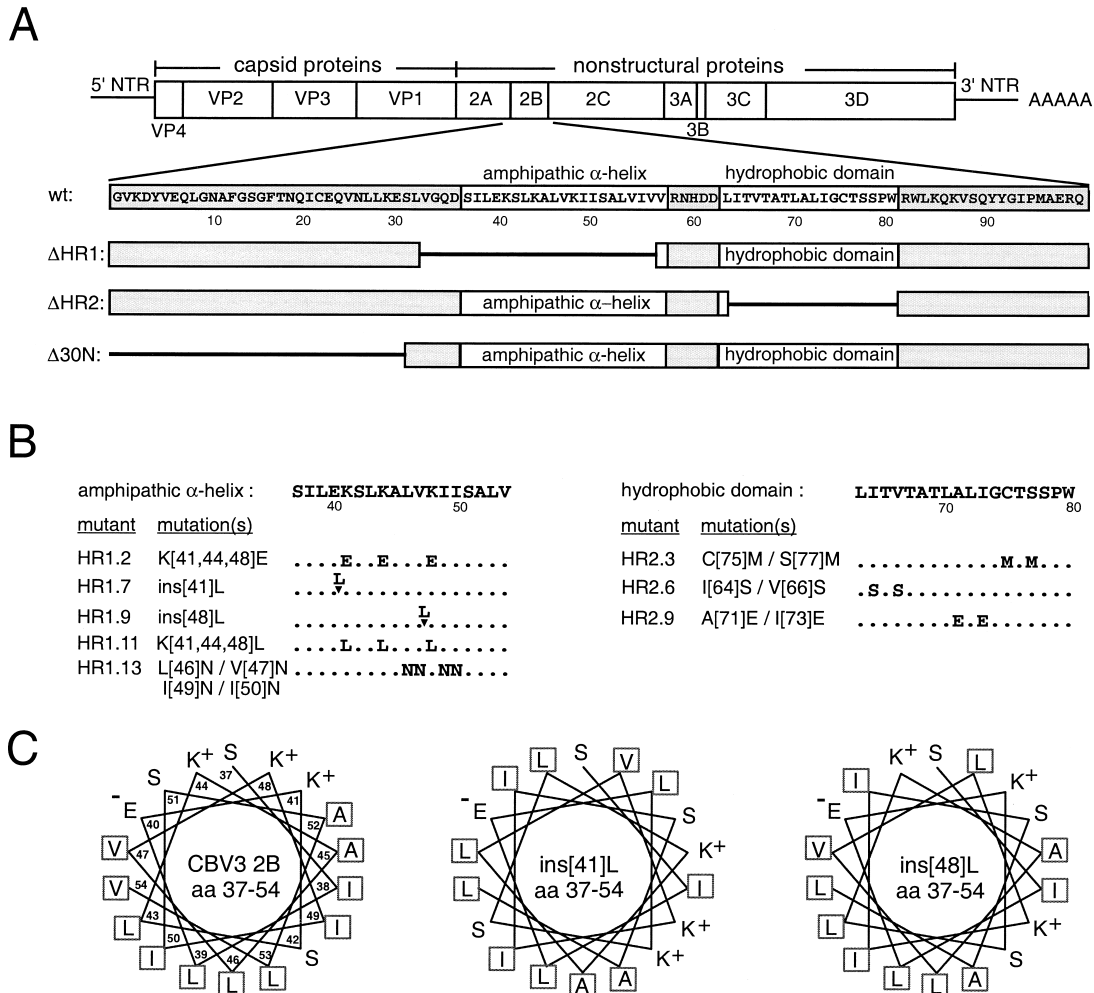


Fig. 1. (A) Schematic representation of the 7.5 kb enterovirus RNA genome. The polyprotein-encoding region (boxed) is flanked by the 5' nontranslated region (NTR) and the 3' NTR, which contains a polyadenylate tract at its end. Vertical lines within the polyprotein indicate proteinase cleavage sites. The 2B protein is enlarged and the 2B amino acid sequence is shown. The amphipathic  $\alpha$ -helix (also referred to as HR1) and the second hydrophobic domain (also referred to as HR2) are indicated. The positions of the deletions  $\Delta$ HR1 (deletion of aa 34–56),  $\Delta$ HR2 (deletion of aa 64–80) and  $\Delta$ 30N (deletion of the N-terminal 30 aa) are indicated. As a result of the construction procedure, leucine-63 is changed into a valine in the  $\Delta$ HR2 construct. (B) Amino acid sequences of the amphipathic  $\alpha$ -helix (HR1) point mutants and the second hydrophobic domain (HR2) point mutants used in this study. Periods indicate unchanged residues and triangles indicate locations in which amino acids have been inserted, not substituted. (C) Helical wheel representations of the putative amphipathic  $\alpha$ -helix formed by 2B aa 37–54 and the corresponding domains of the insertion (ins) mutants ins41[L] and ins48[L]. Charged residues are indicated. Boxed residues represent hydrophobic residues.

merization reactions between the nonstructural proteins were observed. Among the interactions of the P2 region proteins were homomultimerization reactions of the 2B and 2BC proteins and heteromultimerization reactions between the 2B, 2C and 2BC proteins. These proteins are localized at the outer surface of the virus-induced membrane vesicles that accumulate in the cytoplasm of the infected cell and are the sites at which vRNA replication takes place (Bienz *et al.*, 1994). The 2BC protein is the viral protein responsible for the proliferation and accumulation of these membrane vesicles (Barco & Carrasco, 1995; Bienz *et al.*, 1983; Cho *et al.*, 1994), possibly in conjunction with the 3A protein (Suhy *et al.*, 2000). A number

of functions have been ascribed to the 2B and 2C proteins but the relevance of these functions for the virus life-cycle remains to be established. The 2B protein is a small protein that contains two hydrophobic domains, of which one is predicted to form an amphipathic  $\alpha$ -helix (Fig. 1C) (van Kuppeveld *et al.*, 1996). The 2B protein has been implicated in the modification of membrane permeability (Aldabe *et al.*, 1996; Doedens & Kirkegaard, 1995; van Kuppeveld *et al.*, 1997a, c), the inhibition of protein secretion (Doedens & Kirkegaard, 1995; van Kuppeveld *et al.*, 1997c) and the disassembly of the Golgi complex (Sandoval & Carrasco, 1997). The identification of 2B mutations that interfere with virus growth but do not disturb

the ability of the 2B protein to inhibit protein secretion or increase membrane permeability argues for additional functions of the 2B protein or its precursor 2BC (van Kuppeveld *et al.*, 1997c). The 2C protein is a nucleoside triphosphatase that is endowed with RNA binding capacities (Mirzayan & Wimmer, 1994; Rodriguez & Carrasco, 1993, 1995), and therefore may be the viral protein that mediates attachment of the vRNA to the membranous replication complex. Moreover, a role for the 2C protein in the initiation of negative-strand RNA synthesis has been proposed (Barton & Flanagan, 1997).

In this study, we investigated multimerization reactions of the 2B, 2C and 2BC proteins of coxsackie B3 virus (CBV3) using a mammalian two-hybrid system. Mammalian two-hybrid systems are used to confirm putative protein:protein interactions *in vivo* that were initially identified by the yeast two-hybrid system or by *in vitro* biochemical methods. The mammalian two-hybrid system allows characterization of protein:protein interactions within a cellular environment that more closely mimics the native protein environment. Differences exist between yeast and mammalian cells in patterns of post-translational modifications as well as in intracellular localization of proteins. These differences, as well as other unique factors or modulators present in mammalian cells, may influence the ability of protein domains to interact. Here, we report the homomultimerization and heteromultimerization characteristics of the CBV3 proteins 2BC, 2B and 2C in mammalian cells and the identification of the domains and amino acids involved in these interactions.

## Methods

■ **Cells.** COS-1 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal bovine serum, 100 units penicillin/ml and 25 µg streptomycin/ml. Cells were grown at 37 °C in a 5% CO<sub>2</sub> incubator.

■ **Plasmids.** The Checkmate Mammalian Two-Hybrid System (Promega) includes plasmids pACT, pBIND and pG5*luc*. The pACT and pBIND plasmids contain the herpes simplex virus (HSV) type 1 VP16 activation domain and the yeast GAL4 DNA binding domain, respectively, followed by a multiple cloning site (which is the same in both plasmids). In addition, the pBIND plasmid includes the *Renilla* luciferase gene driven by the SV40 early promoter and enhancer, which allows monitoring of the transfection efficiency. The pG5*luc* plasmid is a reporter plasmid containing five GAL4 binding sites upstream of a minimal TATA box that precedes the firefly luciferase gene.

The coding sequences of the CBV3 2BC, 2B and 2C proteins (wild-type or mutant) were amplified by PCR, using mutant pCB3/T7 plasmids (van Kuppeveld *et al.*, 1995, 1996) as template, and introduced in the expression plasmid pBIND as described below. The nucleotide sequence of the pBIND inserts was verified by sequence analysis. The inserts were excised from the pBIND plasmids and directly cloned in the pACT plasmids (benefiting from the fact that both plasmids contain the same multiple cloning site).

The 2BC coding sequence was amplified with primers that introduced a *Bam*HI site at the upstream end (forward primer p115-14; 5' gatcaaggatccaggagtggaaggactatgtg 3') and a stop codon (underlined) plus a *Sal*I site at the downstream end (reverse primer p115-3; 5'

tttgatctctgtatagctgacttactggaacagtgcctc 3'). The 2BC products were cloned into pBIND cut with *Bam*HI and *Sal*I. The 2B coding sequence was amplified by PCR with forward primer p115-14 and a reverse primer that introduced a stop codon (underlined) plus a *Sma*I site at the downstream end (p115-7; 5' aagccaccgggctattggcggttcagcatagg 3'). The 2B products were cloned into pBIND cut with *Bam*HI and *Eco*RV. The 2C coding sequence was amplified by PCR with primers that introduced a *Bam*HI site at the upstream end (forward primer p115-15; 5' atggctggatccaaaacaatagctggccttaag 3') and reverse primer p115-3. The 2C product was cloned in pBIND cut with *Bam*HI and *Sal*I.

For the construction of pBIND-2BCΔHR1 and pBIND-2BCΔHR2, we made use of pCB3/T7-2BΔHR1 and pCB3/T7-2BΔHR2 constructs, which contain in-frame deletions of 2B amino acids (aa) 34–56 (HR1) or aa 64–80 (HR2), respectively (F. J. M. van Kuppeveld and others, unpublished data). For the construction of pBIND-2BCΔ30N and pBIND-2BΔ30N constructs, forward primer p115-22 (5' gtaaccggatccaagaatcactagtggtgctca 3') was used.

■ **Transfection.** COS cells were grown in 24-well tissue culture plates to 70% confluency. Cells were transfected with a total of 0.75 µg plasmid DNA (1:1:1 mix of the pBIND:pACT:pG5*luc* plasmids) using the FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche). For each transfection, 3 µl of FuGENE 6 reagent was added to 100 µl of serum-free medium and incubated for 5 min at room temperature. This mix was added dropwise to the 0.75 µg plasmid DNA preparation described above and incubated at room temperature for 15 min. After this incubation, the FuGENE 6 reagent/DNA mixture was added drop-wise to the cells. The cells were grown at 37 °C until further analysis.

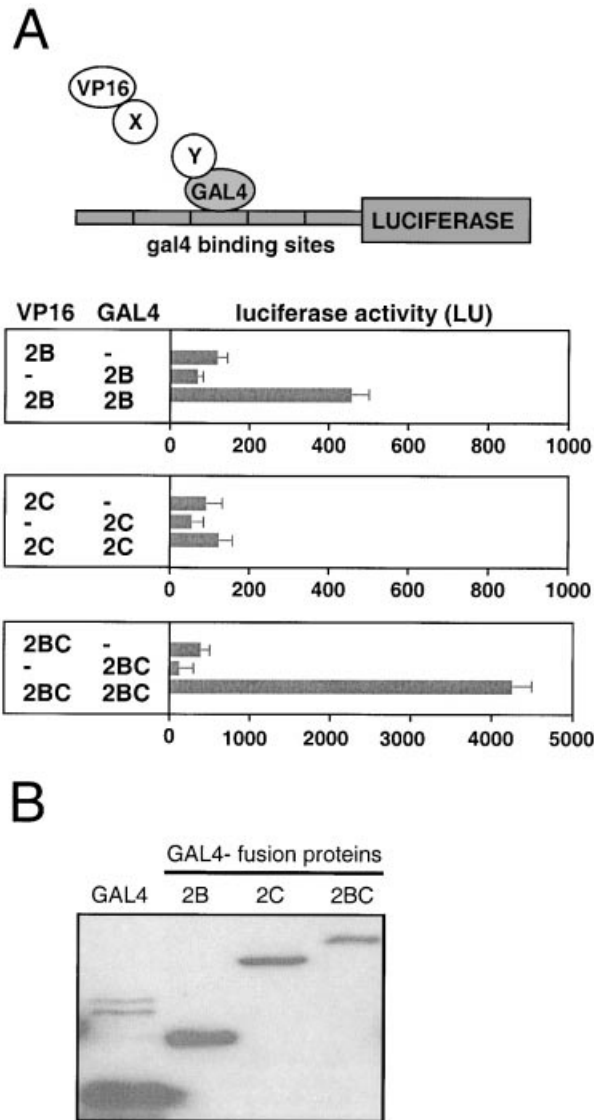
■ **Analysis of luciferase activities.** At 48 h post-transfection, cells were lysed and both the firefly luciferase and *Renilla* luciferase enzyme activities were measured from the same cell lysate sample using the Dual-Luciferase Reporter Assay System, according to the manufacturer's instructions (Promega). Luciferase activities were measured in a Bio-Orbit 1251 luminometer. Measurement of the *Renilla* luciferase production revealed only small differences among different samples from the same experiment. Because these small differences merely reflected variations in the luciferase measurement, we did not normalize for transfection efficiency.

■ **Western blot analysis.** Cell lysates were prepared at 48 h post-transfection. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunodetected using a monoclonal antibody against the GAL4 DNA binding protein (Clontech). Proteins were visualized using a chemoluminescent detection system (Amersham Pharmacia Biotech).

## Results

### Description of the mammalian two-hybrid system

Testing of putative interacting proteins using the mammalian two-hybrid system requires cotransfection of three plasmids into eukaryotic cells. The pACT plasmid provides the activation domain of HSV type 1 VP16 (aa 411–456) and an added nuclear localization sequence, followed by a multiple cloning site for the insertion of the DNA sequence of interest. The pBIND plasmid provides the DNA binding domain of a yeast GAL4 gene product (aa 1–147), which contains an endogenous nuclear localization sequence, followed by a multiple cloning site. Both plasmids contain the CMV



**Fig. 2.** (A) Homomultimerization reactions of the CBV3 proteins 2B, 2C and 2BC expressed as fusion proteins to the HSV VP16 activation domain (as encoded by the pACT plasmids) or the yeast GAL4 DNA binding domain (as encoded by the pBIND plasmids). COS-1 cells were transfected with the indicated constructs and assayed for luciferase production at 48 h post-transfection. A hyphen indicates the expression of a unfused VP16 or GAL4 protein. LU, light units. Values represent means  $\pm$  standard errors of measurements of four independent experiments. (B) Western blot analysis of the expression of the GAL4 fusion proteins. COS-1 cells were transfected with the indicated constructs and assayed for protein expression at 48 h post-transfection. Cell lysates were prepared, separated by SDS-PAGE and transferred to nitrocellulose membranes. The blot was probed with a monoclonal antibody against the GAL4 DNA binding domain.

immediate-early promoter to drive high-level expression of the VP16 and GAL4 fusion proteins. A chimeric intron and the late SV40 polyadenylation signal sequence serve to provide efficient processing and optimized steady-state levels of the transcribed RNA. The VP16 and GAL4 chimeric proteins expressed in the transfected cells are tested for functional

protein:protein interactions in combination with the reporter plasmid pG5*luc*, which contains five GAL4 binding sites upstream of a minimal TATA box that precedes the firefly luciferase gene (Fig. 2A).

### Homomultimerization reactions of the 2B, 2C and 2BC proteins

The CBV3 proteins 2B, 2C and 2BC were cloned in the two-hybrid plasmids pACT and pBIND and tested for homomultimerization reactions. The amounts of firefly luciferase produced by combination of the VP16-fusion protein and the GAL4-fusion protein were compared to the amounts of firefly luciferase produced by the combination of the VP16-fusion protein and the non-fused GAL4 binding domain (provided by the empty pBIND plasmid) and the combination of the GAL4-fusion protein and the non-fused VP16 activation domain (provided by the empty pACT plasmid). These two controls were included to exclude the possibility that the firefly luciferase production is due to an interaction of either one of the fusion proteins with the non-fused VP16 or GAL4 domains.

Fig. 2(A) shows that a strong homomultimerization reaction was observed with the 2BC protein. The amount of firefly luciferase produced in COS cells by the VP16–2BC and GAL4–2BC fusion proteins was in all experiments about 10- to 20-fold higher than observed with the two control transfections. A weak interaction was observed between the VP16–2B and GAL4–2B fusion proteins. The amount of firefly luciferase produced by the VP16–2B and GAL4–2B fusion proteins was about 3- to 4-fold higher than observed with the two control transfections. No interaction was observed between the VP16–2C and GAL4–2C fusion proteins. Western blot analysis showed that transfected COS cells correctly expressed fusion products of the expected molecular mass (Fig. 2B).

### Heteromultimerization reactions of the 2B, 2C and 2BC proteins

To test for heteromultimerization reactions of the 2B, 2C and 2BC proteins, COS cells were transfected with all possible pair-wise combinations and assayed for protein:protein interactions (Fig. 3). The VP16–2B fusion protein was found to interact with the GAL4–2BC fusion protein, but not with the GAL4–2C fusion protein (Fig. 3A). The VP16–2C fusion protein also interacted with the GAL4–2BC fusion protein, but not with the GAL4–2B fusion protein (Fig. 3B). The VP16–2BC fusion protein displayed strong interactions with both the GAL4–2B and the GAL4–2C fusion proteins (Fig. 3C). The 2BC protein was also tested for interactions with the CBV3 3A protein, a small (89 aa) hydrophobic viral protein. No interaction was observed between the VP16–2BC fusion protein and the GAL4–3A fusion protein (Fig. 3C), a fusion protein that was efficiently expressed (data not shown).

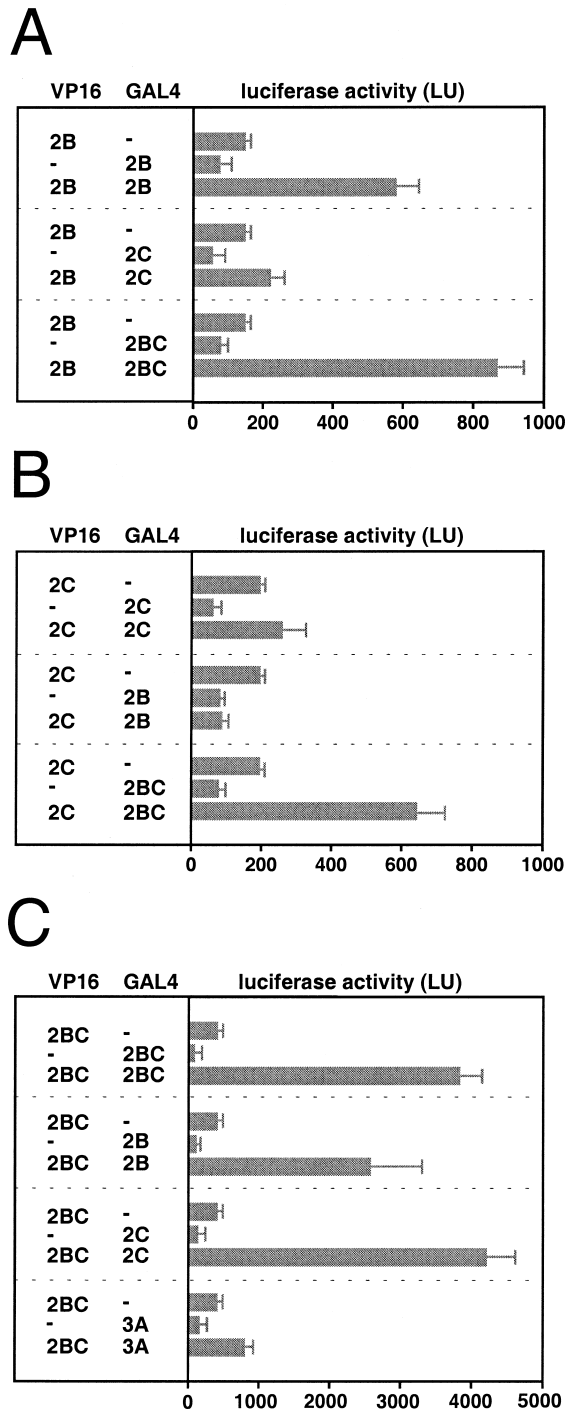


Fig. 3. Heteromultimerization reactions of CBV3 proteins (A) 2B, (B) 2C and (C) 2BC. COS cells were transfected with all possible pair-wise combinations of pACT and pBIND plasmids that drive expression of 2B, 2C or 2BC either as fusion protein to the HSV VP16 activation domain or the yeast GAL4 DNA binding domain, respectively. To test the specificity of the 2BC heteromultimerization reactions, interaction with the 3A protein was assayed also. Values represent means  $\pm$  standard errors of measurements of two independent experiments.

Collectively, these results provide evidence for specific heteromultimerization reactions between proteins 2BC and 2B and between proteins 2BC and 2C, but not between proteins 2B and 2C.

The data in Fig. 3 demonstrate that the strength of both the 2BC:2B interaction and the 2BC:2C interaction was vector-dependent. The 2BC:2B and 2BC:2C interactions were always stronger when the 2BC protein was fused to the VP16 activation domain than to the GAL4 DNA binding domain. Vector-dependence (also referred to as 'directionality' or 'polarity') has been reported in many two-hybrid system studies and was also observed in the yeast two-hybrid interaction analysis of the PV P2 region proteins (Cuconati *et al.*, 1998).

### Construction of mutant 2BC and 2B fusion proteins

To gain more insight into the molecular determinants involved in the homomultimerization and heteromultimerization reactions of the 2BC and 2B proteins, several pACT and pBIND plasmids driving expression of 2BC and 2B fusion proteins containing either in-frame deletions or point mutations in the 2B coding region were generated. In the deletion mutants, either the N-terminal 30 aa (30N), the amphipathic  $\alpha$ -helix (i.e. the first hydrophobic region, HR1) or the second hydrophobic region (HR2) were deleted (Fig. 1A).

The point mutants contained well-characterized amino acid substitutions or insertions in HR1 or HR2 (Fig. 1B). The amphipathic  $\alpha$ -helix mutants are designated HR1.2, HR1.7, HR1.9, HR1.11 and HR1.13. In mutant HR1.2, the three lysines in the hydrophilic part of the amphipathic  $\alpha$ -helix are replaced with glutamic acid residues (mutation K[41,44,48]E). Mutants HR1.7 and HR1.9 contain insertions of leucine residues at 2B positions 41 or 48 (mutations ins[41]L and ins[48]L, respectively). These insertions lead to a dispersion of the charged residues and, as a consequence, a decrease in the amphipathic character of the  $\alpha$ -helix, without disturbing the overall hydrophobicity of the domain (Fig. 1C). Mutant HR1.11 contains a replacement of the three lysines in the hydrophilic part of the amphipathic  $\alpha$ -helix with leucine residues (mutation K[41,44,48]L). Mutant HR1.13 contains a replacement of four hydrophobic residues in the hydrophobic part of the amphipathic  $\alpha$ -helix with polar asparagine residues (mutation L[46]N/V[47]N/I[49]N/I[50]N). Viral RNAs carrying these HR1 mutations were all nonviable due to a primary defect of the 2B protein in vRNA replication (van Kuppeveld *et al.*, 1996; unpublished results).

The second series of hydrophobic domain mutants was designated HR2.3, HR2.6 and HR2.9 (Fig. 1B). In mutant HR2.3, cysteine-75 and serine-77 are replaced with more hydrophobic methionine residues (mutation C[75]M/S[77]M). In mutants HR2.6 and HR2.9, the overall hydrophobic character of HR2 is diminished. In mutant HR2.6, isoleucine-64 and valine-66 are replaced with polar serine residues (mutation I[64]S/V[66]S). In mutant HR2.9, alanine-71 and isoleucine-73

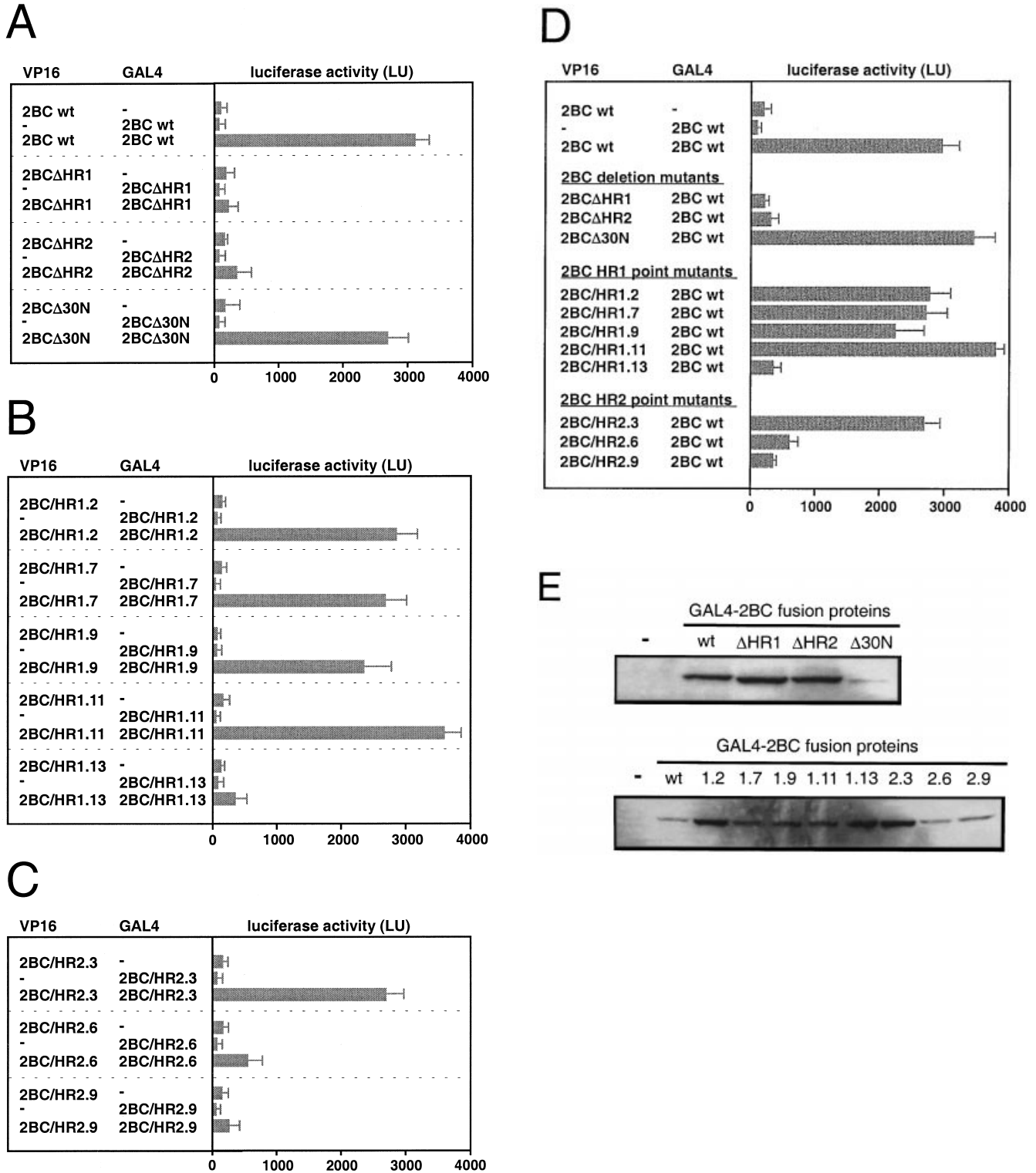


Fig. 4. Homomultimerization reactions of (A) 2BC deletion mutants, (B) 2BC point mutants carrying amino acid alterations in the amphipathic  $\alpha$ -helix, (C) 2BC point mutants carrying amino acid alterations in the second hydrophobic domain and (D) mutant VP16–2BC fusion proteins with the wild-type GAL4–2BC fusion protein. The 2BC deletion mutants (Fig. 1A) lacked either the amphipathic  $\alpha$ -helix ( $\Delta$ HR1), the second hydrophobic domain ( $\Delta$ HR2) or the N-terminal 30 aa ( $\Delta$ 30N) of the 2B region. The 2BC point mutants (Fig. 1B) contained specific amino acid substitutions or insertions in either the amphipathic  $\alpha$ -helix (HR1 mutants) or the second hydrophobic domain (HR2 mutants). Values represent means  $\pm$  standard errors of measurements of three independent experiments. (E) Western blot analysis of the expression of the GAL4–2BC deletion mutants and point mutants. Western blot analysis was performed as described in the legend to Fig. 2.

are replaced with glutamic acid residues (mutation A[71]E/I[73]E). All of these HR2 mutations caused a primary defect in vRNA replication, rendering the viral RNA either quasi-infectious (mutation C[75]M/S[77]M) or nonviable (mutations I[64]S/V[66]S and A[71]E/I[73]E) (van Kuppeveld *et al.*, 1995).

### Homomultimerization reactions of mutant 2BC fusion proteins

Analysis of the homomultimerization reactions of the 2BC deletion mutants showed that the N-terminal 30 aa are not required for this activity (Fig. 4A). Deletion of either HR1 or HR2, however, abolished the homomultimerization reaction of the 2BC protein, suggesting that the integrity of each of these domains is required for the formation of 2BC homomultimers. All 2BC deletion mutants were efficiently expressed (Fig. 4E), arguing that the lack of interaction of the 2BC $\Delta$ HR1 and 2BC $\Delta$ HR2 proteins is not due to impaired protein production or stability.

Analysis of the homomultimerization reactions of the amphipathic  $\alpha$ -helix point mutants (Fig. 4B) showed that mutants HR1.2, HR1.7, HR1.9 and HR1.11 efficiently formed homomultimers. Thus, neither the cationic character nor the amphipathic character of the  $\alpha$ -helix is absolutely required for the ability of the 2BC protein to form homomultimers. Mutant HR1.13, however, was unable to form homomultimers, indicating that the hydrophobic character of the amphipathic  $\alpha$ -helix is required for the homomultimerization reaction of the 2BC protein. Analysis of the homomultimerization reactions of the second series of hydrophobic domain mutants (Fig. 4C) showed that mutant HR2.3 efficiently formed homomultimers. Mutants HR2.6 and HR2.9, however, were no longer capable of forming homomultimers, indicating that the hydrophobic character of the second hydrophobic domain is also required for the ability of protein 2BC to homomultimerize. Similar results were obtained when the VP16–2BC mutants were tested for interactions with the GAL4–2BC wild-type protein (Fig. 4D). Western blot analysis showed that all 2BC point mutants were correctly expressed (Fig. 4E). Taken together, these data provide evidence that the homomultimerization reaction of the 2BC protein depends on hydrophobic determinants in both the amphipathic  $\alpha$ -helix and the second hydrophobic domain of the 2B protein.

### Homomultimerization reactions of mutant 2B fusion proteins

Analysis of the homomultimerization reactions of the 2B deletion mutants showed that the N-terminal 30 aa are not required for this activity (data not shown). Analysis of the homomultimerization reactions of 2B point mutants (Fig. 5A, B) showed that mutants HR1.2, HR1.7, HR1.9 and HR1.11 and HR2.3 had retained the ability to form homomultimers.

Mutants HR1.13, HR2.6 and HR2.9, however, displayed a severe defect in the ability to homomultimerize. Similar results were obtained when the VP16–2B point mutants were tested with the wild-type GAL4–2B fusion protein (data not shown). All point mutants were correctly expressed (Fig. 5C), indicating that the differences observed in the multimerization reactions are not due to impaired protein production or stability. Thus, the homomultimerization reaction of the 2B protein also depends on hydrophobic determinants in the amphipathic  $\alpha$ -helix and the second hydrophobic domain.

### Heteromultimerization reactions between mutant 2BC and 2B fusion proteins

To investigate the 2BC:2B heteromultimerization determinants, interactions were assayed between mutant VP16–2BC fusion proteins and the wild-type GAL4–2B fusion protein (Fig. 6A) and between the VP16–2BC wild-type protein and GAL4–2B point mutants (Fig. 6B). Essentially, the same results were observed as for the 2BC:2BC and 2B:2B homomultimerization reactions (Figs 4 and 5, respectively). Intriguing results, however, were observed with the VP16–2BC wild-type protein and the GAL4–2B point mutants 2B–HR2.3 and 2B–HR2.6 (Fig. 6B). Mutant 2B–HR2.3 was only partially efficient ( $\sim 50\%$  relative to 2B wild-type) in forming heteromultimers with the 2BC protein. This finding is surprising because the 2BC–HR2.3 protein showed a wild-type phenotype in the 2BC:2B heteromultimerization reaction (Fig. 6A), and because proteins 2BC–HR2.3 and 2B–HR2.3 showed efficient homomultimerization reactions (Figs 4 and 5). Mutant 2B–HR2.6 was also partially efficient ( $\sim 40\%$  relative to 2B wild-type) in forming 2BC:2B heteromultimers, which is surprising because the 2BC–HR2.6 protein was severely disabled in forming 2BC:2B heteromultimers (Fig. 6A) and because proteins 2BC–HR2.6 and 2B–HR2.6 were unable to form homomultimers. These results suggest that the 2BC:2B heteromultimerization reaction may not simply reflect a 2B:2B homomultimerization reaction.

### Heteromultimerization reactions between mutant 2BC fusion proteins and the 2C fusion protein

The 2BC protein also showed a strong heteromultimerization reaction with the 2C protein (Fig. 3). To investigate the role of the 2B region in this heteromultimerization reaction, interactions between mutant VP16–2BC fusion proteins and the wild-type GAL4–2C fusion protein were assayed (Fig. 7). Deletion of the N-terminal 30 aa of the 2BC protein had no effect on the interaction with the 2C protein, whereas deletion of either HR1 or HR2 completely abolished the 2BC:2C interaction. Analysis of the heteromultimerization reactions between the 2BC point mutants and the 2C protein showed that the mutants HR1.2, HR1.7, HR1.9, HR1.11 and HR2.3 efficiently interacted with the GAL4–2C fusion protein. Mutants HR1.13, HR2.6 and HR2.9, however, failed to form heteromultimers with the 2C protein. Thus, the same mutations

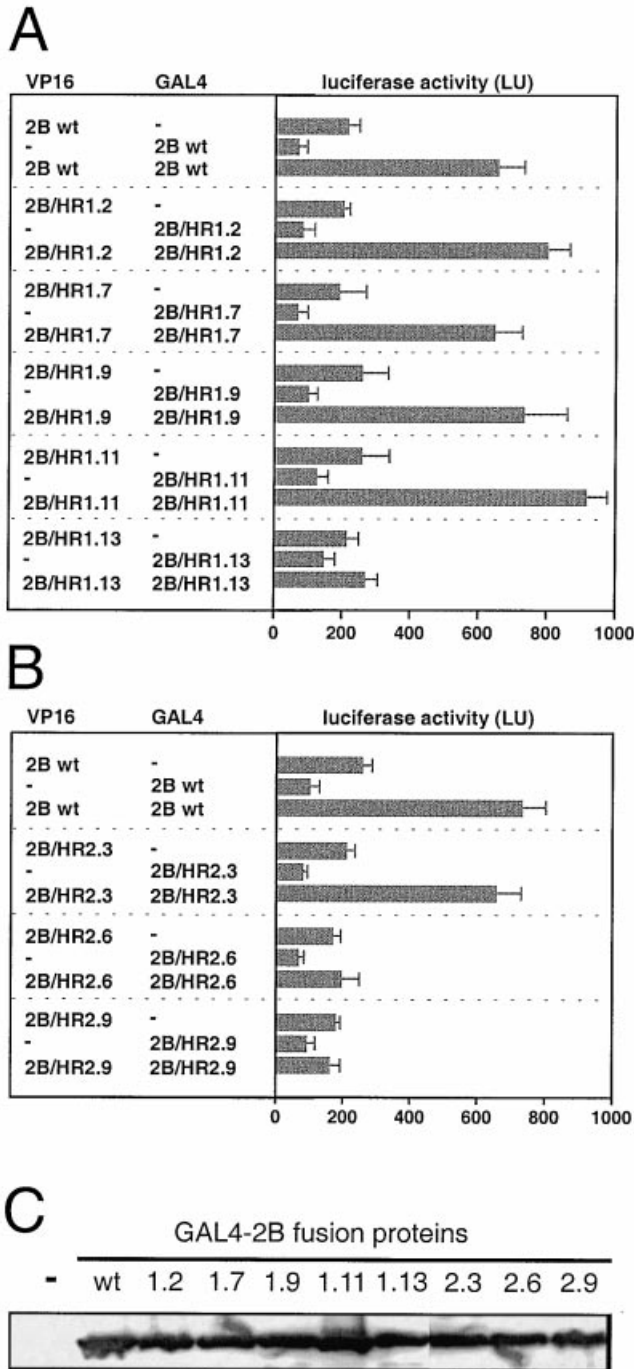


Fig. 5. Homomultimerization reactions of 2B point mutants carrying amino acid alterations in (A) the amphipathic  $\alpha$ -helix or (B) the second hydrophobic domain. Values represent means  $\pm$  standard errors of measurements of two independent experiments. (C) Western blot analysis of the expression of the GAL4–2B point mutants. Western blot analysis was performed as described in the legend to Fig. 2.

that disrupted the formation of 2BC homomultimers (Fig. 4) also abolished heteromultimerization reactions between the 2BC and 2C proteins.

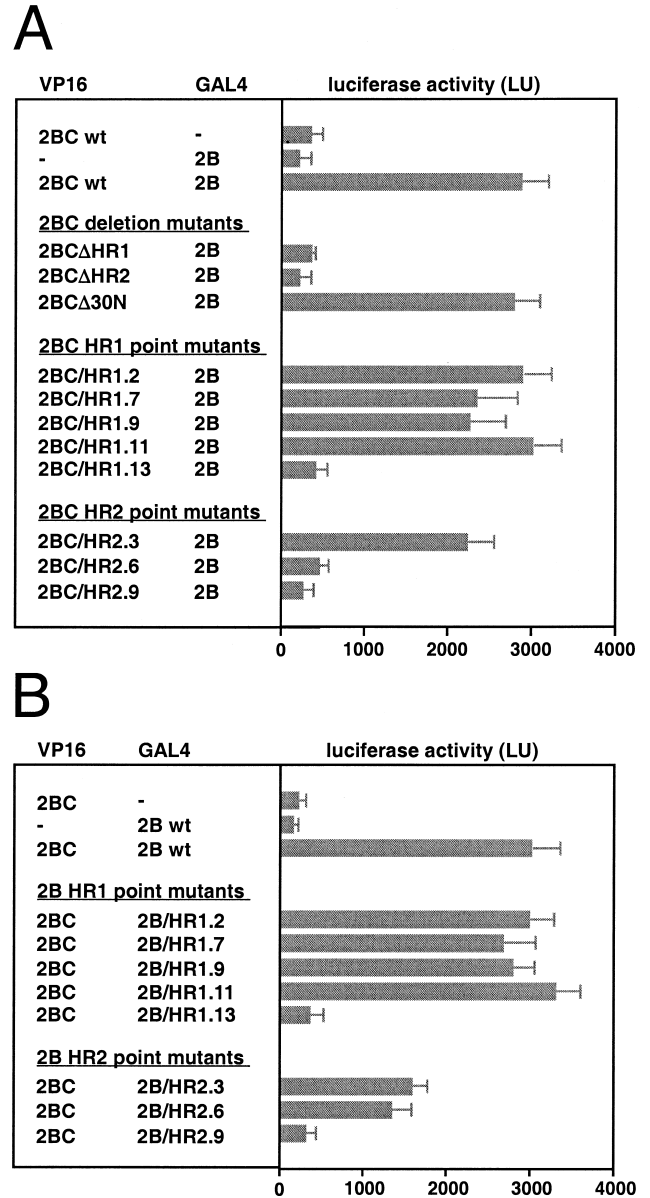


Fig. 6. Heteromultimerization reactions of (A) VP16–2BC deletion mutants and point mutants with the wild-type GAL4–2B fusion protein and (B) VP16–2BC wild-type fusion protein and GAL4–2B point mutants. Values represent means  $\pm$  standard errors of measurements of three independent experiments.

## Discussion

In this study, we have characterized homomultimerization and heteromultimerization reactions of the CBV3 nonstructural 2B, 2C and 2BC proteins using a mammalian two-hybrid system, which allows characterization of protein:protein interactions within a cellular environment that provides the native protein environment with regard to post-translational modifications and subcellular localization of the viral proteins. We performed the mammalian two-hybrid analysis in COS

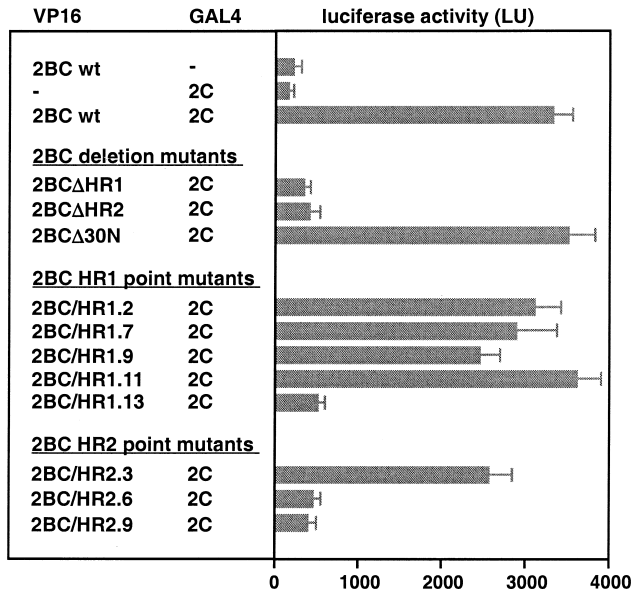


Fig. 7. Heteromultimerization reactions of VP16–2BC deletion mutants and point mutants with the GAL4–2C fusion protein. Values represent means  $\pm$  standard errors of measurements of two independent experiments.

cells, a cell line that permits CBV3 replication and thereby provides the appropriate host cell proteins that may be involved in regulating or modulating viral protein interactions.

### Homomultimerization reactions of the 2BC, 2B and 2C proteins

Homomultimerization reactions were observed with the 2BC protein and, albeit weakly, with the 2B protein, but not with the 2C protein. Western blot analysis demonstrated the efficient expression of the 2C protein arguing that it is unlikely that the failure to detect a 2C:2C interaction was due to impaired protein synthesis or stability. The possibility that either the GAL4–2C or the VP16–2C fusion protein was misfolded or unable to be transported to the nucleus seems also unlikely because protein:protein interactions were observed with both the GAL4–2C fusion protein (in combination with VP16–2BC) and the VP16–2C fusion protein (in combination with GAL4–2BC). Taken together, these data strongly suggest that the 2C protein is unable to form homomultimers. Multimerization of the poliovirus 2C protein was suggested by Tolskaya *et al.* (1994) on the basis of complementation experiments. It should be stated, however, that *trans-rescue* of a genetic defect merely provides evidence for the requirement of a functional version of the protein rather than that it provides proof for the requirement of homomultimerization.

Analysis of mutant proteins carrying deletions or well-defined point mutations in the 2B region revealed that both the 2BC:2BC and 2B:2B homomultimerization reactions were dependent on hydrophobic determinants located in both the cationic amphipathic  $\alpha$ -helix (aa 37–54) and the second

hydrophobic domain (aa 63–80) of protein 2B. Deletion of the amino-terminal 30 aa of the 2B protein, as well as mutations that interfered with the cationic or the amphipathic character of the predicted cationic amphipathic  $\alpha$ -helix, which is well conserved among all enterovirus 2B proteins (van Kuppeveld *et al.*, 1996), did not interfere with the homomultimerization reactions of the 2BC and 2B proteins. The observations that (i) the 2BC and the 2B homomultimerization reactions are sensitive to the same mutations in the 2B region and (ii) the 2BC:2BC interaction was completely inhibited by specific point mutations in the 2B region, strongly suggest that the homomultimerization reactions of the 2BC proteins depend on an intermolecular 2B:2B interaction mediated by hydrophobic determinants located in the amphipathic  $\alpha$ -helix and the second hydrophobic domain.

Do these results correlate with the yeast two-hybrid results obtained by Cuconati *et al.* (1998)? These authors also observed homomultimerization reactions of the PV 2B and 2BC proteins, but not of the 2C protein. These authors, however, found that the 2BC:2BC interaction may be aided by, or is even dependent on, interaction of the 2C moieties and does not occur exclusively through 2B:2B or 2B:2C interactions. In this study, no evidence for an absolute dependence of the 2C protein was observed. As mentioned above, specific point mutations in the 2B region completely abolished the homomultimerization reaction of CBV3 protein 2BC. If there were a contribution of the 2C protein in the 2BC:2BC interaction, then a residual interaction should have been expected. Our observations, however, do not exclude the possibility that an efficient 2BC homomultimerization reaction is dependent on both the 2B and 2C moieties, whereby specific determinants in the 2B moiety catalyse the initiation of the 2BC:2BC multimerization reaction, whereas the 2C moiety serves to stabilize this intermolecular interaction. This possibility is in agreement with our observation that the homomultimerization reaction of the 2BC protein was much stronger than the homomultimerization reaction of the 2B protein, even though the 2B protein was expressed in much higher amounts than the 2BC protein. However, it should be emphasized that care should be taken in drawing conclusions from the level of reporter gene transcription, because this does not necessarily reflect the strength of the protein:protein interaction. Factors like fusion protein folding and the efficiency with which the interacting protein complex is transported to the nucleus (which may be different for the 2B and 2BC fusion proteins) may be of importance as well. Therefore, no conclusions can be drawn on the potential role of the 2C moiety in the 2BC homomultimerization reaction.

### Heteromultimerization reactions of the 2BC, 2B and 2C proteins

In this study, heteromultimerization reactions were observed between the CBV3 2BC and 2B proteins, and

between the 2BC and 2C proteins, but not between the 2B and 2C proteins. Analysis of the 2BC:2B interaction using 2BC point mutants showed that the heteromultimerization reaction was sensitive to the same mutations that disrupted the 2BC:2BC and 2B:2B homomultimerization reactions, indicating that the 2BC:2B interaction may be mediated by an interaction between the 2B moieties. However, analysis of the 2BC:2B heteromultimerization reaction using 2B point mutants identified a mutation (HR2.6, I[64]S/V[66]S) that only partially inhibited the formation of 2BC:2B heteromultimers but completely prevented the formation of 2BC:2BC and 2B:2B homomultimers. This finding indicates that the 2BC:2B interaction may not simply reflect a 2B:2B interaction. Possible explanations for this remarkable observation are addressed below.

The 2BC:2C interaction was sensitive to the same mutations that disrupted the 2BC:2BC and 2B:2B homomultimerization reactions. A possible interpretation for this finding is that the same 2B determinants in the 2BC protein that govern the interaction with another 2B moiety also mediate interaction with the 2C protein. Although this possibility cannot be excluded, it seems unlikely given the absence of a 2B:2C interaction. Moreover, it is difficult to envisage how a certain protein domain mediates specific interactions with two different polypeptides which have no homology in amino acid sequence. Alternative explanations should be considered. A possible explanation is that the folding of the 2BC protein gives rise to new protein interaction domains which are absent in the 2B and 2C polypeptides alone. Disruption of the 2BC protein structure, which may depend on specific hydrophobic determinants in the 2B moiety, may disrupt the formation or the exposure of the 2BC-specific interaction domains and thereby disturb the interaction with the 2C protein. Another alternative explanation is that higher order protein complexes are formed. VP16–2BC fusion proteins may form homomultimers that provide a scaffold for the interaction with the GAL4–2C fusion protein, which may interact either with determinants located in the 2B moiety, the 2C moiety or 2BC-specific interaction determinants. Mutations in the 2B moiety that disturb the 2BC homomultimerization reaction may thereby interfere with the 2BC:2C heteromultimerization reaction.

These alternative explanations may of course also be valid for the 2BC:2B heteromultimerization reaction. The 2B protein may interact with 2BC-specific domains, which may or may not depend on the formation of 2BC homomultimers. Disruption of either the 2BC protein structure or the ability to form homomultimers by mutations in the 2B moiety may thereby account for the disturbance of the interaction with the 2B protein. The possibility that the 2BC:2B heteromultimerization reaction is not simply provided by 2B:2B interaction is consistent with the differential results obtained with mutant HR2.6 (see above).

Further experiments are required to dissect the molecular

determinants that underlie the heteromultimerization reactions between the 2B, 2C and 2BC proteins. An important step would be investigations to assay possible interactions between the 2B and 2C proteins. In the mammalian two-hybrid system, we found no evidence for a 2B:2C interaction. In contrast, Cuconati *et al.* (1998) observed a strong PV 2B:2C interaction in the yeast system. These authors, however, found no evidence for a 2B:2C interaction in the GST pull-down assay. Further investigations are required to shed light on the reason for the discrepancies between the yeast and mammalian systems.

### Implications for the virus life-cycle

Enteroviruses gradually modify the permeability of the ER membrane and the plasma membrane of the host cell (van Kuppeveld *et al.*, 1997a). The 2B protein has been identified as the membrane-active protein responsible for these modifications, and both the amphipathic  $\alpha$ -helix and the second hydrophobic domain have been recognized as major determinants for its membrane-active function (Aldabe *et al.*, 1996; Barco & Carrasco, 1998; Doedens & Kirkegaard, 1995; van Kuppeveld *et al.*, 1997a, c). The membrane alterations induced by protein 2B (or its precursor 2BC) are of major importance for creating the microenvironment required to replicate the vRNA, since mutations in the hydrophobic domains of the 2B protein cause early defects in vRNA replication (van Kuppeveld *et al.*, 1995, 1996). The molecular mechanism by which the 2B protein modifies membrane permeability is as yet unknown. One possible mechanism that may explain the membrane-active function of the 2B protein is the formation of membrane-integral multimers of protein 2B (or 2BC), which may build aqueous pores by exposing the hydrophilic sides of the amphipathic  $\alpha$ -helices towards each other. The finding that both the 2B protein and the 2BC protein formed homomultimers in the mammalian two-hybrid system lends support to this hypothesis. Each of the hydrophobic domains was found to be required for the 2B and 2BC homomultimerization reaction. This suggests that the formation of these membrane-integral pore structures may involve (sequence-specific) synergistic contacts between the two hydrophobic domains, rather than that they act as autonomous entities. This idea is consistent with previous observations that (i) mutations in each of the hydrophobic domains can interfere with the membrane-active function of the 2B protein (van Kuppeveld *et al.*, 1997c) and (ii) neither the amphipathic  $\alpha$ -helix nor the second hydrophobic domain of the CBV3 2B protein can be functionally exchanged with its PV counterpart (van Kuppeveld *et al.*, 1997b). All mutations that disturb the homomultimerization reaction of the 2B protein (mutations I[64]S/V[66]S, A[71]E/I[73]E and L[46]N/V[47]N/I[49]N/I[50]N) abolish the membrane-active function of the 2B protein (A. S. de Jong and others, unpublished), providing evidence that there is a firm correlation between the ability of

the 2B protein to form homomultimers and to permeabilize membranes. Consistent with our results, Cuconati *et al.* (1998) also identified hydrophobic determinants (PV aa I[53] and I[54]) as being important for the formation of PV 2B homomultimers.

Further biochemical and genetic experimentation is required for a better understanding of the complex interplay of homomultimerization and heteromultimerization reactions that mediate regulation and execution of protein functions in the virus life-cycle.

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