Cell surface expression of a functional rubella virus E1 glycoprotein by addition of a GPI anchor

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SUMMARY

Rubella virus (RV) envelope glycoproteins E1 and E2 are targeted to the Golgi as heterodimers. While E2 contains a transmembrane Golgi retention signal, E1 is arrested in a pre-Golgi compartment in the absence of E2, and appears to require heterodimerization in order to reach the Golgi. Various forms of E1 with deletions in the ectodomain or lacking the cytoplasmic (CT) and transmembrane (TM) domains, as well as the 29 C-terminal amino acid residues of the ectodomain were also retained intracellularly. We therefore investigated the possibility of targeting E1 to the plasma membrane by addition of a glycosylphosphatidylinositol (GPI) anchor. We found that E1GPI was transported to the cell surface where it retained the hemadsorption activity characteristic of the wild-type E1/E2 heterodimer. Furthermore, coexpression of a mammalian GPI-specific phospholipase D (GPI-PLD) resulted in the release of E1GPI and in constitutive expression of a soluble form of E1. This study thus demonstrates that the GPI anchor has a dominant effect over the E1 pre-Golgi retention signal and that E1 is sufficient for hemadsorption.

Key words: Rubella virus (RV), Hemagglutinin, GPI anchor

INTRODUCTION

Rubella virus (RV) is an important human pathogen causing severe foetal malformations when infecting a mother during the first trimester of pregnancy. The virion consists of a nucleocapsid enclosing the RNA genome, and an envelope containing viral glycoproteins which are associated with hemagglutination activity. While most enveloped viruses bud at the cell surface, RV was shown to assemble intracellularly in a variety of different cell lines (von Bonsdorff and Vaheri, 1969; Bardeltti et al., 1979). Virus maturation is thought to take place at the site of accumulation of envelope glycoproteins, suggesting that these proteins, like cellular membrane proteins residing in the different compartments of the secretory pathway, carry retention/targeting signals. Although not precisely defined, such signals have been shown to be present in the cytoplasmic or transmembrane domain of several viral proteins which are retained in the early secretory pathway (Pettersson, 1991; Matsuoka et al., 1994).

RV is composed of three structural proteins, a capsid protein (C) and two envelope glycoproteins (E1, E2) embedded in a lipid bilayer (Oker-Blom et al., 1983). The release of particles from the host cell depends both on the cytoplasmic tail of E1 and capsid protein (Hobman et al., 1994a). When expressed by itself, E1, a type I membrane protein, is arrested in an intermediate compartment between the ER and Golgi, and reaches its final destination only in the presence of E2, both proteins being found as heterodimers in the Golgi complex (Hobman et al., 1992, 1993). No retention signal has yet been identified in E1, but the protein could be targeted to the cell surface and was found to be secreted only upon removal of its transmembrane domain and coexpression of E2 (Hobman et al., 1994b; Seto and Gillam, 1994).

In the present study, we investigated whether E1 alone is sufficient for hemagglutination activity. For this purpose, it was important to relieve the intracellular retention of the protein and express it in an active form in the absence of E2. Internal deletions or C-terminal truncations proved inefficient with respect to E1 transport beyond its retention site. To circumvent this problem, we investigated whether a signal for GPI anchor addition could overcome the effect of the putative E1 pre-Golgi retention signal. Indeed, E1 was shown to be transported to the cell surface of transfected COS cells upon replacement of its transmembrane and cytoplasmic domains with sequences responsible for the attachment of a GPI anchor. Importantly, GPI anchored E1 was found to be sufficient for hemagglutination activity. Furthermore, when coexpressed with a mammalian GPI-specific phospholipase D (Scallon et al., 1991) responsible for the cleavage of its anchor domain, E1 was constitutively secreted. This protein might be useful as an antigen for developing diagnostic tests.

MATERIALS AND METHODS

Reagents

Restriction and modification enzymes were purchased from
Boehringer-Mannheim or Gibco BRL. Oligonucleotides were obtained from Mycrosynth, Balgach (Switzerland).

cDNA cloning, plasmids
Rubella virus (M33 strain, ATCC VR-315) E1 cDNA was obtained by RT-PCR using the downstream oligonucleotide 5'-ATGAATTCCTACGACCCGGCTGCGCGAT-3' for first strand cDNA synthesis with the reverse transcriptase SuperScript™ RNase H from Gibco BRL. The same oligonucleotide, together with the upstream primer 5'-ATGAAATTCGGCCGGCCGCGCTCA-3' was used in the subsequent PCR amplification using the Vent™ DNA polymerase (New England Biolabs). The amplified RV genomic sequences (E1ATMCT) corresponded to bp 8,192 to 9,476 of the Genbank sequence M3735 (Dominiguez et al., 1990), and encoded the E1 signal peptide and amino acid residues 1 to 411 (Fig. 1A). Using a synthetic oligonucleotide linker, the original E1 signal peptide was then replaced by a semi-artificial one previously shown to mediate efficient transport of E1 into the cell membrane domain and, in some cases, of adjacent sequences (Scallon et al., 1991) and was obtained from Dr J. Kochan, Hoffmann-La Roche, Nutley.

Antibody preparation
RV E1 sequences encoding B-cell epitopes (Terry et al., 1988; Ner et al., 1991; Chaye et al., 1992; Wolinsky et al., 1993) were obtained using RT-PCR with the upstream primer 5'-CGGAATTTCCACGACCCGGCCCCGGAGGCG-3' and the downstream primer 5'-CGGAATTTCCACGACCCGGCCCCGGAGGCG-3'. The amplification product (E1ep), corresponding to E1 amino acid residues 133 to 359, was cloned into pCB6 (Coyne et al., 1994), under the control of the human CMV immediate early gene promoter, the human growth hormone transcription terminator and a SV40-derived origin of replication for expression in COS cells. A chimeric E1-Thy-1 gene encoding E1GPI was constructed as follows. A 170 bp fragment of the C terminus of mouse Thy-1 cDNA contained in plasmid pTM813 (Fasel and Déglon, 1992) was PCR amplified using the upstream primer 5'-CGGAATTTCCACGACCCGGCCCCGGAGGCG-3' and the downstream primer 5'-CGGAATTTCCACGACCCGGCCCCGGAGGCG-3'. The PCR product was digested with SacI, blunt-ended with T4 DNA polymerase and then digested with EcoRI. The resulting fragment was then inserted into plasmid pCB6/E1ATMCT, which had previously been digested with Xhol and rendered blunt with the Klenow fragment of DNA polymerase I, and finally digested with EcoRI. The E1GPI C-terminal sequence is present in Fig. 1B. Expression vector pBJ1682 containing the complete cDNA of a bovine glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) has been described elsewhere (Scallon et al., 1991) and was obtained from Dr J. Kochan, Hoffmann-La Roche, Nutley.

Cell culture and transfection
COS-1 cells were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal calf serum. For transfection, 4×106 exponentially growing cells were electroporated at 0.4 kV/cm for 5 milliseconds (Bio-Rad Gene Pulser apparatus) in the presence of 25 μg of specific plasmid DNA, and seeded onto a 60 mm Petri dish.

Immunofluorescence staining
Transfected COS cells were washed in PBS 48 hours post-transfection and fixed for 10 minutes with 3% paraformaldehyde in PBS. After three washes in PBS, the second with PBS including 0.1 M glycine, cells were dipped for 10 minutes in 50 mM NH4Cl. For permeabilization, cells were first treated for 10 minutes in 0.1% Triton X-100 and then dipped for 10 minutes in 50 mM NH4Cl. GP anti-E1ep (1:150) and fluorescein-conjugated goat anti-GP IgG (1:64; Sigma) were diluted in PBS containing 5% nonfat dry milk as a blocking agent. For intracellular visualization, diluted conjugate also contained 0.01% Evans blue as a counterstain, which fluoresced red in all permeabilized cells, creating a marked contrast of immunoreactive structures fluorescing yellow-green, against a red cellular background (Newkirk and Mack, 1992). Incubations were for 60 minutes at room temperature. Preparations were observed and photographed on a Zeiss Axioshot microscope using Fujichrome 1600D film.

RESULTS
Expression and localization of E1 deletion mutants
Several viral type I membrane proteins have been shown to be converted into soluble proteins upon removal of their transmembrane domain and, in some cases, of adjacent sequences (Rose and Bergmann, 1982; Singh et al., 1990; Matsuoka et al., 1994). In contrast, anchorless RV E1 was found to be retained mainly in the ER even after removal of its cytoplasmic domain (Hobman et al., 1994b). In order to determine the role in this retention of the C-terminal part of its ectodomain, we con-
constructed a truncated mutant of E1 (E1ΔTMCT). The mutant protein contained amino acid residues 1-411, but lacked the transmembrane and cytoplasmic domains, as well as 29 C-terminal residues of the ectodomain (Fig. 1A). When transiently expressed in COS cells and detected by immunofluorescence staining 48 hours post-transfection, E1ΔTMCT was found to be localized in the perinuclear region with a staining pattern typical for localization in the Golgi or in the intermediate compartment. No other cytoplasmic or cell surface signal was detected (Fig. 2C-F). Thus, all three E1 mutants used in this study were unable either to reach, or go beyond the site where the wild-type protein expressed alone is arrested (Hobman et al., 1992).

**A GPI anchor attachment signal mediates E1 transport to the cell surface**

E1 transport out of the Golgi was only obtained by coexpression of RV E2 glycoprotein and has been suggested to depend on heterodimerization (Baron et al., 1992; Hobman et al., 1994b). To possibly trigger the further transport of E1 along the secretory pathway independently of E2, we investigated the behaviour of E1ΔTMCT after fusion of a GPI cell surface targeting signal to its C terminus (Fig. 1A,B). Thy-1, a glycoprotein of murine thymocytes and neurons was shown to be synthesized as a precursor protein containing all structural requirements for the addition of a glycolipid anchor in its C-terminal 53 amino acid residues (Crise et al., 1989; Beghdadi et al., 1993). Furthermore, addition of a GPI moiety results in the removal of 31 C-terminal amino acids of the precursor, which was also shown to occur on chimeric proteins carrying this domain at their C terminus (Crise et al., 1989; Kaetzel et al., 1990; Salzwedel et al., 1993).

The analysis of permeabilized COS cells transiently transfected with a construct encoding E1ΔTMCT fused to the 53 C-terminal amino acids of Thy-1 (E1GPI) revealed an immunostaining reminiscent of the one observed with other GPI anchored proteins (Moran and Caras, 1991; Fig. 2G). Moreover, a clear signal was seen on the surface of unpermeabilized cells (Fig. 2H). These results show that the addition of a GPI anchor mediates transport of RV E1 to the cell surface.

**E1GPI is anchored in the cell membrane through a GPI moiety**

E1ΔTMCT was found to be transported to the cell surface upon fusion to a GPI attachment signal. In order to show that a GPI molecule had indeed been added and was responsible for E1ΔTMCT anchoring in the cell membrane, we assessed the sensitivity of this protein to *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC). The GPI anchor-hydrolyzing activity of bacterial PI-PLCs has been widely used to detect and characterize GPI-anchored proteins. On intact cells, PI-PLCs were shown to cleave the GPI linkage, releasing proteins in soluble form into the medium (Davitz et al., 1986; Low et al., 1988). When added 48 hours post-transfection to the medium of COS cells transiently transfected with E1GPI, PI-PLC was found to specifically release E1 which migrated at 65-70 kDa as a group of bands characteristic of glycoproteins (Fig. 3).

**E1GPI retains E1 hemagglutination function**

E1 is considered as the major target for humoral immune response during natural RV infection. Anti-E1 antibodies which
inhibit hemagglutination indicate that E1 is also the viral hemagglutinin (Waxham and Wolinsky, 1985; Green and Dorsett, 1986). In order to assess whether E1GPI transported to the cell surface retains the property of its authentic counterpart, we incubated transiently transfected COS cells with human erythrocytes at 48 hours post-transfection and assayed binding activity. Microscopic analysis clearly showed that E1GPI expressing cells adsorbed erythrocytes (Fig. 4A). In order to more quantitatively assess the effect of E1GPI expression on erythrocyte binding, the relative amount of hemoglobin of adsorbed red blood cells was measured. As presented in Fig. 4B, only COS cells transfected with the E1GPI construct showed a peak of absorbance at 540 nm. This result provides direct evidence that E1 alone is sufficient for binding to human erythrocytes and that addition of a GPI anchor to E1 ectodomain does not abolish this property.

**Fig. 2.** Cellular localization of E1 mutants and E1GPI chimeric protein. COS cells were transiently transfected with indicated constructs and fixed at 48 hours post-transfection, with or without permeabilization for intracellular (A,C,E,G) or surface staining (B,D,F,H). Permeabilized cells were counterstained using 0.01% Evans blue, which created a red cellular background. Immunofluorescence staining was performed using a polyclonal antibody, GP anti-E1ep, and a FITC-conjugated secondary antibody.

**Fig. 3.** Sensitivity of E1GPI to PI-PLC. COS cells were transiently transfected with either an empty vector (COS) or an E1GPI encoding construct (COS/E1GPI). At 48 hours post-transfection, cells were washed and incubated in DMEM in the presence (+) or absence (-) of PI-PLC for 1 hour. Both the cell lysates (C) and the culture medium supernatants (S) were collected and prepared for SDS-PAGE and transfer onto nitrocellulose. Immunoblot analysis was performed using a polyclonal antibody, GP anti-E1ep, and a HRP-conjugated secondary antibody. Size standards (in kDa) are shown at the right.

**Fig. 4.** Erythrocyte binding activity of E1GPI. COS cells were transfected with an empty vector (pCB6) or constructs encoding either E1ΔTMCT or E1GPI. At 48 hours post-transfection, cells were treated with trypsin as described in Materials and Methods and incubated with human group O red blood cells. After extensive washes in PBS, they were either photographed (A) or resuspended and absorbance measured at a wavelength of 540 nm (B).

**E1GPI is secreted when coexpressed with a mammalian GPI-specific phospholipase D (GPI-PLD)**

E1GPI, a chimeric form of RV E1 and mouse Thy-1 molecule, was found to be transported to the cell surface. In order to obtain a secreted form of E1, we assessed the effect of coexpression of a mammalian GPI-PLD. Although their physiological role is still only partially understood, mammalian cell-associated GPI-PLDs have recently been shown to mediate the release of GPI-anchored proteins from cell surfaces (Metz et al., 1994; Xie and
Functional GPI anchored RV hemagglutinin

DISCUSSION

RV E1 glycoprotein was found to be arrested in a tubular pre-Golgi compartment when expressed in the absence of E2, the other viral envelope glycoprotein (Hobman et al., 1992). A truncated form lacking both the transmembrane and the cytoplasmic domains was also shown to be cell-associated (Hobman et al., 1994b). Our results show that a similarly truncated mutant (E1ΔTMCT) lacking 70 C-terminal amino acid residues accumulated in a perinuclear site distinct from the ER, producing an immunostaining pattern very reminiscent of the one observed with the full-length protein by Hobman et al. (1992). Thus, in contrast to what was observed for several ER or Golgi resident proteins (Machamer et al., 1990; Swift and Machamer, 1991; Matsuoka et al., 1994), we confirmed that the region important for E1 retention does not lie in its transmembrane or cytoplasmic domain. Since E1ΔTMCT also lacks 29 C-terminal amino acid residues of the ectodomain, our results suggest that the retention mechanism does not involve any sequences lying very close to the membrane-spanning domain. Both the E1 mutants E1ΔNE and E1ΔXho I, which lack additional sequences of the ectodomain, were found to accumulate in the ER. However, this localization is very unlikely to be related to the retention mechanism of the natural full-length protein, but rather reflects an arrest due to misfolding. Indeed, both these mutants lacked N-linked glycosylation sites, which are known to influence protein conformation (Machamer and Rose, 1988; Vidal et al., 1989). Thus, deletions in the ectodomain failed to provide information on E1 transport. However, pre-Golgi retention of E1ΔTMCT may reflect specific features of the ectodomain, which are likely to be involved in RV budding from inside the cell. It has been suggested that E1 heterodimerization with E2 is required for the former protein to be correctly folded and transported to the Golgi (Baron et al., 1992; Hobman et al., 1993), where the complex accumulates due to a Golgi retention signal in the membrane-spanning domain of E2 (Hobman et al., 1995). By fusing a potent cell surface targeting signal to the C terminus of its ectodomain, we showed that E1 could be rendered transport-competent in the absence of E2, suggesting that the retention mechanism was bypassed in this context. Thus, in agreement with previous observations (Hobman et al., 1992), our results strongly suggest that E1ΔTMCT was not blocked in the intermediate compartment due to misfolding. Indeed, the hemadsorption property of chimeric E1-Thy-1 protein showed that, upon transport to the cell surface, the biological activity of the E1/E2 heterodimer was maintained in E1 alone.

Addition of a GPI anchor has been used in several membrane proteins, in order to study the role of individual domains in oligomerization and transport (Crise et al., 1989; Salzwedel et al., 1993) and shown to influence folding (Barboni et al., 1995). In this communication, we show that this approach can also be used to trigger the transport of an integral membrane protein blocked in the secretory pathway. GPI anchor addition was found to act as a dominant cell surface targeting signal and maintain the hemadsorption property of the protein. GPI anchor addition has been shown to occur very early after protein translocation into the ER (Conzelmann et al., 1987). Thus, like E1/E2 heterodimerization, it precedes E1 arrival into the pre-Golgi compartment where the protein is retained in the absence of E2. If the retention was mediated by the binding of E1 to another protein, perhaps via sequences in the ectodomain, this interaction might be prevented by oligomerization with E2 or fusion to a GPI anchor. In the latter case, interactions with a putative retention factor might only partially occur, or not occur at all, as a consequence of a probable sorting of the GPI-anchored proteins in the ER by clustering them in membrane subdomains enriched in cholesterol and sphingolipids (Brown and Rose, 1992; Gascard et al., 1993). By pursuing its route towards the plasma membrane, E1GPI would then enter a subcellular compartment or vesicles containing the coexpressed phospholipase, which would act at a post-Golgi site or at the plasma membrane (Metz et al., 1993).

Our results show that fusion of a glycolipid anchor attachment signal can render transport competent intracellularly retained proteins which are naturally co-expressed with other molecules.

Fig. 5. Determination of E1GPI secretion in the presence of GPI-PLD. COS cells were transiently transfected with either an empty vector (COS) or an E1GPI encoding construct (COS/E1GPI), and cotransfected (+), or not (-), with a plasmid encoding GPI-PLD cotransfected in trans. At 48 hours post-transfection, the cells were washed and incubated in DMEM lacking FCS. Culture medium supernatants (S) were collected after 2 or 16 hours (S2, S16) to detect accumulation of secreted E1GPI. Cell lysates were collected after 16 hours. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose. Immunoblot analysis was performed using a polyclonal antibody, GP anti-E1ep, and a HRP-conjugated secondary antibody. Size standards (in kDa) are shown at the right.

Low, 1994). In contrast to bacterial PI-PLCs, these enzymes are inactive when secreted into the medium, and they appear to act intracellularly by hydrolyzing GPI-anchored molecules in a post-Golgi compartment (Metz et al., 1993). This enzymatic activity was also observed upon cotransfection of cDNAs coding for a GPI-PLD and a GPI-anchored protein (Scallon et al., 1991). Furthermore, the soluble form of Thy-1 found in the cerebrospinal fluid was proposed to result from a GPI-PLD cleavage (Almqvist and Carlsson, 1988). Indeed, when COS cells were transiently transfected with expression plasmids encoding E1GPI and a bovine GPI-PLD, a specific product migrating at 65-70 kDa was found to accumulate in the culture medium, as detected by immunoblot analysis (Fig. 5, lanes C and F). The cell-associated specific signal observed in the absence of GPI-PLD dramatically decreased in the presence of the enzyme (Fig. 5, compare lanes C and F), suggesting the constitutive release of E1GPI as the result of GPI-PLD activity.
required for their stability, as has also been demonstrated for T cell antigen receptor heterodimers (Lin et al., 1990). We anticipate that such fusions will be useful for further studies on the sorting mechanisms along the secretory pathway. Furthermore, although the system might be limited by the availability of the preassembled anchor and the saturation of the machinery for anchor attachment as discussed for example by Moran and Caras (1992), the production of large amounts of such proteins in secreted form might be feasible by coexpressing the ectodomain of these proteins fused to a GPI anchor attachment signal, together with a GPI-specific phospholipase.

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