

Induction of Antibodies Binding to the Membrane Proximal External Region of gp36 of HIV-2

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Key Words

HIV-2 · Vaccine · Neutralizing antibodies · Transmembrane envelope protein

Abstract

Objective: The ability to induce neutralizing antibodies may be the most important feature of an antiretroviral vaccine, preventing infection of target cells and subsequent integration of the virus into the cellular genome where the virus may persist. Broadly neutralizing antibodies directed against conserved epitopes in the membrane proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1 such as the monoclonal antibodies (mAb) 2F5 and mAb 4E10 have been found in infected individuals; however, all attempts to induce such antibodies failed. In individuals infected with HIV-2 such antibodies were not yet reported. **Methods:** Two recombinant proteins corresponding to the ectodomain of the TM protein gp36 of HIV-2 were produced, rats were immunized and sera were analyzed for binding and neutralizing antibodies. **Results:** Although binding antibodies were induced, none of the sera neutralized HIV-2. Most interestingly, epitope mapping showed specific binding of the antibodies to the MPER of gp36, to a region homologous to the binding site of the mAb 4E10 in gp41 of HIV-1. **Conclusions:** Although MPER-specific anti-

bodies were induced by vaccination with gp36, these antibodies did not neutralize HIV-2. This is similar to the situation with HIV-1, but in contrast to that with gammaretroviruses.

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Broadly neutralizing antibodies, recognizing epitopes in the membrane proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1, such as the monoclonal antibodies (mAb) 2F5 and mAb 4E10 have been isolated from HIV-1 infected individuals [1–3]. mAb 2F5 and mAb 4E10 neutralize a wide range of different HIV-1 group M subtypes with high efficacy [4]. The MPER of gp41 was shown to be one of the most conserved regions within Env of HIV-1 [5] and therefore represents an excellent target for neutralizing antibodies. Although unsuccessful until now (for a review, see [6]), the induction of mAb 2F5/4E10-like neutralizing antibodies is a major goal in HIV-1 vaccine research. In contrast, neutralizing antibodies against the porcine endogenous retrovirus (PERV), the feline leukemia virus (FeLV) and the Koala retrovirus (KoRV) could be easily induced, immunizing with their TM proteins p15E [7–10]. The induced antibodies recognized two epitopes, one located in the fusion peptide proximal region (FPPR) of the N-terminal part (epitope 1, E1), and the other in the C-terminal MPER

Table 1. Primers and probes used for the cloning of gp36 and detection of virus expression

Name	Location	Sequence (5'-3')
HIV-2-RT-For	530–548 ^a	CTGTATAAATGTACCCGCT
HIV-2-RT-Rev	721–739 ^a	ACTTGCTTCTAATTGGCAG
HIV-2-RT-probe	571–587 ^a	FAM-CTGCGGAGAGGCTGGCA-BHQ1
hGAPDH-For	365–385 ^b	GCGGATGCTGGCGCTGAGTAC
hGAPDH-Rev	495–513 ^b	TGGTCCACACCCATGACGA
hGAPDH-probe	407–430 ^b	HEX-TTCACCACCATGGAGAAGGCTGGG-BHQ
HIV-2-gp36-For ^c	8279–8307 ^a	GGGACTTTCCAGAAGGGGCT
HIV-2-gp36-Rev ^d	8693–8719 ^a	CCAGGCGGCGACTAGGAGAG
His-For		TATGCATCACCATCACCATCAGG
His-Rev		CGTGATGTGATGGTGTATGCAT

FAM = 6-Carboxy-fluorescein; HEX = 5'-hexachloro-fluorescein phosphoramidite; BHQ = Black Hole Quencher.

^a Location refers to accession number L36874. ^b Location refers to accession number AF261085. ^c Primer comprises *Bam*HI restriction site. ^d Primer comprises *Eco*RI restriction site and a stop-codon.

(E2) of the TM protein p15E. The location of the E2 epitopes corresponds to the location of the mAb 2F5/4E10 epitopes in gp41 of HIV-1. Despite the evolutionary distance between HIV-1 on the one hand, and PERV, FeLV as well as KoRV on the other hand, a partial sequence homology in the E2 epitope was observed (F/YEGWFN in the case of gammaretroviruses; NWFNIT, the mAb 4E10 epitope in the case of HIV-1; identical amino acids are underlined). Using ELISA, epitope mapping and surface plasmon resonance analysis it was recently shown that the presence of a peptide corresponding to the FPPR of gp41 of HIV-1 increased binding of mAb 2F5 and mAb 4E10 to their epitopes located in the MPER [11]. An interaction between the FPPR and the MPER of gp41 during infection has also been described [12–14]. However, immunization experiments using the recombinant ectodomain of gp41 of HIV-1 containing E1 and E2 – analogous to the successful immunization experiments using the ectodomain of gammaretroviruses – failed to induce such antibodies [unpubl. results]. To our knowledge, there is no information about mAb 2F5/4E10-like neutralizing antibodies in patients infected with HIV-2 and only a few attempts have been undertaken to induce such antibodies [15].

To immunize with the ectodomain of the TM protein gp36 of HIV-2, two recombinant gp36 constructs were designed. The sequence corresponding to the ectodomain of gp36 (aa511–684) was lacking the fusion peptide and the membrane-spanning domain, and was amplified by PCR from cells infected with HIV-2_{7312A} (accession No. L36874) using the primers HIV-2-gp36-For and HIV-2-gp36-Rev (table 1). The amplicon was ligated either into the expres-

sion vector pCal-n (Stratagene, La Jolla, Calif., USA) or pCal-n-His using the restriction sites *Eco*RI and *Bam*HI. In pCal-n-His, the CREB-binding protein (CBP) tag was removed by cleavage with the enzymes *Bam*HI and *Nde*I. Oligonucleotides comprising the sequence of a 6x His-tag (His-For and His-Rev; table 1) and sticky ends for *Bam*HI and *Nde*I were ligated to the CBP-deleted pCal-n. The plasmids pCal-n and pCal-n-His harboring the sequence of the ectodomain of gp36 were used for expression in the *E. coli* strain BL21. The recombinant protein gp36-CBP was insoluble under physiological conditions and could not be purified since the CBP tag does not allow purification under denaturing conditions. Thus, to eliminate the soluble contaminating proteins, bacteria were pelleted after expression (20,000 g/10 min/4°) and sonicated in 10 ml PBS on ice (pulse: 3 × 20 s, break: 30 s with a Branson Sonifier II 250; G. Heinemann, Schwäbisch Gmünd, Germany). After collecting the insoluble gp36-CBP protein by centrifugation (20,000 g/10 min/4°) the resulting pellet was washed overnight with 500 ml PBS rotating slowly at 4°. The next day gp36-CBP was collected again by centrifugation (20,000 g/10 min/4°). The protein pellet was resuspended in 10 ml PBS and used for immunization. gp36-His was also insoluble but could be purified under denaturing conditions using 8 M urea and Ni-NTA-agarose (Qiagen, Hilden, Germany) according the manufacturer's protocol. The purity of both proteins was analyzed by PAGE and Western blot analysis applying a cross-reacting serum from a SIVmac-infected rhesus monkey and an antipenta-His-antibody (Qiagen, Hilden, Germany; fig. 1). Female Wistar rats (Charles River, Sulzfeld, Ger-

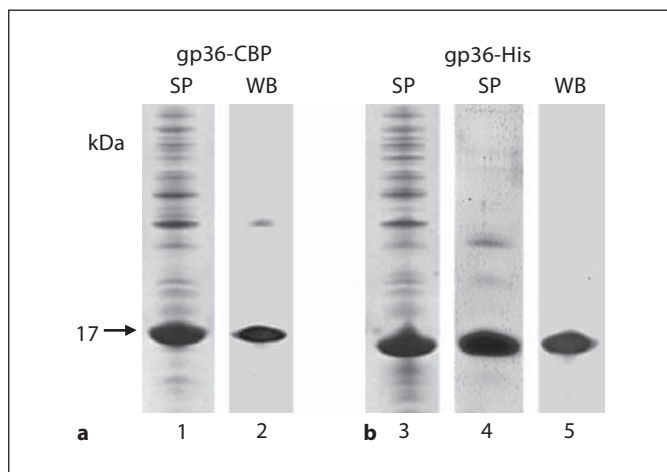


Fig. 1. SDS-PAGE (SP) and Western blot (WB) analysis using a cross-reactive serum from a SIVmac-infected animal (1:1,000) for gp36-CBP (**a**); an antipenta-His-antibody for gp36-His (**b**). Columns 1 and 2 are partially purified gp36-CBP, 3 is unpurified gp36-His, 4 and 5 are affinity-purified gp36-His.

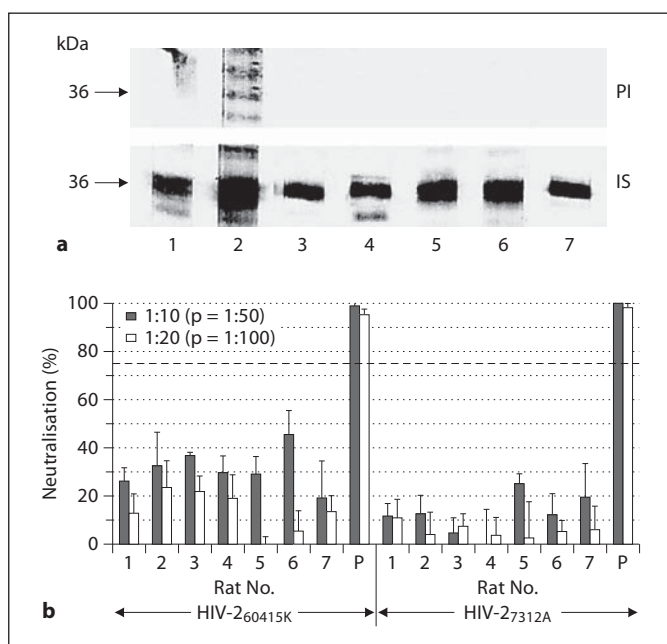
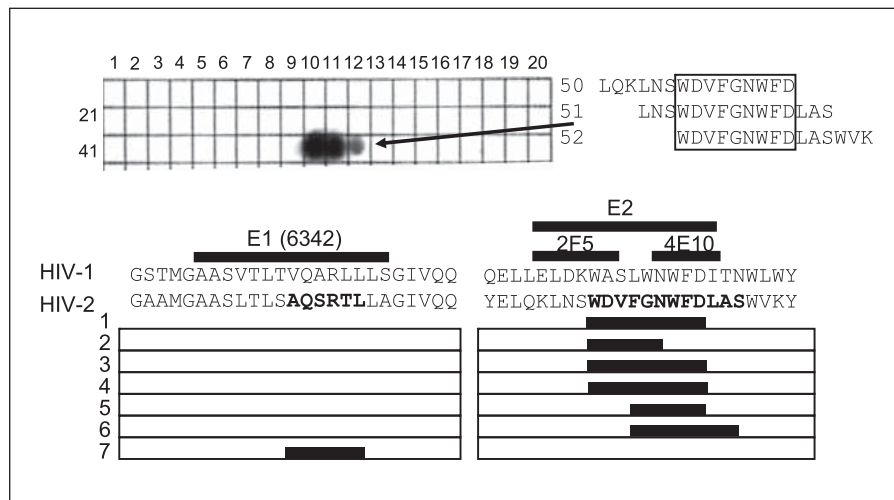


Fig. 2. Reactivity of 7 rat sera before and after immunization with gp36-CBP and gp36-His. **a** Western blot using a lysate of HIV-2_{7312A}-infected H9 cells. Sera were diluted 1:1,000, the secondary anti-rat-POD antibody was diluted 1:4,000. PI = Preimmune sera, IS = immune sera. **b** Neutralizing activity of these sera in an assay using HIV-2_{6045K} and HIV-2_{7312A}. All values are means of 3 replicates with SD. All sera were diluted 1:10 and 1:20. A pool of sera (P) from 3 patients infected with HIV-2 was diluted 1:50 and 1:100 and used as a positive control. The dashed line indicates the threshold at 75% neutralization.

many) were immunized twice, on day 0 and day 21, each with 250 μ g of recombinant protein in 0.4 ml PBS and 0.4 ml of Freund's adjuvant (Pierce, Rockford, Ill., USA). Blood samples were taken on day 35 after primary immunization and the sera were characterized by ELISA and Western blot [16]. To perform ELISA, 200 ng of purified gp36-His were coated per well of a 96-well Nunc-Immuplate (Nalge Nunc, Denmark) at 37° overnight. Sera No. 1, 2 and 4 (gp36-CBP) showed a gp36-specific titer of 64,000; serum No. 3 (gp36-CBP) as well as No. 5 and 7 (gp36-His) of 256,000 and serum No. 6 (gp36-His) of 128,000. Western blot analysis was performed using a lysate from H9 cells infected with HIV-2_{7312A} (TCID₅₀ = 10^{4.9}/100 μ l). Cells were lysed with radioimmunoprecipitation assay buffer (25 mM Tris HCl pH 7.6; 150 mM NaCl; 1% (v/v) NP-40; 1% sodium deoxycholate; 0.1% SDS) for 30 min at 4°, and cleared by centrifugation (14,000 g/5 min/4°). SDS-PAGE was performed using a 14% polyacrylamide gel. All 7 sera showed a strong reaction with gp36 of HIV-2 (fig. 2a), and they were able to bind to native gp36 on H9 cells infected with HIV-2 as investigated by flow cytometry (not shown). To analyze the neutralizing activity in these sera, a newly developed assay was used, measuring neutralization as reduced provirus integration determined by real-time PCR [17]. Two HIV-2 strains were used, HIV-2_{7312A} (the antigen used for immunization corresponds to this virus) and HIV-2_{60415K}, both obtained through the AIDS Research and Reference Reagent Program [18]. Briefly, after incubation of 20 μ l serum dilution with 80 μ l virus for 30 min at 37°, C8166 cells (100 μ l, 5 \times 10⁵ cells/ml) were added. After 65 h, the supernatant was aspirated and the cells were lysed by 3 freeze-thaw cycles (20 min at -80° followed by 5 min at 60°), followed by treatment with proteinase K (20 μ g/ml, Invitrogen) for 3 h at 60°. The enzyme was then inactivated at 95° for 20 min. The lysate was used for quantification of provirus integration by an HIV-2-specific real-time PCR, using the primers HIV-2-RT-For and HIV-2-RT-Rev and the HIV-2-RT-probe (table 1). Amplification was performed using a real-time cycler MX-4000 (Stratagene, La Jolla, Calif., USA) as follows: 95°/10 min for 35 cycles: 95°/45 s + 52°/60 s + 72°/20 s. To monitor possible toxic effects of the serum, a duplex real-time PCR was performed that simultaneously amplified the sequence in the viral LTR and a single copy sequence of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) primers hGAPDH-For, hGAPDH-Rev and the hGAPDH-probe (table 1). Significant neutralization was defined as a reduction of provirus integration above 75%. Neutralisation (NT) was calculated using the formula: NT = 100 - 100/2 ^{Δ Ct}. None

Fig. 3. a Epitope mapping of the serum from rat 1 immunized with gp36-CBP. Three peptides (50–52) were recognized and the epitope was defined as WDVFGN-WFD. **b** Sequences of the FPPR and MPER regions of gp36 of HIV-2 and schematic presentation of the epitopes recognized by sera from rats 1–7. For comparison, the corresponding sequence of gp41 from HIV-1_{MN} (Gene Bank: M17449) is aligned. The localization of the epitopes of the monoclonal antibodies mAb 2F5 and mAb 4E10 in the E2 region of gp41 and of the E1 peptide No. 6342 located in the FPPR and interacting with the MPER are marked above the HIV-1 sequence. The HIV-2 sequence corresponds to isolate 7312A (Gene Bank: L36874).



of the induced rat sera was able to reduce integration of proviruses significantly, indicating a lack of neutralizing antibodies (fig. 2b).

To map the epitopes, synthetic overlapping peptides corresponding to the ectodomain of gp36 of HIV-2_{7312A} spotted on a membrane (JPT, Berlin, Germany) were used. Sera were diluted 1:1,000 in blocking solution (PBS + 10% (w/v) fetal bovine serum) and incubated with the membrane for 3 h. The membrane was washed 3 times for 15 min with Tris-buffered saline, pH 7.5 containing 0.05% (v/v) Tween 20 (Sigma, Munich, Germany). After incubation for 2 h with a peroxidase-conjugated secondary antibody diluted 1:10,000 in blocking solution, binding was detected using a chemiluminescence detection solution (ECL, Amersham Biosciences, Piscataway, N.J., USA). Using this method, the epitope WDVFGN-WFD, localized in the MPER of the TM protein gp36 (fig. 3), was recognized by sera from rats 1, 3 and 4. Serum from rat 2 recognized WDVFGN, serum from rat 5 recognized FGN-WFD and serum from rat 6 recognized FGN-WFD-LAS, all located in the MPER and showing partial sequence homology to that of mAb 4E10 (NWFN/DIT) in gp41 of HIV-1 (identical amino acids are underlined). However, no cross-neutralization of HIV-1 by the sera was observed. Only one serum (rat 7) reacted with the epitope AQSRTL, localized in the FPPR, but did not react with the E2 region (fig. 3b).

Although 6 out of 7 sera reacted with a domain in the MPER of gp36 corresponding to the epitope domain of the mAb 4E10 neutralizing HIV-1 [3], and to the epitope domain of specific antibodies neutralizing the gamma-retroviruses PERV [7] and FeLV [8, 9] we could not ob-

serve neutralization of HIV-2 by these sera. Whereas immunization with the TM proteins of PERV, FeLV and KoRV always resulted in neutralizing antibodies recognizing epitopes in the FPPR (E1) and MPER (E2), immunizations with the TM protein gp41 of HIV-1 induced neither MPER-specific nor neutralizing antibodies [unpubl. data]. A negative result was also obtained when goats and rats were immunized with the TM protein gp48 of the feline foamy virus (FFV), no antibodies neutralizing FFV were induced [19]. Potential reasons for the difference in inducing neutralizing antibodies to PERV, FeLV, and KoRV on the one hand, and HIV-1, HIV-2 and FFV on the other hand, using a similar immunization strategy, may be the following: the TM proteins of HIV-1, HIV-2 and FFV are larger and highly glycosylated whereas the TMs of gammaretroviruses are smaller and not glycosylated. Obviously, the large size, the high complexity and the strong glycosylation of the TM proteins of HIV-1, HIV-2 and FFV did not support conformations able to induce neutralizing antibodies when immunizing with the entire recombinant ectodomain. Nevertheless, broadly neutralizing antibodies such as mAb 2F5 and mAb 4E10 have been isolated from HIV-infected individuals. This supports the view that the (glycosylated) gp41 of HIV-1 undergoes conformational changes during infection and that one of these conformations is able to induce neutralizing antibodies.

It was therefore surprising to find antibodies binding to the MPER of gp36 of HIV-2 after immunization with the recombinant ectodomain. Although immune sera raised against a linear peptide derived from the MPER of gp36 and conjugated to a carrier protein have been re-

ported to show neutralizing activity [15], the sera obtained here did not neutralize. Until now there are no reports showing the existence and prevalence of mAb 2F5/4E10-like neutralizing antibodies in HIV-2-infected patients. Serum IgA from HIV-2-infected patients was described that bound to the peptide MYELQKLNSWD-VFGN [20] overlapping the epitopes described here (fig. 3, WDVFGNWFGNWFDLAS, identical amino acids are underlined). This sequence contained the epitopes recognized by the sera from rats immunized with the ectodomain of gp36 (fig. 3). Although the IgA population contained neutralizing antibodies, it is unclear if the antibodies binding to the peptide were neutralizing [20]. A more detailed characterization of sequences involved in neutralization is needed. Although individuals infected

with HIV-2 progress slower towards AIDS, the outcome of an HIV-2 infection is also fatal. Generating an HIV-2 vaccine would be worthwhile, especially for regions of high incidence. Based on the successful immunization experiments with the TM proteins of different gamma-retroviruses, new strategies to design vaccines against HIV-1 and HIV-2 should be developed.

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