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Immunoreactivity of chimeric proteins carrying the HIV-1 epitope IGPGRAF
Correlation between predicted conformation and antigenicity

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Abstract Sera from HIV-1 infected individuals were examined for their reactivity to the principal neutralizing domain, IGPGRAF sequence, of the V3-loop of HIV-1. Four hybrid proteins carrying this sequence inserted in four different outer loops of a protein that makes up the capsid of an insect virus were used as antigen in a Western blot assay for this survey. All the four antigens showed different activity: sera that recognise all antigens to sera that reacted with only one of them. Competition experiments indicated that the antibodies recognised these proteins with different affinity. Molecular modelling of the hybrid proteins predicted that the inserted sequence adopted different conformations in each position. Comparison of predicted most stable conformations for IGPGRAF indicated that there is a close relationship between conformational similarity to a V3-loop reference structure and the degree of reactivity with sera.

Key words: Epitope presentation system; HIV-1; V3 loop; Epitope conformation; Serum immunoreactivity

1. Introduction

The V3 domain of the HIV-1 glycoprotein (gp120) is a strong immunodominant epitope capable of inducing an antibody response in at least 90% of the sera of patients infected with HIV [1]. It is the single most important epitope, defined to date, that can invoke a neutralizing response in vaccinated animals or humans [2–9]. However, as it is a very variable domain, it induces a type-specific immune response. LaRosa et al. [10] found that the amino acid sequence at the tip of the V3-loop, GPGR, is far more conserved than the rest of the sequence. This is probably due to the structural constraints imposed by gp120 functionality. It was subsequently demonstrated that human monoclonal antibodies directed against this conserved sequence exhibit a rather broad neutralisation range [11,12]. It has also been reported that a single amino acid substitution in this sequence can completely alter its conformation and prevent the attachment of neutralizing monoclonal antibodies [13]. Until now, the evaluation of anti-V3 antibodies has been mostly made using peptides containing the core sequence as well as the flanking regions, which make it difficult to discriminate the origin of the immunoresponse. Furthermore, free peptides can adopt a wide set of low energy conformations, except in the simpler antibody response to a linear sequence, and hence they are of low value for the discrimination of the type of humoral response. We have developed a new epitope-presenting system based on the capsid protein of the insect virus Flock house virus (FHV). This protein scaffold was used as a carrier for an HIV-1 V3-loop epitope. The X-ray structure of this FHV protein is known at 2.8 Å resolution [14], and this allows the choice of the positions where the foreign HIV sequence will be located. Fig. 1 shows a ribbon diagram of the 3D structure of the FHV capsid precursor protein. The sites chosen for insertion (L1, L2, L3, I3) are indicated at the outer loops of the protein which connect β-sheets of the eight stranded β-barrel structure. In this way the inserts are placed on the exterior surface of the protein. These antigens were extremely useful in studying the influence of the stereochemistry of the presenting molecule in the recognition of a short heterologous amino acid sequence by the human immune system. Our results demonstrate how the reactivity of a foreign sequence, in this case IGPGRAF of HIV 1, is affected by its location in the carrier protein. Molecular modelling studies indicated that different environments impose different conformations on the core sequence IGPGRAF. There was a good correlation between the similarity of the modelled structure of each conformer and those of the V3-loop reference structure and their corresponding reactivity with HIV-1-positive sera.

2. Material and methods

2.1. Plasmid construction and protein expression

The construction of the plasmids carrying the FHV capsid precursor and the genetic manipulation performed to produce the hybrid genes (carrying the IGPGRAF sequence) used will be described in detail elsewhere (Scodeller et al., unpublished). Briefly, the RNA2 of FHV was mutagenized to create the sites L1, L2, L3, I3, and to introduce the foreign sequence IGPGRAF of HIV1 (Fig. 1). BL21(DE3) *E. coli* carrying a chromosomal copy of the phage T7 polymerase gene under the control of the inducible lacUV5 promoter was used as host for expression of hybrid proteins from pET vectors (Novagen, Madison, WI). Expression and purification of recombinant proteins were performed as described [15].

2.2. Immunoassay

To evaluate the reactivity of the expressed proteins with sera from HIV-1-positive individuals or anti-FHV rabbit sera, the proteins were transferred to nitrocellulose membranes as described [16]. Protein bands on the membrane were identified by Ponceau red staining and

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subsequently narrow strips containing the bands, corresponding to the hybrid antigens or the wild-type FHV protein, were cut out. The strips were blocked for 1 h in 5% (w/v) non-fat dry milk in PBS and then incubated for 2–4 h with the different sera, diluted in PBS-0.5% Tween 20 (1 : 100 dilutions of human sera and 1 : 2000 dilutions of rabbit anti-FHV serum). Bound antibodies were detected by incubation of the strips with either horseradish peroxidase-conjugated anti-human or anti-rabbit antibody, and developed with hydrogen peroxide-3′-3-diaminobenzidine tetrahydrochloride.

2.3. Patient sera

Sera from HIV-1-infected individuals were obtained from Cattinara Hospital, USL No. 1, Trieste, Italy. The sera were classified as positive, based on a twice-repeated reactivity in an ELISA test assay (Abbott Recombinant HIV1–2, 3rd Generation EIA-Abbott) and confirmed with Western blot (New Lav Blot 1-Diagnostics Pasteur). All the sera were also tested by Abbott Envacor HIV 1 EIA-Abbott and all were found to be positive.

ELISAs were performed as previously described [2] using 20 amino acid synthetic peptides representative of the principal neutralizing domain (PND) of HIV-1 strains MN, SF2 and SC, obtained from American Bio-Technologies Inc. (Cambridge, MA).

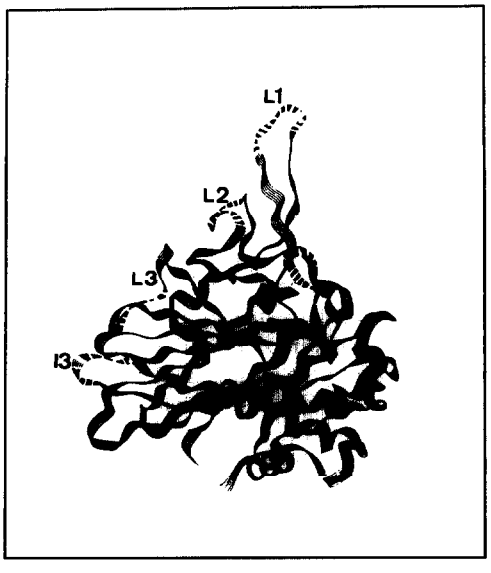
2.4. Molecular modelling

The evaluation of the total energy of the calculated molecular systems was performed by the method of molecular mechanics with cvff (consistent valence force field) [18] of the Insight II package (Biosym Technologies) [18]. The total energy was considered as the sum of bonding terms (r, j, q) and non-bonding terms (dispersion, repulsion and electrostatics). The geometries of individual structures were optimised using a conjugate gradient method [19] starting from different initial geometries and searching for absolute minima of energy. The starting geometry of the FHV capsomer protein, for computer modelling, was created from X-ray coordinates of BBV capsomer protein [20]. As the primary structures of the FHV and BBV capsomer proteins are highly homologous [21], differing residues in the structure of BBV have been replaced to fit the FHV sequence. The whole structure of the protein was then optimised by molecular mechanics, thus obtaining a reference geometry for the FHV capsomer protein (Fig. 1). This was the base for the modelling of structural modifications introduced by the insertion of IGPGRAF at the sites L1, L2, L3 and I3.

3. Results and discussion

The HIV sequence to be inserted in the FHV capsomer protein was IGPGRAF, which represents amino acid 314–320 within the HIV-1 V3-loop of the strains MN, SC and SF2. According to extensive sequence analysis studies, this sequence is present in 52% of the circulating strains in the United States and Europe [10,22]. The insertion of this sequence in sites L1, L2 and L3, resulted in the deletion of 4 or 5 amino acids of the original FHV sequence, concurrently with the insertion of the HIV sequence. In the case of position I3, the HIV sequence was inserted without any deletions in the FHV sequence. However, the genetic handling to introduce the new sequence stretch created an extra glutamic acid at the carboxy-terminus of the insertion (Fig. 1). Fig. 2A shows the Coomassie blue staining of a gel of the FHV capsid protein and the hybrids derived from it which carry the sequence IGPGRAF. As expected, all of the proteins reacted with anti-FHV-specific rabbit antiserum. The same samples, blotted onto nitrocellulose, were probed with sera from 50 HIV-1-infected patients. 62% of the HIV-1-positive sera analysed recognized at least one of the hybrid protein antigens and failed to react with the wild-ype FHV capsid protein without any HIV insert. Five characteristic patterns of reactivity were obtained (Fig. 2B): (i) all four hybrids were reactive (22.6%), (ii) L2, L3, I3 reactive (3.2%), (iii) L1, L2 and L3 reactive (9.7%), (iv) L2, L3 reactive (3.2%), and (v) only L2 (61.7%). The total number of sera that reacted with one or more of the hybrid proteins is in agreement with the known number of patients infected with HIV-1 strains that carry the IGPGRAF sequence [10]. When tested by ELISA against synthetic peptides carrying sequences representative of several HIV-1 strains (MN, SF2 and SC), 90% of the sera gave positive results. This result is in complete agreement with those of other labs [1]. Instead, reactivity against IGPGRAF might be more stringent because of the restraint imposed on the sequence by the structure of the FHV capsid protein. It should also be considered that with antigens carrying the IGPGRAF sequence, antibodies directed against the flanking region of this sequence are not detected.

L2 seems to be the most reactive hybrid since it is recognized by all positive sera. Instead, L1 and L3 are recognized by only 32% of the patients. I3 is the less reactive hybrid (17%). These results could be explained by a hierarchical order of recognition based on the differential affinity of human antibodies for related but not identical antigens. Another possibility is that the sera of HIV-1 infected patients contain several different antibodies which bind selectively only to some of the hybrid antigens. If the different recognition is based on a different affinity for the antigens, then these proteins should compete for the specific antibodies present in the sera. That this is the case is demonstrated by the results in Fig. 2C which show the reactiv-



Positions	aa deleted	aa insertion
L1	205-209 (5)	IGPGRAF
L2	268-272 (5)	IGPGRAF
L3	131-134 (4)	IGPGRAF
I3	303 (0)	IGPGRAFE

Fig. 1. 3D ribbon structure of FHV capsomer protein showing the positions of L1, L2, L3 and I3 sites chosen for the insertion of IGPGRAF sequence. Amino acid position in FHV and the modifications done for inserting the HIV-1 sequence in the different sites.

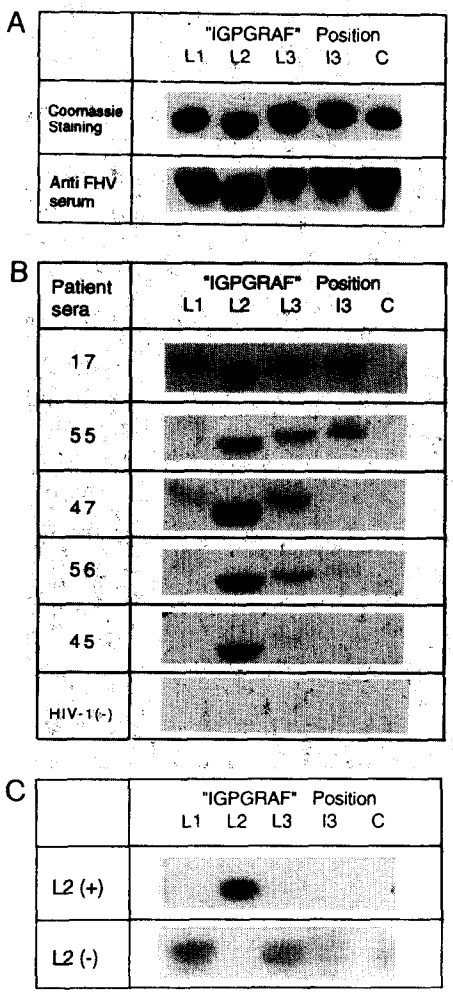


Fig. 2. Reactivity of the hybrid proteins (FHV-IGPGRAF) transferred to nitrocellulose to (A) anti-FHV. (B) HIV-I positive patients. (C) Serum 45 in the presence or absence of L2 protein.

ity of serum 45 in the presence or absence of L2 protein. When L2 is present, it captures all the reactive antibodies, instead when it is absent, L1 and L3 become positive. This would indicate that although L1 and L3 are able to bind specific antibodies present in the serum, they can not compete for binding when L2 is present. This is in agreement with the fact that L2 is the most reactive antigen, followed by L1 and L3 (with similar reactivity), and I3 (the least reactive). Sera with the highest titers of anti-IGPGRAF have enough antibodies to detect even antigens of low affinity, like I3, but when the titer of these specific antibodies is low the reactivity is restricted to more suitable antigens, like L1 and L3 or only to L2.

The different conformations that the HIV-specific sequence can adopt in each position of the FHV capsid protein can present different affinities. To test this hypothesis, the possible structures that the sequence IGPGRAF may adopt in the different positions of the protein carrier were calculated by molecular mechanics preceded by short molecular dynamic runs. The most stable conformers inserted in the FHV capsid protein at the sites L1, L2, L3 or I3 are shown in Fig. 3. From the many different conformations obtained for the protein structure with IGPGRAF at each of the positions, those with the lowest energy (most stable) were selected. In all four cases, it was found that the structure of the protein is affected mostly in regions adjacent to the inserted IGPGRAF segment (around 10 residues on both sides). Apart from the region of insertion, the overall changes of the hybrid protein are not significant. As to the IGPGRAF segment itself, its conformation varies significantly according to the sites of insertion (Fig. 3). There are differences in the backbone orientation of the segment. In L1 and I3 the inserted segments present a sharp turn at P-G residues. L2 and L3 show two turns. It can be assumed that the structure which best resembles the conformation of IGPGRAF within gp120 would be the most reactive with HIV antibodies. Since the X-ray structure of whole gp120 has not yet been determined, a direct comparison is not possible with the predicted structures shown in Fig. 3. However, the X-ray structure of a complex between a segment of the V3-loop peptide containing the sequence IGPGRAF and an HIV-1-neutralizing Ab was recently published [23] and can be used for comparison. In this complex, the V3-loop segment has an S-shaped conformation with two turns at P-G and R-A. The similarity between this structure and the structure of IGPGRAF at L2 or L3 is higher than when IGPGRAF is at L1 or I3. The similarity, clearly shown by the ribbons in Fig. 3, can be quantified by RMS (root mean squares) of the differences of the corresponding coordinates of all atoms, taking the V3 portion of the structure (of the peptide–antibody complex) as a reference. The following order of similarity between IGPGRAF inserted in FHV and the reference structure was thus obtained:

Positions:	L2	> L1	> L3	≥ I3
RMS:	2.12	2.67	3.01	3.15

The orientation of the side chains is in all cases somewhat different, probably due to alterations conferred by the antibody to the V3 structure and possible flexibilities of the individual insertions. Nevertheless, there is a clear correlation between the predicted similarity of our constructs to a putative V3 reference structure and their reactivity with HIV-1-specific antibodies.

In the present studies we analysed proteins previously denatured as required by the SDS-PAGE technique. It can be argued that the protein structure is changed and therefore any conclusions about reactivity and conformation are too speculative. However, it has been demonstrated that activity can be regained, after SDS-PAGE, for a number of enzymes and DNA-binding proteins [24–26]. This would not be possible without the partial or full renaturation of these proteins to their original native states. A similar process of renaturation after electrophoresis could account for our findings.

Knowledge of the 3D structure of the FHV capsid protein allows the possibility of inserting epitope sequences in different outer regions of the carrier protein. Our results demonstrate the influence of the surrounding structure on the reactivity of foreign sequences introduced in carrier proteins with specific antibodies. The predicted most stable conformations for IGPGRAF indicated that there is a close relationship between conformational similarity to a V3-loop reference structure and the degree of reactivity with sera when this sequence is properly located in the FHV carrier protein.

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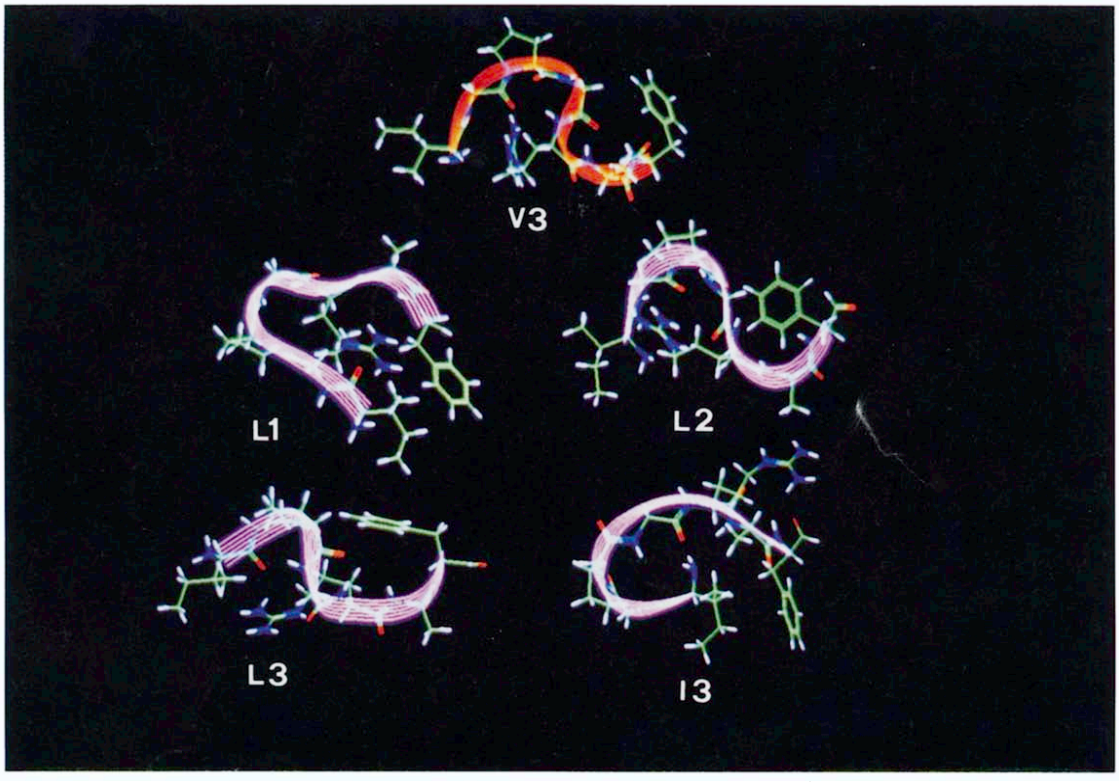

Fig. 3. Comparison between HIV-1 IGPGRAF sequence conformation and the most stable structures of IGPGRAF inserted in sites L1, L2, L3 and I3 in the FHV capsomer protein.

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