

## Analysis of the *Drosophila gypsy* endogenous retrovirus envelope glycoprotein

Yolande Misseri,<sup>1</sup> Martine Cerutti,<sup>2</sup> Gérard Devauchelle,<sup>2</sup> Alain Bucheton<sup>1</sup> and Christophe Terzian<sup>1,3</sup>

Correspondence  
Christophe Terzian  
cterzian@snv.jussieu.fr

<sup>1</sup>Institut de Génétique Humaine CNRS, 141 rue de la cardonille, 34396 Montpellier cedex 5, France

<sup>2</sup>Laboratoire de Pathologie Comparée, 30380 Saint Christol les Ales, France

<sup>3</sup>Ecole Pratique des Hautes Etudes – UMR 7625, Université Paris 6, cc237, 7 quai Saint Bernard, 75252 Paris cedex 05, France

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*gypsy* is the only endogenous retrovirus of *Drosophila* whose infectious properties have been reported. Previous studies have shown an unexpected relationship between the gene encoding the putative envelope glycoprotein (Env) of *gypsy* and genes encoding the fusion protein of several baculoviruses. The fact that fusion proteins mediate membrane fusion suggests that Env of insect retroviruses might also have fusogenic properties. The results reported here indicate that *gypsy* Env mediates cell-to-cell fusion. Cleavage of the Env precursor was also studied; it is shown that this polypeptide is cleaved at a furin-like cleavage site. This is the first report that the *env*-like gene of insect retroviruses encodes a fusion protein.

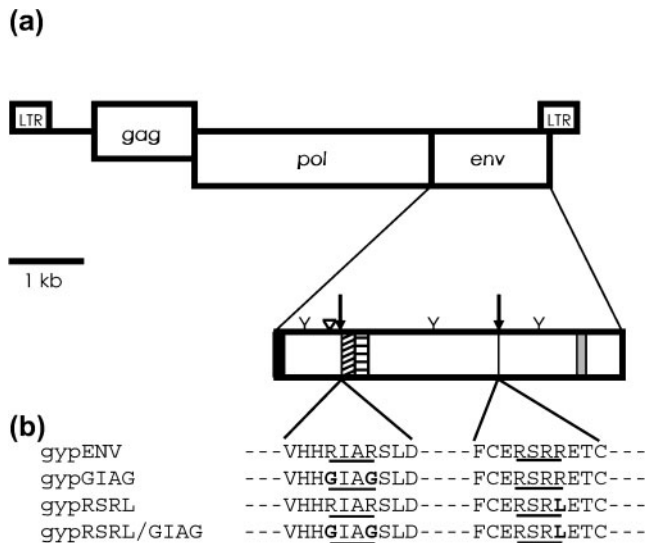
### INTRODUCTION

Endogenous retroviruses (ERVs) are present in the genomes of many eukaryotes. Because they are present in the germ line, they propagate vertically as cellular genes (Löwer *et al.*, 1996). They share a common genomic structure with exogenous retroviruses, consisting of two long terminal repeats (LTRs) flanking an internal region. The latter contains one to three major ORFs encoding structural and enzymic functions that are necessary for their replication. ERVs and exogenous retroviruses also have essential steps in their replication process in common: they are transcribed into an mRNA, which is reverse-transcribed into a cDNA in a pseudo-viral or viral particle and inserted into a new locus.

It is usually assumed that ERVs originate from exogenous retroviruses that once integrated into the germ-line chromosomes (Löwer *et al.*, 1996). This 'endogenization' event is at the heart of important differences between exogenous retroviruses and ERVs. ERV replication is a non-infectious process, as these elements are transmitted vertically as part of the host genome. Their behaviour is therefore very similar to that of transposable elements, known as LTR retrotransposons. Exogenous retroviruses replicate only in the cells of infected individuals. They are not present in the germ line and therefore propagate horizontally as a result of their infectious properties. Virus entry is mediated by the envelope glycoprotein (Env), which is encoded by the retroviral genome. Hence, the fact that most ERVs are defective for the *env* gene can be explained by the non-essential role of Env in their propagation.

However, it has been shown that several ERVs have an intact envelope and retain the ability to encode Env proteins. This is the case for the human ERVs HERV-K (Tönjes *et al.*, 1996) and HERV-Fc (Bénet *et al.*, 2003), porcine ERVs (Czauderna *et al.*, 2000), ovine ERV (Carlson *et al.*, 2003) and insect ERVs (Terzian *et al.*, 2001). Unexpectedly, infectious properties have been demonstrated experimentally for porcine ERV (Niebert & Tönjes, 2003) and for the *gypsy* insect ERV (IERV) that is present in *Drosophila melanogaster*. *gypsy* infectivity has been demonstrated by experiments in which larvae lacking active copies of *gypsy* were fed with either crude extracts (Kim *et al.*, 1994) or purified particles (Song *et al.*, 1994) that contained actively transposing *gypsy* ERVs: new copies of *gypsy* were observed in the genomes of the progeny of the treated flies. In addition, it has been shown that particles of the Moloney murine leukaemia virus pseudo-typed with *gypsy* Env are able to infect cultured *Drosophila* cells, albeit with a low efficiency (Teyssset *et al.*, 1998). One can ask why some ERV genomes have kept the ability to encode a functional envelope. One step towards an understanding of the evolutionary history of infectious ERVs is to estimate the fusogenic properties of their Env proteins and to map their functional domains.

*gypsy* Env is produced from a subgenomic spliced RNA (Péllisson *et al.*, 1994). Several putative domains that are typical of vertebrate retroviral glycoproteins have been described in this protein, namely a signal peptide, glycosylation sites and an RSRR endopeptidase cleavage site, although the latter is not followed by the hydrophobic



**Fig. 1.** (a) Organization of the *gypsy* ERV. Two LTRs flank a central region, which contains three ORFs corresponding to the *gag*, *pol* and *env* genes. The predicted 54 kDa protein encoded by the *env* gene is represented with potential *N*-glycosylation sites (Y) and the signal peptide (black box), followed by two putative furin-like cleavage sites (arrows), a putative fusion peptide (diagonally hatched box), a coiled-coil domain (horizontally hatched box) and a transmembrane domain (grey box). A putative subtilase-like cleavage site is indicated by a white arrowhead (see Discussion). (b) Sequence of the putative cleavage sites of the Env protein of *gypsy* and location of mutations. The sequences of the N and C terminus cleavage sites are shown. The RIAR and RSRR cleavage sites are underlined and the introduced mutations are shown in bold.

region that is characteristic of a fusogenic region (see Fig. 1a). Moreover, two reports have shown that there are significant similarities between IERV *env* gene products and a baculovirus fusion protein (Malik *et al.*, 2000; Rohrmann & Karplus, 2001). These findings led us to reconsider the structural analogy with the surface/transmembrane subunit glycoproteins of vertebrate retroviruses that was suspected previously. Hence, a comparative analysis between *gypsy* Env and the fusion protein of baculoviruses allowed us to map putative functional domains (Rohrmann & Karplus, 2001; Terzian *et al.*, 2001). Alignment of the predicted envelope fusion proteins of a number of baculoviruses, errantiviruses and paramyxoviruses has led to the identification of a region of high similarity (Misseri *et al.*, 2003). This region contains an RIAR furin-like consensus motif, contiguous with a hydrophobic sequence. This hydrophobic sequence contains a set of conserved amino acids followed by a predicted coiled-coil region, suggesting that it could be the fusion peptide.

In this paper, we have described a quantitative fusion assay for *gypsy* Env by using a baculovirus expression system in lepidopteran cells. The results indicated that this Env has fusogenic properties. We also identified a furin-like cleavage

site used for the processing of this protein that is different from the RSRR site that was described previously.

## METHODS

**Cell lines, viruses and antibodies.** *Spodoptera frugiperda* (Sf9) cells were maintained at 28 °C in TGV 3 rich medium (TC-100 modified medium) containing 5% fetal calf serum. Recombinant baculoviruses were propagated in Sf9 cells. For infection, cells ( $4 \times 10^6$  per 25 cm<sup>2</sup> flask) were inoculated with a viral suspension at  $5 \times 10^8$  p.f.u. After 1 h adsorption at room temperature, the viral inoculum was removed and fresh culture medium was added. Cells were further incubated at 28 °C.

The mAb 8E7 (Song *et al.*, 1994) was provided by Victor G. Corces (Johns Hopkins University, Baltimore, MD, USA). Generation and purification of the polyclonal antibody E78P were done by synt:em (www.syntem.com). The following peptides corresponding to different parts of *gypsy* Env were synthesized: (1) RQIAINSETQKQINKLTD, (2) SIQNLMDVSESGSPRLWF and (3) FNMTEGDGHKLEGGVNN. Two rabbits were immunized with the three peptides according to the manufacturer's protocol. Fractions containing the immunopurified antibodies were detected by measuring  $A_{280}$  and mixed. Immunoreactivity was verified by ELISA and shown to be restricted to the two most C-terminal peptides (2 and 3).

**Construction of recombinant baculoviruses.** For construction of the wild-type *gypsy* Env, a 1879 bp fragment encoding *gypsy* Env derived from the pDm111 plasmid (Baye *et al.*, 1984) by PCR amplification using synthetic primers WT *Bgl*III (5'-GAAGATCTG-AAAAGCATGTTCAACCCTCATGA-3') and WT *Acc*65I (5'-AAGG-TACCATTATCACTAGCCTTGTGGCGATA-3') was produced. The resulting fragment contained *Bgl*III and *Acc*65I restriction sites (underlined) and was cloned into the pGmAc 217 vector (Gaymard *et al.*, 1996) under the control of the polyhedrin gene promoter. This construct was named pGmEnv2. The integrity of the Env fragment was confirmed by sequencing.

For construction of *gypsy* Env mutants, two putative furin-like cleavage sites (RxxR) that have been described previously (Misseri *et al.*, 2003) were mutated. The RSRR putative cleavage site was mutated from pGmEnv2 by using a QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The mutation (shown in bold) was chosen in order to change Arg to Leu (RSRR→RSRL). Primers used were 5'-GAGCGTCTCGGCTCGA-AACTTGCGTG-3' and 5'-CACGCAAGTTTCGAGCCGAGACCGC-TC-3' and the plasmid obtained was named pGmRSRL. The RIAR putative cleavage site was mutated to GIAG by PCR amplification from pGmEnv2 using the primers Mut *Eco*RV (5'-GCTGATATCA-TGGAGATAAT-3') and Mut *Xba*I (5'-ATCTAGACTTCCAGCT-ATTCCATGGAGGAC-3'), which contained *Eco*RV and *Xba*I restriction sites (underlined), respectively. The template pGmEnv2 and PCR product were then digested by *Eco*RV and *Xba*I and ligated together. The resulting plasmid was named pGmGIAG. The double mutant pGmRSRL/GIAG was obtained by digesting the pGmGIAG and pGmRSRL plasmids with *Eco*RV and *Xba*I and inserting the fragment containing the GIAG mutation into the pGmRSRL plasmid. The mutation sequences were confirmed by sequencing.

Recombinant *gypsy* Env baculoviruses were generated in Sf9 cells by cotransfection of Sf9 cells with recombinant baculovirus transfer vectors and *Autographa californica multipolyhedrosis virus* (AcMNPV) DNA, using the lipofection method (Felgner & Ringold, 1989). Recombinant baculoviruses were plaque-purified by standard techniques (O'Reilly *et al.*, 1992) and designated gypENV, gypRSRL, gypGIAG and gypRSRL/GIAG.

**Env expression and maturation.** One hour prior to infection, cells were seeded in 25 cm<sup>2</sup> flasks in 4 ml serum-free medium to allow attachment to the flask. Cells were then infected with a viral suspension at an m.o.i. of 10 and left for 1 h for virus adsorption. Next, 4 ml serum-rich medium was added and cells were incubated at 27 °C. Cells were collected 3 days later and washed with 500 µl PBS. A 10 µl aliquot was deposited in each well of an eight-well slide and treated for immunofluorescence.

**Immunofluorescence.** Cells deposited in eight-well slides were fixed for 2 min in methanol at -20 °C and washed three times for 10 min each with PBT (0.5% BSA, Triton X-100 in PBS). Cells were incubated with 5% horse serum in PBT for 1 h and incubated overnight with the primary antibody E78P. Samples were then washed in PBT four times for 10 min each and incubated for 30 min with an fluorescein isothiocyanate (Jackson ImmunoResearch Laboratory)-conjugated anti-rabbit secondary antibody. The slide was mounted by using Mowiol (Calbiochem) for observation with a Leica SP2 DMIRB confocal microscope.

**Western blotting.** Infected and uninfected cells were washed and resuspended in 200 µl RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid sodium salt, 0.1% SDS, 50 mM Tris/HCl (pH 8), 1 mM dithiothreitol] and left for 30 min on ice. Samples were sonicated twice for 5 s each. A 100 µl aliquot of each sample was deglycosylated with *N*-glycosidase F (PNGase F; New England Biolabs) and 5 µl of each sample was mixed with 5 µl 2 × Laemmli. A 5 µl aliquot of each protein extract was run on a 12% acrylamide gel and electroblotted onto nitrocellulose (Optitran BA-S 83; Schleicher & Schuell BioScience). Env was detected by using the mAb 8E7, provided by V. G. Corces (1:100 dilution for 1 h at room temperature). Antigen detection following incubation of blots with a horseradish peroxidase-conjugated second antibody (anti-mouse Ig from sheep, 1:3000 dilution for 20 min at room temperature; Amersham Biosciences) was carried out using ECL (Amersham Biosciences).

Ovaries were extracted from flies as described previously (Song *et al.*, 1994). Proteins from ovaries were extracted following the same protocol as for cells.

**Cell labelling and fusion assay.** For labelling of cells, infected and uninfected Sf9 cells were collected and resuspended in a serum-free medium. Calcein (485/525) acetoxymethyl (AM) ester and Vybrant DiI (549/565) were obtained from Molecular Probes. To monitor dye redistribution as a result of fusion, gypENV-expressing Sf9 cells were loaded with calcein-AM (green fluorescence) following the manufacturer's protocol. The AM ester penetrates into cells, where it is hydrolysed to form the impermeant dye. Uninfected cells grown in the same way were harvested and labelled with Vibrant DiI, a fluorescent lipophilic tracer (orange fluorescence). Once applied to cells, the dyes diffuse laterally within the plasma membrane, resulting in staining of the entire cell. Transfer of this probe between intact membranes is usually negligible.

Calcein-AM (0.5 µl of a 0.5 µg µl<sup>-1</sup> solution in DMSO) and Vybrant DiI (5 µl of a 1 mM solution) were added to 1 ml infected and uninfected Sf9 cells, respectively. Cells were incubated for 15–20 min at 27 °C in the dark. Cells were then washed twice with serum-free medium and resuspended in serum-rich medium.

For the quantitative fusion assay, infected and uninfected cells were mixed in a 24-well culture dish at a final concentration of 5 × 10<sup>5</sup> cells and incubated for at least 2.5 h at 28 °C. Fusion was observed at pH 6. Different ratios of infected and uninfected cells were tested. Cells were then washed and resuspended in 500 µl PBS. Fusions were observed by using a confocal microscope and quantified by using a Becton Dickinson FACScan analytic flow cytometer.

Unlabelled cells were handled similarly and used as controls for intrinsic fluorescence. Compensation was made for optimal separation of fluorescence signals using labelled infected and uninfected Sf9 cells. Cell debris was excluded from the analysis. Each analysis was carried out on 10 000 cells.

## RESULTS

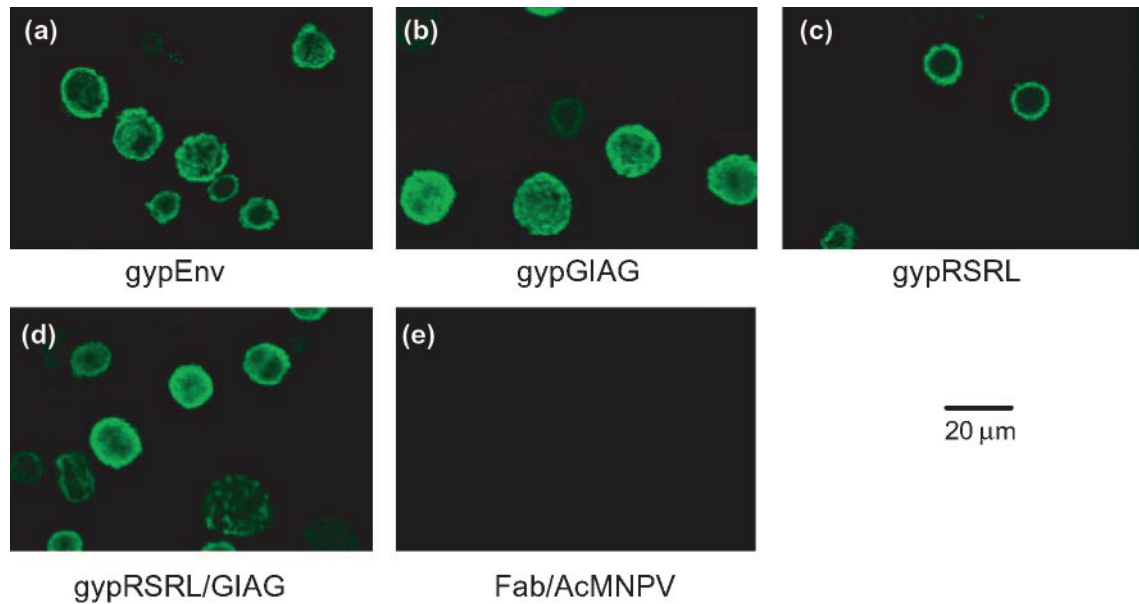
### Mutations at the putative cleavage sites of the *gypsy* Env do not affect its localization to the cell membrane

The *env* gene of *gypsy* (Fig. 1a) was inserted into the AcMNPV baculovirus genome as described in Methods to obtain a recombinant baculovirus named gypENV. In order to study the role of the two putative furin-like cleavage sites, recombinant baculoviruses harbouring mutated *gypsy* Env proteins were constructed (Fig. 1b). Three mutants were obtained. The first had a mutation in the RSRR site, where the last Arg was substituted by a Leu (RSRR→RSRL). In the second mutant, the two Arg residues of the RIAR putative cleavage site were replaced by two Gly residues (RIAR→GIAG). The third mutant had both cleavage sites mutated. The three new recombinant baculoviruses obtained were named gypRSRL, gypGIAG and gypRSRL/GIAG, respectively.

Sf9 cells were infected with each recombinant baculovirus. Three days after infection, cells were fixed and analysed for expression of the different Env proteins by using the E78P antibody raised against the C terminus of the protein. Observations using a confocal microscope (Fig. 2a) showed large amounts of wild-type Env expressed at the surface of cells infected with gypENV. Immunofluorescent detection also showed the presence of the mutated Env proteins at the surface of cells infected with gypRSRL, gypGIAG or gypRSRL/GIAG (Fig. 2b–d, respectively). No staining was detected in the negative control recombinant baculovirus Fab/AcMNPV, which expressed the Fab protein under the control of the polyhedrin promoter (Fig. 2e) (Bès *et al.*, 2001). The signal and localization observed with the mutant proteins were identical to those observed with wild-type Env. The mutations did not seem to affect the general conformation of the protein significantly, as the antibody was able to detect each mutant protein at the cell surface.

### *gypsy* Env is glycosylated and processed in the ovaries of flies

We studied the expression of *gypsy* Env in the ovaries of flies. In *D. melanogaster*, *gypsy* is controlled by the host gene *flamenco* (*flam*) (Prud'homme *et al.*, 1995). In order to identify the proteins of *gypsy*, we carried out Western blot experiments on total protein extracts of ovaries from females of MG stocks that were homozygous for a permissive allele of *flam* and contained several active proviruses of *gypsy* in their genome (Pélissou *et al.*, 1994; Prud'homme *et al.*, 1995). *gypsy* is known to be highly expressed in the ovaries of such females and to transpose at high frequency



**Fig. 2.** Immunofluorescent analysis of cells infected with wild-type gypEnv (a), mutant (b, c, d) and negative control Fab/AcMNPV (e) recombinant baculoviruses. Localization of *gypsy* Env was revealed by using the E78P antibody as described in Methods. The figure shows optical sections of cells obtained with a Leica SP2 DMIRB confocal microscope (excitation wave 488 nm, emission wave 500–535 nm).

in their progeny. We compared the products of the *env* gene from *MG* ovaries with those from the ovaries of females of the *wOR(P)* stock, which does not contain an active copy of *gypsy* (Chalvet *et al.*, 1999). The results are shown in Fig. 3(a). No bands were detected in protein extracts of the ovaries of *wOR(P)* females (Fig. 3a, lanes 3 and 4). In contrast, extracts of the ovaries from *MG* homozygous females were rich in actively transposing copies of *gypsy* (Fig. 3a, lanes 1 and 2) and Env was detected in these extracts. Three bands were detected in lane 1, which contained non-deglycosylated extracts. The largest may correspond to the uncleaved native protein. This was the glycosylated form of the protein, as it disappeared when the extract was deglycosylated (Fig. 3a, lane 2). The second-largest band observed (Fig. 3a, lanes 1 and 2) may correspond to the non-glycosylated form of the native protein, which has a theoretical size of 54 kDa. Interestingly, the third band (slightly less than 50 kDa; Fig. 3a, lane 2) corresponded to the expected size of the processed protein when cleaved at the RIAR site, as the theoretical size of the native C-terminal polypeptide is 42.9 kDa.

#### ***gypsy* Env is glycosylated and cleaved at the RIAR site in cultured Sf9 cells**

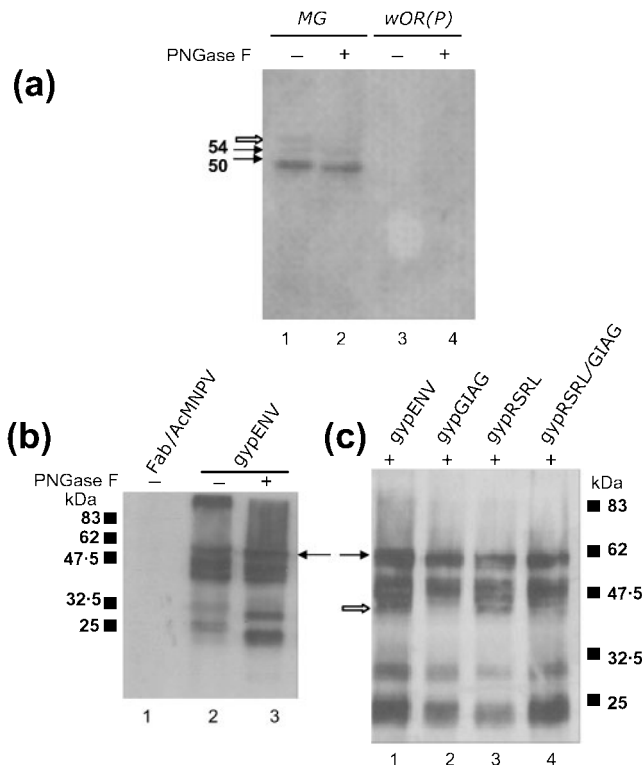
Protein expression in cells infected with the recombinant baculoviruses was analysed by Western blotting. Expression was detected by using mAb 8E7. No signal was observed in total protein extracts of cells infected with the control recombinant baculovirus Fab/AcMNPV (Fig. 3b, lane 1). Lanes 2 and 3 in Fig. 3(b) show the *gypsy* Env proteins

with or without deglycosylation treatment with PNGase F endoglycosidase, respectively. A band of 54 kDa was detected that may correspond to the native protein observed in ovary extracts (Fig. 3a). A faster-moving band with a size greater than 40 kDa could correspond to the product produced by cleavage at the RIAR site and another band of between 20 and 25 kDa to the product produced by cleavage at the RSRR site, as the theoretical sizes of these polypeptides are 42.9 and 20 kDa, respectively. When the extract was deglycosylated, a significant band-shift was observed for the faster-moving bands, whereas no shift was observed for the bands of between 40 and 60 kDa. Lanes 1–4 in Fig. 3(c) show expression of the gypENV, gypRSRL, gypGIAG and gypRSRL/GIAG deglycosylated proteins, respectively. The pattern appeared to be complex, but no variation between gypENV and gypRSRL was detected, indicating that the RSRR site did not seem to be involved in processing of the protein. However, the band of greater than 40 kDa that was present in lane 1 (gypENV) disappeared in the gypGIAG and the gypRSRL/GIAG mutants. These results suggested that, in Sf9 cells, *gypsy* Env is glycosylated and that the mutations that we designed did not affect glycosylation (data not shown).

Taken together, these results showed that the *in vivo* cleavage site of *gypsy* seems to correspond to RIAR.

#### ***gypsy* Env has fusogenic properties**

We studied the fusogenic properties of *gypsy* Env in Sf9 cells. Cells infected with recombinant baculovirus expressing either the Env protein of *gypsy* or the Fab control



**Fig. 3.** (a) Western blot analysis of protein extracts from the ovaries of *MG* homozygous females, which contain approximately 15 active copies of *gypsy*, and from the ovaries of *wOR(P)* homozygous females, which lack active *gypsy* elements. Extracts were deglycosylated with PNGase F (+) or not (-). Protein extracts were analysed by SDS-PAGE and immunoblotted with the anti-Env mAb 8E7. The protein band present in the extract not treated with PNGase F and missing in the deglycosylated extract is indicated by an open arrow. Sizes are indicated in kDa. (b) Expression of *gypsy* wild-type Env proteins in Sf9 cells infected with *gypsy* wild-type recombinant baculoviruses. Extracts were treated (+) or not (-) with PNGase F as indicated. Fab/AcMNPV, negative control. (c) Expression of *gypsy* wild-type and mutant Env proteins in Sf9 cells infected with *gypsy* wild-type recombinant baculoviruses. The 54 kDa protein that may correspond to the native protein is indicated by a black arrow and the protein missing in the gypGIAG- and gypGIAG/RSRL-infected cell extracts is indicated by an open arrow.

protein were labelled with a cytoplasmic green fluorescent dye (Calcein-AM) as described in Methods. Uninfected cells were labelled with a membrane red fluorescent dye (Vybrant DiI). After 2.5 h co-culture of infected and uninfected cells, single-stained cells and double-fluorescent fused cells were observed by confocal microscopy. Several fused cells were observed, indicated by their increased size and dual fluorescence (data not shown).

The baculovirus vector has its own fusion protein, GP64, which has already been shown to be fusogenic (Monsma *et al.*, 1996). Hence, cells infected with gypEnv had two

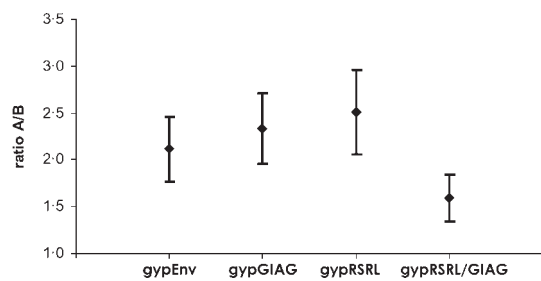
glycoproteins at their membranes: GP64 and *gypsy* Env. Quantification of fusion was thus necessary to determine the effects of the presence of *gypsy* Env.

FACS analysis was carried out after 2.5 h co-culture of infected and uninfected cells. Sf9 cells labelled with Vybrant DiI and cells labelled with calcein-AM were analysed as controls for non-specific dye transfer or cellular autofluorescence. To determine the optimal fusion conditions, co-cultures with varying ratios of uninfected and infected Sf9 cells with gypENV or Fab/AcMNPV were prepared. The highest percentage of fusion was obtained with a ratio of 2:3 of infected versus uninfected cells (data not shown).

The ratio of percentage of fused cells after infection with gypENV to percentage of fused cells after infection with Fab/AcMNPV was determined. Seven independent repeats of the experiment were done and the mean  $\pm$  SEM was calculated. The results indicated that cells expressing both envelope glycoproteins, GP64 and *gypsy* Env, were twice as fusogenic as those expressing only GP64 or Fab/AcMNPV at their surface. Non-parametric statistical analyses of these results using both Kendall's and Spearman's tests gave highly significant scores (Kendall's test:  $z=2.553$ ,  $P=0.01$ ; Spearman's test:  $z=2.187$ ,  $P=0.03$ ). Therefore, the *gypsy* Env was able to mediate cell-to-cell fusion.

The fusion capabilities of the putative cleavage site mutants were also quantified in the same way. Six independent experiments were done for each mutant. The results showed that none of the mutants lost its fusion capabilities (Fig. 4; Kruskal-Wallis test,  $\chi^2=3.1$ ,  $df=3$ ,  $P>0.30$ ).

All of these results indicated that *gypsy* Env has fusion



**Fig. 4.** Quantitative flow-cytometry analysis of fusion. Infected and uninfected cells were labelled green and red, respectively, and mixed as described in Methods. Fusion events were analysed by flow cytometry. The ratio of the percentage of fused cells after infection with the recombinant baculovirus expressing *gypsy* Env (wild-type or mutated) (A) to the percentage of fused cells after infection with Fab/AcMNPV (B) was determined. The mean value  $\pm$  SEM of the ratio A/B, obtained after six independent repetitions of the experiment, is shown. No statistically significant difference in fusogenic properties was observed between the wild-type and mutant Env-expressing cells.

properties that are not affected by mutations at the RIAR or RSRR sites.

## DISCUSSION

In this report, we have demonstrated that *gypsy* Env is a glycosylated fusion protein that is localized at the plasma membranes of Sf9 cells infected with gypEnv recombinant baculoviruses. To our knowledge, this is the first demonstration that the Env protein of an IERV is able to induce membrane fusion.

The results also give new insight into the structure and presence of functional domains in *gypsy* Env. It was assumed previously that the cleavage site of *gypsy* Env was RSRR, by analogy with vertebrate retroviral surface and transmembrane subunit glycoproteins (Péllisson *et al.*, 1994). In contrast to this assumption, our results indicated that cleavage occurs at the RIAR site in lepidopteran cells and suggest that this site is also used in *Drosophila* ovaries. However, gypEnv, the wild-type Env protein, and gypGIAG, gypRSRL and gypGIAG/RSRL, which contained mutations affecting the putative cleavage sites of this protein, had similar abilities to cause cell-to-cell fusion, suggesting that cleavage of the protein at these sites is not required for its fusogenic properties.

The fact that cleavage at the RIAR site is not necessary for the fusogenic properties of *gypsy* Env is unusual for an envelope protein. Indeed, other data have shown that these proteins are synthesized as inactive precursors that must be cleaved by host proteases for activity. It has been reported that the baculoviral fusion proteins LD130 and SE8 are truncated downstream of the RRHR and RSKR furin-like cleavage sequences, respectively, and that cleavage is necessary for fusogenic activity of LD130 (Ijkel *et al.*, 2000; Pearson *et al.*, 2002). However, it was shown recently that foamy viral cores that have incorporated cleavage-deficient Env proteins can be transferred from cell to cell (Heinkelein *et al.*, 2003). Three hypotheses could explain why the mutations that we introduced into the putative cleavage sites of *gypsy* Env did not affect the fusogenic properties of this protein: (i) cell-to-cell fusion does not require cleavage of *gypsy* Env, whereas this cleavage is required for virus-to-cell fusion, which was not taken into account in our experimental procedures; (ii) the overproduction of uncleaved *gypsy* wild-type and mutant Env proteins in the baculovirus expression system permitted cell-to-cell fusion, which would not be the case in physiological conditions; (iii) cleavage does not occur at the RIAR site, but an alternative site is recognized and cleaved by an endoprotease that is active only under specific conditions. In fact, detailed sequence analysis of *gypsy* Env allowed us to detect a subtilase-like cleavage sequence RKLL just upstream of RIAR (Fig. 1b). This sequence is well-conserved among the Env proteins of *gypsy* from various *Drosophila* species. It was shown recently that the glycoprotein precursors of three different viruses were

processed proteolytically by the subtilase SKI-1 (Beyer *et al.*, 2003; Lenz *et al.*, 2001; Vincent *et al.*, 2003), and a subtilase-like sequence is present in the *D. melanogaster* genome database (GenBank accession no. AAM20922). It would thus be interesting to test whether this site is cleaved in *gypsy* Env *in vivo*.

Why an ERV has maintained the capacity to encode a fusogenic glycoprotein remains a puzzling question. It has been shown previously that *gypsy* Env does not seem to be required for *gypsy* proviral multiplication in the germ line (Chalvet *et al.*, 1999). One can suggest that *gypsy* of *D. melanogaster* is an infectious retrovirus that has only recently been 'endogenized'. Hence, the evolutionary history and function of *gypsy* Env remain to be determined.

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## REFERENCES

- Bayev, A. A., Jr, Lyubomirskaya, N. V., Dzhumagaliev, E. B., Ananiev, E. V., Amiantova, I. G. & Ilyin, Y. V. (1984). Structural organization of transposable element mdg4 from *Drosophila melanogaster* and a nucleotide sequence of its long terminal repeats. *Nucleic Acids Res* **12**, 3707–3723.
- Bénit, L., Calteau, A. & Heidmann, T. (2003). Characterization of the low-copy HERV-Fc family: evidence for recent integrations in primates of elements with coding envelope genes. *Virology* **312**, 159–168.
- Bès, C., Cerutti, M., Briant-Longuet, L. & 9 other authors (2001). The chimeric mouse-human anti-CD4 Fab 13B8.2 expressed in baculovirus inhibits both antigen presentation and HIV-1 promoter activation. *Hum Antibodies* **10**, 67–76.
- Beyer, W. R., Pöpplau, D., Garten, W., von Laer, D. & Lenz, O. (2003). Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. *J Virol* **77**, 2866–2872.
- Carlson, J., Lyon, M., Bishop, J. & 7 other authors (2003). Chromosomal distribution of endogenous Jaagsiekte sheep retrovirus proviral sequences in the sheep genome. *J Virol* **77**, 9662–9668.
- Chalvet, F., Teyssset, L., Terzian, C., Prud'homme, N., Santamaria, P., Bucheton, A. & Péllisson, A. (1999). Proviral amplification of the *Gypsy* endogenous retrovirus of *Drosophila melanogaster* involves env-independent invasion of the female germline. *EMBO J* **18**, 2659–2669.
- Czuderna, F., Fischer, N., Boller, K., Kurth, R. & Tönjes, R. R. (2000). Establishment and characterization of molecular clones of porcine endogenous retroviruses replicating on human cells. *J Virol* **74**, 4028–4038.
- Felgner, P. L. & Ringold, G. M. (1989). Cationic liposome-mediated transfection. *Nature* **337**, 387–388.
- Gaymard, F., Cerutti, M., Horeau, C., Lemaillet, G., Urbach, S., Ravallec, M., Devauchelle, G., Sentenac, H. & Thibaud, J.-B. (1996). The baculovirus/insect cell system as an alternative to *Xenopus*

- oocytes. First characterization of the AKT1 K<sup>+</sup> channel from *Arabidopsis thaliana*. *J Biol Chem* **271**, 22863–22870.
- Heinkelein, M., Rammling, M., Juretzek, T., Lindemann, D. & Rethwilm, A. (2003). Retrotransposition and cell-to-cell transfer of foamy viruses. *J Virol* **77**, 11855–11858.
- IJkel, W. F. J., Westenberg, M., Goldbach, R. W., Blissard, G. W., Vlak, J. M. & Zuidema, D. (2000). A novel baculovirus envelope fusion protein with a proprotein convertase cleavage site. *Virology* **275**, 30–41.
- Kim, A., Terzian, C., Santamaria, P., Pélisson, A., Prud'homme, N. & Bucheton, A. (1994). Retroviruses in invertebrates: the *gypsy* retrotransposon is apparently an infectious retrovirus of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **91**, 1285–1289.
- Lenz, O., ter Meulen, J., Klenk, H.-D., Seidah, N. G. & Garten, W. (2001). The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. *Proc Natl Acad Sci U S A* **98**, 12701–12705.
- Löwer, R., Löwer, J. & Kurth, R. (1996). The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proc Natl Acad Sci U S A* **93**, 5177–5184.
- Malik, H. S., Henikoff, S. & Eickbush, T. H. (2000). Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res* **10**, 1307–1318.
- Misseri, Y., Labesse, G., Bucheton, A. & Terzian, C. (2003). Comparative sequence analysis and predictions for the envelope glycoproteins of insect endogenous retroviruses. *Trends Microbiol* **11**, 253–256.
- Monsma, S. A., Oomens, A. G. P. & Blissard, G. W. (1996). The GP64 envelope fusion protein is an essential baculovirus protein required for cell-to-cell transmission of infection. *J Virol* **70**, 4607–4616.
- Niebert, M. & Tönjes, R. R. (2003). Molecular cloning and functional characterization of infectious PERV and development of diagnostic tests. *Curr Top Microbiol Immunol* **278**, 217–237.
- O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1992). *Baculovirus Expression Vectors: a Laboratory Manual*. New York: W. H. Freeman.
- Pearson, M. N., Russell, R. L. Q. & Rohrmann, G. F. (2002). Functional analysis of a conserved region of the baculovirus envelope fusion protein, LD130. *Virology* **304**, 81–88.
- Pélisson, A., Song, S. U., Prud'homme, N., Smith, P. A., Bucheton, A. & Corces, V. G. (1994). *Gypsy* transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila flamenco* gene. *EMBO J* **13**, 4401–4411.
- Prud'homme, N., Gans, M., Masson, M., Terzian, C. & Bucheton, A. (1995). *Flamenco*, a gene controlling the *gypsy* retrovirus of *Drosophila melanogaster*. *Genetics* **139**, 697–711.
- Rohrmann, G. F. & Karplus, P. A. (2001). Relatedness of baculovirus and *gypsy* retrotransposon envelope proteins. *BMC Evol Biol* **1**, 1.
- Song, S. U., Gerasimova, T., Kurkulos, M., Boeke, J. D. & Corces, V. G. (1994). An env-like protein encoded by a *Drosophila* retroelement: evidence that *gypsy* is an infectious retrovirus. *Genes Dev* **8**, 2046–2057.
- Terzian, C., Pélisson, A. & Bucheton, A. (2001). Evolution and phylogeny of insect endogenous retroviruses. *BMC Evol Biol* **1**, 3.
- Teyssset, L., Burns, J. C., Shike, H., Sullivan, B. L., Bucheton, A. & Terzian, C. (1998). A Moloney murine leukemia virus-based retroviral vector pseudotyped by the insect retroviral *gypsy* envelope can infect *Drosophila* cells. *J Virol* **72**, 853–856.
- Tönjes, R. R., Löwer, R., Boller, K. & 12 other authors (1996). HERV-K: the biologically most active human endogenous retrovirus family. *J Acquir Immune Defic Syndr Hum Retrovirol* **13** (Suppl. 1), S261–S267.
- Vincent, M. J., Sanchez, A. J., Erickson, B. R., Basak, A., Chretien, M., Seidah, N. G. & Nichol, S. T. (2003). Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J Virol* **77**, 8640–8649.