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Baculoviral Display of Functional scFv and Synthetic IgG-Binding Domains

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Viral vectors displaying specific ligand binding moities such as scFv fragments or intact antibodies hold promise for the development of targeted gene therapy vectors. In this report we describe baculoviral vectors displaying either functional scFv fragments or the synthetic Z/ZZ IgG binding domain derived from protein A. Display on the baculovirus surface was achieved via fusion of the scFv fragment or Z/ZZ domain to the N-terminus of gp64, the major envelope protein of the *Autographa californica* nuclear polyhedrosis virus, AcNPV. As examples of scFv fragments we have used a murine scFv specific for the hapten 2-phenyloxazolone and a human scFv specific for carcinoembryonic antigen. In principle, the Z/ZZ IgG binding domain displaying baculoviruses could be targeted to specific cell types via the binding of an appropriate antibody. We envisage applications for scFv and Z/ZZ domain displaying baculoviral vectors in the gene therapy field.

The display of foreign proteins on the surface of viruses or cells provides a tool for the analysis and design of biomolecules and their interactions (1, 2). Display technology has made great progress over the last ten years and covers applications ranging from basic research to diagnostics and therapy. One of the most successful examples of display technology is the isolation of antibodies from large combinatorial libraries displayed on the pIII coat protein of the filamentous bacteriophages (reviewed in 3). Such antibodies that recognize cell-surface markers promise to be useful tools for cell-specific targeting with applications in both research and the clinic. Recently, for example, it has been shown that filamentous phage displaying an anti-receptor (ErbB2) antibody, isolated from a phage antibody library selected on tumour cells, were capable of targeted gene delivery via receptor-mediated endocytosis into ErbB2 overexpressing cells (4).

The display of foreign proteins on the surface of the baculoviral virion has also been demonstrated (5–7). Such display has been achieved by fusion of the target protein to the N-terminus of gp64, the major envelope protein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), or to the gp64 transmembrane domain. The baculoviral display approach holds promise for the surface display of complex, glycosylated proteins which are not suitable for expression in the procaryotic environment used for phage display. The potential of this approach for the generation and screening of a eukaryotic expression library (8) or for the production of monoclonal antibodies (9) has also been demonstrated.

The baculovirus AcNPV has traditionally been used as a means of initiating the expression of heterologous proteins in insect cells (10), or as the basis of a biological insecticide (11). More recently, the ability of the baculoviral virion to efficiently mediate gene transfer and expression in mammalian cells has been demonstrated (reviewed in 12). Initially these reports indicated such gene transfer was specific for cells of hepatic origin (13, 14). Viral vectors are in general the most efficient way to mediate gene transfer (15, 16). The target cell specificity of this transfer varies depending on the type of viral vector used and maybe altered by engineering of the virion surface (reviewed in 17). One approach to engineering the viral vector surface is to display an antibody on the viral particles specific for the target cell type of interest.

In this study we have investigated the ability of the AcNPV envelope protein gp64 to display functional single-chain antibody (scFv) fragments and antibody binding proteins on the baculoviral surface. As examples of scFv fragments we have chosen a murine scFv specific for the hapten 2-phenyloxazolone (Ox) (18) and

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a human scFv specific for carcinoembryonic antigen (CEA). In order to display intact antibodies on the baculovirus surface, we fused either one (Z) or two (ZZ) synthetic immunoglobulin binding domains (19, 20) to the N-terminus of gp64. Here we report that such constructs result in functional fusion proteins, either able to bind hapten/antigen, in the case of scFv fragments, or intact antibodies, in the case of the IgG binding domains. The ability to display specific ligand binding moieties such as scFv fragments or antibodies on the baculovirus particle may allow targeting of baculoviral mediated gene transfer and hence have applications in the field of gene therapy.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* (Sf9 and Sf21AE) cells were grown in monolayer and/or suspension cultures in SF900-II medium (Gibco-BRL, MD) with antibiotics at 27°C. Cells were infected by each recombinant baculovirus at a multiplicity of infection (MOI) of 1–10 and the products analyzed 3 days post infection (p.i.). Viral stocks were produced by infecting cells at MOI 0.1 and harvesting virus after 3 days. Insect cell culture and baculoviral procedures followed standard protocols (10). For storage at 4°C, stock virus samples had FCS added to 2%.

Isolation of CEA binding scFv by phage display. A human scFv specific for carcinoembryonic antigen (CEA) was isolated from a mixture of scFv/kappa (κ) and scFv/lambda (λ) phage libraries created from gene repertoires from non-immunized humans (construction of the phage libraries will be described elsewhere).

For isolation of CEA binding scFvs, the scFv/κ and scFv/λ phage libraries were mixed in the ratio 1:2 and about 10^{11} phage particles (100 μl) of this mixed phage library was preincubated for 30 min at room temperature in a microtitre plate well coated and blocked with 0.5% BSA in 10 mM phosphate buffer/0.15 M NaCl pH 7.4 (PBS) to decrease the amount of non-specific binding phages. After preincubation the phage library was incubated for 1 h at room temperature in a microtitre plate well coated with 1 μg (100 μl/well) of CEA (Calbiochem, Cat. No. 219369) and blocked with 0.5% BSA/PBS (300 μl/well). Unbound phages were removed by washing the well several times with PBS using a microtitre plate washer. The bound phages were eluted with 0.1 M HCl pH 2.0 (100 μl/well) and neutralized with 2M Tris. The eluted phage pool was then amplified by infection of XL-1 Blue *E. coli* cells and applied for the next round of selection. The CEA binding scFv used in this work was isolated from the fifth round of panning.

Specificity of the CEAscFv. The DNA encoding the CEA binding scFv was isolated (DNA isolation kit, QIAGEN) and sequenced. For an efficient production of a soluble CEA binding scFv, the expression unit was cloned into the pKK223-3 tac vector (21) and transformed into *E. coli* strain RV 308. Induction was performed as described earlier (18), and the culture supernatant was collected. For testing the specificity of the CEA binding scFv, microtitre plate wells were coated with 1 μg of the antigen (120 μl/well) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C and blocked for 1 h at room temperature with 300 μl of 0.5% BSA in PBS. Increasing concentrations of CEA were added to the wells containing 60 μl of the culture supernatant (total volume 120 μl) and incubated for 2 h at room temperature. The scFv produced contained the myc-peptide tag at the C-terminus and the binding was detected by adding the anti-myc peptide antibody (9E10) followed by alkaline-phosphatase-conjugated anti-mouse IgG (H+L) (Bio-Rad, CA). Both antibodies were incubated for one hour at room temperature. After addition of the substrate *p*-nitrophenylphosphate in diethanolamine-$MgCl_2$-buffer (2 mg/ml) (Orion) the absorbance of each well was measured at 405 nm.

Plasmid construction and production of recombinant baculoviruses. The construction of the plasmid pFLAGgp64 has been described previously (6). The plasmids pOxscFv/L1gp64 and pOxscFv/L4gp64 were constructed by PCR of two murine single-chain antibodies (scFv) specific for the hapten 2-phenyloxazolone (Ox) (18) (5′ primer, TAC CAG ATC TGT CAG GTG CAG CTG AAG, 3′ primer, TAG CGA ATT CCA GCT CCA GCT TGG T) and cloning of the products into the BglII/EcoRI sites of pFLAGgp64. The resultant plasmids encode the fusion proteins FLAG-OxscFv/L1-AcNPVgp64 and FLAG-OxscFv/L4-AcNPVgp64, where the linker peptide joining the V_H and V_L domains was either a 28 amino acid interdomain linker peptide from the fungal cellulase CBHI (L1) or the 15 amino acid $(GGGGS)_3$ peptide (L4), respectively (18). The plasmid pCEAscFvgp64 was constructed by PCR of the human scFv specific for CEA (containing the L4 linker, 5′ primer, TAG AGA ATT CCA GGT GCA GCT GGT GC, 3′ primer, TAA AGA ATT CTC TAG AGG CCG CAC GTT TGA TCT CCA G), which was isolated from the phage display library as described above, and cloning of the product into the EcoRI site of pFLAGgp64. The resultant plasmid encodes the fusion protein FLAG-CEAscFv-AcNPVgp64. The plasmids pZgp64 and pZZgp64 were constructed by PCR of the Z (5′ primer, TAC CGG ATC CTG CAG GCG CAA CAC GAT GAA GC, 3′ primer, TAA AGA ATT CCC GGG TTT CTA GAT CTA CTT TCG GCG CCT GAG C) and ZZ (5′ primer, same as for the Z domain, 3′ primer, TAA AGA ATT CCC GGG TTT CTA GAT TCG CGT CTA CTT TCG G) domain coding sequences (19, 20) from the plasmid pEZZ18 (Pharmacia Biotech, Uppsala, Sweden) and cloning of the products into the BglII/EcoRI sites of pFLAGgp64. The plasmids pZgp64 and pZZgp64 encode the fusion proteins FLAG-Z-AcNPVgp64 and FLAG-ZZ-AcNPVgp64, respectively. All the 3′ primers used for the PCR based cloning of the CEAscFv, Z and ZZ domains, include a XbaI site, which is unique in the plasmids pCEAscFvgp64, pZgp64 and pZZgp64. This site can be used to insert an oligonucleotide encoding a linker peptide to separate the displayed protein domain (scFv, Z or ZZ) from the gp64 viral envelope protein. The recombinant baculoviruses AcFLAGgp64, AcOxscFv/L1gp64, AcOxscFv/L4gp64, AcCEAscFvgp64, AcZgp64 and AcZZgp64 were produced by homologous recombination after co-transfection of the respective recombinant transfer plasmid with Bsu 36I digested BacPak6 baculoviral DNA (Clontech, CA).

SDS–PAGE and immunoblots. Protein extracts from baculovirus infected insect cells were prepared by solubilization of infected Sf9 or Sf21 cells in SDS–PAGE sample buffer with 2-ME and boiled for 5 min before electrophoresis in a 7.5% or 10% SDS–PAGE gel. Baculovirus samples for immunoblot analysis were prepared from infected cell supernatants by centrifugation of medium (100,000*g*, 30 min at 4°C) which had previously been clarified by low speed centrifugation (6,000*g*, 15 min at 4°C). The viral pellet was directly solubilized in SDS–PAGE sample buffer with 2-ME and boiled before electrophoresis. After electrophoresis, cell and baculoviral extracts were transferred to nitrocellulose and probed with either the anti-AcNPV gp64 mAb B12D5 (Dr. Loy Volkman) or the anti-FLAG mAb M1 (22, Sigma, MO), and developed with an alkaline phosphatase-conjugated anti-mouse antibody according to the manufacturer's instructions (Bio-Rad, CA). Wild type AcNPV and the recombinant AcFLAGgp64 (6) baculoviruses were used as controls.

ELISA of gp64 fusion proteins and baculoviruses. The binding activity of the different gp64 fusion proteins was confirmed with an ELISA, by initially coating 96-well microtitre plates with 10 μg/ml of bovine IgG or CEA, or with 40 μg/ml of Ox_{19}BSA in 0.1M carbonate buffer, pH 9.6 (100 μl/well). Following an overnight incubation at 4°C, the wells were washed three times with PBS and blocked with 1% BSA in PBS (200 μl/well) for 1 h at room temperature. The cell lysates (diluted in 1% BSA in PBS) were added to the wells (100 μl/well), and the plates incubated for 1 h at room temperature. The wells were washed as described above and the bound gp64 fusion

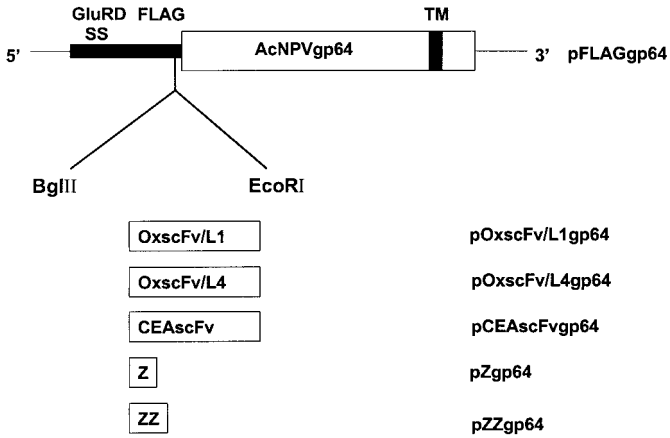

FIG. 1. Expression cassette structure of the AcNPVgp64 fusion vectors. The pFLAGgp64 vector was used for construction of the scFvgp64 fusion vectors [pOxscFv/L1gp64, pOxscFv/L4gp64, and pCEAscFvgp64] and the IgG-binding domain fusion vectors [pZgp64, and pZZgp64]. The signal peptide (SS) used is derived from the glutamate receptor GluR-D. The FLAG peptide sequence (DYKDDDDK) is derived from the pFLAGgp64 vector and located at the N-terminus of the fusion proteins. TM, transmembrane segment of the AcNPVgp64 gene. OxscFv/L1 and OxscFv/L4, 2-phenyloxazolone specific scFvs with the L1 or L4 peptide linkers (18), respectively. CEAscFv, carcinoembryonic antigen specific scFv. Z, synthetic IgG-binding domain.

proteins detected by subsequent incubation with mouse anti-gp64 mAb (in 1% BSA in PBS, 100 μl/well) for 1 h at room temperature, followed by washing with PBS, and incubating with alkaline phosphatase-conjugated goat anti-mouse antibody (in 1% BSA in PBS, 100 μl/well) for 1 h at room temperature. After the addition of the substrate (100 μl/well), the absorbance of each well was measured at 405 nm. Lysates of insect cells infected with either wild type AcNPV or recombinant AcGFPgp64 (6) baculoviruses were used as controls.

The binding specificity of the gp64 fusion baculoviruses was determined by a competition ELISA, which differed from the assay described in the previous paragraph in the following respects: (1) the assay buffer following the binding of test proteins to the microtitre plates was in 10 mM Tris buffer/0.15 M NaCl pH 7.4 (TBS), and following incubation with the detection antibody, in TBS/2 mM $CaCl_2$; (2) viral samples were incubated for 1 h with increasing concentrations of competitive ligand (0, 0.1, 1 or 10 μg/ml of CEA, Ox_{19}BSA or bovine IgG) prior to addition to microtitre plates; (3) the bound gp64 fusion baculoviruses were detected with the Ca^{2+} dependent mouse anti-FLAG mAb (M1). The AcGFPgp64 baculovirus displaying the green fluorescent protein (GFP) on the surface (6) was used as a control.

RESULTS

We have previously constructed the vector pFLAGgp64 (6) to enable fusion of heterologous proteins to the baculovirus AcNPV major envelope glycoprotein, gp64. Such fusion proteins are incorporated into and displayed on the surface of the budded baculovirus particle (5–7). We have now used the pFLAGgp64 vector to obtain baculoviral display of single-chain antibody fragments (scFv) and either one (Z) or two (ZZ) synthetic immunoglobulin binding domains (19, 20) (Fig. 1). As examples of scFv fragments we have selected a murine scFv specific for the hapten 2-phenyloxazolone (Ox) (18) and a human scFv specific for CEA (Fig. 2). Fusion proteins consisting of the OxscFv and gp64 were created with the V_H and V_L domains of the scFv connected by two different linker peptides, either the 28 amino acid interdomain linker peptide from the fungal cellulase CBH1 (L1), or the $(GGGGS)_3$ (L4) peptide linker (18). The human scFv specific for CEA was isolated after five rounds of panning on CEA antigen. The DNA fragment encoding the CEA binding scFv was cloned into the vector pKKtac and transformed into RV308 for efficient production of the antibody fragment. The expression of the scFv was confirmed from both the culture supernatant and the periplasmic fraction by immunoblot using the anti-myc peptide antibody (9E10) for detection (data not shown). The specificity of the binding was tested by competitive ELISA by adding increasing amounts of CEA in the culture supernatant containing a constant concentration of the scFv. The binding of the scFv to CEA was inhibited by the antigen in a concentration dependent manner confirming the specificity of the interaction (Fig. 2).

The gp64 fusion plasmids shown in Fig. 1 were used to generate the corresponding recombinant baculoviruses. These viruses were used to infect Sf21 insect cells, and the resulting cell pellets were analyzed by SDS-PAGE followed by probing with the FLAG epitope specific antibody, M1 (Fig. 3). The results indicate that the gp64 fusion baculoviruses are producing the correct fusion proteins, as bands were detected corresponding to the following proteins: FLAGgp64, 66kDa; OxscFv/L1gp64, 91.5kDa; OxscFv/L4gp64, 90kDa; CEAscFvgp64, 92.5kDa; Zgp64, 72kDa; and ZZgp64, 79kDa. Lower molecular weight immunoreactive products, possibly the result of proteolytic breakdown, can also be seen for some of the viruses. The proteins which were detected with

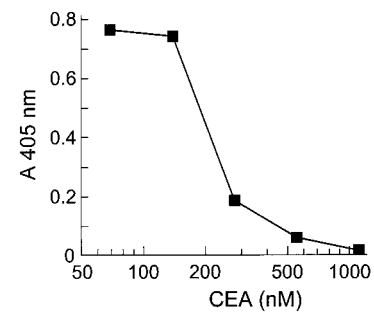

FIG. 2. Antigen binding activity of the CEAscFv determined by competitive ELISA. The induced overnight culture medium of RV 308 was incubated in CEA-coated microtiter plate wells in the presence of increasing amounts of soluble CEA in a total volume of 100 μl. The bound scFv was detected by the monoclonal antibody 9E10, specific for the myc-peptide, followed by an alkaline phosphatase antibody conjugate.

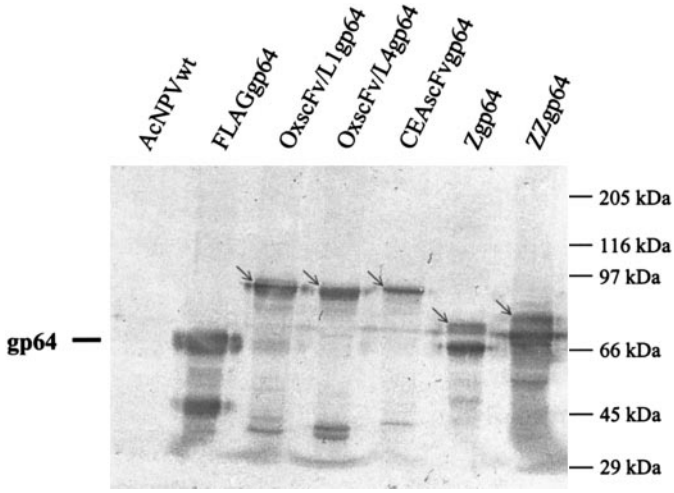

FIG. 3. Western blot of gp64 fusion baculovirus infected Sf21 cells. The nitrocellulose membrane was probed with the FLAG epitope specific mAb, M1. The viruses used are indicated. The position of the protein molecular mass standards is indicated on the right of the figure. An arrow indicates the positon of the individual gp64 fusion proteins.

the M1 antibody also reacted with an anti-gp64 antibody (data not shown).

To investigate the level of incorporation of the gp64 fusion proteins into the AcNPV virion we collected budded virus from infected Sf21 cells and prepared viral pellets by centrifugation. The purified viral pellets were analyzed by SDS-PAGE and probing with either the M1 or an anti-gp64 antibody (Fig. 4). A comparison of the results for the scFvgp64 fusion proteins indicates that the L1 linker peptide fusion protein is minimally incorporated into the AcNPV virus particles, where as the L4 linker fusion proteins (OxscFv/L4gp64 and CEAscFvgp64) are easily detected in the virus particles. Both of the IgG-binding domain (Z or ZZ) gp64 fusion viruses incorporated the corresponding gp64 fusion proteins into the virus to clearly detectable levels (Fig. 4).

The ability of the scFv or IgG binding domain gp64 fusion proteins to bind to their respective ligands was tested in ELISA assays. Initially, antigen or IgG binding was demonstrated using the cell lysates from virus infected insect cells (Fig. 5). The only fusion protein which failed to give a signal above the background level was the OxscFv/L1gp64 fusion. The specificity of antigen or IgG binding of the different gp64 fusion proteins was demonstrated in competition ELISA assays utilizing tissue culture supernatants collected from virally infected insect cells (Fig. 6). The OxscFv/L1gp64 protein failed to bind Ox_{19}-BSA, consistent with the low level of incorporation of this fusion protein into the virus particle (Fig. 4). Although the Zgp64 fusion protein was capable of binding to IgG (Fig. 5), it gave only a very low signal when viral supernatants were tested in an ELISA assay (data not shown). The CEAscFvgp64 and ZZgp64 baculoviruses retained CEA and IgG binding activity, respectively, after pelleting through a 25% sucrose cushion (data not shown), confirming the viral incorporation of the gp64 fusion proteins.

DISCUSSION

The studies reported here suggest that scFv fragments and the synthetic Z/ZZ IgG binding domain of protein A are displayed in a functional form on the baculoviral surface via fusion to the N-terminus of the AcNPV envelope protein gp64. Recently, it has been shown that a number of foreign proteins including glutathione-*S*-transferase (GST) and the human immunodeficiency virus type 1 (HIV-1) gp120 protein (5), the HIV-1 gp41 protein (7), the green fluorescence protein (GFP) and rubella virus spike proteins (6), and human nuclear receptors (9), are all expressed on the

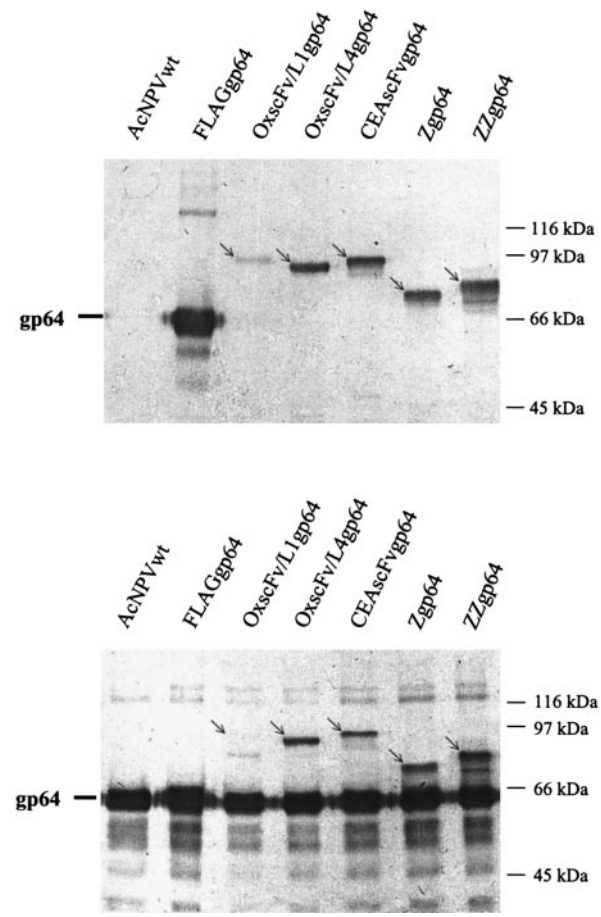

FIG. 4. Western blots of gp64 fusion baculoviruses. Nitrocellulose membranes were probed with either the FLAG epitope specific mAb, M1 (upper panel), or the anti-gp64 mAb, B12D5 (lower panel). The viruses used are indicated. The position of the protein molecular mass standards is indicated on the right of the figure. An arrow indicates the position of the individual gp64 fusion proteins.

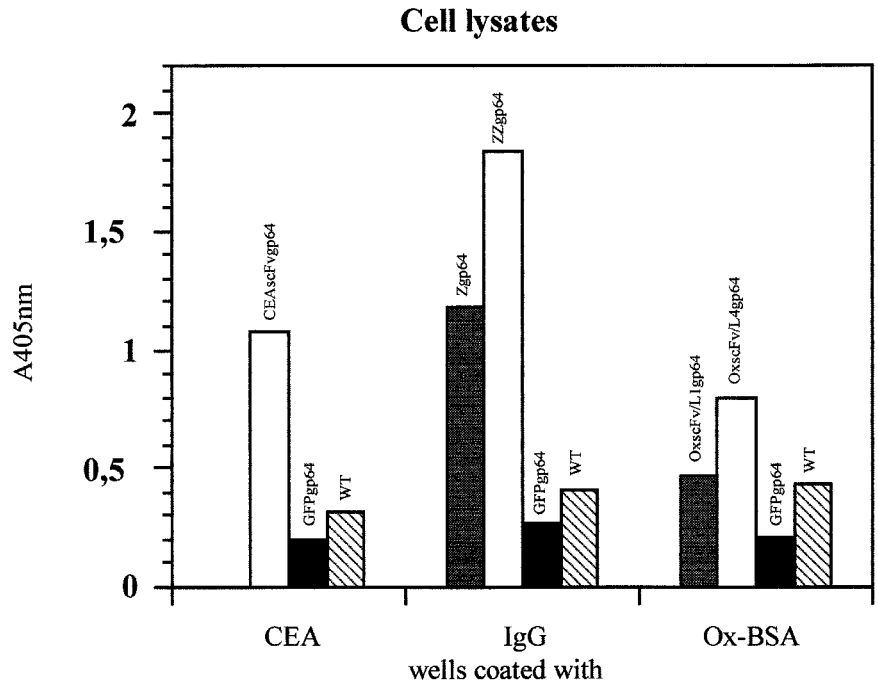

FIG. 5. Antigen or IgG binding of the gp64 fusion proteins. ELISA assays utilizing virally infected cell lysates were performed as described under Materials and Methods. Wild type AcNPV and AcGFPgp64 (6) baculoviruses were used as controls.

surface of the baculovirus virion when fused to the N-terminus of gp64. To our knowledge this is the first time that scFv fragments or antibody binding proteins (the Z domain) have been displayed on the surface of the baculovirus.

Of the scFvgp64 fusion proteins used in this study the CEAscFvgp64 protein, which was originally isolated from an antibody phage display library, showed the highest antigen binding activity based on ELISA measurements. In our earlier studies the OxscFv/L1 and OxscFv/L4 fragments showed similar hapten binding properties when produced as soluble antibodies in bacteria (18). Here we observed hardly any hapten binding activity in the case of the OxscFv/L1gp64 protein. The L1 linker peptide used to join the variable domains of the heavy and light chains is a linker peptide of the cellobiohydrolase I (CBHI) of the filamentous fungus *Trichoderma reesei*. The linker peptide is known to be highly O-glycosylated (23). Possibily the hyperglycosylation of the linker may prevent the proper folding of the V domains and thus effect the binding activity of the scFv. Furthermore, hyperglycosylation of the linker may also disturb incorporation of the protein into the baculovirus particle resulting in the low amount of the OxscFv/L1gp64 protein observed in the virus particle.

The Z domain, which is a synthetic variant of the staphylococcal protein A (19, 20), binds strongly to the Fc domain of IgG antibodies. The Z domain is an individually folding domain, which can be used as a target for patch engineering (1) to create binders with different specificities, as shown by studies where combinatorial libraries of the Z domain were constructed and displayed on phage to select proteins or "affibodies" with novel binding specificities (24, 25). In our study the results suggest that the amounts of the Zgp64 and ZZgp64 incorporated into the virus particle are comparable, while higher IgG binding activity was detected in the case of the ZZgp64. This can at least partly be due to the bivalency of this two domain protein.

The ability to display specific ligand binding proteins on the baculovirus surface may allow targeting of baculoviral-mediated gene transfer to specific cell types via the targeting of particular entry and uptake pathways. Initial reports of baculoviral gene transfer to mammalian cells indicated that the transfer was specific for hepatic cells (13, 14). Further studies however have indicated that baculoviruses are capable of mediating gene transfer to a wide range of cell types (26–28). The reason for the discrepancy between these studies is not clear. However, these studies have demonstrated the potential of virion surface engineering (27) and the importance of promoter selection (28) in baculoviral-mediated gene transfer to mammalian cells. A hypothetical cell type specific baculoviral vector may need to combine both the virion surface engineering approach with a target cell specific promoter.

A number of viral vectors which have been utilized in gene transfer studies have also been modified with the aim of obtaining directed gene therapy vectors (reviewed in 17). Retroviral vectors have the longest history of such attempts, and both the display of scFv

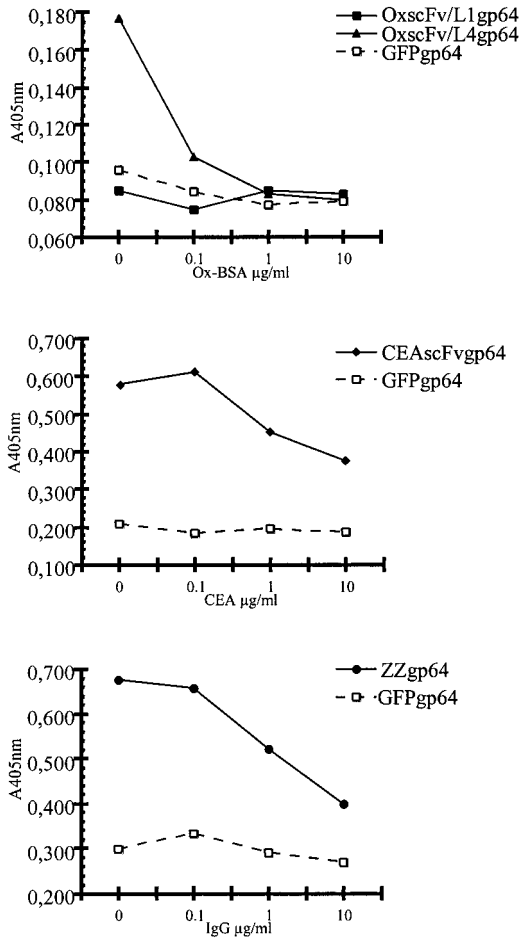

FIG. 6. Antigen or IgG binding of the gp64 fusion baculoviruses. Competition ELISA assays utilizing viral supernatants were performed as described under Materials and Methods. The AcGFPgp64 baculovirus used as a control has been described previously (6).

fragments (29–35) and the IgG binding domain of protein A (36) has been achieved. Recently, a Sinbis virus vector has been generated displaying the IgG-binding domain of protein A (37), and this vector has established the principle of a multiply-targeted vector via the use of different target cell specific monoclonal antibodies. Adenovirus mediated gene transfer has been used in many gene therapy studies and recently success has been achieved in specific targeting of adenoviral vectors (38–43). At least some of these studies rely on the ability to ablate the native tropism of the adenoviral vector for the coxsackievirus/adenovirus receptor or for specific cell surface integrins, and to simultaneously introduce a new receptor binding moiety (38, 40, 42). Before it is possible to apply a similar approach to baculoviral vectors much more will need to be known about the mechanism of baculovirus uptake by mammalian cells. In the interim period the usefulness of gp64 fusion baculovirus vectors for gene therapy applications needs to be investigated. In this context, the AcCEAscFvgp64 virus may be useful in targeting tumour cells expressing CEA antigens, eg colorectal carcinoma cells (33), whereas the AcZZgp64 virus may be targeted to any cells where an antibody is available to a cell specific antigen, eg the EGF receptor (42).

Although baculoviral vectors can be highly efficient vehicles to mediate gene transfer to mammalian cells *in vitro* (13, 14, 26–28), *in vivo* gene delivery is hampered by viral inactivation by complement (44–46). One approach to overcome this problem is to block activation of the complement pathway, and this has been demonstrated with a blocking antibody against complement component 5 (45). Therefore, one potential application of the AcZZgp64 virus would be to bind and display a complement blocking monoclonal antibody to enable the use of baculoviral-mediated gene transfer in the *in vivo* setting.

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