The EMBO Journal vol.14 no.14 pp.3349–3364, 1995

A human virus protein, poliovirus protein 2BC, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces cerevisiae*

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Inducible synthesis of poliovirus protein 2BC in *Saccharomyces cerevisiae* arrests cell growth in the G_2 phase of the cell cycle, while no effects are observed upon expression of poliovirus genes 2B or 2C, either individually or in combination. Expression of 2BC induces a number of morphological modifications in yeast cells, one of the most striking being the proliferation of small membranous vesicles that fill most of the cytoplasm. These vesicles are morphologically similar to the cytopathic vacuoles that proliferate during the infection of human cells by poliovirus. The transport and processing of several yeast proteins, including vacuolar carboxypeptidase Y, aminopeptidase I or yeast α-mating factor, is hampered upon expression of poliovirus 2BC, suggesting that transport of proteins through the Golgi apparatus is impaired by this viral protein. Finally, a number of 2BC variants were generated and the effects of their expression on yeast growth, cellular morphology and protein processing were analyzed. 2BC variants defective in the NTPase activity were still able to interfere with yeast growth and the exocytic system, while deletion of 30 amino acids at the N-terminus of 2BC impairs its function. These findings lend support to the idea that 2BC, but not 2B or 2C, is the protein responsible for vesicle proliferation in poliovirus-infected cells. In addition, the activity of a human virus protein in yeast cells opens new avenues to investigate the exact location at which poliovirus 2BC interferes with the vesicular system and to test the action of other animal virus proteins potentially involved in modifying the vesicular system in mammalian cells.

Key words: exocytic pathway/membrane proliferation/poliovirus protein 2BC/vesicular system

Introduction

The transport and sorting of proteins in eukaryotic cells relies upon the continuous flow of membranes and vesicles through the different cellular compartments. (Pfeffer and Rothman, 1987; Waters *et al.*, 1991; Pryer *et al.*, 1992) The function of this continuous vesicle flow is to transport proteins from the endoplasmic reticulum (ER), where they are synthesized, to different cellular compartments. The best understood model system for vesicular transport in genetic terms is the yeast *Saccharomyces cerevisiae* (Pryer *et al.*, 1992). Extensive genetic analysis of this system has identified a number of genes involved in protein transport (Novick *et al.*, 1980; Kaiser and Schekman, 1990). Understanding the function and the molecules involved in the exocytic pathway in mammalian cells has benefited from the analysis of both normal and animal virus-infected cells. Some animal viruses profoundly modify the normal morphology as well as the function of the vesicular system (Dales *et al.*, 1965; Bienz *et al.*, 1983; Griffiths *et al.*, 1989). Thus, during the replication of cytoplasmic animal viruses, a number of membranous structures are induced in different locations of the cytoplasm. The exact function of these new structures is not known, but they have been implicated in the synthesis of new viral genomes (Caliguiri and Tamm, 1969; Guinea and Carrasco, 1990) and in the morphogenesis of virus particles (Sodeik *et al.*, 1993). In the case of picornaviruses, most of the cytoplasm becomes filled by vesicles of various sizes (50–400 nm) at late times of infection, while the Golgi apparatus is no longer recognized in these cells (Dales *et al.*, 1965; Bienz *et al.*, 1983). Picornaviruses are positive single-stranded RNA viruses that synthesize their genomes on the newly generated membranous vesicles that proliferate from the mid-phase of infection (Semler *et al.*, 1988). Both the function and the generation of these vesicles have been the object of intense research for >20 years (Carrasco, 1995). Elegant electron microscopy studies showed that poliovirus RNA replication complexes were surrounded by a rosette-like structure of membranous vesicles (Bienz *et al.*, 1992). The generation and correct trafficking of the newly formed vesicles is blocked by inhibitors of phospholipid synthesis, such as cerulenin, or by the antibiotic brefeldin A. These agents are also potent inhibitors of poliovirus RNA synthesis (Guinea and Carrasco, 1990; Maynell *et al.*, 1992; Irurzun *et al.*, 1993). Therefore, both continuous membrane proliferation and vesicular traffic are necessary to replicate poliovirus genomes. These findings provided clues to understanding the function of the viroplasm during the virus replication cycle: the newly made vesicles are required for viral RNA synthesis. However, it remains a challenge to understand how membrane proliferation and the synthesis of viral nucleic acids are biochemically coupled.

With regard to the generation of these membranous structures and the viral proteins involved in this process, it was found that apart from the viral RNA polymerase, $3D^{pol}$, other viral non-structural proteins were tightly associated with the poliovirus replication complexes (Girard and Baltimore, 1967; Butterworth *et al.*, 1976; Bienz *et al.*, 1983; Tershak, 1984). Of interest in this regard was the association of 2BC and 2C not only with the membrane vesicles, but also with the viral nucleic acids (Butterworth *et al.*, 1976; Bienz *et al.*, 1983; Tershak, 1984). Initial studies localized several poliovirus proteins in these membranous vesicles and suggested that the precursor 2BC could play an important part in the generation of the vesicles (Bienz *et al.*, 1983). Recent support

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Table I. Expression plasmids used in this work

Expression plasmid	Promoter	Selection marker	Characteristics	Reference
pEMBLyex4	UAS_{GAL}-CYC1	*LEU2d, URA3*	Galactose-inducible multicopy expression vector	Murray *et al.*, 1987
pEMBL.2B	UAS_{GAL}-CYC1	*LEU2d, URA3*	2B poliovirus sequence cloned in pEMBLyex4	Barco and Carrasco, 1995
pEMBL.2B(–B)	UAS_{GAL}-CYC1	*LEU2d, URA3*	pEMBL.2B minus *Sma*I, *Bam*HI and *Xba*I sites	This study
pEMBL.2C	UAS_{GAL}-CYC1	*LEU2d, URA3*	2C poliovirus sequence cloned in pEMBLyex4	Barco and Carrasco, 1995
pEMBL.2BC	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence cloned in pEMBL.2B(-B)	This study
pEMBL.2bC(S)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2bC(D)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(*Eco*RI)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(*Sal*I)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(*Eco*RI/*Sal*I)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2bc(Δ*Sph*I)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(ΔGKS)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(Δ*Bam*HI)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2bC(Δ30N)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pEMBL.2Bc(Δ*Xba*I)	UAS_{GAL}-CYC1	*LEU2d, URA3*	2BC poliovirus sequence with the mutation indicated	This study
pINTyex4	UAS_{GAL}-CYC1	*URA3*	Integrative plasmid from pEMBLyex4	This study
pINT.2B	UAS_{GAL}-CYC1	*URA3*	Integrative plasmid from pEMBL.2B	This study
pINT.2C	UAS_{GAL}-CYC1	*URA3*	Integrative plasmid from pEMBL.2C	This study
pYcDE8	ADHI	*TRP1*	Constitutive multicopy expression vector	Klein and Roof, 1988
pYc.2B	ADHI	*TRP1*	2B poliovirus sequence cloned in pYcDE8	This study
pYc.2C	ADHI	*TRP1*	2C poliovirus sequence cloned in pYcDE8	This study

for this idea has come from the analysis of the effects of 2BC or 2C expression by means of recombinant vaccinia viruses (Cho *et al.*, 1994; Aldabe and Carrasco, 1995). The individual expression of each of these two poliovirus proteins induced membrane proliferation and vesicle formation in vaccinia virus-infected cells, although the vesicles induced by protein 2BC were more similar to those that appear during the infection of human fibroblasts by poliovirus (Cho *et al.*, 1994; Aldabe and Carrasco, 1995). These findings are of paramount interest for understanding the function of poliovirus proteins 2BC and 2C, but the major drawback of the system used was that vaccinia virus infection itself also modifies the vesicular system (Sodeik *et al.*, 1993). In addition, some vaccinia virus proteins complement poliovirus gene products (Pal-Ghosh and Morrow, 1993), making it impossible to analyze the function of these poliovirus proteins in the absence of other animal virus products. Poliovirus protein 2C has been shown recently to possess NTPase activity and to bind to nucleic acids (Rodríguez and Carrasco, 1993, 1994, 1995). These findings led us to suggest that protein 2C was involved in trafficking viral nucleic acids through the cytoplasm, a process mediated by membrane vesicles (Rodríguez and Carrasco, 1993). Viral genome traffic through the vesicular system could be required not only for viral RNA synthesis, but also for virus assembly (Li and Baltimore, 1990; Rodríguez and Carrasco, 1993).

We have taken advantage of the ease of genetic manipulation in the yeast *S.cerevisiae* and have cloned poliovirus 2B, 2BC, 2C and several mutated proteins in this eukaryotic microorganism. Strikingly, our results indicate that the inducible expression of poliovirus protein 2BC, but not 2B or 2C alone or in combination, is able to produce this effect, reinforcing the idea that yeast and mammalian proteins that participate in vesicular transport are interchangeable. In addition, our studies could benefit from the extensive characterization of vesicular transport at the molecular level in yeast (Pryer *et al.*, 1992). Certainly, *S.cerevisiae* is the best understood organism as regards the complexity and functioning of the genes involved in protein transport and sorting (Kaiser and Schekman, 1990; Pryer *et al.*, 1992). The action of poliovirus protein 2BC on vesicular transport and protein processing in yeast cells suggests that 2BC profoundly distorts both processes at a stage located between the ER and the Golgi apparatus.

Results

Cloning and expression of poliovirus proteins 2B, 2C and 2BC in S.cerevisiae

The infection of human cells by poliovirus produces a number of alterations in cellular morphology and metabolism, including the modification of the vesicular system (Bienz *et al.*, 1983). Our aim in cloning and expressing poliovirus proteins in *S.cerevisiae* was to detect the viral proteins which are responsible for cytotoxicity. To this end, most of the poliovirus non-structural proteins were expressed in an inducible manner in yeast (Barco and Carrasco, 1995). This work already indicated that poliovirus protein 2A was very toxic for *S.cerevisiae* cells due to its protease activity. To analyze the actions of poliovirus proteins 2B, 2C and 2BC on yeast cells, the sequences encoding each of these proteins were amplified by PCR from the whole poliovirus genome and cloned in the plasmid pEMBLyex4 under the inducible promoter UAS_{GAL}-CYC1. A summary of the different constructs used in the present work is shown in Table I. The yeast strain W303-1B was transformed with these constructs. To analyze the expression of these poliovirus proteins, yeast cells were placed in galactose-containing medium and collected 8 h after induction. Proteins were separated by SDS–PAGE and immunoassayed by Western blot with a mixture of specific polyclonal antisera against poliovirus proteins 2B or 2C; the precursor 2BC is detected by immunoblot analysis using both polyclonal antisera

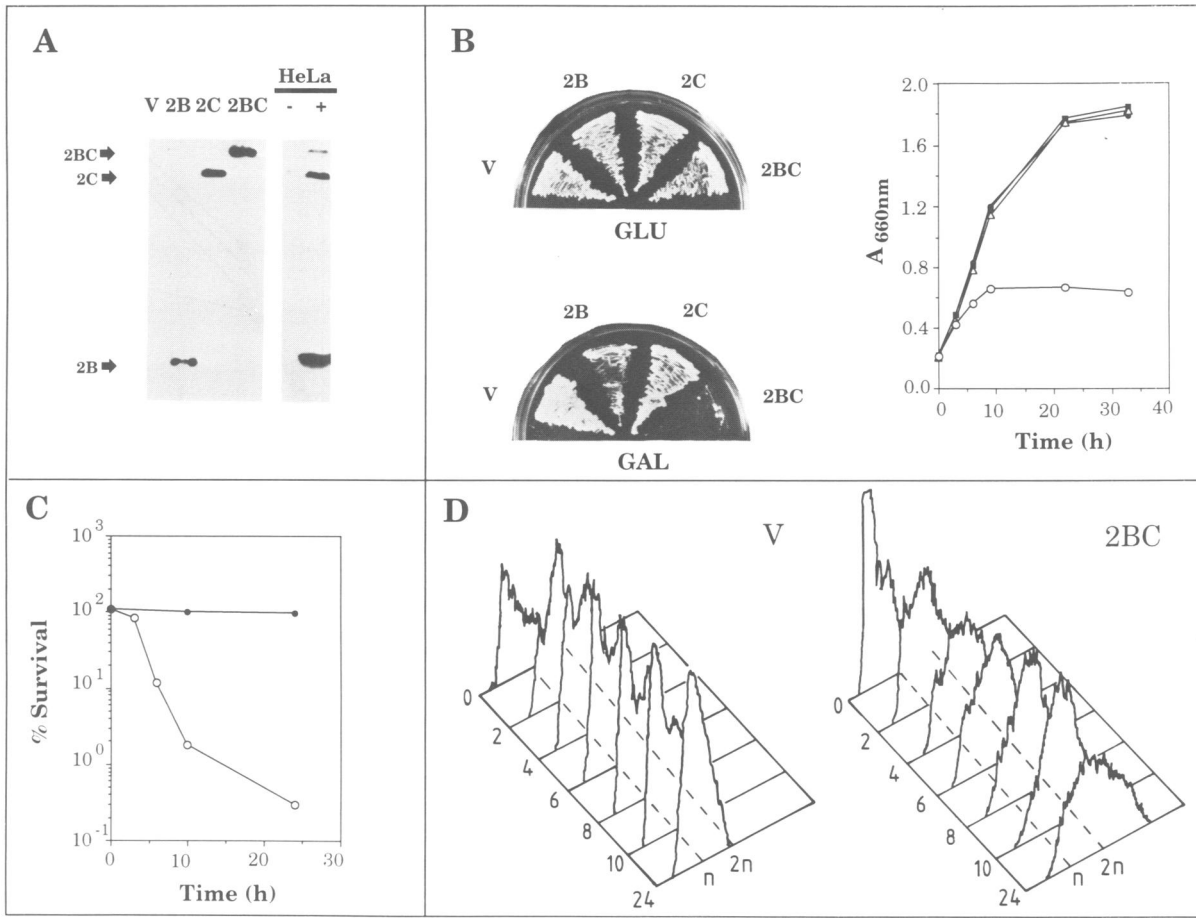


Fig. 1. Growth of yeast cells expressing the poliovirus proteins 2B, 2C and 2BC. (**A**) Immunoblot analysis of poliovirus proteins. Yeast cells containing pEMBLyex4 (V) that contain vector alone without insert or the expression plasmids coding for the indicated poliovirus protein, were grown in YNB.LGal medium to induce the synthesis of the proteins. Crude extracts were obtained as described in Materials and methods 8 h after induction, separated by SDS–PAGE, transferred to nitrocellulose membranes and immunoreacted with a mix of rabbit antisera directed against 2B (1:5000) and 2C (1:1000). The reaction was performed as described in Materials and methods. Uninfected HeLa cells (–) or poliovirus-infected HeLa cell (+) extracts were used as controls. The position of recombinant poliovirus proteins is indicated by arrows. (**B**) Growth of yeast cells bearing genes 2B (△), 2C (■) or 2BC (○) proteins streaked on YNB.Glu medium that does not induce expression (upper plate), or YNB.Gal medium that induces expression of poliovirus genes (lower plate) or grown in YNBLGal medium. Yeast cells transformed with plasmid pEMBLyex4 (V, ●) were used as control. (**C**) Survival of yeast cells that express 2BC protein. Yeast cells transformed with pEMBLyex4 (●) or pEMBL.2BC (○) were grown in YNB.LGal medium (galactose was added at time zero) to induce poliovirus protein synthesis. Aliquots of cultures were placed in YNB.Glu plates to repress 2BC expression. The number of the initial cells was calculated from A_{660} data and the number of viable cells was obtained by counting colonies after 3 days of incubation at 30°C. (**D**) Measurement of cellular DNA content of control yeast cells (bearing pEMBLyex4, left panel) or cells expressing 2BC (right panel) by flow cytometry. Cells were withdrawn at the indicated times post-induction (0–24 h as indicated in the figure), fixed, stained with propidium iodide and analyzed by flow cytometry. Two cell populations are observed; those that contain a normal amount of DNA after cell division (n) and those cells containing the replicated genome ($2n$).

(Figure 1A). These antibodies do not recognize any protein in uninfected HeLa cells, while 2C and 2BC proteins (α-2C antiserum) or 2B and 2BC (α-2B antiserum) are recognized in extracts from poliovirus-infected cells (Barco and Carrasco, 1995). These antisera also do not react with any protein in yeast cells induced with galactose bearing the vector pEMBLyex4 without an insert. Figure 1A shows the immunoblot analysis using both polyclonal antisera. These results indicate that the expression of poliovirus proteins 2B, 2C and 2BC is tightly controlled using the pEMBLyex4 vector and galactose induction. On the other hand, proteins are already detected as soon as 2 h after induction, reaching levels of the induced proteins which are comparable with a poliovirus infection of HeLa cells. Proteins 2C and 2BC are also clearly identifiable by [^{35}S]methionine labeling (results not shown).

Inhibition of yeast growth by poliovirus 2BC expression

Figure 1B shows that yeast bearing plasmids encoding protein 2B, 2C or 2BC grow well in solid medium containing glucose as the carbon source, while growth is hampered for recombinant yeast cells that encode protein 2BC in galactose-containing medium. The presence of galactose induces the synthesis of 2B, 2C or 2BC, while these proteins are not detected in glucose medium. Growth of cells encoding protein 2B in solid medium is partially reduced under these conditions. Recombinant yeast clones that express proteins 2B or 2C grow to levels similar to control cells bearing only the vector pEMBLyex4 in liquid medium that contains galactose, whereas growth of cells that express poliovirus 2BC declines 4–5 h after induction and growth is arrested after 10 h (Figure 1B). Cell survival

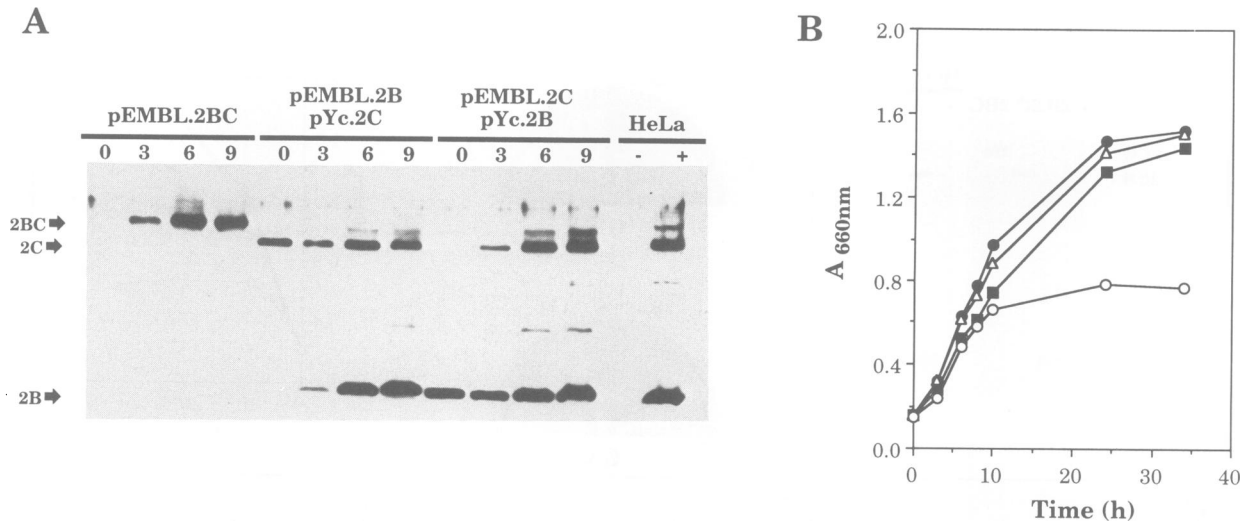


Fig. 2. Kinetics of 2B and 2C co-expression. (**A**) Yeast cells containing the indicated plasmids were grown in YNB.LGal medium for the time indicated. The extracts were prepared and immunoblot analysis was performed as described in the legend of Figure 1A. Uninfected HeLa cells (–) or poliovirus-infected HeLa cell (+) extracts were used as controls. The position of recombinant poliovirus proteins is indicated by arrows. (**B**) Growth of yeast cells co-expressing 2B and 2C proteins. Growth of yeast cells induced in YNB.LGal medium bearing the plasmids: pEMBLyex4 and pYcDE8 (●), pEMBL2B and pYc.2C (△), pEMBL.2C and pYc.2B (■), and pEMBL.2BC and pYcDE8 (○).

of the recombinant clone encoding poliovirus protein 2BC after different times post-induction is shown in Figure 1C. Certainly, induction with galactose is detrimental to the formation of yeast colonies 5 h after induction, even when the cells are returned to glucose medium, although it should be noted that the effect is reversible during the first 2 h, even if the expression of 2BC has been triggered, and the protein synthesized is stable in the cell for some time after synthesis.

To ascertain the precise step blocked by poliovirus 2BC in the yeast cell cycle, flow cytometry studies were conducted. Control cells bearing plasmid without an insert contain a haploid number of chromosomes (*n*); 2 h later most of the cells have doubled their DNA content and, at the stationary phase (24 h post-induction), the cells contain a haploid genome content again. The recombinant clone that encodes poliovirus 2BC shows a different behavior; the passage to the diploid state also occurs when these cells are placed in galactose-containing medium, but these cells retain the 2*n* chromosome number, suggesting that the cells are blocked between the S and the M phases, most probably at the G_2 phase of the cell cycle. In conclusion, the expression of poliovirus 2BC, but not 2B or 2C, arrests growth of yeast cells, which are unable to form colonies and divide. These cells are probably halted at the G_2 phase of the cell cycle (Figure 1D). Of interest, yeast cells mutated in the *YPT1* gene, contrary to other *sec* mutants (Novick *et al.*, 1980), are also arrested after the S phase of the cell cycle (Segev and Botstein, 1987). The *YPT1* gene encodes a GTP binding protein implicated in vesicle consumption between the ER and the Golgi (Pryer *et al.*, 1992). Notably, 2BC is endowed with GTPase activity (Rodriguez and Carrasco, 1993) and seems to behave like the deletion of the *YPT1* gene in this respect, i.e. halting yeast cells at the G_2 phase.

During our initial experiments on the inducibility of poliovirus proteins, it became evident that 2BC expression was deleterious for yeast growth, while the expression of proteins 2B or 2C was well tolerated. To determine whether these inhibitory effects were due to a specific 2BC function or to the simultaneous expression of 2B and 2C, both proteins were co-expressed. Co-expression of 2B and 2C to similar levels was achieved from yeast cells simultaneously bearing multicopy plasmids pEMBL.2B and pYc.2C or pEMBL.2C and pYc.2B (Figure 2A). Cells that express 2B and 2C simultaneously grow to control levels in galactose-containing medium (Figure 2B). The same result was obtained when yeast cells containing the 2B or 2C sequences integrated, that inducibly synthesize these poliovirus proteins, were transformed with the constitutive expression plasmids pYc.2C and pYc.2B, respectively. To analyze the level of expression of 2BC or 2B plus 2C at different times post-induction, yeast extracts were prepared from cells that express 2BC, or 2B constitutively and 2C in an inducible manner or *vice versa* (Figure 2A). The proteins from these extracts were immunoblotted against a mixture of 2B plus 2C antibodies. Figure 2A shows that 2BC protein is apparent 3 h post-induction and continues accumulating in yeast cells for at least 9 h. On the other hand, constitutive expression of 2B and induction of 2C or *vice versa* gives levels of synthesis of these proteins comparable with 2BC alone. These findings suggest that the 2BC protein has to be synthesized as such in order to observe the inhibitory effects on yeast cells, while the simultaneous synthesis of 2B plus 2C does not show this effect.

Morphological alterations of yeast cells that synthesize poliovirus protein 2BC

A number of alterations in yeast cellular morphology are apparent upon induction of synthesis of poliovirus protein 2BC. Phase contrast microscopy shows that yeast cells synthesizing 2BC are similar in size, or slightly bigger, than control cells, but remarkably their appearance is different to normal cells, particularly when viewed by Nomarski microscopy (Figure 3, upper panels). Cells induced to synthesize poliovirus 2BC show a number of granules, clearly apparent on the cell surface, while the

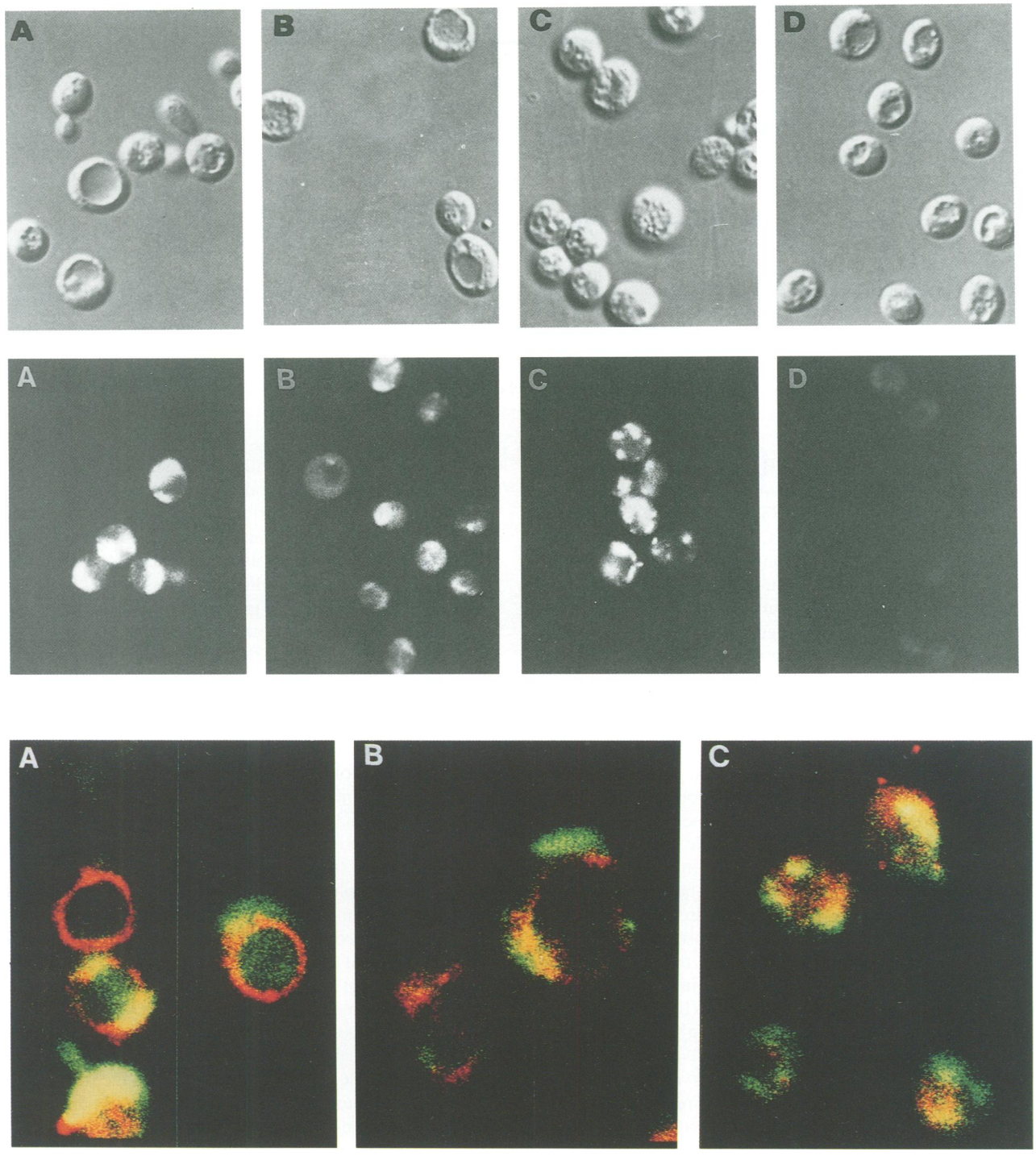


Fig. 3. Nomarski (upper panels) and immunofluorescence (middle and lower panels) microscopy of yeast cells bearing plasmid pEMBL.2B (**A**), pEMBL.2C (**B**), pEMBL.2BC (**C**) or pEMBLyex4 (**D**) after 6 h growth in YNB.LGal to induce the expression of poliovirus genes. Spheroplasts were obtained and examined under a Nomarski lens at 600× magnification. The spheroplast preparation was used for the immunofluorescence analysis as described in Materials and methods. In the middle panel, cells were fixed and stained with rabbit antisera, directed against 2B (A, C and D) or 2C (B). In the lower panel, the cells were doubly stained with these antisera (green) and with the mouse monoclonal antibody against the V-ATPase 60 kDa subunit (red) and visualized with a LSM Zeiss confocal microscope. The staining of negative control and 2BC-expressing cells is similar with α-2C or α-2B antisera.

vacuole is not observed. The morphology of cells that express poliovirus 2B or 2C genes is normal. As regards the intracellular distribution of poliovirus proteins 2B, 2C and 2BC, Figure 3 shows that all three proteins usually appear clustered in one or two cellular regions, although the distribution of 2BC is more scattered and appears in several clusters in the cytoplasm (Figure 3, middle panels).

Confocal immunofluorescence microscopy of doubly stained cells with a cellular marker for the vacuole, i.e. the 60 kDa subunit of the vacuolar yeast ATPase and α-2B or α-2C antisera, shows that 2B or 2C expression does not modify the vacuole. Remarkably, the vacuole appears disaggregated and can no longer be observed intact in yeast cells that express 2BC (Figure 3, lower

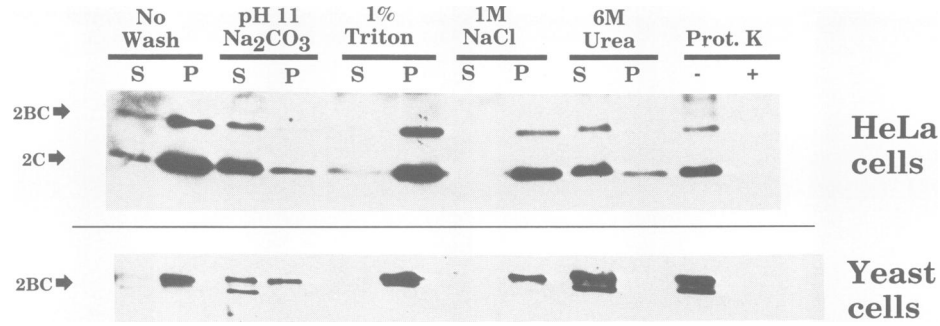


Fig. 4. Characterization of the association of 2BC with the P100 subcellular fraction. Poliovirus-infected HeLa cells (5 h post-infection at 50 p.f.u./cell) or yeast cells expressing 2BC (5 h post-induction) were lysed as described in Materials and methods. The lysate was cleared of unbroken HeLa cells or yeast spheroplasts by centrifuging for 5 min at 500 *g*. Then, this supernatant was centrifuged for 15 min at 15 000 *g* and the resulting supernatant (S15) was treated for 15 min at 4°C with the following reagents; buffer alone, 100 mM Na_2CO_3 (pH 11), 1% Triton X-100, 1 M NaCl or 6 M urea. The S15 was treated (+) or not (–) with proteinase K (100 μg/ml) for 30 min at 37°C. After this treatment, the samples were centrifuged for 30 min at 100 000 *g* to generate the supernatant (S) and pellet (P) fractions as indicated. Immunoblot analysis was performed with α-2C antibodies.

panels). All three proteins 2B, 2C and 2BC seem to interact with membranes in mammalian cells (Butterworth *et al.*, 1976; Bienz *et al.*, 1983; Tershak, 1984). To test this possibility in yeast cells, we employed standard differential centrifugation techniques to fractionate yeast lysates from spheroplasts (Bankaitis *et al.*, 1989). After fractionation of mitochondria and nuclei at 15 000 *g*, the supernatant (S15) was centrifuged at 100 000 *g*. The three proteins 2B, 2C and 2BC appear associated with the sediment in this cellular fractionation (results not shown). To better understand the nature of the association of 2BC with microsomes, the S15 fraction was treated with various reagents and centrifuged at 100 000 *g*. In addition, the behavior of 2BC in poliovirus-infected HeLa cells was also tested for comparative purposes. A proportion of 2BC appeared in the supernatant after treatment with 100 mM sodium bicarbonate or 6 M urea, while incubation with 1 M NaCl or 1% Triton X-100 had little effect on the association of 2BC with microsomes, both in yeast and HeLa cells (Figure 4). Proteinase K treatment destroys 2BC, suggesting that the protein is exposed on the surface and not inside the vesicles. Curiously, the solubilization of 2BC gives rise to two products that immunoreact with the anti-2C antibodies in yeast cells (Figure 4). These findings suggest that 2BC associates with a protein complex. In addition, it is possible that, apart from this interaction of 2BC with other proteins or with itself, a portion of 2BC might also associate directly with membranes. Our results agree well with previous observations on the resistance to the solubilization by sodium deoxycholate of 2BC from poliovirus-infected cells (Bienz *et al.*, 1983).

Ultrastructural alterations of yeast cells that synthesize poliovirus protein 2BC

To analyze the modifications in the morphology of cellular components in yeast cells expressing 2BC in more detail, they were observed by transmission electron microscopy. A central vacuole that occupies a large proportion of the cytoplasm, in addition to the nucleus and several mitochondria, is usually clearly visible in control yeast cells (Figure 5A). This normal morphology is also apparent in cells that express poliovirus 2B or 2C after 20 h of induction (Figure 5B and C), or in cells bearing the plasmid pEMBL.2BC without induction (Figure 5D). However, 6 h post-induction, most of the cells that express poliovirus 2BC show a different morphology (Figure 5F), with the cytoplasm containing small vesicles of ~50–400 nm diameter and an abnormal vacuolar morphology. Although most of the cells show this morphology 6 h after induction of 2BC (Figure 5F), a few of them show the beginning of this process with the vacuole starting to collapse (Figure 5E). At 20 h post-induction, yeast cells that express poliovirus 2BC show a morphology profoundly different from control cells, with most of the cytoplasm occupied by vesicles of ~50–400 nm diameter and the vacuole no longer recognizable (Figure 5G and H). This morphology very much resembles that observed in human cells infected with poliovirus (Dales *et al.*, 1965; Bienz *et al.*, 1983), where most of the cytoplasm becomes occupied by vesicles of a size similar to those observed in yeast cells (see below, Figure 6). Moreover, this vacuolated morphology is exacerbated in the case of yeast cells synthesizing poliovirus protein 2BC, as compared with human fibroblasts. These striking findings show for the first time that a human virus protein is able to reproduce the formation of the so-called viroplasm in an organism as distantly related to human cells in evolutionary terms as are yeast cells.

To determine in more detail the intracellular location of poliovirus 2BC and its association with the membranous structures, immunogold analyses using antibodies against 2B or 2C were performed both in poliovirus-infected HeLa cells and in yeast cells bearing pEMBL.2BC after induction with galactose. The results shown in Figure 6 with α-2B antiserum indicate that proteins 2B or 2BC preferentially localize to the cytopathic vesicles that proliferate in HeLa cells after poliovirus infection (Figure 6A); similar results were obtained with α-2C antisera (results not shown). These findings are in good agreement with the localization of proteins 2BC and 2C in poliovirus-infected HeLa cells using α-2C antibodies (Troxler *et al.*, 1992). To our knowledge, no such studies have been described using anti-2B antiserum. Yeast cells that synthesize 2BC clearly contain this protein associated with the membranous vesicles that fill most of the cytoplasm (Figure 6B, lower panel). These vesicles are not marked with the α-V-ATPase or α-KAR2 antibodies (a marker of

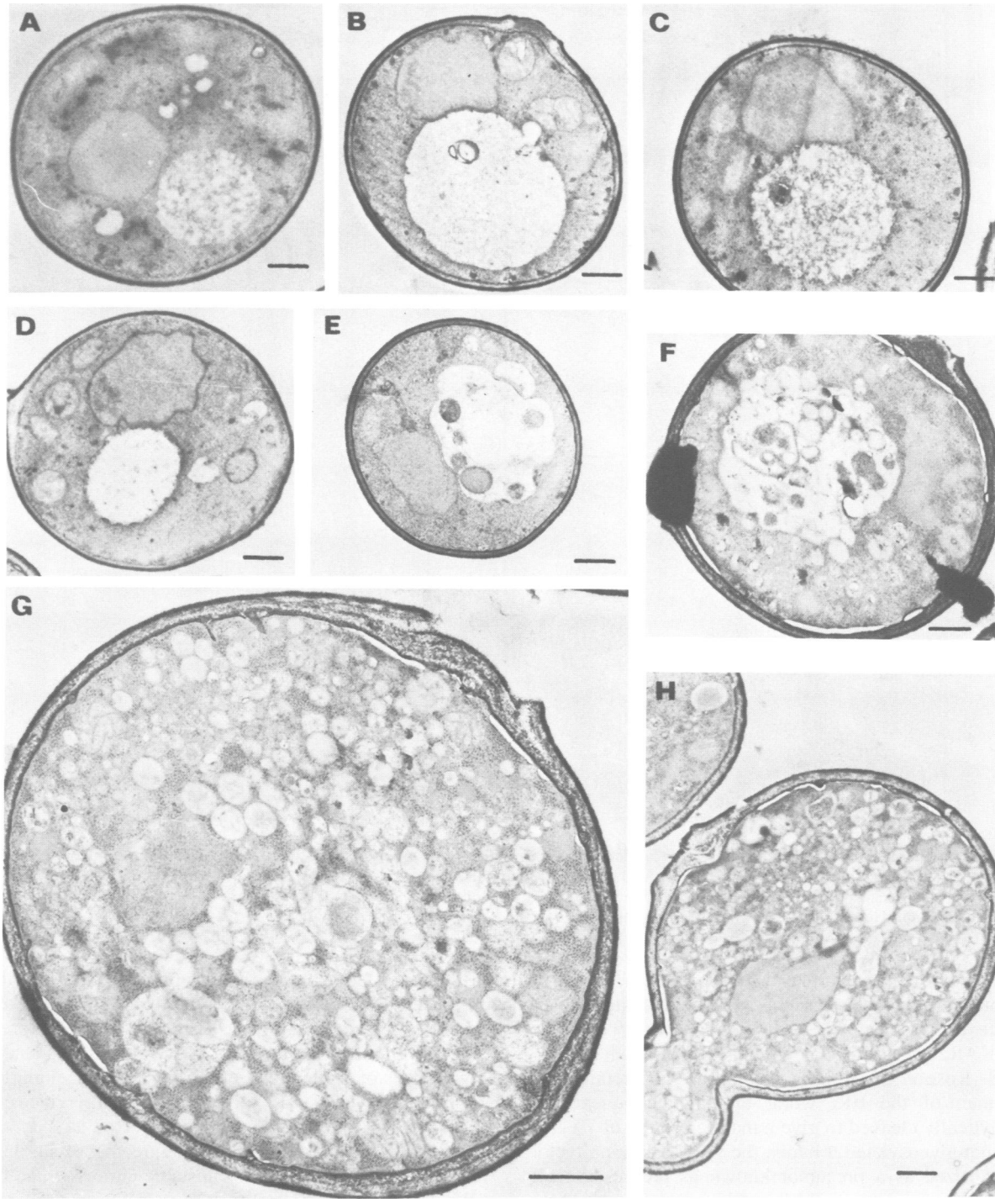


Fig. 5. Thin section electron microscopy of yeast cells expressing 2B (**B**), 2C (**C**), 2BC (**D, E, F, G** and **H**) or transformed with the plasmid pEMBLyex4 (**A**). Cells were fixed at 0 (D), 6 (E and F) or 20 h post-induction (A, B, C, G and H) and were processed for electron microscopy as described in Materials and methods. Thin sections were prepared and photographed. Bar = 1 μm.

the rough ER) as observed by immunofluorescence (results not shown).

Effects of poliovirus protein 2BC on the secretory pathway

Proteins synthesized in the ER are transported to the different cellular destinations through the vesicular system. During this transport, many proteins undergo post-translational modifications, including proteolytic processing of the protein. To analyze the action of poliovirus 2BC on protein transport, the processing of four different yeast proteins was assayed. The vacuolar carboxypeptidase Y (CPY) is synthesized as a proenzyme in the ER (67 kDa) and is core glycosylated in this organelle (Banta *et al.*, 1988; Graham *et al.*, 1993). During passage through the Golgi apparatus, CPY is further glycosylated and the protein is finally cleaved on arrival at the vacuole to generate the mature form of the enzyme, mCPY (61 kDa) (see scheme in Figure 8B). Aminopeptidase I (API) is transported to the yeast vacuole by direct translocation of

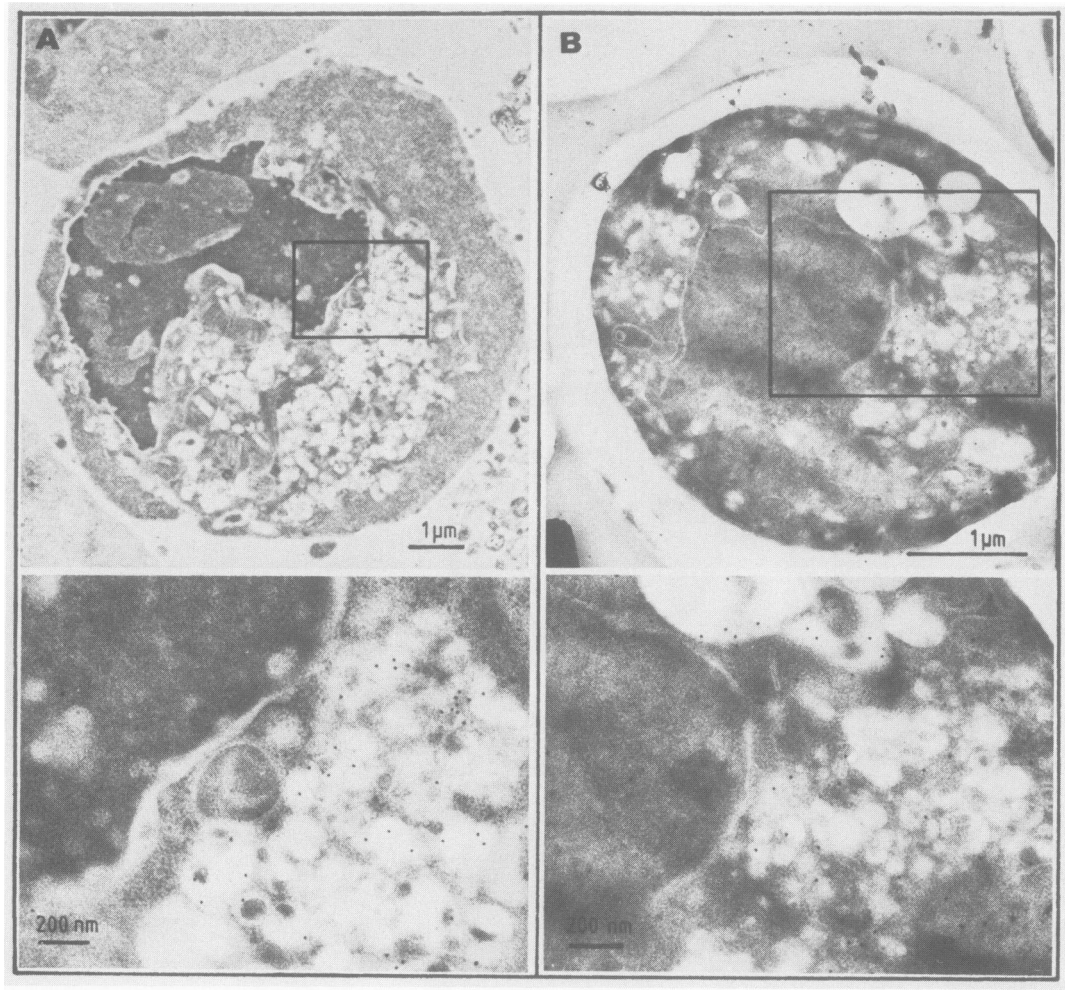


Fig. 6. Electron micrographs of HeLa and yeast cells labeled with α-2B. Cells were fixed and labeled with α-2B antibodies and protein A–gold as described in Materials and methods. (**A**) HeLa cells 5 h post-infection with poliovirus 50 p.f.u./cell. (**B**) Yeast cell expressing 2BC 20 h post-induction. The lower panels show the detail marked in the upper panels. Note the size and structural similarities between the vesicles generated by poliovirus infection in HeLa cells and 2BC-expressing yeast cells.

the protein from the cytoplasm to the vacuole, followed by proteolytic cleavage of the N-terminal fragment to generate the mature API (Klionsky *et al.*, 1992). The yeast KAR2 of ~82 kDa, similar to mammalian BiP/GRP78 (Rose *et al.*, 1989), is imported and retained in the lumen of the ER, while the signal sequence is proteolytically cleaved to give a mature protein of 79 kDa that is not glycosylated. Finally, the yeast α-mating factor is synthesized as a precursor known as prepro-α-factor that is processed extensively to produce the mature pheromone of only 13 residues that is secreted to the medium (Julius *et al.*, 1984). If the secretory pathway were affected by expression of 2BC one might expect that the precursor forms of these four proteins would accumulate. Moreover, the specific action on the processing of each of these proteins might indicate the step and the vesicular compartment affected by 2BC expression.

To test whether protein transport in the secretory pathway was affected in 2BC-expressing cells, an aliquot of cells was taken 4 h post-induction, labeled with [^{35}S]-methionine and CPY was immunoprecipitated using a specific antiserum (Figure 7). 2BC-expressing cells specifically accumulated the core-glycosylated, p1 precursor of CPY, suggesting that protein transport from the ER was inhibited. We could not detect CPY in the extracellular fraction, indicating that the CPY was not secreted (results not shown). The effect of 2BC expression on the processing of the yeast α-factor was also analyzed: two forms of the α-factor accumulated intracellularly, the core glycosylated ER form and the hyperglycosylated Golgi form. These forms are not detected after a 30 min chase in 2BC-expressing cells, in agreement with the idea that these products are very unstable (Julius *et al.*, 1984). We could not detect any mature α-factor, suggesting that the block induced by 2BC preceded the Kex2 protease compartment.

To further analyze the effects of poliovirus 2BC on the synthesis and maturation of CPY, API, KAR2 and α-mating factor, yeast cells were collected at various times after induction of 2BC synthesis with galactose, the proteins were separated by SDS–PAGE and analyzed by Western blot using specific antisera against each protein. 2BC synthesis is detected as soon as 2 h after induction and increases in yeast cells until ~8 h after induction (Figure 8A). The major form of CPY detected in uninduced cells, or in cells induced with galactose that do not express 2BC, is the mature form, mCPY, while cells that synthesize poliovirus 2BC clearly accumulate the precursor form of

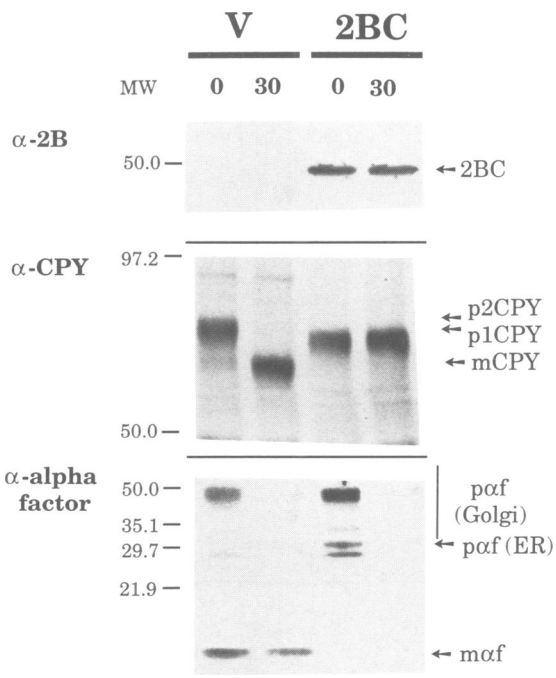


Fig. 7. Effects of poliovirus protein 2BC on the exocytic pathway. Yeast cells 4 h post-induction in YNB.LGal medium were labeled for 10 min with [^{35}S]methionine as described in Materials and methods and chased for 0 or 30 min. Immunoprecipitation of 2BC, CPY and α-mating factor of control cells (bearing pEMBLyex4) or 2BC-expressing cells. mCPY: mature CPY; p2CPY and p1CPY: precursor forms of CPY. mαf: mature mαf; pαf: precursor mαf (see scheme Figure 8B).

CPY from 2 h post-induction. The conversion of pCPY to mCPY is thought to occur in the vacuole after transport through the Golgi compartment; our results indicate that this transport is blocked in 2BC-expressing cells, but not in 2B- or 2C-expressing cells, individually or simultaneously (see below, Figure 9). The prepro-α-factor, which is the major form detected in uninduced cells, is glycosylated after synthesis, while the precursor underglycosylated form of pro-α-factor is clearly detectable in yeast cells synthesizing poliovirus 2BC. A similar inhibition of protein processing is observed with AP1, although in this case direct translocation of the precursor form of AP1 to the vacuole has been postulated (Klionsky *et al.*, 1992). Therefore, these results indicate that maturation of AP1 is hampered by poliovirus 2BC. Finally, the processing of KAR2 is also affected in cells that express 2BC, but later than the other proteins analyzed, suggesting that the normal functioning of the ER is modified by the poliovirus protein 2BC at later times. These results reinforce the idea that the synthesis of 2BC interferes with the normal functioning of the exocytic pathway in yeast, and suggest that the early compartments of the secretory pathway are targets of 2BC action (Figure 8B).

Identification of the regions of the 2BC molecule involved in yeast growth inhibition, membrane proliferation and interference with the exocytic pathway

As a first step in identifying the portions of the 2BC molecule involved in growth arrest of yeast cells, membrane proliferation and blockade of the exocytic pathway, a number of 2BC mutants were generated. Figure 9 depicts the structure of protein 2BC, the different deletions and point mutations introduced into the 2BC molecule and the nomenclature of these mutants. Both proteins 2B and 2C interact with membranes, although the regions involved in membrane interaction have not yet been characterized. Some regions in 2B are highly conserved among different picornaviruses, such as the hydrophobic tract comprising amino acids 51–54 (LVII). Two charged amino acids were introduced into this region. A region of the N-terminus of protein 2C with similarities to apolipoprotein C-III has been predicted by computer analysis of this sequence to form an amphipathic helix, suggesting that this region of 2C is involved in membrane interaction (Paul *et al.*, 1994). Our analyses of protein 2B also predict a similar amphipathic helix at the N-terminus of 2B. The amino acids 17–34 of 2B are equivalent to the amphipathic region (aa16–33 of 2C or 117–136 of 2BC) defined by Paul *et al.* (1994; results not shown). On the other hand, the two domains of 2C involved in GTPase activity, the GKS (aa 231–233) and DD (aa 273–274) motifs were modified to GEF and VD. Deletions spanning the 2B–2C junction [mutant 2bc(ΔSph)] or the entire GKS motif, or the initial 30 amino acids of 2B containing a putative amphipathic helix as well as long deletions at the C-terminus of the 2C molecule were generated.

Initially, the ability of the different 2BC variants to arrest yeast growth was tested both in liquid medium and in agar medium. Inhibition of cell growth on agar medium (YNB.Gal) clearly occurs, not only with wild-type 2BC, but also with most of the mutants, except 2bC(Δ30N), and a smaller effect was observed with 2Bc(ΔGKS) and 2Bc(ΔXbaI). The results obtained for growth in liquid medium (YNB.LGal) are different: all the 2BC variants, except 2bC(Δ30N), inhibited yeast growth, although inhibition by mutants 2Bc(ΔXbaI), 2Bc(ΔGKS) and 2bC(D) was delayed. We have also analyzed the effect of expression of these mutant proteins on CPY processing, 4 h post-induction. As indicated in Figure 9, expression of 2BC blocks the processing of CPY, leading to some accumulation of pCPY. The ratio of pCPY to mCPY was calculated by densitometry and this value was taken as 100%. The relative proportion of pCPY was also calculated for the rest of the mutants. In general, all of them interfere with the processing of pCPY to different extents (Figure 9). At 8 h post-induction, all the mutants, except 2bC(Δ30N), exhibit a clear defect in CPY processing (results not shown). Finally, the cellular location of the mutant proteins was analyzed by cellular fractionation and immunofluorescence. All variant proteins appeared in the membrane fractions (results not shown) and were located in internal membranes. The patterns of expression 6 h post-induction are similar to that for wild-type 2BC protein, although less scattered in the case of 2Bc(ΔXbaI), 2Bc(ΔGKS) and 2bC(D) mutants. The results of Nomarski microscopy and α-V-ATPase staining with these mutants are also intermediate between control and 2BC-expressing cells. The pattern obtained with the mutant 2bC(Δ30N) is similar to that for 2B or 2C protein, individually or in combination (results not shown). Figure 10 shows the Nomarski and immunofluorescence patterns of some representative mutants. Three different morphologies can be differentiated (Figure 10). The first one corresponds to

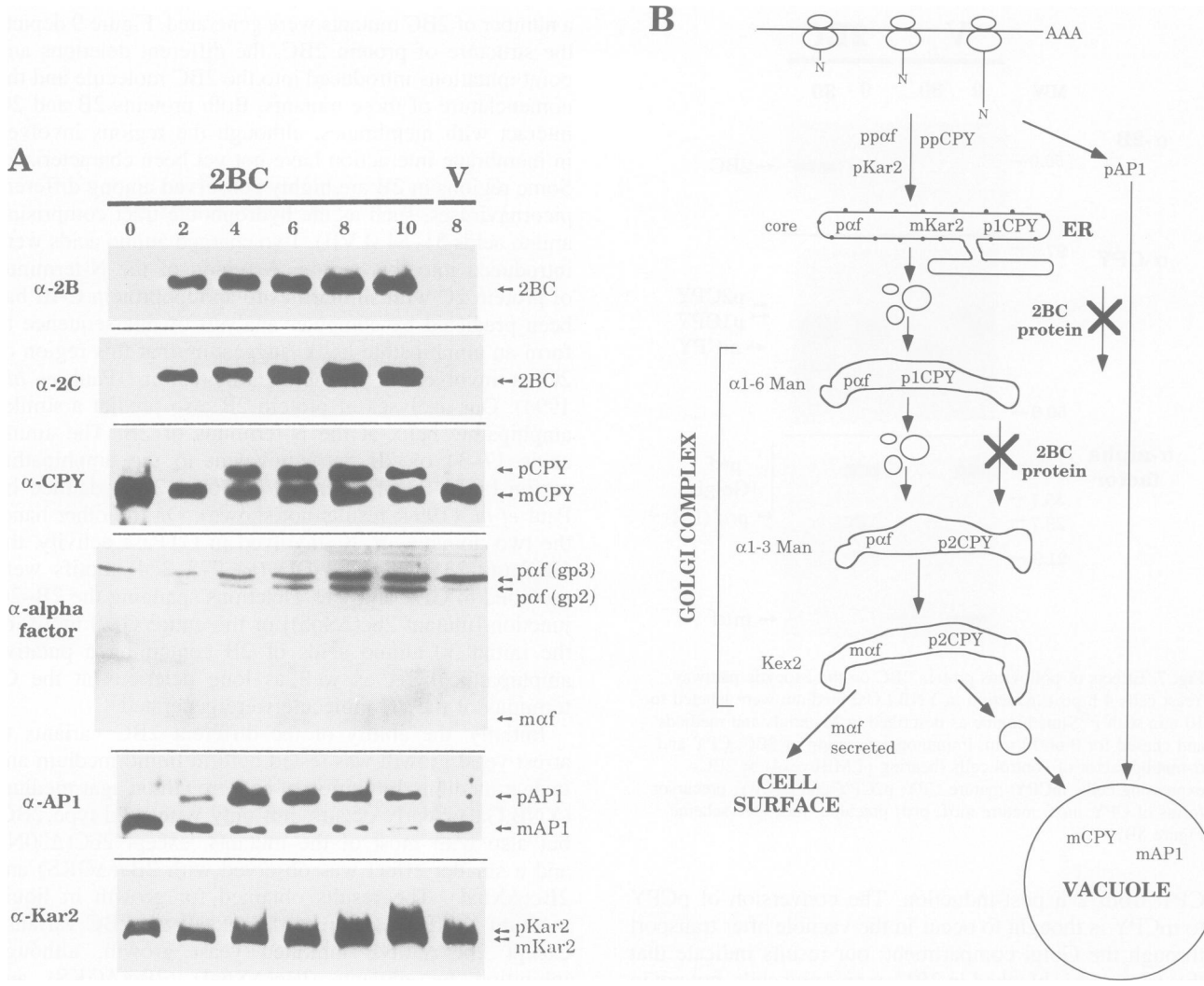
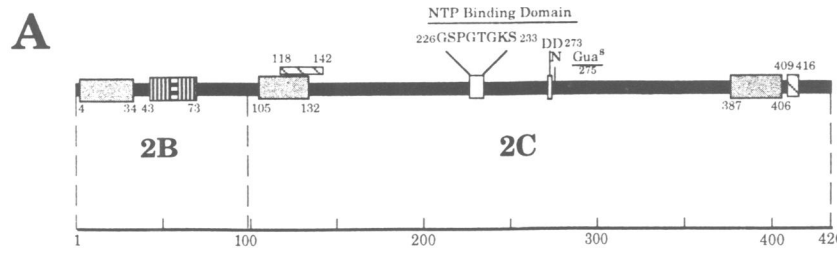


Fig. 8. Effects of poliovirus protein 2BC on the exocytic pathway. (**A**) Kinetics of 2BC expression and accumulation of precursor forms of different marker proteins. Western blots with α-2B, α-2C, α-CPY, α-mating factor, α-AP1 and α-KAR2 were carried out with extracts of yeast cells bearing the plasmid pEMBL.2BC (2BC) or pEMBLyex4 (V) at the post-induction times indicated. gp3 and gp2, pro-α-factor harboring three- and two-core oligosaccharide chains, respectively. (**B**) Schematic diagram representing protein transport in *S.cerevisiae* and the site of action of 2BC (modified from Graham *et al.*, 1993).

control yeast cells, where the nucleus appears normal after DAPI staining and the vacuole is clearly visualized, both by Nomarski microscopy and by immunofluorescence with antibodies against the 60 kDa subunit of the V-ATPase (Figure 10A). This normal morphology is also observed with the 2BC deletion mutant that lacks the initial 30 amino acids of 2B (Figure 10F). The second type of morphology corresponds to yeast cells that express wild-type poliovirus 2BC (Figure 10B), where the vacuole is not apparent and the 2BC protein is scattered in cytoplasmic clusters. This morphology is shared by the mutant that lacks 20 amino acids in the junction between 2B and 2C (mutant ΔSphI; Figure 10C). Finally, the third kind of morphology corresponds to cells showing a rather normal nuclear shape and the vacuole not totally disorganized (Figure 10D and E).

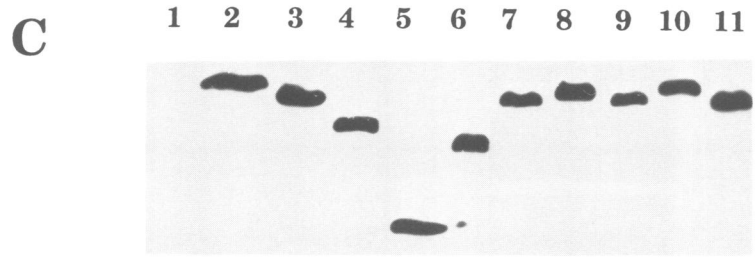
These results indicate that sequences present in both 2B and 2C moieties affect the 2BC inhibitory activity. Notably, deletion of the initial 30 residues of 2B abrogates the activity of 2BC. The finding that cell growth is halted by mutant 2Bc(ΔBamHI) to a similar extent as wild-type 2BC would suggest that portions of the 2C molecule beyond residue 257 are not involved in this effect, although the result found with mutant 2Bc(ΔXbaI) indicates that some regions of the C-terminus could be necessary for adequate folding of other domains involved in the toxic activity.

Mutations in the 2B residues LVII (aa 51–54) only partially affect the inhibition of cell growth. The involvement of the GKS motif in cell growth arrest is not clear cut. Mutant 2Bc(ΔGKS) lacking residues 226–269 interferes less with yeast growth than does wild-type 2BC, while point mutations in the GKS and DD motifs only partially affect this property. Nevertheless, it appears that the NTP binding motif is not essential for the effects of 2BC observed in yeast cells. Addition of guanidine to the medium has no effect on the phenotype shown by 2BC-expressing cells. These findings are in good agreement with the results of Cho *et al.* (1994) who found membrane proliferation in mammalian cells with a mutant lacking the GKS motif and no inhibition by guanidine.



B

Protein expressed	Characteristics	Schematic representation	Growth agar	Growth liquid	% pCPY
none	-		+++	+++	<1
2B	1-97		++	+++	<1
2C	98-426		+++	+++	<1
2B + 2C	1-97 + 98-426		++	+++	≤1
2BC	1-426		-	-	100.0
2bc(ΔSphI)	Δ86-108		-	-	47.3
2Bc(ΔGKS)	Δ226-269	DD / VD	+	±	13.6
2Bc(ΔBamHI)	1-257(+9)		-	-	53.0
2Bc(ΔXbaI)	1-353		++	±	28.5
2Bc(EcoRI)	K232E, S233F	GKS / GEF	-	-	37.1
2Bc(SalI)	D273V	DD / VD	-	-	52.9
2Bc(EcoRI/SalI)	K232E, S233F, D273V	GKS DD / GEF VD	-	-	67.5
2bC(S)	V52D	LV / LD	-	-	ND
2bC(D)	V52D, I54K	LVII / LDIK	-	±	12.0
2bC(Δ30N)	31-426		+++	+++	<1



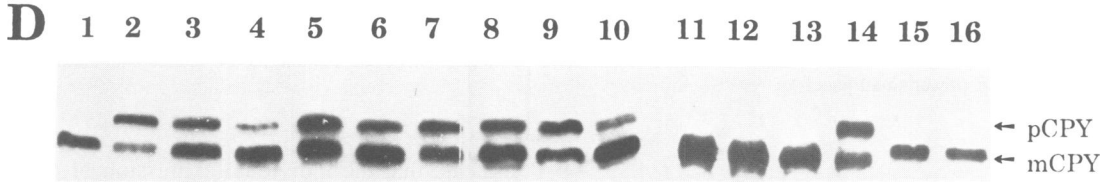


Fig. 9. Effects of 2BC mutant proteins on yeast growth and CPY processing. (**A**) A linear map of poliovirus protein 2BC. ⊡ Amphipatic helix, ▤ hydrophilic turn, ▥ hydrophobic tract and ▧ RNA binding domain (according to Rodriguez and Carrasco, 1995). (**B**) Schematic representation of 2BC mutants and effects of their expression in yeast cells. (**C**) Detection of mutant proteins. Extracts of control cells (bearing pEMBLyex4; 1), or 2BC wild-type (2), 2bc(Δ*Sph*I) (3), 2Bc(ΔGKS) (4), 2Bc(Δ*Bam*HI) (5), 2Bc(Δ*Xba*I) (6), 2Bc(*Sal*I) (7), 2Bc(*Eco*RI) (8), 2Bc(*Eco*RI/*Sal*I) (9), 2bC(D) (10) and 2bC(Δ30N) (11) expressing cells, were prepared and inmunoblot analysis was performed as described in Materials and methods. (**D**) pCPY accumulation. The same extracts used in the immunoblots in (C) (lanes 1–11), Figure 1A (lanes 12 and 13, corresponding to 2B- and 2C-expressing cells, respectively) and Figure 2A (lanes 14, 15 and 16, corresponding to induced cells bearing the plasmids pEMBL.2BC and pYcDE8, pEMBL.2B and pYc.2C or pEMBL.2C and pYc.2B, respectively) were assayed with antibodies against CPY. Lanes 1–10 and lanes 11–16 correspond to extracts of cells 4 and 8 h post-induction, respectively.

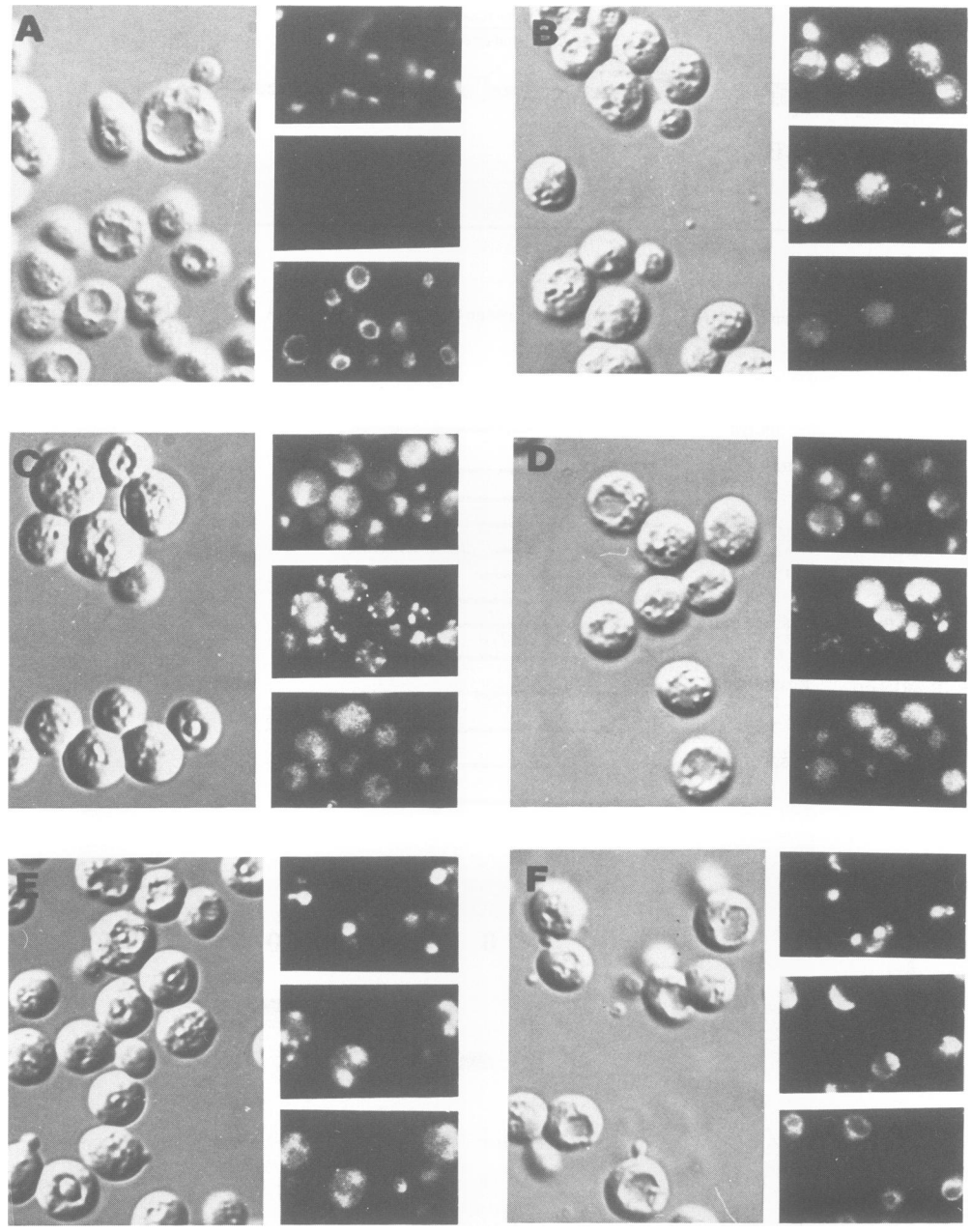


Fig. 10. Nomarski (larger panels) and immunofluorescence (smaller panels) microscopy of control cells (**A**), or 2BC wild-type (**B**), 2bc(Δ*Sph*I) (**C**), 2Bc(Δ*Xba*I) (**D**), 2bC(D) (**E**) and 2bC(Δ30N) (**F**) expressing cells after 6 h growth in YNB.LGal. The same cell samples were stained with DAPI (upper small panels), α-2B rabbit antiserum, except in F where α-2C was used (middle small panels), and mouse monoclonal antibody against the V-ATPase 60 kDa subunit (lower small panels).

Discussion

Induction of vesicle proliferation by poliovirus protein 2BC

Unraveling the complexity of vesicular transport in eukaryotic cells has benefited greatly from the isolation and characterization of yeast strains containing mutations in the components of the vesicular system (Banta *et al.*, 1988; Kaiser and Schekman, 1990; Pryer *et al.*, 1992). These yeast mutants can be complemented successfully *in vitro* by addition of mammalian extracts or by isolated proteins, since yeast and mammalian proteins that participate in vesicular transport are interchangeable in cell-free systems (Pryer *et al.*, 1992). Our present findings indicate that the individual expression of poliovirus 2BC in yeast cells mimics the proliferation observed in human fibroblasts after poliovirus infection (Dales *et al.*, 1965), reinforcing the idea that proteins that function in the vesicular system of mammalian cells also act on yeast cells. The use of yeast cells to analyze the function of other proteins from animal viruses that potentially interfere with the vesicular system is now possible. On the other hand, detailed analysis of the functions modified by these viral proteins, including poliovirus 2BC, on yeast vesicular transport provides a new model system for understanding its exact functioning. The present results indicate that 2BC induces profound alterations in the vesicular system, the transport of proteins and the morphology of the yeast

vacuole. The expression of poliovirus protein 2BC, but not 2B or 2C individually or in combination, is sufficient to induce vesicle formation, illustrating that the poliovirus precursor protein 2BC, which is very abundant in infected cells, is endowed with an activity not present in the mature products 2B or 2C.

The phenotype shown by 2BC-expressing cells resembles the specific activity of 2BC and does not seem to be an indirect effect of the expression of a toxic protein (Nigro *et al.*, 1992; Boschelli, 1993). Our results on the toxicity of poliovirus $2A^{pro}$ expression in yeast cells indicated that cellular morphology was also profoundly modified by the expression of $2A^{pro}$ (Barco and Carrasco, 1995), in such a way that the nucleus appeared fragmented, the microtubules were disorganized and electron-dense granules and autophagic bodies appeared in the cytoplasm (unpublished results). However, we did not observe any selective effect by this protease on the secretory pathway or vesicular proliferation similar to that found with 2BC expression.

Recently, the capacity of poliovirus 2C and 2BC to induce vesicle proliferation in vaccinia virus-infected fibroblasts has been described (Cho *et al.*, 1994; Aldabe and Carrasco, 1995). The proliferation of membranes observed with protein 2C was not exactly the same as that produced by 2BC. Thus, 2C induced longer vesicles and myelin-like structures, while the vesicles that appeared in cells expressing 2BC resemble more closely those present in poliovirus-infected cells (Cho *et al.*, 1994). Unfortunately, it is possible that the proteins expressed by vaccinia virus itself may affect the activity of 2C or 2BC. This possibility is not unlikely, considering that vaccinia virus infection also affects membrane formation (Sodeik *et al.*, 1993). In addition, some poliovirus proteins, such as the protease $2A^{pro}$, can be complemented by vaccinia virus by a still unknown mechanism (Pal-Ghosh and Morrow, 1993).

From this point of view, the analysis of the poliovirus proteins involved in membrane proliferation and vesicle formation in yeast cells constitutes a better system, without the background of an ongoing permissive viral infection. One potential drawback of the yeast system is the large evolutionary distance between yeast and mammalian cells. Despite this evolutionary distance, a protein from a human virus induces membrane proliferation in a fashion similar to that observed in human cells, providing another example of the molecular relatedness between the vesicular system from both types of cells. In addition, the ease of performing genetic studies with yeast makes this system ideal for the isolation and characterization of 2BC mutants unable to induce membrane proliferation, multicopy suppressors or yeast mutants resistant to 2BC. The genetic analysis of these mutants will aid our understanding of the target of 2BC action.

Interference with protein processing and export

The present findings indicate that membrane flow and vesicular transport are modified profoundly by poliovirus 2BC, in such a way that the membranes and proteins arising from the ER are no longer transported through the Golgi apparatus. In principle, this effect may be due to a blockade at the level of the Golgi apparatus or to an inhibition of vesicles that arise from the ER to fuse with the *cis*-Golgi. The processing and glycosylation of proteins synthesized in the ER is hampered greatly by the presence of 2BC, as indicated by the processing of CPY and α-mating factor. Moreover, not only is the transport of proteins through the Golgi inhibited, but also the direct translocation of API to the vacuole is halted. API, like α-mannosidase (Yoshihisa and Anraku, 1990), is a protein that is transported by direct translocation from the cytoplasm to the vacuole, without migrating through the Golgi complex (Klionsky *et al.*, 1992). There are several possibilities to explain our findings that 2BC blocks the translocation of API to the vacuole. One is that API travels through vacuoles of the secretory pathway not affected by the mutants used by Klionsky *et al.* (1992). Another possibility is that the vacuole itself becomes largely modified by poliovirus 2BC, in such a way that proteins are no longer targeted to this organelle. The finding that the vacuole becomes disorganized very early upon 2BC expression supports the second possibility. Finally, the processing of KAR2 is also affected, but at later times, suggesting that the translocation of proteins into the ER is not affected early during expression of 2BC. Thus, the step blocked by 2BC seems to be located after the ER. Additional work aimed at isolating *ts* mutants of 2BC incapable of modifying the secretory pathway and disorganizing the yeast vacuole would provide a new model system to study the reorganization of the vesicular system and the yeast vacuole.

Poliovirus genome replication. A function for poliovirus protein 2BC

A key question in poliovirus biology concerns the elucidation of the exact function of viral proteins that induce the membranous vesicles that constitute the viroplasm. Understanding the functioning of these viral proteins at the molecular level will provide clues to understanding the generation of new membranes and the replication of viral genomes. According to our results, the precursor 2BC is needed for membrane proliferation. Little is yet known with regard to the exact function of proteins 2B, 2C or their precursor 2BC during the poliovirus replication cycle. No biochemical function for protein 2B has ever been suggested, although genetic studies indicate that 2B (or 2BC) is required for efficient viral genome replication (Bernstein *et al.*, 1986; Sarnow *et al.*, 1990; Wimmer *et al.*, 1993). Sequence analysis of picornavirus protein 2C predicted that it was an NTPase (Dever *et al.*, 1987). Indeed, the initial isolation of this protein provided evidence that 2C was endowed with GTPase activity and bound to RNA (Rodríguez and Carrasco, 1993). We advanced the suggestion that 2C could transport the viral RNA through the vesicular system (Rodríguez and Carrasco, 1993), a process that would be required for poliovirus genome replication and the assembly of RNA into virion particles. Not only the formation, but also the correct trafficking of these vesicles is required for efficient poliovirus RNA synthesis, since cerulenin, an inhibitor of phospholipid synthesis, immediately blocks poliovirus RNA synthesis (Guinea and Carrasco, 1990). A similar potent inhibition is observed with brefeldin A, an antibiotic that affects the vesicular system (Maynell *et al.*, 1992; Irurzun *et al.*, 1993).

As a working hypothesis, we propose that the function

of 2BC is to interact with membranes to modify vesicular traffic, most probably between the ER and the Golgi complex. The inhibition of membrane traffic at this point would redirect membranes to the formation of a new dead-end compartment formed by these small vesicles. 2BC could play an active function in the genesis of these vesicles, i.e. 2BC could possess an intrinsic activity to generate these vesicles. It must be kept in mind that 2C has GTPase activity and shows some homology to the small GTP binding proteins (Rodríguez and Carrasco, 1993). It is theoretically possible that 2C is analogous to these proteins and thus 2BC, by virtue of its GTPase function, can modify the directionality of the vesicular transport. Alternatively, 2BC may interact with a cellular protein involved in the secretory pathway to modify or inhibit its function. In this regard it is of interest to note that a viral protein such as HIV-1 Nef is able to interact with β-COP (Benichou *et al.*, 1994). For example 2BC could inhibit the function of a cellular protein involved in recognition and fusion of donor and acceptor vesicles (Rothman and Orci, 1992), leading to a blockade of the vesicular system at a given step resulting in accumulation of donor vesicles.

Materials and methods

Microbial strains

Escherichia coli DH5 (Sambrook *et al.*, 1989) was used for the construction of all expression plasmids described. The *S.cerevisiae* strain used was W303-1B (*Matα, Ade2, His3, Leu2, Trp1, Ura3*), generously provided by Dr J.P.G.Ballesta (Centro de Biología Molecular; Madrid, Spain).

General recombinant DNA protocols

Construction of vectors was carried out by standard procedures (Sambrook *et al.*, 1989). Sequences obtained by PCR reactions were sequenced following the dideoxy method (Sambrook *et al.*, 1989). The oligodeoxyribonucleotides used in this work were the following. 5′2B.B2: 5′-GGCCGGCCCGGGATCACCAATTAC ATAGAG, contains a *Sma*I restriction site and the poliovirus sequences 3833–3853. 5′2B.31: 5′-GCGGGATCCATGGTGACCAGTACCATCACTG, contains a *Bam*HI restriction site and the poliovirus sequences 3923–3944. 5′2B.(S-X): 5′-CATATCCTCTCTAGATATTAT/AAACTAGG, contains a *Spe*I restriction site and the poliovirus sequences 3833–3853. 5′2C.4424: 5′-ATACAGAAACTAGAGCATACT, contains the poliovirus sequences 4424–4444. 5′2C.EcoRI: 5′-GAACAGGTGAATTCGT AGCAACCAA, contains an *Eco*RI restriction site and the poliovirus sequences 4518–4540. 5′2C.SalI: 5′-GTGATTATGGTCGACCTGAAT, contains a *Sal*I restriction site and the poliovirus sequences 4640–4660. 3′2C.D129–172: 5′-CAGGTCGACC ATAATCAC ATGTACTAGCAAACATAC, contains a *Sal*I restriction site and the poliovirus complementary sequences 34657–4640 and 4507–4490. 3′2C.B2: 5′-GGGCCCAAGC-TTA CTA TTGAAACAAAGCCTCCATAC, contains a *Hin*dIII restriction site, two stop codons and the poliovirus complementary sequence 5110–5091. All the oligonucleotides are written in the 5′→3′ direction. The 5′ oligonucleotides contain poliovirus coding sequences, the 3′ oligonucleotides contain complementary sequences. The nucleotides mutated are indicated with bold letters, italic letters indicate silent mutations.

Constructions using pEMBLyex4

The yeast expression plasmid used in this work was the yeast–*E.coli* shuttle vector pEMBLyex4 (Cesareni and Murray, 1987), a 2 μ plasmid derivative. Poliovirus sequences were amplified by PCR from a cloned cDNA of poliovirus type 1 (generously provided by E.Wimmer, Stony Brook, USA). The plasmids pEMBL.2B and pEMBL.2C have been described (Barco and Carrasco, 1995). pEMBL.2B(–B) is as pEMBL.2B but the polylinker sites *Sma*I, *Bam*HI and *Xba*I were deleted to facilitate the construction of 2BC mutants. To construct pEMBL.2BC, oligos 5′.2B.B2 and 3′.2C.B2 were used., The PCR product was digested with *Spe*I and *Hin*dIII and cloned in pEMBL.2B(–B) digested with the same enzymes. Plasmids pEMBL.2B [or (–B)], pEMBL.2BC and pEMBL.2C encoded the indicated poliovirus proteins with an additional methionine at the N-terminus.

Construction of plasmids encoding 2BC mutant proteins

pEMBL.2bc(ΔSphI). Starting with the plasmid pTM1 without *Sph*I sites (Moss *et al.*, 1990), pTM1.2BC was first generated, digested with *Sph*I and religated to obtain pTM1.2bc(ΔSphI). The *Spe*I–*Bam*HI fragment of this plasmid was cloned in pEMBL.2BC cut with the same enzymes.

pEMBL.2Bc(ΔGKS). This plasmid was constructed by the overlap extension method. PCR1 was carried out with primers 5′2B.B2 and 3′2C.Δ129–172. PCR2 was carried out with primers 5′2C.*Sal*I and 3′2C.B2. The overlap created by PCR1+PCR2 is extended in a PCR3 carried out with primers 5′2B.B2 y 3′2C.B2. The PCR3 product is *Spe*I–*Sal*I digested and ligated to pEMBL.2Bc(*Sal*I) treated with the same enzymes.

pEMBL.2Bc(ΔBamHI). pEMBL.2BC was digested with *Bam*HI and *Hin*dIII, and treated with Klenow enzyme. Thus, the deletion mutant of 2BC obtained contains nine residues: QLMIQYQRK and two stop codons after amino acid 257 from 2BC.

pEMBL.2Bc(ΔXbaI). pEMBL.2BC was digested with *Xba*I and treated with Klenow enzyme. The deletion mutant of 2BC obtained contains one serine residue and one stop codon after amino acid 353 from 2BC.

pEMBL.2Bc(EcoRI). This plasmid was constructed using the methodology of overlap extension. PCR1 was carried out with primers 5′2C.*Eco*RI and 3′2C.B2. PCR2 was performed with primers 5′2B.B2 and 3′2C.B2 using as template the overlap between the pEMBL.2BC *Sac*I–*Bam*HI restriction fragment and the PCR1 product. This PCR2 product is *Spe*I–*Bam*HI digested and ligated to pEMBL.2BC digested with the same enzymes. Clones with the *Eco*RI site were selected.

pEMBL.2Bc(SalI). This plasmid was also constructed using the methodology of overlap extension. PCR1 was carried out with primers 5′2C.*Sal*I and 3′2C.B2. PCR2 was carried out with primers 5′2C.4424 and 3′2C.B2 using as template the overlap between the 2BC *Sph*I–*Xba*I restriction fragment and the PCR1 product. This PCR2 product was digested with *Bam*HI–*Xba*I and ligated to pEMBL.2BC digested with the same enzymes. Clones with the *Sal*I site were selected.

pEMBL.2Bc(EcoRI/SalI). The *Bam*HI–*Hin*dIII fragment of pEMBL.2Bc(*Sal*I) was cloned in the same sites of pEMBL.2Bc(*Eco*RI).

pEMBL.2bC(S and D). The oligos 5′.2B(S-X) and 3′.2C.B2 were used, the amplified PCR product was digested with *Xba*I and *Bam*HI and cloned in pEMBL.2BC digested with *Spe*I and *Bam*HI. Clones without the *Spe*I site were selected. The single (S) and double (D) mutants were identified by sequencing.

pEMBL.2bC(Δ30N). For the construction of these mutants, we used the oligos 5′.2B.31 and 3′.2C.B2. The PCR product was digested with *Bam*HI and *Hin*dIII and cloned in pEMBLyex4, subsequently the fragment *Spe*I–*Hin*dIII was replaced by the same fragment of pEMBL.2BC previously sequenced.

Constructs in pYcDE8. To co-express proteins 2B and 2C, the constitutive vector pYcDE8 was used (Klein and Roof, 1988). pEMBL.2B and pEMBL.2C were digested with *Asp*718 and *Bam*HI (the 2C sequence contains an internal *Bam*HI site, thus partial digestion with *Bam*HI was carried out), the fragments bearing the 2B or 2C sequences were cloned in the same sites of pYcDE8, giving rise to plasmids pYc.2B and pYc.2C respectively.

Integrative constructs. pINTyex4, pINT.2B and pINT.2C are integrative plasmid derivatives of pEMBLyex4, pEMBL.2B and pEMBL.2C, respectively. These plasmids contain the 2 μ origin deleted after digestion with *Hin*dIII and *Nae*I, Klenow treatment and recircularization. Linearization with *Stu*I was used to direct integration into the *URA3* locus (Murray *et al.*, 1987).

Yeast media, growth, transformation and induction

Yeast cells were transformed and selected on minimal YNB plates containing 0.67% yeast nitrogen base without amino acids, and 2% of either glucose (YNB.Glu) or galactose (YNB.Gal) plus 20 mg/l of the required amino acids or bases according to the auxotrophic markers. For induction of UAS_{GAL}-CYC promoter, cells were grown in YNB.Lac [0.67% yeast nitrogen base without amino acids, 2% lactic acid, 0.1% glucose, 5 mM K_2HPO_4, 10 mM $(NH_4)_2SO_4$, adjusted to pH 6 with KOH]. Galactose (2%) was added directly to YNB.Lac and this medium

is referred to as YNB.LGal. Transformation of yeast by the lithium acetate procedure was performed as previously described (Rose *et al.*, 1990).

Growth kinetics, flow cytometry and cell viability

For growth kinetics and flow cytometry assays, overnight yeast cultures grown at 30°C in YNB.Lac were diluted in YNB.Lac or YNB.LGal medium, and cells were harvested at 1–2 h intervals. Absorbance at 660 nm (A_{600}) was measured to quantitate cell density. For flow cytometry analysis, cells were fixed, stained and tested as described previously (Segev and Botstein, 1987). Cell viability was assessed by plating aliquots of cell cultures on selective plates of YNB.Glu at different times post-induction.

Immunoprecipitation and Western blot analysis

The α-2B or α-2C sera were obtained by inoculation of the maltose binding protein–2B fusion protein (MBP.2B) and MBP.2C (Rodríguez and Carrasco, 1993) in rabbits. The background of the immunoblot assay was reduced by pre-absorbing antisera with acetone powders of W303-1B host cells. The antisera against KAR2, CPY (used in immunoprecipitations), α-mating factor and API were generously given by R.Schekman (University of California, Berkeley), D.Gallwitz (MPI of Biophysical Chemistry, Gottingen), J.A.Rothblatt (Department of Biological Sciences, Hanover) and B.Segui (Centro de Biología Molecular, Madrid). The monoclonal antibodies against the 60 kDa subunit of the V-ATPase or CPY (used in Western blot) were purchased from Molecular Probes, Inc.

Cell labeling and immunoprecipitations were carried out as described (Graham *et al.*, 1993). Yeast extracts for Western blot analysis were prepared as previously indicated (Yaffe and Schatz, 1984). Subcellular fractionation of 2BC and study of 2BC association with the P100 fraction were performed as described (Horazdovsky and Emr, 1993); HeLa cells were treated as yeast spheroplasts. For standard immunoreactions, dilutions (1:1000) of sera in phosphate-buffered saline with 0.05% Tween 20 (PBST) were used. Proteins were separated on SDS–PAGE gels and transferred onto nitrocellulose [or immobilon membranes (Millipore) in the case of the α-mating factor]. After incubation in PBST containing 5% non-fat dry milk, the blots were incubated with the corresponding polyclonal or monoclonal antibodies. Goat anti-rabbit or anti-mouse biotinylated antibodies (diluted 1:10 000 in PBST) were used as secondary antibodies and a third step of amplification used streptavidin POD–conjugate (diluted 1:20 000). A 1:1 mixture of 100 mM Tris–HCl (pH 8), 2.5 mM luminol and 78 μM luciferin with 100 mM Tris–HCl (pH 8) and 5 mM H_2O_2 was used as a substrate for the chemiluminescence reaction.

Immunofluorescence, electron microscopy and immunogold labeling

For immunofluorescence analyses, cells were treated as described (Roberts *et al.*, 1991). The protocol for electron microscopy was adapted from that described by Wright *et al.* (1988), with two modifications: yeast cells were fixed for 2 h at 4°C with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8 and finally embedded in LR white (The London Resin Co. Ltd, Cardiff, UK). For immunogold labeling, HeLa and yeast cells were fixed with 0.1 M phosphate buffer (pH 7.4) containing 8% paraformaldehyde and treated as described (Alcalde *et al.*, 1994).

Acknowledgements

The expert technical assistance of Mr M.A.Sanz is acknowledged. We thank Carmen San Martín for advice and assistance in the electron microscopy experiments and Alberto Alvarez for his help and advice in the flow cytometry analyses. We would like to thank R.Schekman, D.Gallwitz, J.A.Rothblatt and B.Segui for generously providing antiserum samples, R.D.Klein for providing the pYcDE8 plasmid, and R.Aldabe and P.Rodríguez for their collaboration and stimulating discussion. I.Sandoval and B.Seguí are acknowledged for critical reading of the manuscript and for stimulating discussions. A.B. is the holder of a Formacion de Personal Investigador fellowship. Plan Nacional project number BIO 92–0715, DGICYT project number PB90–0177 and the institutional grant to the CBM of Fundación Ramón Areces are acknowledged for financial support.

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Received on January 17, 1995; revised on May 8, 1995