Soluble ectodomain of rabies virus glycoprotein expressed in eukaryotic cells folds in a monomeric conformation that is antigenically distinct from the native state of the complete, membrane-anchored glycoprotein

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Rabies virus glycoprotein (G) is a trimeric type I transmembrane glycoprotein that mediates both virus receptor recognition and low pH-induced membrane fusion. G can assume three different states: the ‘native’ state (N) detected at the virus surface, which is responsible for receptor binding, the activated hydrophobic state (A), which interacts with the target membrane as a first step in the fusion process, and the fusion-inactive conformation (I). These three states, which are structurally different, are in a pH-dependent equilibrium. This equilibrium is shifted toward the I state at low pH. This paper includes an investigation of the structure of the ectodomain of the PV strain of rabies virus when it is synthesized as a soluble form (G1–439) lacking the transmembrane and intracytoplasmic domains (residues 440–505). It is shown that, whatever the extracellular pH, G1–439 is secreted as a monomer that has the antigenic characteristics of the I state. This I-like state is not acquired in the acidic compartments of the Golgi but directly in the endoplasmic reticulum. Finally, membrane anchorage by the G transmembrane domain (G1–461) is sufficient for the G ectodomain to be folded into the native N form. These results emphasize the role of the G transmembrane domain in the correct folding of the ectodomain.

Introduction

Most glycoproteins are translated on membrane-bound ribosomes and inserted co-translationally into the endoplasmic reticulum (ER) in an unfolded form. In the case of transmembrane proteins, folding occurs in three topologically and biochemically distinct environments: the ER lumen, the ER membrane and the cytosol. It is therefore supposed that the ectodomain, the transmembrane domain and the cytoplasmic domain constitute independent folding domains within glycoproteins. This view is supported by the efficient surface expression of many chimeric proteins when these domains are exchanged between different glycoproteins and also by the successful expression and secretion of soluble ectodomains of cellular receptors and viral glycoproteins. However, in most cases, the structure of the chimeric or soluble glycoproteins was not analysed in detail, because transport and/or secretion was considered to be proof of correct folding. It is therefore conceivable that some more or less subtle differences may exist between the soluble and the membrane-anchored ectodomains of a glycoprotein. Indeed, it has been suggested that the ER ‘quality control’ is not so stringent for soluble proteins and that correct oligomerization is not required for secretion (Singh et al., 1990). In the case of class II MHC molecules, it has been also shown that mutations in the transmembrane domain of α or β chains resulted in incorrect folding of the ectodomains of the proteins (Cosson & Bonifacino, 1992).

Rabies virus glycoprotein (G) is a type I membrane glycoprotein. It is a trimer (3 × 65 kDa) that forms a spike extending 8–3 nm from the virus membrane (Gaudin et al., 1992; Whitt et al., 1991). The native glycoprotein contains 505 amino acids (Anilionis et al., 1981). It is responsible for viral receptor recognition (Tuffereau et al., 1998; Wunner et al., 1984) and for the low pH-induced fusion of the viral envelope with the endosomal membranes (Gaudin et al., 1993; Whitt et
al., 1991). The pH threshold for fusion is about 6.3 and pre-incubation of the virus below pH 6.75 in the absence of a target membrane results in inhibition of virus fusion properties. This inhibition is reversible by re-incubating the virus above pH 7 (Gaudin et al., 1993). This behaviour, shared with another rhabdovirus, vesicular stomatitis virus (Clague et al., 1990; Pak et al., 1997), is different from that observed with other viruses fusing at low pH, for which low pH-induced fusion inactivation is irreversible (Gaudin et al., 1995b).

We have previously demonstrated that G can assume at least three different states: the 'native' state (N) detected at the virus surface, which is probably responsible for receptor binding, the activated hydrophobic state (A), which interacts with the target membrane as a first step in the fusion process, and the fusion-inactive conformation (I). There is a complex pH-dependent equilibrium between these states. This equilibrium is shifted toward the I state at low pH. By using electron microscopy, it has been shown that the I conformation is 3 nm longer than the N state, from which it is also antigenically distinct (Gaudin et al., 1993). We have proposed that G is transported through the Golgi apparatus in an I-like conformation to avoid undesirable fusion during its transport through the acidic Golgi vesicles (Gaudin et al., 1995a).

In order to study in detail the low pH-induced structural transitions of G by spectroscopic techniques, we decided to express a soluble ectodomain of G. We were encouraged in this approach by the recent work of Wojczyk et al. (1995), who have demonstrated that a soluble form of G, constructed by insertion of a stop codon just before the transmembrane domain, was efficiently expressed and secreted in transfected CHO cells. In this study, using well-characterized monoclonal antibodies (MAbs), we have analysed the folding and transport of the soluble ectodomain of G (G1–419) and compared it with the folding of the complete G (Gwt) and that of a glycoprotein lacking only its intracytoplasmic domain (G1–461) in transfected cells. Our results indicate that, in the absence of the transmembrane domain, the ectodomain of G folds in a monomeric I-like conformation and is not able to adopt the N conformation, whatever the experimental conditions.

Methods

**Cells, viruses and MAbs.** BSR cells, a clone of BHK 21 (baby hamster kidney) cells, were grown in minimal essential medium (Gibco-BRL) supplemented with 10% calf serum. The PV strain of rabies virus was cultivated and purified as described previously (Gaudin et al., 1992). Recombinant vaccinia virus (VT7-3) containing the T7 RNA polymerase gene has been described previously (Fuerst et al., 1986) and was kindly provided by B. Moss (National Institutes of Health, Bethesda, MD, USA). MAbs 30AA5, 29EC2 and 17D2 have been described previously (Gaudin, 1997) and their properties are summarized in Table 1. They were used as mouse ascites preparations.

**Plasmid construction.** Recombinant plasmids based on pcDNA I (Invitrogen) and expressing Gwt, G1–419 and G1–461 of the PV strain of rabies virus were constructed as follows. Total RNA from PV-infected BSR cells was isolated 20 h post-infection and the PV G mRNA was amplified by RT–PCR with oligonucleotides Y1 (5′ GCCGGATCCGAGCCAGCCTCTCA 3′) and X1 (5′ GCCGGATCCAGATCTGAGGAAAAGATG 3′), which contain BamHI sites (in italics) and hybridize to the 3′ end and to the complementary sequence of the 5′ end of the G mRNA, respectively. In the same manner, the fragment of PV G mRNA encoding the ectodomain of the glycoprotein was amplified by RT–PCR with oligonucleotides X2 and Y2 (5′ GCCGGATCCCATCTAGTTCCCTGATTTGGG 3′), which hybridizes to the 3′ end of the mRNA region encoding the ectodomain and allows the addition of a stop codon (underlined) at the end of the ectodomain coding sequence. The amplified DNA fragments were digested with BamHI and inserted into pcDNA I to make pcDNA I-Gwt and pcDNA I-G1–419 encoding Gwt and G1–419, respectively. To construct pcDNA I-G1–461, we took advantage of the presence of a unique BspHI restriction site situated in the G gene upstream of the transmembrane domain coding region and a unique Xhol restriction site in the vector located 76 bp downstream of the G gene. The fragment of plasmid pcDNA I-Gwt encoding the transmembrane domain of G was amplified by PCR with oligonucleotides X3 (5′ TCCATCATGACCAGACAGTG 3′), corresponding to nucleotides 921–945 of the G mRNA and containing the BspHI site (in italics), and Y3 (5′ GCCGCTTAGATTCCAGATCTGAT 3′), which contains the complementary sequence of nucleotides 1428–1440 of the G mRNA plus a stop codon (underlined) and the Xhol site (in italics). The amplified DNA fragment was digested with BspHI and Xhol and inserted into pcDNA I-G1–415 digested with the same enzymes, to make pcDNA I-G1–461.

**DNA transfection.** Proteins were transiently expressed by using a T7 vaccinia virus expression system according to the method of Fuerst et al. (1986). Briefly, BSR cells were grown in 3.5 cm dishes and about 80% confluent and were infected with VTF7-3 at an m.o.i. of 5 p.f.u. per cell. After 1 h of adsorption, the cells were transfected with 5 µg supercoiled plasmid DNA by the calcium phosphate co precipitation procedure (Parker & Stark, 1979).

**Pulse–chase experiments and immunoprecipitations.** At 20 h post-transfection, the culture medium was replaced with methionine- and cysteine-free medium (ICN) and the cells were incubated for 1 h at 37 °C. The cells were then labelled for 5 min with 500 µl pre-warmed methionine- and cysteine-free medium supplemented with 200 µCi [35S]methionine/[35S]cysteine (Promix, Amersham). Pre-warmed normal growth medium containing 5 mM cold methionine and 1 mM cold cysteine was then added for the chase period. The cells were then washed in cold MD buffer (173 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 25 mM Tris–HCl, pH 7.5) and lysed on ice in 1 ml MD containing 1% CHAPS and an antiprotease cocktail (2 µg/ml leupeptin, 2 µg/ml antipain, 2 µg/ml pepstatin, 2 µg/ml chymostatin and 16 µg/ml aprotonin). The lysates were spun in an Eppendorf centrifuge for 5 min at 12000 g and 4 °C. The supernatant was divided into three and each third was incubated for 1 h at 4 °C with one of the anti-G MAbs used in the study (30AA5, 29EC2 or 17D2). Protein A–Sepharose was then added and the mixture was incubated for 45 min at 4 °C. The resulting immune complexes were centrifuged and washed three times with MD containing 1% CHAPS and the antiprotease cocktail. The complexes were then boiled for 5 min in Laemmli buffer before analysis by 10% SDS–PAGE and autoradiography. Quantification of radioactivity was performed by using a phosphorimager ( Molecular Dynamics).

When cells were transfected with plasmids encoding G1–419, the cellular supernatant containing the secreted G1–419 was retained at the end of the chase and divided into three parts. Each part was incubated for 1 h at 4 °C with one of the MAbs used in the study. Protein A–Sepharose
Fusion-inactive G (I)

were collected from the bottom of each gradient. Secreted G
for 16 h at 4°C were immunoprecipitated by 29EC2 and 30AA5, respectively,
viral G were immunoprecipitated by 29EC2 and 30AA5, respectively,

coefficient markers. The gradients were then centrifuged at 35000 r.p.m.
catalase (10 µg/ml) was added and the mixture was incubated for 45 min at 4°C. The
resulting immune complexes were then treated and analysed as above.

Endoglycosidase H (EndoH) treatment. Immune complexes
associated with protein A–Sepharose were incubated in phosphate–
citrate buffer, pH 5–6, containing 1% SDS for 5 min at 100°C. The
supernatant was divided in two (2 x 30 µl) and half of the sample was treated
with 8 mU EndoH for 14 h at 37°C. The remaining half was incubated at 37°C in the absence of EndoH and was used as a control.

Bafilomycin, brefeldin A and low temperature treatments.
Brefeldin A and bafilomycin were used at concentrations of 5 and 0.4 µM, respectively. They were present during the methionine
and cysteine deprivation, the pulse and the chase. In the experiments where
transport was inhibited by low temperature treatment, the Petri dishes
were incubated at 15°C immediately after the end of the pulse and
maintained at this temperature until the end of the chase. In these
experiments, the chase medium was already at 15°C when it was added to

was then added and the mixture was incubated for 45 min at 4°C. The
resulting immune complexes were then treated and analysed as above.

Oligomerization analysis. For sedimentation analysis, linear
sucrose gradients were prepared from stocks of 5 and 20% sucrose in
20 mM NaCl and 20 mM Tris–HCl, pH 8, supplemented with the
antiprotease cocktail. Three gradients were run in parallel. Cellular
medium (250 µl) containing metabolically labelled Gwt was loaded on
the first gradient. Metabolically labelled complete G (PV strain),
solubilized from the virus membrane with 1% CHAPS (250 µl) as
described by Gaudin et al. (1992), was loaded on the second (in this case,
the gradient contained 1% CHAPS). BSA (4.3 S), aldolase (7.6 S) and
catalase (10.6 S) were loaded on the third gradient as sedimentation
coefficient markers. The gradients were then centrifuged at 35 000 r.p.m.
for 16 h at 4°C in an SW 41 rotor (Beckman). Fractions (12 x 1 ml) were
collected from the bottom of each gradient. Secreted G1–1439 and
viral G were immunoprecipitated by 29EC2 and 30AA5, respectively,
before analysis by SDS–PAGE.

Results

In order to study the folding, transport and secretion of
G1–1439, we used a transient expression system to obtain a high
level of the proteins in BSR cells. Cells were infected with the
VTF7-3 recombinant vaccinia virus encoding the bacteriophase T7 RNA polymerase (Fuerst et al., 1986) and then
transfected with plasmids encoding either G1–1439 or the
complete glycoprotein (Gwt) under the T7 promoter.

Folding and transport of Gwt

We first analysed the folding and transport of Gwt. For this
purpose, transfected cells were labelled with [35S]methionine and
[35S]cysteine for 5 min and ‘chased’ in cold medium
for the times indicated before lysis in TDD buffer containing 1% CHAPS.
G in the lysates was then immunoprecipitated with the MAbs indicated and
analysed by 10% SDS–PAGE under reducing conditions followed by
 autoradiography. NT, untrimmed G; GT, fully trimmed G.

Table 1. Characteristics of the MAbs used in the study

<table>
<thead>
<tr>
<th>MAb</th>
<th>Epitope location</th>
<th>SDS-treated G*</th>
<th>Recognition of:</th>
<th>Fusion-inactive G (I)</th>
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<td>Native G (N)</td>
<td>On the virus</td>
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<td>On the virus</td>
<td>After solubilization</td>
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<td>17D2</td>
<td>255–270</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>29EC2</td>
<td>10–15</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>30AA5</td>
<td>Site II</td>
<td>+</td>
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* As judged by a Western blot assay.
Folding and transport of $G_{1-439}$

The folding and transport of $G_{1-439}$ was then analysed. For this purpose, transfected cells were labelled with $[^{35}S]$methionine and $[^{35}S]$cysteine for 5 min and ‘chased’ for periods of up to 160 min. Cell supernatants and cell lysates were then immunoprecipitated with 17D2, 29EC2 and 30AA5. The immune complexes were analysed by SDS–PAGE.

The kinetics of epitope acquisition were studied by immunoprecipitation of the cell lysates (Fig. 2a). MAb 29EC2 recognized $G_{1-439}$ immediately after the pulse. At this time, only the untrimmed glycoprotein (NT) was detected. During the chase, NT was converted into the mature GT. The amount of ectodomain immunoprecipitated by 29EC2 reached a maximum after about 20 min and then decreased. This decrease of cellular $G_{1-439}$ was due to secretion of soluble ectodomain (see below) into the cell supernatant and also to degradation of unfolded proteins. MAb 17D2 also recognized $G_{1-439}$ immediately after the pulse. However, after 10 min or more of the chase, the amount of G immunoprecipitated by 17D2 was much smaller (about threefold less) than the amount of G immunoprecipitated by 29EC2 (Fig. 2b). Finally, MAb 30AA5 did not recognize $G_{1-439}$. At most, 5% of the amount immunoprecipitated by 29EC2 was pelleted by 30AA5, and this was probably nonspecific, because a band of the same intensity was detected when the cellular extracts were immunoprecipitated with an unrelated MAb (anti-rabies phosphoprotein) (Fig. 3).

After 40 min of the chase, the soluble ectodomain was detected in the cell supernatant after immunoprecipitation by 29EC2 (Fig. 2c). After 160 min, about 30–40% of the pulse-labelled $G_{1-439}$ was found in the supernatant and immunoprecipitated by 29EC2. This extracellular ectodomain was not recognized by 30AA5. As mentioned above, the faint band (at most 5% of the amount immunoprecipitated by MAb 29EC2) detected in the gel after immunoprecipitation with 30AA5 corresponded to nonspecific pelleting. This result indicated that $G_{1-439}$ present in the cell supernatant was antigenically distinct from the native complete glycoprotein. The ratio of extracellular ectodomain immunoprecipitated by 17D2 versus 29EC2 increased with the length of the chase, and after 160 min about 25% of the amount of $G_{1-439}$ immunoprecipitated by 29EC2 was immunoprecipitated by 17D2. As 17D2 recognized G after denaturation, this suggested that secreted $G_{1-439}$ was partially denatured in the cellular supernatant.

EndoH resistance of intracellular and extracellular $G_{1-439}$ was then analysed (Fig. 4). After 150 min of the chase, a significant proportion (about 40%) of intracellular $G_{1-439}$ was still EndoH sensitive and might correspond to unfolded $G_{1-439}$ sequestered in the ER. However, extracellular $G_{1-439}$ was totally EndoH resistant, indicating that secretion occurred via the normal Golgi pathway and was not due to cellular lysis.

As neither 17D2 nor 30AA5 recognized the mature form (GT) of $G_{1-439}$, all these results are consistent with $G_{1-439}$ being transported through the Golgi apparatus and secreted in a conformation that was antigenically similar to the I state. The initial recognition by 17D2 reflects the ability of this MAb to bind folding intermediates. As the same results were obtained when cell lysis and immunoprecipitation were performed at pH 8.7 (data not shown), the I conformation appears to be the more stable state of the soluble ectodomain, whatever the pH.

$G_{1-439}$ is monomeric

The quaternary structure of $G_{1-439}$ was also analysed. For this purpose, supernatant containing metabolically labelled $G_{1-439}$ was loaded on a linear 5–20% sucrose gradient and centrifuged (see Methods). $G_{1-439}$ present in the fractions was immunoprecipitated by 29EC2. $G_{1-439}$ sedimented slightly more slowly than BSA (Fig. 5a). Its sedimentation coefficient was about 4 S, indicating that $G_{1-439}$ was probably a monomer. By comparison, in a similar gradient supplemented with 1% CHAPS, the major part of the complete glycoprotein solubilized from the virion (see Methods) sedimented with a sedimentation coefficient of about 10 S. This form of the glycoprotein has been shown to be a trimer (Gaudin et al., 1992).

These results indicate that a putative oligomeric structure of $G_{1-439}$, if it exists, would be much more unstable than the native trimeric structure of the complete glycoprotein. Consistent with this view, attempts to cross-link potential oligomers of $G_{1-439}$ were unsuccessful (data not shown).

We have previously shown that the complete glycoprotein solubilized from the virion with Triton X-100 was monomeric (Gaudin et al., 1992). It was thus interesting to know whether this monomeric form of G behaves similarly to the soluble...
Folding of the soluble ectodomain of rabies virus G

Fig. 2. (a) Recognition of intracellular pulse-labelled G<sub>1–439</sub> by MAbs 29EC2, 17D2 and 30AA5. BSR cells transfected with pcDNA I-G<sub>1–439</sub> were pulse-labelled with [35S]methionine/[35S]cysteine for 5 min and then ‘chased’ in cold medium for 1 h before lysis in TD containing 1% CHAPS. G in the lysates was then immunoprecipitated with the MAbs indicated and analysed by 10% SDS–PAGE under reducing conditions followed by autoradiography. NT, untrimmed G; GT, fully trimmed glycoprotein. (b) Kinetics of G<sub>1–439</sub> recognition by MAbs. The amount of radioactivity contained in the bands representing G<sub>1–439</sub> was quantified with a phosphorimager. The graph shows the means of five independent experiments identical to that shown in Fig. 3(a). In each experiment, the maximum amount of radioactivity was found in the bands of 29EC2-immunoprecipitated G after 20 min of the chase and was defined as 100%; the other values were calculated as percentages thereof. Error bars indicate standard deviations. ●, 29EC2; ○, 17D2; ■, 30AA5. (c) Kinetics of secretion of G<sub>1–439</sub>. The supernatant of BSR cells transfected with pcDNA I-G<sub>1–439</sub> and pulse-labelled with [35S]methionine/[35S]cysteine for 5 min (same samples as in Fig. 3(a)) was collected after the indicated length of chase and secreted G<sub>1–439</sub> was immunoprecipitated with MAbs 30AA5 (30), 17D2 (17) and 29EC2 (29) as described in Methods. G in the lysates was then immunoprecipitated with the MAbs indicated and analysed by 10% SDS–PAGE under reducing conditions followed by autoradiography.

G<sub>1–439</sub>. Results are presented in Fig. 5(b) and indicate that, after solubilization with 0.5% Triton X-100, G is recognized by 29EC2, 17D2 and 30AA5. This monomeric form of G is still able to undergo a low pH-induced conformational change, after which it is no longer recognized by MAbs 17D2 and 30AA5 (Fig. 5b, pH 6.4). Therefore, although monomeric, Triton X-100-solubilized G has the antigenic characteristics of the trimer and thus behaves differently from G<sub>1–439</sub>.
Is $G_{1-439}$ folded directly in an I-like state or in a metastable N-like conformation?

Among the known fusogenic glycoproteins, rhodovirus glycoproteins are the only ones for which the low pH-induced conformational change is reversible. Other glycoproteins (such as influenza virus HA) are synthesized in a metastable conformation (Ruigrok et al., 1986; Carr et al., 1997) and acquire their stable conformation after the low pH-induced structural transition. It was therefore possible that, in the absence of the transmembrane and intracytoplasmic domains, the native structure of rabies virus glycoprotein was metastable. In other words, it was possible that the soluble ectodomain was first synthesized in an N-like conformation, which would be rapidly converted to the I-like state after passage through the acidic compartment of the Golgi apparatus. Alternatively, $G_{1-439}$ could fold directly in an I-like state. To investigate this point, cells were treated either with drugs known to increase the pH of intracellular compartments or with drugs known to inhibit transport from the ER to the Golgi apparatus.

Bafilomycin A1 is a specific inhibitor of the ATPases involved in the acidification of intracellular compartments (Bowman et al., 1988; Yoshimori et al., 1991). Therefore, we studied the effect of bafilomycin A1 on the recognition of $G_{1-439}$ by the different MAbs. As shown in Fig. 6(a), the results obtained with bafilomycin A1-treated cells were similar to those obtained with untreated cells (i.e. there was no recognition by 30AA5). However, in the presence of the drug, the appearance of GT was delayed and the band corresponding to this form was much more diffuse [see Fig. 6(a) after 80 min of the chase], probably because perturbation of intraorganellar pH affects transport and protein traffic (Henkel & Weisz, 1998). Finally, $G_{1-439}$ synthesized in bafilomycin-treated cells was also secreted and, as in the absence of the drug, was essentially only recognized by MAb 29EC2 (not shown). Similar results were obtained when cells were treated by 20 mM NH$_4$Cl, a weak base that also increases the pH of intracellular compartments (not shown).

As it was possible that treatment with bafilomycin A1 and with NH$_4$Cl did not increase the pH of the intracellular compartments sufficiently, we also used brefeldin A, which is a fungal metabolite that causes disassembly of the Golgi, resulting in a blockage of transport between the ER and Golgi (Fujiiwara et al., 1988). In the presence of brefeldin A (Fig. 6(b)), there was no secretion of soluble ectodomain into the culture medium and no intracellular mature form (GT) of $G_{1-439}$ was immunoprecipitated by 29EC2, indicating that the transport of $G_{1-439}$ to the Golgi apparatus was indeed blocked. Despite this, $G_{1-439}$ was still not recognized by 30AA5, suggesting that $G_{1-439}$ was in an I-like state before reaching the acidic Golgi compartments. However, as it has been shown that brefeldin A induces retrograde transport from the medial Golgi to the ER, we could not exclude a potential progressive acidification of the ER compartment (Doms et al., 1989). Therefore, we decided to block transport from the ER to the Golgi complex by incubating the cells at 15 °C (Saraste et al., 1986; Tartakoff, 1986) during the chase, which, in this case, was extended to 4 h (Fig. 6(c)). Here again, neither an extracellular soluble ectodomain nor an intracellular mature form of $G_{1-439}$ (GT) was immunoprecipitated, indicating that the low temperature had blocked transport from the ER to the Golgi. However, once again, $G_{1-439}$ was not recognized by 30AA5 (Fig. 6(c)).

All these results were consistent with $G_{1-439}$ being in an I-like conformation before reaching the acidic compartments of the Golgi apparatus. Therefore, $G_{1-439}$ was folded directly in an I-like conformation and an N conformation of $G_{1-439}$ could never be detected.

Folding of $G_{1-461}$

A mutant lacking only the cytoplasmic domain of G was then constructed to see whether the presence of the transmembrane domain was sufficient to stabilize the N conformation of rabies glycoprotein. For this purpose, cells transfected with a plasmid encoding $G_{1-461}$ were pulse-labelled with $[^{35}S]$methionine and $[^{35}S]$cysteine for 5 min and ‘chased’ for periods of up to 160 min. Cell lysates were then
Fig. 6. Effect of bafilomycin, brefeldin A and low temperature on folding and transport of G$_{1-439}$. (a), (b) BSR cells transfected with pcDNA I-G$_{1-439}$ were incubated in the presence of 0–4 µM bafilomycin (a) or 5 µg/ml brefeldin A (b), pulse-labelled with [35S]methionine/[35S]cysteine for 5 min and then ‘chased’ in cold medium for the times indicated before lysis in TD plus 1% CHAPS (see Methods). G in the lysates was then immunoprecipitated with the MAbs indicated and analysed by 10% SDS–PAGE under reducing conditions followed by autoradiography. (c) Transfected BSR cells were pulse-labelled with [35S]methionine/[35S]cysteine for 5 min and then ‘chased’ in cold medium at 15 °C for the times indicated before lysis in TD plus 1% CHAPS (see Methods). G in the lysates was immunoprecipitated with the MAbs indicated and analysed by 10% SDS–PAGE under reducing conditions followed by autoradiography. NT, untrimmed G; GT, fully trimmed G. The dots to the right of (b) and (c) indicate the position of GT if it had been present.

Fig. 7. Recognition of pulse-labelled G$_{1-461}$ by MAbs 29EC2, 17D2 and 30AA5. BSR cells transfected with pcDNA I-G$_{1-461}$ were pulse-labelled with [35S]methionine/[35S]cysteine for 5 min and then ‘chased’ in cold medium for the times indicated before lysis in TD plus 1% CHAPS (see Methods). G in the lysates was immunoprecipitated with the MAbs indicated and analysed by 10% SDS–PAGE under reducing conditions followed by autoradiography. NT, untrimmed G; GT, fully trimmed G. The immune complexes were analysed by SDS–PAGE (Fig. 7). The kinetics of acquisition of epitopes of G$_{1-461}$ were similar to those of complete G. Particularly, (i) 17D2 immunoprecipitated G with the same efficiency as 29EC2, even after long periods of chase, (ii) both 17D2 and 30AA5 recognized the mature G$_{1-461}$ (GT) and (iii) phosphorimager quantification revealed that after 40 min of the chase, 30AA5 immunoprecipitated about 45% of the maximum amount of G$_{1-461}$ immunoprecipitated by 17D2 and 29EC2. All these data indicated that G$_{1-461}$ behaves like the complete glycoprotein and that the presence of the transmembrane domain was sufficient to stabilize the N conformation of the glycoprotein.

Discussion

In this paper, we have investigated the structure of the ectodomain of the PV strain of rabies virus when it is synthesized as a truncated form (G$_{1-439}$) lacking the transmembrane and intracytoplasmic domains. Four major conclusions can be drawn. Firstly, as shown by sedimentation
Y. Gaudin and others

analysis in sucrose gradients, G_{1-439} is monomeric. Secondly, G_{1-439} is secreted in a conformation that has the antigenic characteristics of the fusion-inactive state (I). Thirdly, this I-like state is not acquired in the acidic compartments of the Golgi but directly in the ER and, in fact, an N-like state for this soluble ectodomain does not seem to exist. Finally, membrane anchorage by the transmembrane domain is sufficient for G to exhibit the behaviour of the complete glycoprotein (i.e. folding in an N-like structure recognized by 30AA5). These conclusions are different from those of Wojczyk et al. (1995), who have suggested that the soluble ectodomain of G of the ERA strain, when expressed in CHO cells, has the same antigenicity as the complete glycoprotein. However, it was clear from their study that antibodies 719-3, 509-6 and 61-105, which recognized the complete glycoprotein, were not able or were only poorly able to recognize the soluble ectodomain. However, it was clear from their study that antibodies 719-3, 509-6 and 61-105, which recognized the complete glycoprotein, were not able or were only poorly able to recognize the soluble ectodomain (Wojczyk et al., 1995). Interestingly, MAb 719-3 is directed against antigenic site II (Lafon et al., 1983; Préhaud et al., 1988), which is known to be absent in the inactive state (I) of G (Gaudin et al., 1993). Thus, the results described here are not a peculiarity of the soluble ectodomain (G_{1-439}) of the PV strain expressed in BSR cells. Indeed, we have obtained similar results with the soluble ectodomain of the CVS strain expressed in BSR cells and with both PV and CVS strains in lepidopteran cells when G_{1-439} is expressed by a recombinant baculovirus (not shown).

It is generally supposed that glycoprotein ectodomains are independent folding domains. In many cases, expression of soluble ectodomains from membrane-anchored glycoproteins has been successful and has provided powerful tools for biochemical and biophysical studies, e.g. CD4 (Davis et al., 1990; Wu et al., 1997), herpes simplex virus type 1 glycoprotein D (Sisk et al., 1994) and La Crosse virus glycoprotein G1 (Pekosz & Gonzalez-Scarano, 1996). However, in an increasing number of cases, the absence of the transmembrane domain results in secretion of an ectodomain with an aberrant quaternary structure. This has been shown for the anchor-free spike glycoprotein S of transmissible gastroenteritis virus which, although secreted, was unable to trimerize (Godet et al., 1991) and for anchor-free influenza virus HA, which leaves the ER as a monomer and forms trimers and higher-order aggregates in the late compartment of the Golgi pathway (Singh et al., 1990). Furthermore, in the case of influenza virus HA, when the pH of the expression medium in which the cells were grown was adjusted to 8.5, no aggregation was observed and most of the protein remained monomeric (Vanlandschoot et al., 1996), suggesting that HA aggregation was the result of the low pH of the Golgi compartments. To explain these results, it has been proposed that the ER ‘quality control’ is not as stringent for soluble proteins and that correct oligomerization is not required for secretion (Singh et al., 1990). Alternatively, as suggested by Vanlandschoot et al. (1998), it is possible that soluble HA adopted a folded, stable structure that was different from the ectodomain structure in complete HA and did not need to oligomerize to be secreted. In the case of rabies virus, the structure of G_{1-439} is different from that of Triton X-100-solubilized, membrane-anchored G, which is also monomeric but has the antigenic characteristics of trimeric G.

One obvious role for the transmembrane domain in the folding of the ectodomain could be to facilitate the oligomerization of the whole molecule. Firstly, transmembrane domains may assist oligomerization simply by restricting the movement of the glycoproteins in the two dimensions of the membrane, thus increasing the apparent association constant of the subunits. Secondly, transmembrane domains may act in a more specific manner by nucleating the oligomerization process. This is the case for the class II MHC molecules (Cosson & Bonifacino, 1992), in which the oligomerization of the x and β chains is probably initiated by an interaction between the transmembrane domains, involving several glycine residues on the same face of a putative x-helix. In the case of rabies virus G, there is a threonine in position 449, right in the middle of the putative hydrophobic transmembrane domain. The burial of the polar hydroxyl group in the hydrophobic interior of the bilayer would be very unfavourable from an energetic point of view. Therefore, this hydroxyl group could be involved in interchain hydrogen bonds in the trimeric structure. Together with our results, this suggests a direct interaction between the transmembrane domains of rabies virus G that would assist the correct folding of the ectodomain in the N form.

Finally, this work shows that, although it may provide some structural information on the I state, the soluble ectodomain of rabies virus G expressed in cells will be of little help for the study of low pH-induced structural transitions and is probably not the best tool for biophysical investigations. An alternative approach could be to cleave the soluble ectodomain by limited proteolysis of the glycoprotein at the virus surface. However, despite numerous attempts, this approach has been unsuccessful (Y. Gaudin and C. Tuffereau, unpublished results).

This, of course, greatly impairs the eventual structural study of rabies virus G and consequently our understanding of its functions at a molecular level. However, it remains possible that some smaller G fragments may provide relevant structural data. Such an approach has been successful for soluble subdomains of influenza virus HA2 (Chen et al., 1995) and human immunodeficiency virus gp41 (Weisshorn et al., 1996).

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References


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