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In vitro binding of simian immunodeficiency virus matrix protein to the cytoplasmic domain of the envelope glycoprotein

Julieta M. Manrique, José L. Affranchino, Silvia A. González *

Laboratorio de Virología, Facultad de Ciencias Exactas y Naturales, Universidad de Belgrano (UB), Villanueva 1324, C1426BMJ Buenos Aires, Argentina

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Abstract

Incorporation of the envelope (Env) glycoprotein into budding virions is a key step in the replication cycle of lentiviruses. Previously, we provided genetic and biochemical evidence indicating that Env packaging into simian immunodeficiency virus (SIV) particles is mediated by the association of the Env cytoplasmic domain (CD) with the matrix (MA) domain of Gag. In this study, we developed an in vitro binding assay that, based on recombinant proteins expressed in bacteria, allowed us to demonstrate the physical interaction between the SIV Env CD and the MA in the absence of other viral or cellular proteins. We show that this association is blocked by mutations in each of the interacting domains that have been reported to interfere in vivo with the incorporation of Env into SIV virions. Moreover, we determined that the binding of SIV MA to the Env CD is saturable with a dissociation constant of 7×10^{-7} M. Interestingly, the SIV MA is capable of specifically interacting in vitro with the human immunodeficiency virus type 1 Env CD, but not with that of the distantly related feline immunodeficiency virus. Our results strongly support the notion that the association between the SIV MA and Env CD plays a central role in the process of SIV Env incorporation into Gag-made particles.
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Keywords: Simian immunodeficiency virus; Matrix protein; Envelope glycoprotein cytoplasmic domain; Virus assembly

Introduction

The retroviral Gag proteins bear all the structural elements necessary for particle assembly (Hunter, 1994). In the case of the primate lentiviruses human and simian immunodeficiency viruses (HIV and SIV, respectively), Gag is first synthesized as a polyprotein precursor which, subsequent to virus budding, is proteolytically processed by the viral protease into the matrix (MA), capsid (CA), nucleocapsid and p6 proteins, as well as the p2 and p1 spacer peptides (Henderson et al., 1992). In addition to Gag processing, the formation of infectious lentivirus particles requires the incorporation of the viral envelope (Env) glycoprotein into the budding virions. The functional Env complex, composed of the surface (SU; gp120) and the transmembrane (TM; gp41) glycoproteins, is responsible for the specific binding of virions to the cellular CD4 and chemokine receptors, which triggers the fusion of the viral and target cell membranes and thus mediates virus entry (Chan and Kim, 1998).

The MA domain of the SIV Gag precursor has been shown to be implicated in a number of key roles during the virus life cycle: it provides the primary determinants for the proper targeting and association of the Gag precursor with the plasma membrane and is necessary for viral particle assembly as well (González and Affranchino, 1995, 1998; González et al., 1993, 1996). In addition, the SIV MA protein appears to be involved in the process of Env incorporation into virions. In this regard, we have provided genetic and biochemical evidence supporting the notion that the interaction between the SIV TM cytoplasmic region, which is 164-amino acid long, and the MA domain of Gag mediates the packaging of Env into SIV virions. Indeed, we have previously shown that truncations or in-frame deletions affecting the C-terminus of the SIV TM cytoplasmic domain (CD) inhibit Env incorporation into virions (Celma et al., 2001; Manrique et al., 2001), and that removal of the C-terminal 20 amino acids of the SIV TM protein is sufficient to impair Env packaging

* Corresponding author. Fax: +54 11 4963 9278.
E-mail address: sigonzal@ub.edu.ar (S.A. González).

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into particles (Manrique et al., 2001). Likewise, certain mutations within the SIV MA-coding region block Env incorporation into particles (González et al., 1996; Manrique et al., 2003). Interestingly, that an association between the MA and TM proteins is necessary for Env incorporation into virions has also been shown for HIV-1 (Dorfman et al., 1994; Freed and Martin, 1995, 1996; Kalia et al., 2003; Murakami and Freed, 2000; Wyma et al., 2000; Yu et al., 1992). Of note, we have demonstrated that mutations within the SIV MA region spanning the N-terminal α-helices H1 and H2 confer a differential ability to Gag to associate with the Env glycoprotein, thereby modulating either negatively or positively the levels at which Env is incorporated into virions (Manrique et al., 2003). Based on these precedents, and given the relevance of Env incorporation into virions to virus infectivity, we developed an in vitro association assay that allowed us to demonstrate the physical interaction between the SIV MA and the TM CD in the absence of other viral or cellular proteins.

Results

Establishment of an in vitro assay for the association between the SIV MA and the Env cytoplasmic domain

To study the interaction between the MA protein and the Env CD of SIV, the latter was expressed in *Escherichia coli* as fusion with *Schistosoma japonicum* glutathione *S*-transferase (GST) (Fig. 1A), immobilized onto a glutathione-coupled Sepharose resin, and used as bait in GST pull-down assays. As prey protein, we used recombinant SIV MA purified from *E. coli* extracts as described in Materials and methods. As shown in Fig. 1B, the SIV MA protein bound in a specific manner to the GST-CD_{SIV} fusion protein, but not to beads coated with GST alone, indicating that the SIV MA and the Env CD are capable of establishing a direct physical interaction.

We have previously shown that mutant CD104, a mutant SIV Env glycoprotein lacking the C-terminal 60 residues, is not incorporated into Gag particles (Manrique et al., 2001). Interestingly, this mutant Env CD was unable to interact with the SIV MA in the pull-down assays (Fig. 1B). Moreover, an SIV MA protein carrying the L31E single amino acid substitution did not associate in vitro with the GST-CD_{SIV} protein (Fig. 1C), which is in agreement with the Env incorporation-defective phenotype exhibited by the virus bearing this single point mutation in the MA-coding region (Manrique et al., 2003). By contrast, a specific interaction between the GST-CD_{SIV} protein and the mutant R22L/G24L MA protein was observed (Fig. 1D). Furthermore, introduction of the R22L/G24L mutation in the context of the mutant L31E MA protein partially restored SIV Env CD-MA association (Fig. 1E). These results are in line with our previous data reporting that the SIV Env glycoprotein is efficiently incorporated into the R22L/G24L MA mutant virions and that this double amino acid substitution in SIV MA can substantially compensate for the Env incorporation and infectivity defects exhibited by the L31E mutant (Manrique et al., 2003).

The assays described above are based on the elution in Laemmli buffer of the MA protein bound to the GST-CD_{SIV} beads. To provide further evidence for the specificity of this assay, the GST-CD_{SIV}-coated beads were first incubated with the MA protein and then treated in parallel with thrombin in phosphate-buffered saline (PBS) or with PBS alone. Subsequently, both samples were centrifuged to sediment the resin, and the presence of viral proteins in the supernatants was analyzed by Western blotting. Since there is a thrombin recognition site between the GST and CD moieties in the fusion protein, treatment with thrombin would be expected to release the CD-MA protein complexes. As shown in Fig. 2, both the MA and the Env CD were detected in the supernatant of the binding reaction subjected to thrombin treatment, whereas neither of these proteins were observed in the supernatant fraction obtained after incubation of the resin-coupled proteins in PBS. This result therefore confirms that in these pull-down assays the MA protein specifically interacts with the CD moiety of the GST-CD_{SIV} fusion protein immobilized on the glutathione resin.

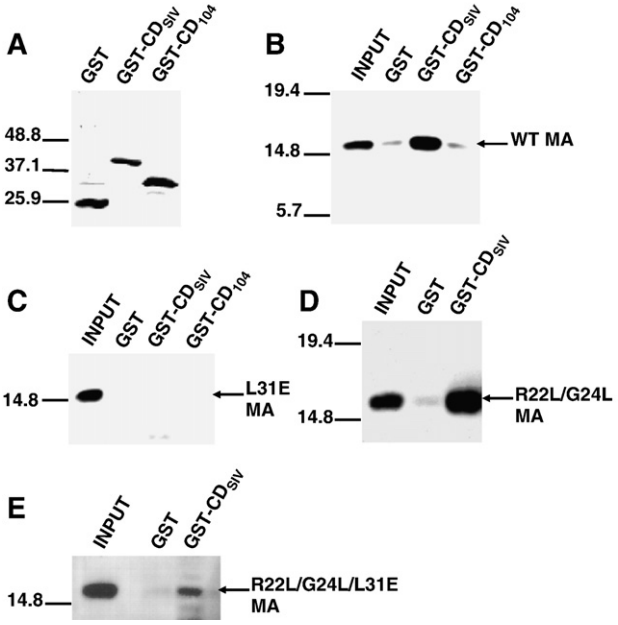

Fig. 1. In vitro binding assay for the interaction between the SIV MA and the Env CD. (A) The wild-type (CD_{SIV}) or mutant (CD104) SIV Env CD polypeptides were expressed as GST fusion proteins, immobilized on glutathione-Sepharose beads, resolved by SDS-gel electrophoresis on a 10% polyacrylamide gel and stained with Coomassie G-250. Equivalent amounts of immobilized GST, GST-CD_{SIV}, or GST-CD104 were incubated with recombinant wild-type SIV MA (B), or the mutant MA proteins L31E (C), R22L/G24L (D), or R22L/G24L/L31E (E) as described in Materials and methods. The protein complexes coupled to the resin were resolved by SDS-PAGE (12% polyacrylamide gel) and detected by Western blotting using the monoclonal antibody KK59 (B), or a polyclonal antiserum specific for the SIV MA (C, D and E). INPUT: As control, one-tenth of the total amount of MA protein used in each binding reaction was loaded on each gel. The mobilities of the wild-type and mutant MA proteins are shown, as are the positions of the molecular weight standards.

Determination of the binding constant for the interaction between the SIV MA and the Env cytoplasmic domain

The interaction between two proteins is governed by the binding constant (*Kd*) which reflects the strength of their

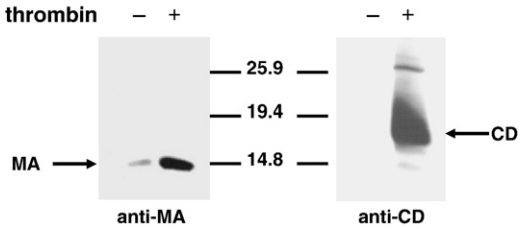

Fig. 2. Elution of the SIV MA-CD complexes by thrombin treatment. Equivalent amounts of the GST-CD_{SIV} fusion protein were coupled to glutathione-Sepharose beads and incubated with recombinant SIV MA. After extensive washings, the beads were incubated for 3 h either with PBS alone (−) or with PBS containing 0.25 U thrombin (+). The proteins in the supernatants resulting from resin centrifugation were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first incubated with the anti-SIV MA polyclonal serum (Left panel), and then reprobed with the antiserum specific for the SIV Env CD (Right panel). The mobilities of the MA and Env CD polypeptides are shown, as are the positions of the molecular weight standards.

association. Protein affinity chromatography is extensively used to estimate binding constants (Phizicky and Fields, 1995). The binding reaction is performed using various concentrations of the immobilized protein that are in excess over that of its ligand. Under these experimental conditions, the minimal concentration of the immobilized protein that retains 50% of the ligand corresponds to the *Kd* value (see Materials and methods; Formosa et al., 1991; Mason and Lis, 1997; Phizicky and Fields, 1995). We therefore made use of this method to determine the *Kd* of the SIV MA-Env CD interaction. In these experiments, increasing amounts of the GST-CD_{SIV} fusion protein immobilized on the glutathione-Sepharose resin were incubated with a constant and limiting amount of the SIV MA protein followed by a single washing. The percentage of bound SIV MA was determined by Western blotting as described in Materials and methods. The binding curve obtained with the data from three independent experiments allowed us to estimate that the SIV MA interacts in vitro with the Env CD with a *Kd* of 7×10^{-7} M (Fig. 3).

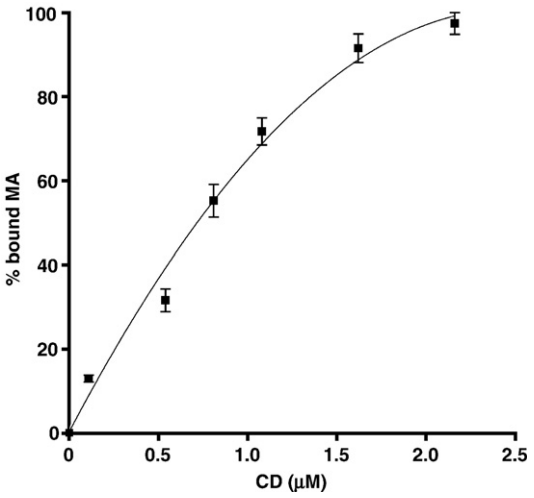

Fig. 3. Saturation curve for the interaction between the SIV MA and the Env CD. Increasing amounts of the GST-CD_{SIV} fusion protein coupled to glutathione-Sepharose beads were incubated with a fixed and limiting concentration of the SIV MA, and the amount of bound MA for each point was determined as described in Materials and methods.

Binding of the SIV MA protein to the Env cytoplasmic domain of related lentiviruses

The SIV MA protein shares only 50% amino acid sequence identity with that of HIV-1. However, X-ray crystallographic data indicate that these proteins exhibit a high degree of structural similarity (Hill et al., 1996; Rao et al., 1995).

We therefore sought to investigate whether the SIV MA was capable of interacting with the CD of the HIV-1 Env glycoprotein. For comparison, we also tested the potential association of the SIV MA with the Env CD of a distantly related lentivirus such as feline immunodeficiency virus (FIV), which is unusually short (approximately 53 amino acids) compared to its primate lentiviral counterparts (Celma et al., 2007). The CDs of the HIV-1 and FIV Env glycoproteins were expressed in *E. coli* as GST fusion proteins (Fig. 4A), immobilized onto glutathione-Sepharose beads, and incubated with the SIV MA protein. Fig. 4B shows that the amount of SIV MA bound to the HIV-1 Env CD was similar to that detected when the SIV Env CD was used as bait protein in the pull-down assays. By contrast, the FIV Env CD only

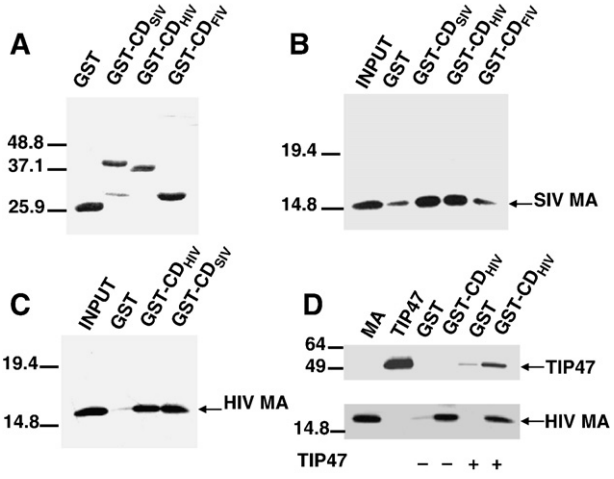

Fig. 4. In vitro binding assay to test the interaction of the SIV MA with the Env CDs of HIV-1 and FIV. (A) The CDs of the HIV-1 and FIV Env proteins were expressed as GST fusion proteins in *E. coli*, purified by affinity chromatography, resolved by SDS-gel electrophoresis on 10% polyacrylamide gels, and visualized by Coomassie G-250 staining. (B) Equivalent amounts of GST, GST-CD_{SIV}, GST-CD_{HIV}, and GST-CD_{FIV} immobilized on gluathione-Sepharose beads were incubated with recombinant SIV MA. Protein complexes resulting from the binding reactions were resolved by SDS-PAGE (12% polyacrylamide gel) and the bound MA in each case was detected by Western blotting using a polyclonal antiserum specific for the SIV MA. INPUT: one-tenth of the total amount of SIV MA protein used in the binding reaction was loaded on the gel. (C) In vitro association of HIV-1 MA with SIV and HIV-1 Env CDs. Biotinylated HIV-1 MA was incubated with equivalent amounts of purified GST, GST-CD_{SIV}, and GST-CD_{HIV} as decribed in Materials and methods. HIV-1 MA binding was analyzed by Western blotting with horseradish peroxidase-conjugated streptavidin. INPUT: one-tenth of the total amount of biotinylated HIV-1 MA protein used in the pull-down assays was run in the gel. (D) Effect of TIP47 on HIV-1 MA-Env CD association. Biotinylated HIV-1 MA was incubated with equivalent amounts of GST and GST-CD_{HIV} in the absence (−) or presence (+) of recombinant His-TIP47. Binding of HIV-1 MA and TIP47 in the pull-down assays was analyzed by Western blotting with horseradish peroxidase-conjugated streptavidin, or with an antibody specific for penta-His, respectively. As controls, samples corresponding to one-tenth of the total amount of HIV-1 MA and His-TIP47 proteins used in the binding reaction were loaded on the gel.

captured background levels of SIV MA (Fig. 4B). This indicates that the SIV MA is capable of establishing a specific interaction with the HIV-1 Env CD, but not with that of FIV. Based on these results, we next tested the HIV-1 MA for its ability to interact with the SIV Env CD. As shown in Fig. 4C, the HIV-1 MA bound to the SIV Env CD at levels comparable to those detected when the GST-CD_{HIV} fusion protein was coupled to the resin. Recently, the cellular protein TIP47 (Tail-interacting protein of 47 kDa) has been shown to mediate the association between the HIV-1 Gag and Env proteins during particle assembly (Lopez-Vergès et al., 2006). We therefore investigated whether the addition of human TIP47 to the binding reaction had any effect on the in vitro association of the HIV-1 MA with the Env CD. Under our experimental conditions, recombinant His-TIP47 specifically interacted with the GST-CD_{HIV} fusion; however, the presence of TIP47 did not enhance binding of the HIV-1 MA to the Env CD (Fig. 4D). Furthermore, we found that addition of TIP47 in our pull-down assays had no effect on the in vitro interaction between the SIV MA and Env CD proteins (data not shown).

Discussion

Our previous mutagenesis studies of the SIV Gag and Env proteins strongly supported the notion that the process of SIV Env incorporation into virions is mediated by MA-Env CD interactions (Celma et al., 2001; Manrique et al., 2001, 2003). However, the physical interaction between these viral protein domains remained to be demonstrated. To this end, we developed an in vitro system that, based on recombinant proteins expressed in *E. coli*, allowed us to analyze the association between the SIV MA and the Env CD. Our pull-down assays show that the SIV MA is capable of establishing a specific interaction with the Env CD in the absence of other viral or cellular proteins. Moreover, this association is blocked in vitro by mutations in either the MA (mutation L31E) or Env CD (mutation CD104) that have been shown to interfere in vivo with Env packaging into virions (Manrique et al., 2001, 2003).

Estimation by protein affinity chromatography of the binding constant for the SIV MA-Env CD interaction yielded a *Kd* of 7×10^{-7} M. Interestingly, binding constants of a similar order of magnitude have been reported for complexes established by other primate lentiviral proteins. Indeed, HIV-1 gp120 associates with soluble CD4 with a *Kd* of 4.29×10^{-7} M (Biorn et al., 2004) and with CXCR4 with a *Kd* of 5.06×10^{-7} M (Hoffman et al., 2000). Binding of SIVmac gp130 to CD4 has been to shown to occur with a *Kd* of 3.5×10^{-7} M (Ivey-Hoyle et al., 1991). In addition, a *Kd* of 8.7×10^{-7} M has been determined for the HIV-1 Nef-CD4 cytoplasmic domain interaction (Preusser et al., 2001). By contrast, HIV-1 Vpr binds to the p6 domain of Gag with lower affinity (*Kd* of 7.5×10^{-5} M) (Jenkins et al., 2001).

The association between the MA domain of Gag and the Env CD has also been investigated for HIV-1. In this regard, it has previously been reported that the HIV-1 Env CD produced in *E. coli* as a fusion protein with GST captures recombinant HIV-1 MA expressed either in bacteria or in COS-1 cells (Cosson, 1996). In this regard, we found that recombinant HIV-1 MA is capable of interacting with both HIV-1 and SIV Env CDs. However, under our experimental conditions this association does not appear to be either dependent on or enhanced by the addition of TIP47 to the binding reaction. This could be explained by the fact that recombinant TIP47 preparations may contain only a small proportion of active molecules (Krise et al., 2000). On the other hand, our finding that the in vitro interaction between the SIV MA and Env CD proteins proceeds in the absence of TIP47 is consistent with the fact that one of the regions in the HIV-1 MA (DRWE, residues 14–17) that have been reported to be necessary for TIP47 interaction (Lopez-Vergès et al., 2006) is not conserved in SIV or HIV-2. However, we cannot rule out the possibility that, in vivo, cellular factors (including TIP47) may modulate the process of SIV Env incorporation into virions. Further experiments will be necessary to address this issue. That equivalent domains of HIV-1 and SIV Gag polyproteins exhibit different properties is not without precedent: HIV-1 CA protein binds to cyclophilin A whereas that of SIV fails to associate with this cellular protein (Braaten et al., 1996).

An interesting observation stemming from our in vitro binding assays is that the SIV MA is able to associate with the HIV-1 Env CD but not with that of the distantly related lentivirus FIV. Our evidence that the SIV MA interacts with the HIV-1 Env CD is in agreement with the fact that construction of SIV/HIV chimeric viruses (SHIVs) expressing SIV Gag and HIV-1 Env proteins results in replication-competent viruses that are pathogenic in monkeys (Li et al., 1992).

An important consequence of the interaction between the MA domain of Gag and the Env CD is that it not only ensures the incorporation of Env into virions during particle assembly, and, thus, virus infectivity, but also appears to be necessary for regulating the biological functions of each of the interacting partners. Several lines of evidence support this notion: (i) the high endocytosis rate of HIV-1 Env is drastically reduced when coexpressed with Gag, thus favoring Env incorporation into particles (Egan et al., 1996); (ii) Gag-Env interaction in HIV-1 immature particles blocks Env fusogenic activity which is restored upon Gag processing and formation of mature virions (Murakami et al., 2004; Wyma et al., 2004), a mechanism that appears to prevent entry of immature virions into cells; (iii) Gag-Env interaction has been shown to be necessary for Env association with lipid rafts, which constitute the plasma membrane sites where virus assembly takes place (Bhattacharya et al., 2004).

In summary, our GST-pull-down assays using recombinant SIV MA and Env CD proteins, together with our previous in vivo genetic and biochemical studies, suggest that the interaction between these viral protein domains is a necessary step in the process of SIV Env incorporation into virions.

Materials and methods

Cloning, expression and purification of recombinant proteins

The coding regions for the wild-type and mutant L31E, R22L/G24L, and R22L/G24L/L31E SIV MA proteins were PCR amplified from the SIV_{SMM} PBj1.9 or mutant proviral

DNAs using two primers that introduce NcoI and SmaI restriction sites at the 5′ and 3′ ends of the DNA products, respectively. The open reading frame for the HIV-1 pNL4-3 MA protein was obtained using the same strategy. The NcoI/SmaI-digested fragments were cloned into the corresponding sites of the pTYB4 plasmid vector of the IMPACT T7 System (Intein-Mediated Purification with an Affinity Chitin-binding Tag, New England Biolabs). This system allowed the expression of the MA open reading frames as fusions with intein, the protein splicing element from the *Saccharomyces cerevisiae WMA1* gene (Chong et al., 1997), together with the chitin-binding domain. The coding sequences for the Env CD of SIV_{SMM} PBj1.9, SIV mutant CD104 (Manrique et al, 2001), HIV-1 pNL4-3, and FIV-14 Petaluma, were obtained by PCR amplification of the corresponding proviral DNAs using 5′ and 3′ primers that introduce unique BamHI and EcoRI restriction sites, respectively. The amplified DNAs were cloned into the BamHI and EcoRI sites of the pGEX-2T plasmid (GE Life Sciences) for the in-frame translation with GST. The recombinant proteins were produced in *E. coli* BL21 (for the pTYB-derived clones) or DH5α (for the pGEX-2T-derived clones) strains by induction with 1 mM isopropyl-β-D-thiogalactopyranoside essentially as we have previously described (Manrique et al., 2004). After induction, bacterial extracts for the purification of the recombinant MA proteins were obtained by sonication in TNE buffer (20 mM Tris–HCl [pH 8.0]; 500 mM NaCl; 0.1 mM EDTA) containing 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin. Insoluble material was removed by centrifugation for 10 min at 16,000 ×*g*, and the supernatants containing the intein/chitin-binding domain-tagged MA proteins were mixed with a 50% (v/v) slurry of chitin beads and incubated for 60 min at 4 °C. Beads were washed with TNE buffer and recombinant MA proteins were released by incubating the beads in TNE containing 30 mM dithiothreitol overnight at 4 °C. To produce recombinant biotinylated HIV-1 MA, the resin-coupled fusion protein was biotinylated at the C-terminus by intein-mediated protein ligation in the presence of the synthetic Bio-P1 peptide (New England Biolabs) under the conditions recommended by the manufacturer. Supernatants containing the eluted recombinant MA proteins were stored at −80 °C until further use. The GST and GST-CD fusion proteins were purified from bacterial lysates as described previously (Manrique et al., 2004). The cDNA for human TIP47 was amplified by reverse transcriptase-PCR from mRNA purified from 293T cells by using specific primers and cloned into the KpnI and BamHI sites of the pET-30b (+) plasmid (Novagen). Recombinant histidine-tagged TIP47 was purified from *E. coli* BL21 lysates by immobilized metal ion adsorption chromatography according to the manufacturer's protocol (His Microspin Purification Module; GE Life Sciences) and stored at −80 °C in the presence of 10% glycerol (Krise et al., 2000). Protein concentrations were estimated by comparison to known amounts of standard bovine serum albumin (BSA) on sodium dodecyl sulfate (SDS)-polyacrylamide gels stained with Coomassie G-250 SimplyBlue Safe Stain (Invitrogen). Quantitation of the amount of protein on gels was performed by densitometry using the GelWorks 1D software (UVP GDS 8000; Ultra Violet Products, Ltd.).

In vitro binding assays

Six micrograms of GST or GST-CD fusion proteins were pre-bound to glutathione-Sepharose 4B (GE Life Sciences) and incubated with 500 ng purified wild-type or mutant SIV MA proteins in 100 μl PBS containing 0.5% CHAPS, 1% BSA, 1 mM PMSF and 10 μg/ml aprotinin for 2 h at 4 °C. Binding of TIP47 and MA to Env CD was analyzed by incubating the immobilized GST-CD fusion proteins with 2 μg His-TIP47 and 850 ng biotinylated HIV-1 MA in 100 μl PBS containing 0.01% Triton X-100, 1% BSA, 1 mM PMSF and 10 μg/ml aprotinin overnight at 4 °C. Beads were washed 6 times with 40 bed volumes of the corresponding binding buffer. The protein complexes were eluted from the glutathione resin by resuspending the beads in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose membranes. Alternatively, in the case of elution of the protein complexes by thrombin treatment, the resin was resuspended in PBS containing 0.25 cleavage units of thrombin and incubated for 3 h at 25 °C. The Sepharose beads were pelleted by low-speed centrifugation and the eluate was resolved by SDS-PAGE. The presence of the wild-type or SIV mutant MA proteins in the eluted protein complexes was analyzed by Western blotting using the SIV MA-specific KK59 monoclonal antibody (NIH Research and Reference Reagent Program) or a mouse anti-SIV MA polyclonal serum, respectively, and horseradish peroxidase-conjugated anti-mouse immunoglobulin (GE Life Sciences) as secondary antibody. The antisera directed against the SIV MA or the SIV Env CD were obtained in our laboratory by immunization of mice with the purified bacterially expressed polypeptides essentially as we have recently described (Celma et al., 2007). Biotinylated HIV-1 MA was detected by using horseradish peroxidase-conjugated streptavidin (GE Life Sciences). Detection of His-TIP47 in the binding assays was performed by Western blotting using a horseradish peroxidase-conjugated antibody specific for penta-His (QIAGEN). Western blots were developed with an enhanced chemiluminescence and chemifluorescence assay (ECL Plus Reagent, GE Life Sciences).

Dissociation constant determination

Determination of the *Kd* for the SIV MA-Env CD interaction was based on the method described by Phizicky and Fields (1995). Estimation of the binding constant for the interaction between a given protein (P) and its ligand (L) is governed by the following equation which refers to the fraction of L bound to protein P: $[PL]/[L_{TOTAL}]=[P_{FREE}]/[P_{FREE}]+Kd$. If the concentration of protein P is in great excess over that of the ligand, $[P_{FREE}]\approx[P_{TOTAL}]$. Then, $[PL]/[L_{TOTAL}]=[P_{TOTAL}]/[P_{TOTAL}]+Kd$. When 50% of the ligand is bound to protein P ($[PL]/[L_{TOTAL}]=0.5$), then $[P_{TOTAL}]/[P_{TOTAL}]+Kd=0.5$, and, therefore, $[P_{TOTAL}]=Kd$. For the experimental determination of the *Kd* of the SIV MA-Env CD interaction, increasing concentrations (0–216 pmol) of GST-

CD_{SIV} coupled to the glutathione-Sepharose resin were incubated with 50 ng recombinant SIV MA in 100 μl Binding Buffer for 2 h at 4 °C. Beads were then collected by centrifugation, washed once with 50 bed volumes PBS containing 0.1% BSA and 0.5% CHAPS, and the bound proteins were resolved by SDS-PAGE, and subsequently transferred to a nitrocellulose membrane. The percentage of SIV MA recovery for each concentration of GST-CD_{SIV} was determined by comparing to known amounts of SIV MA by Western blotting with the KK59 monoclonal antibody. Quantitation was performed as we have previously described (Manrique et al., 2003).

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