Expression, characterization and purification of simian immunodeficiency virus soluble, oligomerized gp160 from mammalian cells

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The envelope glycoprotein, gp160, of human (HIV) and simian (SIV) immunodeficiency viruses mediates virus-host cell binding followed by fusion of the viral and plasma membranes. The envelope proteins are known to exist as non-covalently associated oligomers on the virus surface. The production of permanent mammalian cell lines that constitutively secrete relatively high levels of soluble forms of SIV gp160 is described and we show that these proteins are secreted predominantly as tetramers with lower levels of dimer forms. Oligomeric forms were purified to greater than 90% purity using a simple gel filtration method. The purified proteins bind CD4 suggesting that they remain in their native conformation. The purified oligomeric proteins provide the basis for more relevant structural, functional and immunological studies than recombinant gp120 as they more closely resemble the envelope protein oligomer.

The envelope glycoprotein (gp160) of human (HIV) and simian (SIV) immunodeficiency viruses is responsible for virus binding and entrance into host cells. Gp160 is proteolytically cleaved by cellular proteinases to form the external glycoprotein gp120 and the transmembrane glycoprotein gp41. Gp120 remains associated with gp41 by non-covalent interactions. Gp120 is responsible for binding CD4 on host cell membranes and gp41 serves as an anchor for gp120 and directs membrane fusion in a pH-independent manner (McClure et al., 1988). A fusogenic domain at the hydrophobic amino terminus of gp41 may be unmasked by a conformational change induced when gp120 binds to CD4 (Kowalski et al., 1987). Cleavage of gp160 is required for activation of the fusogenic domain in gp41 (McCune et al., 1988) but does not affect the CD4 binding potential of gp120 (Earl et al., 1991).

Mature HIV and SIV gp160 have been shown to form oligomers. Evidence from cross-linking studies and gradient centrifugation suggests that HIV-1 gp160 exists as a tetramer consisting of two non-covalently associated dimers (Earl et al., 1990; Schawaller et al., 1989). This structure is similar to that observed previously for paramyxovirus envelope proteins F and HN. It has been shown that HIV-1 gp41 units can exist as tetramers (Pinter et al., 1989) and that the extracellular portion of gp41 contains a functionally conserved domain responsible for oligomerization (Doms et al., 1990). Studies also suggest that HIV-1 gp120 can oligomerize (Weiss et al., 1990) and similar results concerning the oligomerization of HIV-2 and SIV gp160 have been reported (Chakrabarti et al., 1990; Rey et al., 1990). Oligomeric HIV envelope proteins have been shown to exhibit multimeric CD4 binding (Earl et al., 1992) and the ability to cross-link CD4 may be important in the pathogenic potential of HIV (Banda et al., 1992).

Oligomerization of surface glycoproteins on enveloped viruses is a common phenomenon and a likely prerequisite for transport to the surface of infected cells. An understanding of the oligomeric structure of HIV and SIV gp160 would contribute to understanding how gp160 mediates attachment and fusion of viral particles and target cells.

We have expressed, purified and characterized a soluble, oligomerized recombinant form of SIV gp160 from continuous mammalian cells that constitutively produce useful quantities of protein. This protein will be valuable in functional studies and immunization trials.

A mammalian expression vector was designed that was capable of efficient expression and secretion of soluble gp160 (sgp160) derived from a molecular clone of a rhesus macaque isolate of SIV, designated SIVmac239 (Rud et al., 1992). The signal sequence of the SIV envelope gene was replaced with the signal sequence from tissue plasminogen activator (tPA), which has been shown to increase the level of expression of some
secreted, heterologous proteins (Chapman et al., 1991). This necessitated a change in the envelope protein sequence such that the mature protein possessed an N-terminal serine in place of a threonine residue (residue 1), a relatively conservative change. A translation stop codon was introduced at residue 666 by PCR mutagenesis at the junction of the extracellular domain and the trans-membrane domain of gp41. The object of this mutation was to eliminate the trans-membrane portion of the envelope gene so that the external envelope glycoprotein, gp160, would be secreted. The primary and secondary cleavage sites were identified by their homology to the cleavage sites described for other isolates of HIV and SIV (Kieny et al., 1988). In one gene construct, mutations were also placed at the putative cleavage sites between gpl20 and gp41, in order to prevent the dissociation of gpl20 and gp41. The DNA encoding the envelope glycoprotein was cloned into the mammalian expression plasmid pEE14tPA, a derivative of pEE14 (Kingston et al., 1992) to generate the vectors pSIV-P (cleavage sites retained) and pSIV-M (cleavage sites removed).

In order to generate pSIV-M two DNA fragments were generated by PCR, the first using oligonucleotides 1 and 4, and the second using oligonucleotides 2 and 3 (see Fig. 1). These DNA fragments were purified and used as templates in a third PCR using oligonucleotides 1 and 2. The resulting DNA fragment had a nucleotide change at position 8171 (A to G), which changed amino acid 523 (lysine) to glutamic acid abolishing the primary cleavage site at the gp120/41 junction. Also changed were nucleotide positions 8141 (A to G) and 8142 (G to A), which changed amino acid 512 (arginine) to glutamic acid abolishing the secondary cleavage site at the gp120/41 junction. The DNA fragment generated includes nucleotides 6671 to 8665 (amino acids 2 to 665) of SIV gp160 with 5' BamHI and 3' EcoRI sites to facilitate subsequent cloning.

The tPA leader sequence was cloned in pEE14 as a HindIII to BglII fragment (Harris et al., 1986) to create pEE14tPA. The BamHI–EcoRI fragment containing the sgp160 gene was cloned between the BglII and EcoRI sites of pEE14tPA to create pSIV-M. The sgp160 gene thus has a tPA leader sequence immediately preceding the first residue (serine). This cloning step is shown in Fig. 1.
A second plasmid vector was made in an identical way as described above except that the DNA fragment was made by a one-step PCR using oligonucleotides 1 and 2 to amplify DNA from pSIV gp160, and thus the primary and secondary cleavage sites between gp120 and gp41 were retained in the DNA fragment generated. The DNA fragment was cloned into pEE14tPA as described above to give pSIV-P. The sgp160 DNA sequences in plasmids pSIV-M and pSIV-P were shown to be correct by DNA sequencing. The ability of the plasmids pSIV-M and pSIV-P to direct the expression of sgp160 was verified by immunoprecipitation and Western blot analysis of supernatants generated by transient expression using Chinese hamster ovary (CHO) L761h cell lines (data not shown). These plasmids were subsequently used to generate permanent CHO L761h cell lines designated M5 and P5 for large-scale production and purification of SIV envelope glycoprotein.

CHO L761h cells (Cockett et al., 1991) which constitutively express the adenovirus E1a trans-activator [which trans-activates the human cytomegalovirus (HCMV) immediate early promoter] were stably transfected with the plasmids pSIV-M and pSIV-P and selected for resistance to 25 μM-methionine sulfoximine in Glasgow modified Eagle’s medium (Kingston et al., 1992). Surviving clones were individually transferred to 24-well plates, grown to confluence and supernatants were tested for secretion of sgp160 by Western blot and a capture ELISA. The highest producer cell lines, designated M5 (which expressed sgp160 M5 mutated at the cleavage site between gp120 and gp41) and P5 (which expressed sgp160 P5 retaining the cleavage site between gp120 and gp41), expressed sgp160 and were selected for further analysis.

CHO L761h M5 and P5 cells were grown in roller bottles until confluent. The growth medium containing 10% dialysed, heat-inactivated fetal calf serum (dFCS) was then replaced with production medium containing 0.5% dFCS. Medium was removed and replaced at 5 day intervals (up to six times). Five days was found to be optimal as cleavage of the expressed proteins resulted from longer harvest times; this was thought to be due to the release of proteinases from dead cells. Conditioned production medium was filtered through a 0.45 μm filter (Sartorius).

Production levels for the cell lines were determined using a twin-site ELISA carried out essentially by a method described previously (Moore, 1990) but using KK8 antibody (2 μg/ml) (Kent et al., 1991) as a capture antibody. Captured sgp160 was detected using sheep polyclonal antibody D7368 raised against a C-terminal peptide of SIVmac251 gp120 (Repligen) was used as a standard. Production levels measured in this way were 0.40 μg/10^6 cells/day for the M5 cell line and 0.16 μg/10^6 cells/day for the P5 cell line. These figures are similar to those reported for other HIV and SIV envelope proteins in mammalian expression systems (Plannelles et al., 1991; M. Spitali, G. Hutchinson, A. Rhodes, G. Yarranton & P. Stephens, unpublished).

To investigate the oligomeric state of SIV sgp160, culture supernatants containing SIV gp120 (M. Spitali et al., unpublished) and SIV sgp160 M5 and P5 samples were examined on a 4% polyacrylamide gel containing 0.1% SDS under reducing and non-reducing conditions and Western-blotted by standard techniques (Sambrook et al., 1989) using the rabbit antiserum R319 generated against SIVmac251 gp120 at Celltech by a standard immunization protocol (Dunbar & Schwoebel, 1990) and a gold labelled anti-rabbit antibody in combination with an AuroProbe kit (Cambio). The results are shown in Fig. 2, but because of the high M_r range examined on this gel, it was not possible accurately to determine the relative molecular masses under non-reducing con-
no difference in the number and site of glycosylation sites between the two closely related isolates and it appears unlikely that interclone differences can account for the different mobilities. It was also notable that only a SIV \(\text{a2}_{(251)}\) gpl20 contains different levels of glycosylation under reducing conditions SIV \(\text{a2}_{(32mJ.5)}\) gpl20 derived from gpl20 (Weiss et al., 1991). There is no difference in the number and site of glycosylation sites between the two closely related isolates and it appears unlikely that interclone differences can account for the different mobilities. It was also notable that only a fraction of sgp160 P5 protein was cleaved to gp120 and gp41 when it is expressed in CHO cells. This may be due to the lower level of proteinases that bring about the cleavage in CHO cells compared to lymphocytes. It has been shown that gp120/gp41 cleavage efficiency is strongly cell type-dependent (Earl et al., 1991).

To investigate the \(M_c\) of these protein species further, 10-fold-concentrated samples of CHO supernatant were subjected to gel filtration through Sepharose 6B (Sigma). The gel filtration column (60 x 1 cm) was calibrated using a gel filtration marker kit (Sigma, MW-GF-1000) containing \(M_c\) markers in the range 29K to 700K and dextran blue for the determination of the column void volume. The elution profiles of SIV proteins were calculated by collecting 0.5 ml fractions and determining the level of SIV gp 120/sgp160 in each fraction using the ELISA described above. The calibration curve for the gel filtration column is shown in Fig. 3(a) and the elution profile for SIV gp 120 and SIV sgp160 M5 and P5 in Fig. 3(b). From the curve it appeared that the major form of sgp160 existed as a 660K species (a tetramer would be 640K) and there were lower levels of a 330K species (a dimer of sgp160 would be 320K). The dimer form of sgp160 appeared as a shoulder to the tetrameric form in the elution profile. By contrast gp120 existed mainly as a 130K form (monomeric) with a possible 260K dimeric component appearing as a shoulder to the elution profile. The sgp160 P5 also contained a 130K component, presumably due to dissociation of sgp160 to gp120.

Since sgp160 appears to be secreted from CHO cells largely as a tetramer, gel filtration provided a possible method for purification. Protein from 1 litre batches of supernatant was precipitated with 85% ammonium sulphate, resuspended in 30 ml of water and dialysed for 24 h against three changes of PBS (2.5%). Six ml aliquots (containing approximately 0.5 mg sgp160) of the dialysed proteins were separated by gel filtration chromatography using a Sephacryl S-400HR (Pharmacia) column (90 x 2.5 cm). Three ml fractions were collected and each fraction was screened for sgp160 using the ELISA described above. Fractions containing sgp160 were pooled and concentrated by ultrafiltration centrifugation through a Centricon filter with a 10K size exclusion (Amicon, PN4206). Protein storage was at \(-20^\circ\mathrm{C} in 50\text{mm-Tris–HCl pH 7.6, 100 mm-KCl.}

Sgp160 M5 and P5 were more than 90% pure as determined by scanning the density of bands on a Coomassie blue-stained 7.5% polyacrylamide gel. The level of sgp160 was quantified against a known concentration of HIV-1HIV gp120 by comparing the band density on a Coomassie blue-stained polyacrylamide gel using a densitometer. The yield of sgp160 M5 was determined to be 1 mg/l of cell supernatant (40%). A similar level of purity and percentage yield were obtained.
Fig. 4. (a) Analysis of purified SIV sgpl60 M5 on a Coomassie blue-stained 0.1% SDS, 4% polyacrylamide gel. Samples were boiled for 1 min and run under non-reducing conditions (lane 1) and reducing conditions (lane 2). (b) Western blot analysis of purified SIV sgpl60 M5 using antibody R319 (samples were not boiled). Material was analysed on a 0.1% SDS, 4% polyacrylamide gel under reducing conditions (lane 1) and non-reducing conditions (lane 2). Arrows indicate high Mr oligomers.

for sgpl60 P5. The gel filtration elution profile of both purified sgpl60 M5 and P5 was identical to that of sgpl60 in crude cell supernatant (results not shown) indicating that the purified material remained predominantly as a tetramer. Purified material was examined on 4% polyacrylamide gels containing 0.1% SDS under reducing and non-reducing conditions and stained with Coomassie blue. The results using purified sgpl60 M5 are shown in Fig. 4(a), and results for sgpl60 P5 were very similar. Under reducing conditions a band is seen at around 160K. Under non-reducing conditions two high Mr bands exist (indicated by arrows) which indicate that the proteins remain in the oligomerized state. A band can also be seen at around 160K under non-reducing conditions. Western blot analysis indicates that this is the monomeric form of sgpl60 (data not shown) and probably results from the partially denaturing conditions on the gel and the fact that the samples were boiled for 1 min prior to loading. In the absence of boiling this band is much reduced in intensity as seen on the Western blot in Fig. 4(b). It should be noted that this monomeric component is not observed on the gel filtration elution profile of purified sgpl60 M5. The relative intensity of the two high Mr bands varied significantly from one gel to another. This was presumably an artefact of the gel conditions resulting in differing levels of tetramer dissociation to dimers. It has been reported that the interaction between dimer units in a tetramer is relatively weak compared to the interactions between individual sgpl60 molecules in a dimer (Doms et al., 1991). Purified sgpl60 M5 was treated with 5 mM chemical cross-linking agent ethylene glycol bis(succinimidyl succinate), as described previously (Chakrabarti et al., 1990). Samples were electrophoresed under reducing conditions and Western-blotted as described in Fig. 4(b). Results were identical to those described in Fig. 4(b). This indicates the observed oligomerization of sgpl60 M5 was not due to non-specific aggregation.

The N-terminal amino acid sequence of purified sgpl60 M5 and P5 proteins was determined by drying the protein onto a Problot PVDF membrane in a Pro-Spin sample preparation cartridge (Applied Biosystems, PN401256). The membrane was then loaded into the sequencing block cartridge on a 470A Protein Sequencer connected on line to a 120A analyser (Applied Biosystems) operated according to manufacturer’s instructions for 15 cycles of Edman degradation. The first residue of sgpl60 M5 and P5 was a serine, as expected, which indicated that the tPA leader sequence had been accurately removed.

The principal known function of SIV and HIV gp120 is to attach virions to the CD4 antigen found on T helper lymphocytes and macrophages (Sattentau et al., 1988). Binding of gp120 appears to be dependent on the overall conformation of the molecule; reduction, denaturation, or synthesis of gp120 in bacteria or yeast result in proteins that are unable to bind CD4 (McCleire et al., 1988; McDougal et al., 1986; Putney et al., 1986). CD4 binding assays were undertaken using a commercial gp120/CD4 ELISA (DuPont) which consists of microtitre plates with bound human CD4. Gp120/sgpl60 samples (100 µl/well) were diluted in PBS and incubated at 4 °C for 2 h. The plate was washed and aspirated three times with PBS. 100 µl of KK8 antibody (2 µg/ml in PBS) was added to each well and incubated at 4 °C for 2 h. The wells were aspirated and washed three times with PBS. 100 µl/well of horseradish peroxidase-labelled anti-mouse antibody (Binding Site; 2 µg/ml in PBS) was added. Colour was developed and recorded as described previously (Moore et al., 1990). Background (negative control) absorbance values were subtracted from those shown in Fig. 5. Levels of sgpl60 were estimated based on the intensity of bands in Coomassie blue-stained SDS–polyacrylamide gels relative to HIV-1MUB gp120 (Celltech) by densitometric scanning. The concentration of HIV-1MUB gp120 has been previously determined by calculation of the extinction coefficient after amino acid analysis.
macaques against homologous virus challenge. It is likely that SIV sgpl60 is a better immunogen than gpl20 as an envelope glycoprotein, because it was not possible to use saturating concentrations of sgpl60 on the ELISA plate. The CD4 binding curve of sgpl60 M5 has a steeper slope than that of gpl20 M5, indicating that sgpl60 M5 binds CD4 more strongly. If this is the case then the lower CD4 binding affinity may be due to the cleavage of sgpl60 to gpl20 in P5. It is possible then that some gp120 dissociates from sgpl60 and may have a lower CD4 binding affinity if it is not oligomerized.

In conclusion we have demonstrated that CHO-produced SIV sgpl60 protein has characteristics that are similar to those reported for native viral envelope proteins. The protein can be easily purified in large quantities and provides an opportunity to investigate the requirements of subunit vaccines for the generation of a protective immune response against SIV and therefore by implication HIV. Purified sgpl60 is currently being tested for its ability to protect immunized rhesus macaques against homologous virus challenge. It is likely that SIV sgpl60 is a better immunogen than gp120 as an HIV-1 sgpl60 protein has been reported to elicit rabbit antibodies that were enriched for populations that blocked CD4 binding, when compared to rabbit antibodies generated against HIV-1 gp120 (Berman et al., 1989). Improved antigenicity has also been reported for recombinant HIV envelope proteins in which the cleavage sites between gp120 and gp41 had been removed (Kieny et al., 1988).

The availability of oligomerized protein in large quantities provides the opportunity to characterize the oligomeric structure of SIV envelope protein further and to determine the clinical significance of oligomerization. Although it has been shown that oligomerized envelope proteins can bind multiple CD4 molecules (Earl et al., 1992), the availability of the protein in large quantities will facilitate study of possible allosteric mechanisms that may be involved in CD4 binding to oligomerized envelope proteins.

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Fig. 5. CD4 binding assay using (a) sgpl160 M5 and (b) sgpl160 P5.

The graph in Fig. 5(a) shows that sgpl160 M5 expressed by the cell line CHO L761h M5 binds CD4 and Fig. 5(b) shows that sgpl160 expressed by CHO L761h P5 also binds CD4. Actual binding constants were not calculated because it was not possible to use saturating concentrations of sgpl160 on the ELISA plate. The CD4 binding curve of sgpl160 M5 has a steeper slope than that of sgpl160 P5 indicating that sgpl160 M5 binds CD4 more strongly. If this is the case then the lower CD4 binding affinity may be due to the cleavage of sgpl160 to gp120 in P5. It is possible then that some gp120 dissociates from sgpl160 and may have a lower CD4 binding affinity if it is not oligomerized.

In conclusion we have demonstrated that CHO-produced SIV sgpl160 protein has characteristics that are similar to those reported for native viral envelope proteins. The protein can be easily purified in large quantities and provides an opportunity to investigate the requirements of subunit vaccines for the generation of a protective immune response against SIV and therefore by implication HIV. Purified sgpl160 is currently being tested for its ability to protect immunized rhesus macaques against homologous virus challenge. It is likely that SIV sgpl160 is a better immunogen than gp120 as an HIV-1 sgpl160 protein has been reported to elicit rabbit antibodies that were enriched for populations that blocked CD4 binding, when compared to rabbit antibodies generated against HIV-1 gp120 (Berman et al., 1989). Improved antigenicity has also been reported for recombinant HIV envelope proteins in which the cleavage sites between gp120 and gp41 had been removed (Kieny et al., 1988).

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