Baculovirus surface display: construction and screening of a eukaryotic epitope library

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ABSTRACT

The baculovirus expression system was utilized to serve as a tool for ligand selection, demonstrating the applicability of the system to the generation and screening of eukaryotic expression libraries. The HIV-1-gp41 epitope ‘ELDKWA’, specific for the neutralizing human mAb 2F5, was inserted into the antigenic site B of influenza virus hemagglutinin and expressed on the surface of baculovirus infected insect cells. In order to improve the antigenicity of the epitope within the hemagglutinin, and therefore enhance the specific binding of 2F5, we inserted three additional, random amino acids adjacent to the epitope. This pool of hemagglutinin genes was directly cloned into the baculovirus Ac-omega. To identify distinct proteins displayed on the cellular surface, we developed a screening protocol to select for specific binding capacity of individual viral clones. Using fluorescence activated cell sorting (FACS) we isolated a baculovirus clone displaying the epitope with markedly increased binding capacity out of a pool of 8000 variants in only one sorting step. Binding properties of the identified ligand were examined by FACS performing a competition assay.

INTRODUCTION

Expression of foreign proteins on the surface of insect cells (1,2) and in occlusion bodies (3), as well as on the baculovirus surface, has been demonstrated previously (4–6). Fusion proteins with the baculoviral envelope protein gp64 (4,6), with the gp64 membrane anchor sequence (5) as well as different foreign membrane proteins such as the influenza virus hemagglutinin (7–9), have shown to be targeted to the surface of infected insect cells.

Surface display libraries, in general, serve as a powerful tool for selecting binding proteins out of defined pools of protein variants. Prokaryotic expression systems used in phage display technology (10–13) or protein targeting to the cellular surface of Escherichia coli (14), do not allow the functional display of complex genes with a high degree of modification and processing. Boder and Wittrup (15), have suggested to use the yeast Saccharomyces cerevisiae as a eukaryotic display system. To evaluate the feasibility of a viral display tool, we constructed a eukaryotic epitope library which was expressed and screened using the baculovirus insect cell system.

We chose the membrane, associated complex hemagglutinin of influenza A virus as a carrier-molecule to provide membrane anchorage of a specific epitope library on the surface of infected insect cells. The neutralizing human monoclonal antibody (hmAb) 2F5 (16) was identified to specifically recognize the linear, six amino acid (ELDKWA) epitope (17) located in the envelope protein gp41 of human immunodeficiency virus type one (HIV-1). The idea in this approach was not to alter the epitope sequence itself but to change the tertiary structure in context with the glycosylated and highly processed hemagglutinin (cell-surface localisation, trimerisation) around the epitope, resulting in variants with increased specific immunogenicity. The aim of our work was to construct a eukaryotic baculovirus surface display library and select for binders exhibiting increased binding characteristics to hmAb 2F5 using a fluorescence activated cell sorter (FACS).

MATERIAL AND METHODS

Cells and virus

Spodoptera frugiperda cells (Sf9, CRL 1711; ATCC, Rockville, MD, USA) were grown in IPL-41 medium (Sigma-Aldrich Chemical, Deisenhofen, Germany) containing yeastolate and a lipid/sterol cocktail with optional 3 or 10% fetal calf serum at 27°C using T-flasks or spinner flasks. Ac-omega and its derivatives were propagated in Sf9 cells. Viruses were isolated and plaque-purified by standard procedures (18).

DNA manipulations

DNA manipulations were carried out essentially as summarized by Sambrook et al. (19). Restriction enzymes, T4 DNA ligase, calf intestine phosphatase and Taq DNA polymerase were purchased from Boehringer Mannheim (Vienna, Austria) and used according to the manufacturer’s recommendation. All primers were synthesized by Codon (Neusiedl, Austria). Polymerase chain reactions (PCRs) were carried out in a 50 or 100 µl reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 5% dimethyl sulfoxide and 200 µM dNTP) using 20 pmol of each primer and 2.5 U of Taq DNA polymerase. Samples were

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subjected to 30 cycles (at 92°C, 55°C or 60°C and 72°C) on a Model 9600 Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). DNA sequence-analysis were carried out on an Applied Biosystems 373A DNA sequencer, using a Taq DyeDeoxy™ Termination Cycle Sequencing Kit from Applied Biosystems (Foster City, CA, USA).

Transfection efficiency of Ac-omega

For testing the transfection efficiency, the β-galactosidase gene (β-gal) and the green fluorescent protein gene (gfp) were PCR amplified using the primers β-galSceback 5′-gatgtagtaacctatacggctagcggatctagcgaatct-3′ and β-galScefor 5′-gatgtagtaacctatacggctagcggatctagcgaatct-3′ and gfpSceback 5′-cgcggatccgctagggataacagggtaattattatttttgacaccagaccaactggg-3′, and gfpScefor 5′-cgcggatccgctagggataacagggtaattattatttttgacaccagaccaactggg-3′, respectively. The SceI treated DNA mixtures, incubated for 6 days and plaqued. The table indicates different molar ratios (β-gal/gfp) from which β-gal recombinant plaques could still be recovered.

Table 1. Transfection efficiency of Ac-Ω

<table>
<thead>
<tr>
<th>DNA (ng)</th>
<th>Molar ratio</th>
<th>β-gal/gfp</th>
<th>β-gal rec. plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Ω (134 kb)</td>
<td>50</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>gfp (0.7 kb)</td>
<td>270</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>β-gal (3 kb)</td>
<td>1.25</td>
<td>1</td>
<td>1:1000</td>
</tr>
<tr>
<td>Ac-Ω</td>
<td>50</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>gfp</td>
<td>270</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>β-gal</td>
<td>0.125</td>
<td>0.1</td>
<td>1:10000</td>
</tr>
</tbody>
</table>

*Virai Ac-omega (Ac-Ω) DNA was simultaneously ligated with different ratios of two reporter genes, β-gal and gfp. Sf9 cells were transfected with the ligation mixtures, incubated for 6 days and plaqued. The table indicates different molar ratios (β-gal/gfp) from which β-gal recombinant plaques could still be recovered.

Construction of the library and vectors for surface display

The epitope library was constructed by annealing two synthetic oligonucleotides 5′-cagttcctccgagcctatagggtaacctgattactttccttcgagctcccccttcttcgagc-3′ and 5′-atttttcgagctcccccttcttcgagc-3′ encoding the epitope motive flanked by three randomly selected amino acids (XELDK-Arc). After annealing, the two oligonucleotides have been end filled with Klenow enzyme. This double stranded DNA-fragment was then treated with restriction endonucleases PsfI and HindIII, and inserted into the plasmid pT3WSN-HAm1 (20) by replacing the wild-type fragment in this vector. In the resulting plasmid pH-A-Lib, the epitope-motive is located at antigenic site B of influenza hemagglutinin. Construction of pHA-B5: the epitope sequence ELDKWAS was inserted into the plasmid pT3WSN-HAm1 (20) by replacing a PsfI and HindIII fragment with a PCR product which was obtained using pT3WSN-HAm1 as template and the sense and antisense primers 5′-cagttcctccgagcctatagggtaacctgattactttccttcgagctcccccttcttcgagc-3′ and 5′-atttttcgagctcccccttcttcgagc-3′, respectively. Purified plasmids pH-A-Lib and pH-A-B5 were subjected to PCR using primers HASSeback 5′-ggatctctattacccgcttattcctaataagcctggagaccaacactggtcc-3′ and HASSefor 5′-ggatctctattacccgcttattcctaataagcctggagaccaacactggtcc-3′.

Direct gene insertion into Ac-omega

Generated PCR-fragments were gel purified, treated with ScI and ligated to 140 ng of purified, dephosphorylated viral Ac-omega DNA at a molar ratio of 1:40 as described previously (21). After incubating the ligation mixture at 16°C over night, Sf9 cells were transfected by lipofection (22). Insertion of foreign sequences into Ac-omega were verified by PCR, amplifying a specific fragment of viral DNA with Ac-448back 5′-ttacgctgggacagttcgc-3′ and Ac-778for 5′-caacaagcagcagatcgc-3′, respectively. PCR products of individual viral clones were sequenced with primer B A 1719 tansense 5′-ggatctctattacccgcttattcctaataagcctggagaccaacactggtcc-3′ to identify the amino acids flanking the epitope.

Transfection of the epitope library

For transfection, 140 ng of ligated Ac-omega DNA containing the library insert, was added to 20 µl lipofectin, diluted in serum free medium. After 15 min this mixture was transferred to 2.5 × 10⁶ cells and after 6 h completed by the addition of serum containing medium. The transfection was incubated at 27°C for 6 days before the cells expressing the epitope library were analysed and sorted by FACS.

Fluorescent labelling of cells

Six days post-transfection or 48 h after infection, respectively, the cells were harvested and washed twice with phosphate-buffered saline (PBS). Subsequently, cells were incubated with 1.5 µg/ml hmAb 2F5 for 1 h, washed again and stained with anti-human IgG-FITC conjugate (Sigma, Germany) at a dilution of 1:40. After an additional 1 h, the labelled cells were pelleted and resuspended in PBS to give a final concentration of ∼4 × 10⁶ cells/ml and subjected to FACS.

For the competition assay virus infected cells (48 h p.i.), virus infected cells were incubated with 1 µg/ml 200, 40 and 8 ng/ml of hmAb 2F5 in order to determine a titration curve and treated in the same manner as described in the labelling protocol (see above).

For the competition assay virus infected cells (48 h p.i.) were preincubated with 1 µg/ml hmAb 2F5 for 15 min before addition of increasing concentrations (0.2, 1, 5, 25 and 125 molar ratios) of competitor ELDKWAS-alkaline phosphatase fusion protein (23). Staining of cells was carried out as specified above.

Flow cytometric analysis and sorting

Labelled Sf9 cells were analysed on a FACS-Vantage, equipped with a 5 W argon laser (Coherent) tuned to 488 nm, output power 250 mW (Becton Dickinson, San Jose, CA). The 0.5% of the cells with the highest fluorescence signal were expected to contain cells expressing the epitope library were analysed and sorted by FACS.

Hemadsorption assay

Hemadsorption of recombinant human erythrocytes (1% in PBS) to infected cells. Sf9 cells were infected with recombinant HA-PVII and wild-type


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was inserted into the antigenic site B of the hemagglutinin gene randomly selected amino acids C-terminally (XELDKW AXX) flanked by one random amino acid N-terminally and two.

hmAb 2F5 (16) had been shown to be capable of inducing HIV-1 neutralizing antibodies in mice, when these were immunized with the native 2F5 epitope on HIV-1 (17). Though the antigenicity of the native 2F5 epitope on HIV-1 (17) had been investigated by Muster and coworkers (17). The binding capacity of this clone to hmAb 2F5 served as an internal standard for binding requirements in this experiment. We intended to select for a hemagglutinin construct with higher, or at least comparable binding characteristics relative to the HA-B5 clone by screening this baculovirus expression library.

RESULTS AND DISCUSSION

Construction and characterization of an epitope library

The conserved linear epitope ELDKWA of HIV-1, specific for the hmAb 2F5 (16), had been shown to be capable of inducing HIV-1 neutralizing antibodies in mice, when these were immunized with the chimeric influenza virus presenting the epitope associated with the hemagglutinin on the viral surface (17). Previously the amino acid sequence ELDKWA had been inserted into the antigenic site B of influenza virus hemagglutinin to mimic the antigenicity of the native 2F5 epitope on HIV-1 (17). Though the results obtained with the chimeric influenza virus were very encouraging, it was assumed that there was still potential for improved presentation of the epitope. To investigate the influence on immunogenicity of amino acids adjacent to the ELDKWA-motive, a baculovirus epitope library was constructed.

The size of a library is limited by the number of clones. To determine the efficiency of our direct cloning system (21) two reporter genes, gfp and β-gal, were simultaneously ligated into the viral genome of Ac-omega (Table 1). The amount of gfp-DNA was kept constant while the β-gal-DNA was serially diluted by a factor of ten (Table 1). At a 10 000-fold molar excess of gfp-DNA, relative to β-gal-DNA, we were still able to recover β-gal recombinant plaques from the transfection which was performed with 50 ng viral Ac-omega DNA. Results from this experiment indicate that 1 μg of Ac-omega DNA yields 2 x 10⁵ recombinant plaques.

A synthetic oligonucleotide encoding the sequence ELDKWA, flanked by one random amino acid N-terminally and two randomly selected amino acids C-terminally (XELDKWAXX) was inserted into the antigenic site B of the hemagglutinin gene of influenza virus A (Fig. 1). The generated pool of DNA plasmids was subjected to PCR, amplifying the entire, modified hemagglutinin sequence, using Sce1 recognition site containing primers. The pool of generated PCR-fragments was treated with the restriction enzyme Sce1 and directly ligated into the linearized viral DNA of Ac-omega (21). Insect cells were transfected with the ligation mix by lipofection and incubated for 6 days. The derived baculovirus expression library contained various viral clones, each of which displaying the epitope ELDKWA in a slightly different conformation and therefore differing in binding characteristics to hmAb 2F5.

Twenty individual plaques of the initial library were randomly picked, isolated from the transfection supernatant and subjected to PCR and sequence analysis. No contaminating wild-type virus could be detected. All viral clones contained the heterologous DNA insert in the proper orientation (due to the non-palindromic Sce1 restriction site), confirming the method of direct cloning to be efficient for the generation of recombinant viral clones (21). Sequence analysis revealed the required variability of the epitope library (Fig. 2). The viral clone HA-B5 was included in this study, containing an ELDKWA-motive, flanked by the native hemagglutinin wild-type amino acids. This construct also exists as chimeric influenza virus and its potential to induce HIV-1 neutralizing antibodies had been investigated by Muster and coworkers (17). The binding capacity of this clone to hmAb 2F5 served as an internal standard for binding requirements in this experiment. We intended to select for a hemagglutinin construct with higher, or at least comparable binding characteristics relative to the HA-B5 clone by screening this baculovirus expression library.

Affinity selection by FACS

The binding properties of expressed proteins on the surface of infected cells, rather than virus particles itself, were analysed, since insect cells can directly be sorted by FACS, while virus particles give too low fluorescent signals, and are too small for the cell sorter to select. This technique was used to sort a pool of initially transfected cells. Sf9 cells, transfected with the recombinant hemagglutinin constructs, were treated with hmAb 2F5 and anti-human IgG FITC-conjugate. Analysis of 20 different single clones of the library pool revealed that the fluorescence signal intensity spanned a range of one to two orders of magnitude. After a 6 day incubation period the initial pool of transfected cells was analysed and sorted by FACS (Fig. 3). Detected binding capacity of the infected cell-pool to hmAb 2F5 showed a quite homogenic distribution of the fluorescence signal-intensity. Only a small
Figure 3. FACS-affinity selection was carried out from the population of initially transfected cells, representing the entire epitope-library (red graph). Less than 0.5% (M1) of this initial pool of clones was selected. Analysis of sorted fraction, 48 h post-infection showed a markedly increase in binding capacity to mAb 2F5 (green line).

number of individual cells was considered to contain high affinity binders. The gate for FACS-sorting, therefore, was set to include <0.5% of the total cell population. This selected cellular fraction was enriched by re-infection of cells or alternatively directly subjected to a plaque assay to isolate individual viral clones.

Analysis of this enriched fraction showed an increase in binding capacity to hmAb 2F5 of about two orders of magnitude compared to the signal-intensity of the initial cell pool, which was derived after transfection of the library-DNA (Fig. 3). Subsequently 18 randomly picked plaques of the selected population were sequence analysed. All viral clones were found to contain three proline residues flanking the epitope-motive (P-ELDKWA-PP).

Characterization of selected clone

The newly selected construct HA-PVII was compared to initially characterized clones, in terms of its binding profile to hmAb 2F5. The fluorescent signal given by HA-PVII is clearly higher than the signal of the HA-B5 construct or of other library clones (Fig. 4), demonstrating that screening this baculovirus expression library was successful in identifying a molecule of higher binding capacity to hmAb 2F5.

For additional characterization of the binding profiles of selected clones, a titration curve was performed (Fig. 5). We investigated the influence of decending concentrations of hmAb 2F5, ranging from 1 µg/ml down to 8 ng/ml, on the reduction of signal intensity of HA-PVII and HA-B5, respectively. A more rapid decrease in loss of binding capacity was observed for HA-B5. The reactivity of both clones is expressed as relative values on a percentage basis (100% binding at 1 µg/ml 2F5). At a concentration of 8 ng/ml hmAb 2F5 a plateau was reached where the intensity of the signal approached background levels measured for the HA-wild-type construct.

Competition of hmAb 2F5 with recombinant epitope

The binding capacity of HA-PVII was further analysed by a competition assay (Fig. 6). To achieve 50% reduction in specific binding, a 2-fold molar excess of the competing ELDKWAS-fusion protein was required for HA-B5, whereas the same level of reduction was obtained not before a 40-fold molar excess of the competitor was reached in the case of HA-PVII. These results indicate a clear difference in resistance to competition, suggesting a better presentation of the epitope within the selected construct.

Also the titration curve of hmAb 2F5, shown for both clones in Figure 5, confirms that enhanced binding characteristics of HA-PVII seem to be caused by the proline residues flanking the epitope. This effect could be attributed to higher concentration/stability of molecules available for 2F5-binding at the cell surface. Thus, a clone with increased binding properties was identified in only one step of sorting, making this procedure highly useful for identifying eukaryotic surface-exposed molecules with desired functions by simple means of selection.

Hemadsorption assay

HA-PVII infected insect cells were examined to test whether the functionality of the hemagglutinin had been maintained, since antigenic site B is located close to the receptor binding site of this
Figure 4. Results of FACS-analysis are shown of four representative clones (including the HA-B5 construct), defining the range of signal intensity from randomly selected clones out of the initial pool. The green graph represents the signal of the selected HA-PVII, containing the three proline residues flanking the epitope. Its binding capacity to mAb 2F5 shows higher signal intensity compared to viral isolates from the initial library.

Figure 5. The binding capacity of HA-B5 and HA-PVII was evaluated by FACS, diluting hmAb 2F5 to determine individual reduction of signal intensity with descending concentrations of hmAb 2F5.

Figure 6. Competition assay was performed by FACS using increasing concentrations of ELDKWAS-alkaline phosphatase fusion protein as competitor.

protein. Generation of intact chimeric influenza virus, expressing this modified hemagglutinin, is dependent on preserving the authentic structure. Sf9 cells were infected with recombinant HA-PVII clone, and 2 days post-infection the cells were treated with human erythrocytes. Adsorption of erythrocytes to the cell surface confirmed that the biological function of the altered hemagglutinin of HA-PVII was not impaired by inserting the epitope structure into this antigenic site. The next steps will be to generate chimeric influenza viruses, containing the HA-PVII hemagglutinin to study the potential ability of this construct to induce HIV-1 neutralizing antibodies in an animal model.

CONCLUSION
We could demonstrate that the baculovirus insect cell system is highly useful for constructing and screening of surface display libraries, which are of complex, eukaryotic origin. Usually such expression libraries are limited by transfection efficiency and frequency of recombinants. We found the method of directly ligating the genes of interest into the baculoviral genome to provide...
sufficient transfection as well as recombination rates (minimum of 2 x 10^7 µg DNA). Analysing the binding properties of foreign proteins on the surface of infected cells rather than monitoring virus particles directly, seemed to be advantageous in this study. By choosing a very small gate of selection (<0.5%), infected cells could directly be sorted by FACS, yielding a high factor of enrichment. By one step of selection we were able to identify a single viral clone out of 8000 variants, that presented a specific epitope of increased binding capacity to hmAb 2F5, compared to the HA-B5 construct or randomly chosen clones from the initial library. Results from competition assays suggest an improved presentation of the epitope within the selected construct. Also the titration curve of hmAb 2F5 confirmed enhanced binding characteristics to the epitope present in selected clone HA-PVII.

The baculovirus expression system offers eukaryotic protein processing and fullfills the requirements for constructing representative libraries (cloning and transfection efficiency). Surface targeting can be achieved by foreign, membrane representative libraries (cloning and transfection efficiency). Given the power of surface display libraries by expressing fusion proteins with the baculovirus major coat associated proteins, such as the influenza virus hemagglutinin, or Surface targeting can be achieved by foreign, membrane representative libraries (cloning and transfection efficiency).

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