The EMBO Journal vol.15 no.21 pp.5783–5788, 1996

Direct interaction between the envelope and matrix proteins of HIV-1

Pierre Cosson

Basel Institute for Immunology, Grenzacherstrasse 487, Postfach CH-4005 Basel, Switzerland

The incorporation of the envelope (env) glycoprotein of the human immunodeficiency virus type 1 (HIV-1) into budding virions has been proposed to be mediated by an interaction between its cytoplasmic domain and the matrix protein of HIV-1. However, this interaction was never directly demonstrated and its role in the biogenesis of HIV-1 virions is still debated. Here, a direct interaction is reported between the matrix protein of HIV-1 and the cytoplasmic domain of the env protein of HIV-1. No interaction was seen with the env cytoplasmic domain of other retroviruses. The region of the HIV-1 env involved in the interaction was delineated by mutagenesis and is comprised of the C-terminal 67 amino acid residues of env. These results, as well as the analysis of mutants of the matrix protein, suggest that the interaction between the HIV-1 env and matrix proteins accounts for the specific incorporation of the env glycoprotein into HIV-1 virions.

Keywords: cytoplasmic domain/envelope/gag/HIV-1/matrix

Introduction

Enveloped animal viruses are released from infected cells by budding from a cellular membrane. The sorting of the viral glycoproteins to budding virions is generally believed to be mediated by an interaction between their cytoplasmic domain and a component of the viral core (Simons and Garoff, 1980). In certain virus systems, in particular the alphaviruses, this hypothesis is supported by the analysis of the phenotype of many mutant viruses. Thus, for Sindbis virus, mutations at several sites within the cytoplasmic domain of the spike glycoprotein E2 inhibit particle release, presumably by affecting the interaction with the viral capsid (Gaedik-Nitschko and Schlesinger, 1991; Ivanova and Schlesinger, 1993). Studies with chimeric viruses also suggest that there is a complementarity between the capsid protein and the E2 cytoplasmic domain (Lopez *et al.*, 1994). Images from cryoelectron microscopy of purified virus particles show a correspondence of spike trimers and nucleocapsid holes (Cheng *et al.*, 1995), a result consistent with the model in which the nucleocapsid acts as a docking site for the cytoplasmic part of E2. Similar results with various alphaviruses (e.g. Semliki Forest virus) suggest that an interaction between the cytoplasmic domain of the spike glycoproteins and the nucleocapsid is essential for virus release, and accounts for the specific incorporation of viral glycoproteins in budding virions. With the exception of Semliki Forest virus (Metsikkö and Garoff, 1990), this putative interaction was not reconstituted *in vitro*.

The picture is less clear for retroviruses, in particular for the human immunodeficiency virus type 1 (HIV-1) (Hunter and Swanstrom, 1990; Freed and Martin, 1995b). Structural evidence (Rao *et al.*, 1995) or crosslinking experiments (Gebhardt *et al.*, 1984) suggest in some cases a close proximity between the viral glycoprotein(s) and some viral core proteins, but analysis of the phenotype of mutant viruses has been complicated by the fact that expression of env is dispensable for the assembly and release of retroviruses (Hunter and Swanstrom, 1990). The specific incorporation of the env protein of HIV-1 in the virion has been proposed to be achieved through an interaction between its cytoplasmic domain and the viral matrix protein ($p17^{gag}$), but evidence derived from mutagenesis of the env and matrix proteins has accumulated both for and against this model in the past decade (Freed and Martin, 1995b; see also Discussion).

In this study a direct interaction is reported between the cytoplasmic domain of the env protein of HIV-1 and the matrix protein *in vitro*. Analysis of various mutants of the env and matrix proteins suggests that this interaction plays a key role in the specific incorporation of the env protein into budding HIV-1 virions.

Results

To study the interaction between the matrix and envelope proteins of HIV-1 a chimeric protein was constructed by fusing the cytoplasmic domain of the env protein to the C terminus of glutathione-*S*-transferase (GST–Env). The fusion protein was expressed in bacteria and purified on glutathione–Sepharose beads as described in Materials and methods. The matrix protein of HIV-1 was expressed in COS-1 cells, the cells were lysed in CHAPS buffer, and the cell lysates applied to the GST–Env beads. The matrix protein bound to GST–Env beads (Figure 1A), and ~50% of the matrix protein was depleted from the cell lysate (data not shown). The matrix protein did not bind to beads coated with GST or GST–Env3 (Figure 1A), a mutant where the 51 C-terminal amino acid residues of GST–Env have been deleted (see Figure 3A).

To test whether cellular factors were required for this interaction, a 6×His tag was transferred to the C-terminal end of the matrix protein (Ma6H). The protein was expressed in *Escherichia coli* and purified on Ni–NTA resin (Figure 1B). The purified Ma6H bound to GST–Env beads, but not to GST beads (Figure 1C), suggesting that the matrix protein can bind directly to the env cytoplasmic domain in the absence of viral or cellular cofactors.

To analyse more precisely this interaction a more quantitative assay was developed. For this, the *E.coli* β-galactosidase was transferred to the C terminus of the

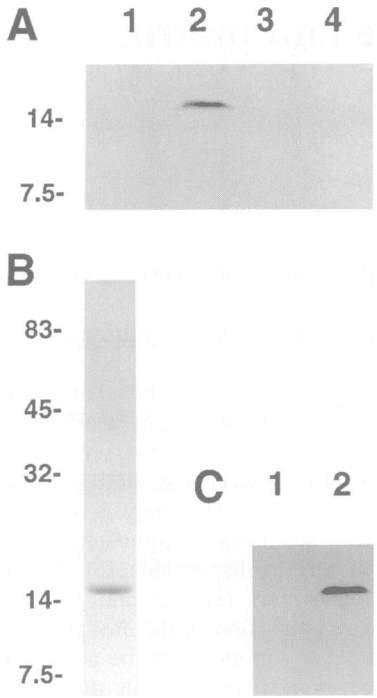

Fig. 1. Interaction of HIV-1 matrix protein with HIV-1 env cytoplasmic domain. (**A**) COS-1 cells expressing the matrix protein of HIV-1 were solubilized in CHAPS buffer and aliquots of the lysate were incubated with beads coated with equivalent amounts of GST (lane 1), GST–Env (lane 2) or GST–Env3 (lane 3). Bound matrix protein was detected by immunoblotting with 8E7, a monoclonal antibody to the matrix protein of HIV-1. No matrix protein was detected when lysates from non-transfected cells were applied to GST–Env beads (lane 4). (**B**) 6×His-tagged matrix protein was produced in bacteria, purified on Ni–NTA gel, and eluted in the presence of 0.5 M imidazole. The purified protein was analysed by SDS–polyacrylamide gel electrophoresis (PAGE; 13% gels) and stained with Coomassie blue R-250. (**C**) Purified Ma6H protein was incubated with beads coated with GST (lane 1) or GST–Env (lane 2) and the bound matrix protein was detected by protein immunoblotting as described above. Protein standards are indicated at left in kDa.

HIV-1 matrix protein (MaGal) and expressed in COS-1 cells. Aliquots of a cell lysate containing MaGal were incubated with beads coated with increasing amounts of GST–Env. Unbound MaGal was detected in the supernatant by a colorimetric assay (Figure 2A), and all the galactosidase activity lost from the supernatant could be detected bound to the beads (data not shown). Binding of MaGal to GST–Env beads was saturable with an apparent K_d of ~30 nM (Figure 2B). No interaction was observed between MaGal and GST (Figure 2A) or between GST–Env and β-galactosidase expressed in COS-1 cells (data not shown).

Making use of this system, the effect of various deletions was tested to delineate the region of the env cytoplasmic domain involved in the interaction (Figure 3A). The most remarkable feature of the env cytoplasmic domain is the presence in its C-terminal region of two putative amphipathic helices extending from residues 64 to 88, and 118 to 150 (Venable *et al.*, 1989; see Figure 3A), though it should be emphasized that the exact boundaries of such amphipathic helices can only be estimated by analysis of the protein sequence. A deletion of the C-terminal 12 residues (GST–Env1) led to a marked decrease of the interaction with MaGal (Figure 3B). Further deletions (GST–Env2, 3 and 4) completely abolished the interaction (Figure 3B). In contrast, deletions of 55 (GST–Env5), 69 (GST–Env6) or 83 (GST–Env7) N-terminal residues did not decrease the ability of the env cytoplasmic domain to interact with MaGal (Figure 3C). The binding of MaGal was diminished by a deletion of 98 residues (GST–Env8) and abrogated by further deletions (GST–Env9, 10 and 11; Figure 3C). In cases where only partial binding of MaGal was observed (GST–Env1 and GST–Env8) the apparent affinity of MaGal for the mutated GST fusion protein could not be determined accurately, but it was at least 100-fold lower than the affinity of MaGal for full-length GST–Env. The insertion of various linker sequences between the GST and the Env portion did not modify the results of the binding experiments (data not shown), suggesting that loss of binding was not due to steric hindrance by the GST protein, but to the deletion of a region of Env essential for the interaction. These results demonstrate that a region composed of the 67 C-terminal residues of the env cytoplasmic domain is responsible for the interaction with the matrix protein. At least one putative amphipathic helix is comprised in this region (Venable *et al.*, 1989). However, several mutants with large segments of amphipathic helices did not show interaction with MaGal (e.g. GST–Env2 and GST–Env9), suggesting that the mere presence of an amphipathic helix in the Env cytoplasmic domain is not sufficient to allow specific interaction with the matrix protein.

Amphipathic helices, usually rich in positively charged amino acid residues, have been identified in the cytoplasmic domain of the env glycoprotein of other lentiviruses (Miller *et al.*, 1991), such as the equine infectious anaemia virus (EIAV). They are not seen in the env cytoplasmic domain of more distantly related retroviruses, such as the human T-cell leukaemia virus type 2 (HTLV-2) (Miller *et al.*, 1991). To test the specificity of the HIV-1 envelope–matrix interaction, the cytoplasmic domain of the env protein of EIAV and HTLV-2 were fused to the C terminus of glutathione-*S*-transferase. The GST–EIAV and GST–HTLV fusion proteins were expressed in bacteria and immobilized on glutathione–Sepharose beads, and lysates containing HIV-1 MaGal were incubated with the beads. No interaction was detected between HIV-1 matrix and either EIAV or HTLV-2 env cytoplasmic domain under the conditions used (Figure 4).

The effect of various mutations of the matrix protein on the envelope–matrix interaction was also tested. Various mutations in the N-terminal 100 amino acid residues of the HIV-1 matrix protein have been reported to affect the incorporation of the envelope into mature virions, while alterations at the C terminus of the matrix domain had no significant effect (Dorfman *et al.*, 1994). Accordingly, a deletion of residues 7–31 (MaGal-A) abrogated the interaction, while a deletion of amino acids 102–132 (MaGal-B) had no noticeable effect (Figure 5). More precisely a point mutation of Leu12 to Glu was shown to impair incorporation of env glycoprotein in virions (Freed and Martin, 1995a). The corresponding mutant (MaGal-C) exhibited markedly reduced *in vitro* binding to GST–Env beads (Figure 5), though some residual binding was still observed. Note that the amounts of GST–Env used in this experiment were higher than in previous experiments and that the apparent affinity of MaGal-C for GST–Env was

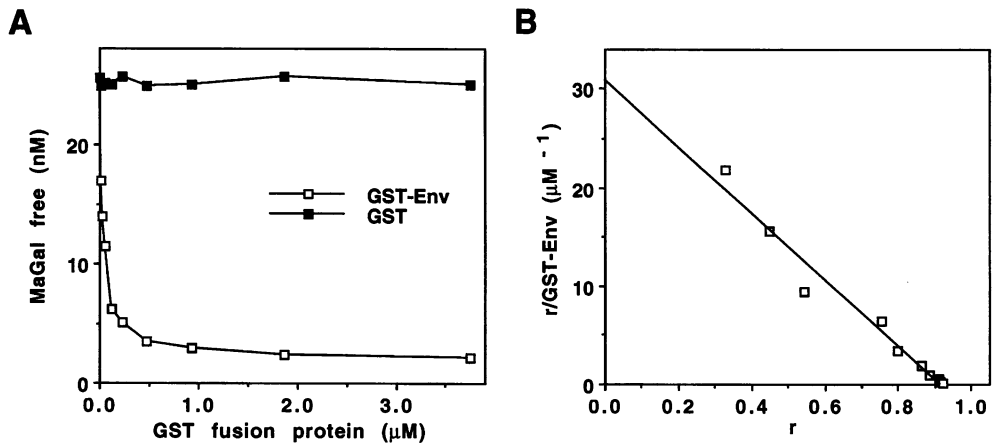

Fig. 2. Interaction of the MaGal fusion protein with the cytoplasmic domain of env. (**A**) COS-1 cells were transfected with DNA encoding a chimeric protein with the β-galactosidase of *E.coli* fused at the C terminus of the matrix protein of HIV-1 (MaGal), lysed in CHAPS buffer, and aliquots of the lysate incubated with glutathione–Sepharose beads coated with increasing amounts of GST (■) or GST–Env (□) fusion protein. Unbound galactosidase activity was measured in the supernatants. All the galactosidase activity lost from the supernatant was detected on the beads after five washes with CHAPS buffer (data not shown). The absolute concentration of MaGal was determined by immunoblotting by comparison with a solution of pure Ma6H of known concentration. (**B**) Scatchard analysis of MaGal binding to GST–Env shows a value for K_d of ~30 nM. r = MaGal free/bound.

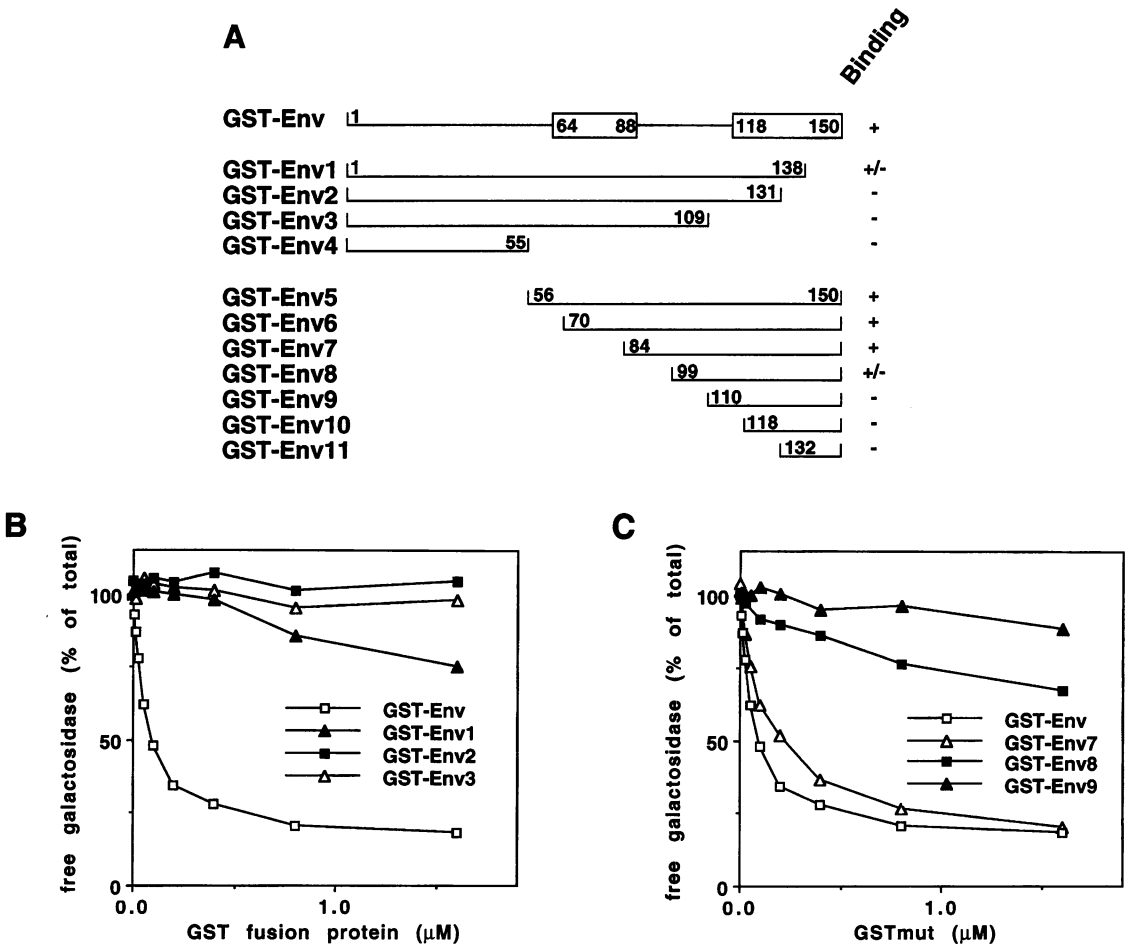

Fig. 3. Role of the C-terminal portion of the env cytoplasmic domain in the interaction with the matrix protein. (**A**) Schematic representation of GST–Env mutants. Position 1 corresponds to the first N-terminal charged residue of the env cytoplasmic domain of HIV-1. Boxes indicate regions with large helical hydrophobic moments (Venable *et al.*, 1991). The ability of various mutants to bind MaGal is indicated. (**B**) Interaction of MaGal with GST–Env mutants with C-terminal deletions. Interaction of MaGal with GST–Env (□), GST–Env1 (▲), GST–Env2 (■) and GST–Env3 (△) was determined as in Figure 2. The free galactosidase activity remaining in the supernatant was measured and is expressed as a percentage of the total activity in the cell lysate. In the conditions used the interaction of GST, GST–Env2, 3 and 4 with MaGal was indistinguishable (data not shown). (**C**) Interaction of MaGal with GST–Env mutants with N-terminal deletions. The interaction of MaGal with GST–Env (□), GST–Env7 (△), GST–Env8 (■) and GST–Env9 (▲) is shown. The interaction between MaGal and GST–Env5, 6 and 7 was identical (data not shown). No interaction was detected between MaGal and GST–Env10 and 11 (data not shown).

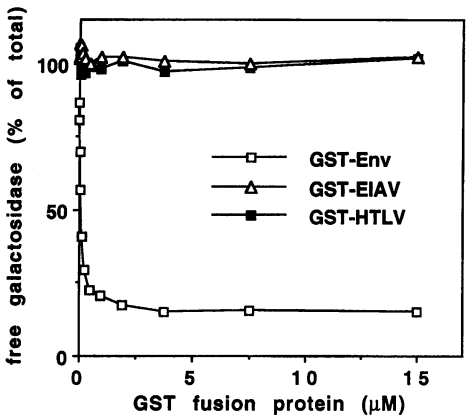

Fig. 4. No interaction between HIV-1 matrix and the env protein of other retroviruses. Binding experiments were done as described in the legend to Figure 2 using beads coated with GST protein fused to the cytoplasmic domain of the env protein of HIV-1 (GST–Env), HTLV-2 (GST–HTLV) or EIAV (GST–EIAV) as indicated. Unbound galactosidase activity was measured in the supernatants.

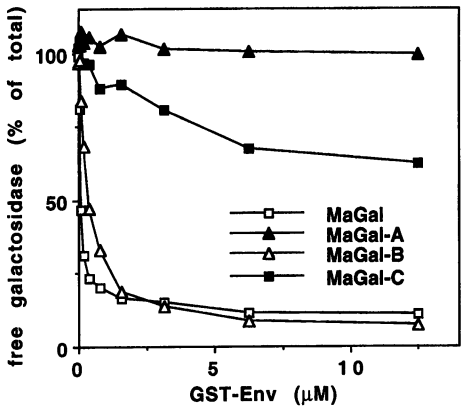

Fig. 5. Role of the N-terminal region of HIV-1 matrix in the interaction with the cytoplasmic domain of env. Lysates from COS-1 cells expressing MaGal (□), MaGal-A (Δ7–31, ▲), MaGal-B (Δ102–132, △), or MaGal-C (Leu12→Glu, ■) were assayed for galactosidase activity. Equivalent amounts of galactosidase activity were applied to GST–Env beads and the binding determined as in the legend to Figure 2. Note that higher amounts of GST–Env were used in this experiment.

at least 100 times lower than the affinity of wild-type MaGal for GST–Env.

Discussion

In this study a direct interaction is characterized *in vitro* between the cytoplasmic domain of the HIV-1 env protein and the viral matrix protein. Deletions in the C-terminal region of the env cytoplasmic domain abrogate the interaction, as well as some mutations in the N-terminal portion of the matrix protein. As discussed below these results show a good correlation with previously published reports where the phenotype of mutant viruses was analysed, and suggest that an interaction with the matrix protein directs the env protein to budding HIV-1 virions.

Various mutations in the viral matrix were reported to inhibit the incorporation of the full-length envelope glycoprotein into HIV-1 virions, in particular a point mutation of Leu12 to Glu (Freed and Martin, 1995a, 1996). The same mutation is shown here to inhibit strongly the ability of the matrix protein to interact with the cytoplasmic domain of the env protein. Similarly, and in agreement with the reported phenotype of other matrix mutants (Dorfman *et al.*, 1994), a deletion in the N-terminal region of the matrix protein abolished binding to the env cytoplasmic domain, while a deletion in the C-terminal region had no effect on the interaction.

The phenotype of mutant HIV-1 virus with deletions in the env cytoplasmic domain is more complex. Various groups have reported that truncations in the cytoplasmic domain of the env protein affect its incorporation into virions (Dubay *et al.*, 1992; Yu *et al.*, 1993; Freed and Martin, 1996). Other authors reported retained *in vitro* infectivity despite truncations in the C-terminal tail of env, though some of the mutants analysed exhibited reduced incorporation of env and reduced infectivity (Wilk *et al.*, 1992). One major reason for such discrepancies in the literature was recently elucidated: several groups reported that env mutants with large cytoplasmic deletions can be incorporated into virions in a matrix-independent manner (Freed and Martin, 1995a; Mammano *et al.*, 1995). It is not clear to date why the incorporation of env protein with short and long cytoplasmic domains should be governed by different rules. One possibility is that a distinct mechanism directs incorporation of env mutants with a short cytoplasmic domain into virions. Another possibility is suggested by the fact that large deletions also affect the endocytic signal(s) contained in the membrane-proximal region of the HIV-1 env cytoplasmic domain, and result in accumulation of endogenously synthesized env protein at the cell surface (Rowell *et al.*, 1995). Non-specific incorporation of env into budding virions might then be sufficient to allow formation of infectious virions. Irrespective of the precise mechanism involved, the current view is that mutated env proteins with short cytoplasmic domains can be incorporated into virions without interacting with the matrix protein, while the incorporation of wild-type HIV-1 env would require an interaction with the matrix protein (Freed and Martin, 1995b). The C-terminal region of the env protein would be crucial for this interaction as truncations of ~20 residues were shown to be sufficient to inhibit markedly the replication of the virus *in vitro* and to decrease the amount of env incorporated into mature virions (Dubay *et al.*, 1992; Yu *et al.*, 1993; Freed and Martin, 1996).

The results presented here demonstrate that a deletion of 12 C-terminal residues in the HIV-1 env cytoplasmic domain affects markedly its ability to interact with the matrix protein, while further truncations abrogate it. Thus, the mutations in the env glycoprotein which were previously reported to affect its incorporation in mature virions are shown here to inhibit the envelope–matrix interaction. The domain in HIV-1 env involved in the interaction with the HIV-1 matrix could not be determined precisely here, and it might comprise a major part of the HIV-1 env cytoplasmic domain including some regions with a very high hydrophobic moment as α-helices (Venable *et al.*, 1989).

Altogether, the results presented here support the notion that an interaction between the env cytoplasmic domain and the matrix protein plays a key role in the incorporation of the env protein into budding HIV-1 virions.

For other retroviruses an interaction between the env

cytoplasmic domain and a component of the viral core has also been proposed to direct the env proteins to budding virions. No clear homology was found between the HIV-1 env cytoplasmic domain and the env cytoplasmic domains of other enveloped viruses, with the exception of the closely related HIV-2 and SIV viruses. Thus, it is not surprising to observe no interaction between the HIV-1 matrix protein and the env cytoplasmic domain of the human T-cell leukaemia virus type 2, an oncovirus of the BLV-HTLV group. Amphipathic helices can be found in the env cytoplasmic domain of several lentiviruses. One of the most clear examples is the equine infectious anaemia virus (Miller *et al.*, 1991), but no interaction was observed between the EIAV env cytoplasmic domain and the HIV-1 matrix. This result illustrates the specificity of the interaction between HIV-1 matrix and env proteins. Further work will be necessary to establish the nature of the cytoplasmic motifs used by other retroviruses to direct their envelope proteins to budding virions.

Materials and methods

Construction and expression of mutant DNAs

cDNAs coding for the HIV-1 matrix protein (gag residues 1–132, NY5 isolate) or a fusion protein of matrix with *E.coli* β-galactosidase were subcloned into a modified version of the pCDM8 expression plasmid (Seed, 1987). All mutant molecules were constructed by polymerase chain reaction mutagenesis (Jones and Howard, 1990). For expression of MaGal constructs COS-1 cells (American Type Culture Collection, Rockville, MD) were plated on 150-mm culture dishes and transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation method as described previously (Hennecke and Cosson, 1993). After 16 h, transfected cells were trypsinized and plated on 35-mm culture dishes and allowed to grow for an additional 48 h. A construct coding for a fusion protein of full-length HIV-1 gag protein with β-galactosidase did not direct the production of significant amounts of soluble protein in transfected cells and could not be used in further experiments (data not shown).

To express 6×His tagged matrix protein (Ma6H), the matrix cDNA was subcloned into the polylinker of the bacterial expression vector pQE60 (Qiagen). Expression and purification of Ma6H on Ni–NTA resin were performed according to the manufacturer's instructions. Briefly, bacteria expressing Ma6H were lysed in phosphate-buffered saline (PBS) containing 1% Triton X-100 (PBS–TX100) and the lysates incubated with Ni–NTA resin. The resin was washed twice with PBS–TX100, twice with PBS, twice with PBS containing 50 mM imidazole, and once with PBS containing 100 mM imidazole; Ma6H was then eluted in PBS containing 0.5 M imidazole. The Ma6H was diluted in CHAPS buffer before incubation with beads coated with GST fusion protein.

To express GST–Env fusion proteins, HIV-1 env coding sequences (bru isolate) were cloned into the polylinker of the bacterial expression vector pGEX-3X (Pharmacia). Expression and purification of GST fusion proteins were carried out as already described (Cosson and Letourneur, 1994). The amounts of GST fusion proteins used in each experiment were normalized by measuring the GST activity in the bacterial lysate (Pharmacia) before its application to glutathione–Sepharose beads. Following the experiment, samples were analysed by SDS–polyacrylamide gel electrophoresis (PAGE; 13% gels) and stained with Coomassie Blue R-250 to determine the absolute concentration of GST fusion protein.

For GST–HTLV and GST–EIAV, synthetic oligonucleotides were cloned into the polylinker of the pGEX-3X vector to reconstitute a sequence coding for the cytoplasmic domain of the env protein of the human T-cell leukaemia virus type 2 (RQIQALPQRLQNRHNQYSLINPETML) or of the equine infectious anaemia virus (RIAGYGLRGLAVIIRICIRGLNLI-FEILRKMLDYIGRALNPG-TSHVSMPQYV), respectively.

Interaction between GST–Env and the matrix protein

COS-1 cells (4×10^6) expressing HIV-1 matrix were lysed in CHAPS buffer [10 mM CHAPS, 150 mM NaCl, 50 mM Tris, pH 7.4, and protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml phenylmethylsulphonyl fluoride)] and centrifuged for 15 min at 20 000 *g*. The addition of iodoacetamide (1.8 mg/ml) to the lysis buffer did not modify the results of the binding experiments (data not shown). Cell lysates were incubated for 2 h at 4°C with GST fusion proteins immobilized on Sepharose beads as indicated. Beads were washed five times in CHAPS buffer, and once in PBS. Proteins were eluted by boiling the beads in reducing SDS sample buffer, separated by SDS–PAGE (13% gels) and transferred to nitrocellulose membranes. Membranes were blocked with PBS containing low-fat milk (5%) and Tween-20 (0.1%) overnight and then incubated with a mouse monoclonal antibody to HIV-1 matrix protein (8E7). All immunoblotting experiments were also performed in parallel with a rabbit antiserum to an HIV-1 matrix peptide (residues 38–57) and yielded identical results (data not shown). Methods for SDS–PAGE (Laemmli, 1970), immunoblotting (Towbin *et al.*, 1979) and immunodetection by enhanced chemiluminescence (Amersham) have been described previously.

To test binding of MaGal to GST–Env beads, COS-1 cells expressing MaGal were lysed in CHAPS buffer and aliquots of the lysate incubated with beads coated with increasing amounts of GST fusion protein, as indicated. Free MaGal was measured in the supernatant by a colorimetric assay as described previously (Cosson *et al.*, 1991). All the galactosidase activity lost from the supernatant was detected on the beads after five washes with CHAPS buffer (data not shown).

Acknowledgements

I thank B.Verrier for the monoclonal antibody 8E7 to HIV-1 matrix; M.Ottman and J.L.Darlix for the DNA encoding HIV-1 matrix; M.Alizon for the DNA encoding HIV-1 env; and K.Ungewiss, F.Letourneur, J.Pieters and C.Steinberg for valuable discussions and critical review of the manuscript. The Basel Institute for Immunology was founded and is supported by F.Hoffmann-La Roche & Co. Ltd, CH-4005 Basel, Switzerland.

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Received on June 17, 1996; revised on July 10, 1996