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The ectodomain of HIV-1 env subunit gp41 forms a soluble, α-helical, rod-like oligomer in the absence of gp120 and the N-terminal fusion peptide

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The human immunodeficiency virus-1 (HIV-1) envelope glycoprotein is composed of a soluble glycopolypeptide gp120 and a transmembrane glycopolypeptide gp41. These subunits form non-covalently linked oligomers on the surface of infected cells, virions and cells transfected with the complete *env* gene. Two length variants of the extracellular domain of gp41 (aa 21–166 and aa 39–166), that both lack the N-terminal fusion peptide and the C-terminal membrane anchor and cytoplasmic domain, have been expressed in insect cells to yield soluble oligomeric gp41 proteins. Oligomerization was confirmed by chemical cross-linking and gel filtration. Electron microscopy and circular dichroism measurements indicate a rod-like molecule with a high α-helical content and a high melting temperature (78°C). The binding of monoclonal antibody Fab fragments dramatically increased the solubility of both gp41 constructs. We propose that gp41 folds into its membrane fusion-active conformation, when expressed alone.

Keywords: conformational change/envelope glycoprotein gp41/HIV-1/membrane fusion

Introduction

The envelope glycoprotein (env) of human immunodeficiency virus-1 (HIV-1) is synthesized as a precursor molecule gp160 and subsequently processed into its subunits gp120 and gp41 (Allan *et al.*, 1985; Veronese *et al.*, 1985). Gp120 is non-covalently associated with gp41 and contains the binding site for CD4 molecules, the cellular receptor of HIV-1 (Dalgleish *et al.*, 1984; Klatzman *et al.*, 1984; Maddon *et al.*, 1986). The gp41 subunit is anchored in the membrane and has a non-polar fusion peptide at its N-terminus (Bosch *et al.*, 1989; Kowalski *et al.*, 1987). The gp120–gp41 molecule forms oligomers on the cell surface and on virions. Evidence for both trimeric and tetrameric oligomeric states has been reported (Gelderbloom *et al.*, 1987; Pinter *et al.*, 1989; Schawaller *et al.*, 1989; Earl *et al.*, 1990; Weiss *et al.*, 1990).

The binding of gp120 to CD4 is thought to result in activation of the membrane fusion activity of gp41, leading to entry of the viral nucleocapsid into a cell (Moore *et al.*, 1991; Sattentau and Moore, 1991). Evidence for a conformational change in the viral glycoprotein upon binding CD4 includes alterations in antibody reactivity, increased protease sensitivity and the dissociation of gp120 (Sattentau and Moore, 1991).

Heptad repeats in the amino acid sequences of many viral glycoproteins adjacent to the N-terminal fusion peptides suggest that α-helical coiled-coils would be a common structural feature of viral membrane fusion proteins (Gallaher *et al.*, 1989; Chambers *et al.*, 1990). A 105 Å long triple-stranded α-helical coiled-coil has been found in the low pH-induced, membrane fusion-active conformation of the influenza virus hemagglutinin (HA) by X-ray crystallography (Bullough *et al.* 1994) and was proposed to project the fusion peptide toward the cellular membrane (Carr and Kim, 1993; Bullough *et al.*, 1994). A soluble fragment of the transmembrane domain of the glycoprotein of Moloney virus expressed in *Escherichia coli* has also been shown by X-ray crystallography to contain a triple-stranded α-helical coiled-coil (D.Fass and P.Kim, personal communication).

Synthetic peptides corresponding to two potential coiled-coil regions in the gp41 sequence have been shown to inhibit the syncitial formation activity of HIV-1 (Wild *et al.*, 1992, 1994a,b), suggesting that the peptides may interfere with a conformational change involving the refolding of a coiled-coil (Carr and Kim, 1993) during the activation of the HIV-1 membrane fusion activity (Wild *et al.*, 1994b; Chen *et al.*, 1995a).

The conformational change that activates the membrane fusion activity of the influenza virus HA has been observed to be irreversible, suggesting that the HA structure found on the virus is metastable and refolds when triggered by low pH to a stable (membrane fusion-active) conformation. The observed irreversibility of the conformational change (Skehel *et al.*, 1982), the increased thermostability of the low pH-induced conformation compared with HA even at neutral pH (Ruigrok *et al.*, 1988), the finding that synthetic segments of the coiled-coil region form α-helical trimers in solution (Carr and Kim, 1993) and the simplicity of the long, α-helical coiled-coil fold of $TBHA_2$ (Bullough *et al.*, 1994) suggested that a soluble fragment of HA_2 would fold spontaneously into the low pH-induced conformation if expressed in *E.coli* because that conformation is more thermodynamically stable. Recently, the soluble domain of the HA_2 subunit of the HA was expressed in *E.coli* and shown to fold spontaneously into the membrane fusion conformation (Chen *et al.*, 1995b).

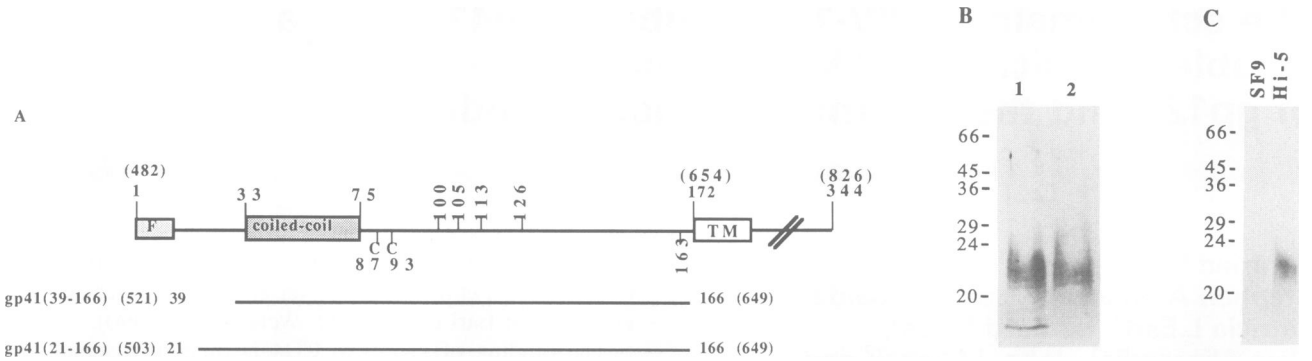

Fig. 1. (**A**) Schematic of gp41. Constructs gp41(39–160) and gp41(21–166) are shown. F = fusion peptide; the predicted coiled-coil domain (Chambers *et al.*, 1990), the cysteines (C) and potential glycosylation sites are shown with amino acid numbers. Numbers in parentheses correspond to the complete env peptide sequence. (**B**) Western blots of secreted gp41(21–166) (lane 1) and gp41(39–166) (lane 2). (**C**) Comparison of expression levels of gp41(21–166) produced in SF9 cells and Hi-5 cells. Cell culture supernatants were separated on 12% SDS–PAGE, blotted, and bands were detected with a rabbit anti-gp41 antiserum. Molecular weight standards are shown.

Here we report the expression of a soluble form of the extracellular domain of gp41 in insect cells. The glycoprotein was secreted and folded spontaneously into oligomers. The molecule was characterized by circular dichroism (CD), thermal denaturation, electron microscopy and antibody reactivity. The soluble oligomers formed highly α-helical rods, suggesting that, in the absence of gp120, gp41 folds into a membrane fusion-active conformation. Reactivity with monoclonal antibodies suggests that this membrane fusion conformation of gp41 is different from the gp41 conformation in gp120–gp41. The solubility of the molecule was greatly enhanced by forming complexes with Fab fragments from monoclonal antibodies.

Results

Expression in insect cells

Gp41 residues 21–166 [gp41(21–166)] and 39–166 [gp41(39–166)] plus an N-terminal threonine and arginine (confirmed by N-terminal sequencing) were expressed in insect cells. The N-terminus of the longer protein begins after the fusion peptide sequence and the shorter one starts at the beginning of the proposed coiled-coil domain (Figure 1A). Recombinant baculoviruses were generated and shown to induce secretion of gp41-specific protein for both constructs in Hi-5 cells. Gp41-specific bands were detected by SDS–PAGE and Western blotting of cell culture supernatants (Figure 1B). Hi-5 cells produced at least five times more gp41 protein than SF9 cells (Figure 1C).

Analysis of the oligomeric state

Chemical cross-linking performed on cell culture supernatants indicated the spontaneous formation of gp41 oligomers. Increasing concentrations of cross-linking reagent revealed a ladder of three prominent bands (Figure 2A and B). At the highest concentration of the cross-linking reagent, most of the protein is cross-linked into the third band. Molecular weight markers are consistent with the interpretation that the second band represents a dimer and the third band a trimer of the gp41 domains. A faint higher molecular weight band could be also detected at

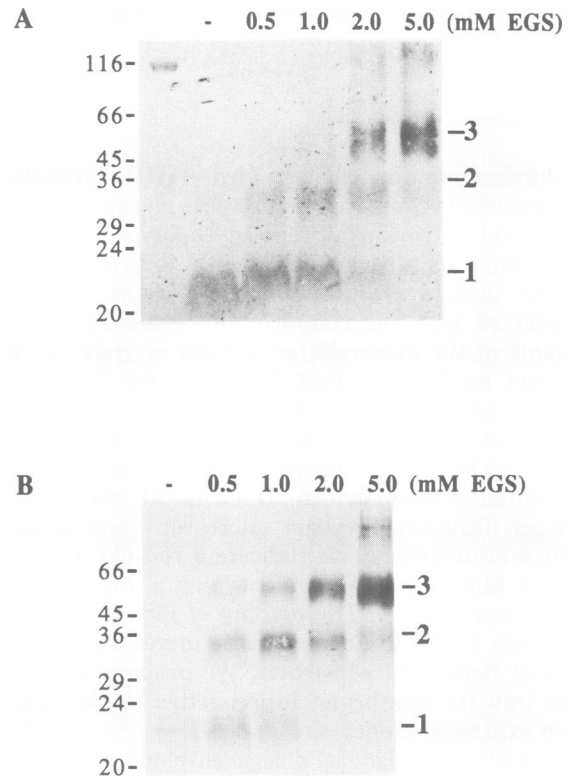

Fig. 2. Chemical cross-linking of (**A**) gp41(21–166) and (**B**) gp41(39–166). Cross-linked products were separated on 12% SDS–PAGE, blotted, and bands were visualized with mAb Md1. The cross-linking concentrations of EGS are shown on top of each lane. Molecular weight standard are shown. Bands corresponding to monomer, dimer and trimer are indicated.

~120 kDa at high concentration of cross-linking reagent; this might represent a dimer of trimers.

After purification (see Materials and methods), both glycoproteins eluted from size exclusion chromatography columns at approximately the same position as bovine serum albumin (66 kDa) (Figure 3A), indicating that the molecular weight of the gp41 oligomers in solution corresponds to that of the third band observed in the cross-linking experiments. The elution peak is broad, possibly indicating the existence of a monomer to oligomer

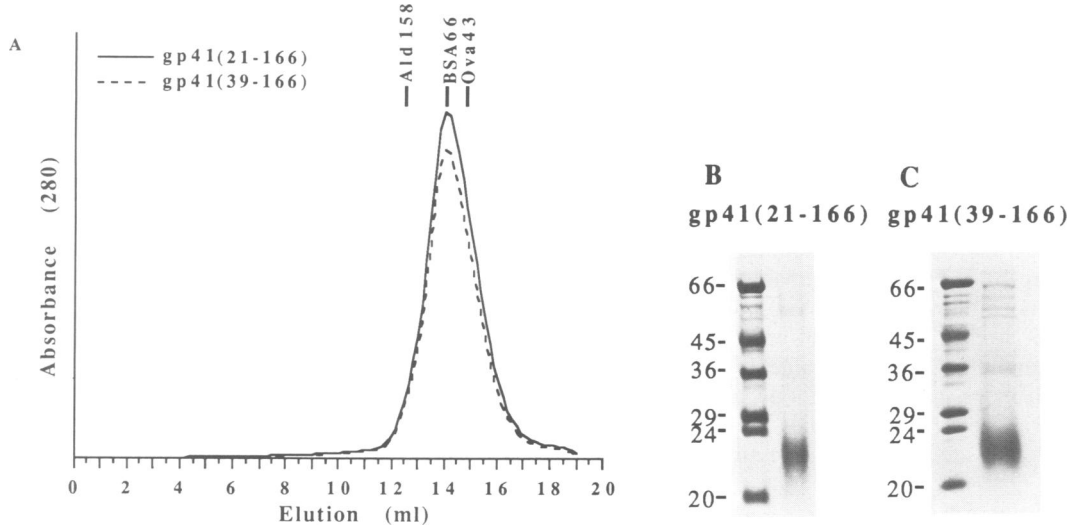

Fig. 3. Gel filtration elution pattern of gp41(21–166) and gp41(39–166). (**A**) The eluted volume is plotted against the absorbance at 280 nm. The elution positions of marker proteins are shown; aldolase 158 kDa, BSA 66 kDa and ovalbumin 43 kDa. (**B**) SDS–PAGE analysis of purified (affinity purification and size exclusion chromatography) gp41(21–166) and (**C**) gp41(39–166). Bands are stained with Coomassie Blue and molecular weight standards are shown.

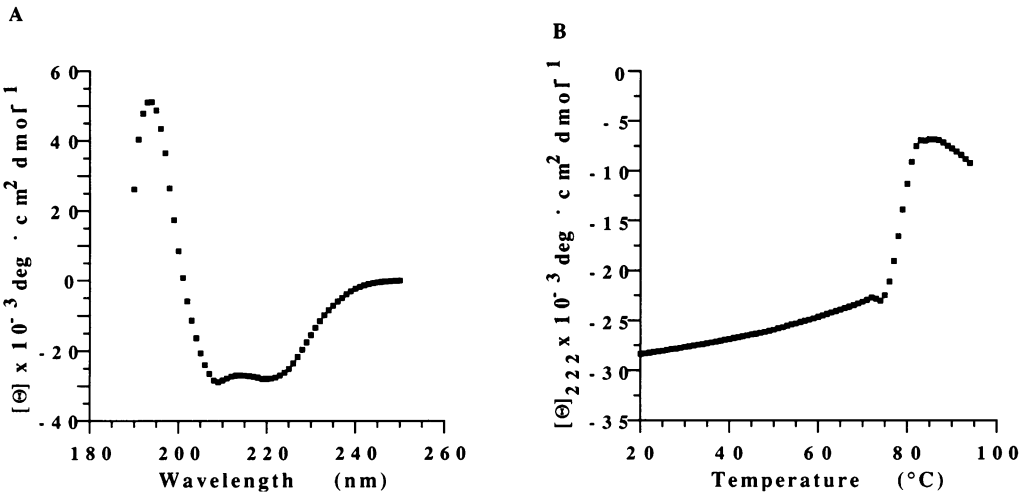

Fig. 4. (**A**) Circular dichroism spectrum of gp41(21–166). Minima at 208 and 222 are characteristics of α-helices. (**B**) Thermal denaturation curve of gp41(21–166) at pH 7.5, recorded at 222 nm with a scan rate of 1°C per min.

equilibrium at the low concentration (0.05–0.1 mg/ml) of the gp41 on the column. Fractions from the gel filtration peak were concentrated to 0.4 mg/ml and analyzed by SDS–PAGE. Bands corresponding to the purified gp41 monomers appear with an estimated mol. wt of 22 kDa (Figure 3B). Complete deglycosylation with peptide *N*-glycosidase F (PNGase F) reduced the molecular weight of gp41(21–166) to its calculated size of 17 kDa (data not shown), indicating that all five potential carbohydrate sites are glycosylated.

α-Helical content and thermodynamic stability

The CD spectrum of gp41(21–166) was measured from 190 nm to 250 nm (Figure 4A). It shows a maximum at 193 nm, a crossover in sign at 202 nm and minima near 208 and 222 nm characteristic of α-helices. The calculated α-helical content is ~80%. Thermal unfolding of gp41(21–166) was observed by monitoring the changes in ellipticity at 222 nm. A single unfolding transition was observed at a melting temperature of 78°C (Figure 4B).

Antibody reactivity

To probe potential structural differences between soluble gp41(21–166) and the ectodomain of gp41 attached to gp120 (gp140), the reactivity of a number of monoclonal antibodies (mAbs) raised against oligomeric gp140 (Earl *et al.*, 1994) was tested (Figure 5). Five mAbs that precipitate both oligomeric and monomeric gp140 also precipitate gp41(21–166) (Figure 5, D16, D38, D40, D41 and D54), suggesting some common structure. Two mAbs that specifically precipitate oligomeric gp140 (T4 and T6) and two that precipitate both monomeric and oligomeric gp140 (D10 and D61) fail to precipitate gp41(21–166). The D61 mAb which does not precipitate gp41(21–166) also interacts with a peptide from the disulfide bond-containing segment of gp41 (Xu *et al.*, 1991), suggesting

a structural difference in that region between gp140 and gp41(21–166).

Electron microscopy

Electron microscopy studies with soluble gp41(21–166) showed a rod-like structure for gp41, expressed by itself. Measurement of 70 molecules indicated an average length of 12.5 ± 1.3 nm (Figure 6A). Gp41(21–146)-2A2 antibody complexes show this mAb binding to one end of the rod (Figure 6B). This is presumably the N-terminus of gp41, as the binding region of mAb 2A2 has been mapped to the N-terminal region of gp41 (K.Javaheirian, personal communication). Oligomeric gp41(21–146) should contain more than one antibody binding site, and complexes of at least two antibodies with the same end of one gp41 rod are visible in electron micrographs (Figure 6B).

Complex with Fab fragments

The soluble gp41 glycoproteins were found to aggregate and precipitate at concentrations above ~0.4 mg/ml, unless they were complexed with Fab fragments of gp41-specific mAbs, which permitted concentrations up to 15 mg/ml. Fab fragments of the gp41-specific mAb 2A2 were generated by papain digestion. Complexes between affinity-purified gp41(21–166) or gp41(39–166) and the Fab fragment were formed at a 1:2 molar ratio. The complex was then concentrated and unbound Fabs were removed by gel filtration chromatography. Elution patterns for gp41(21–166)–Fab-2A2 and gp41(39–166)–FAB-2A2 complexes are shown in Figure 7A. SDS–PAGE analysis of the peak fractions confirmed that both proteins gp41 and Fab were present [Figure 7B and C (the two bands between 29 and 24 kDa correspond to Fab 2A2 heavy and light chain fragments, while the broad band below them represents glycosylated, soluble gp41 domains)]. The complex eluted slightly faster than the marker protein catalase with a mol. wt of 222 kDa. The molecular weight of three Fabs and soluble gp41 domain trimer add up to ~220 kDa. Dynamic light scattering analysis of the complex revealed a monomodal distribution of molecules, indicating that no aggregation occurred upon concentration.

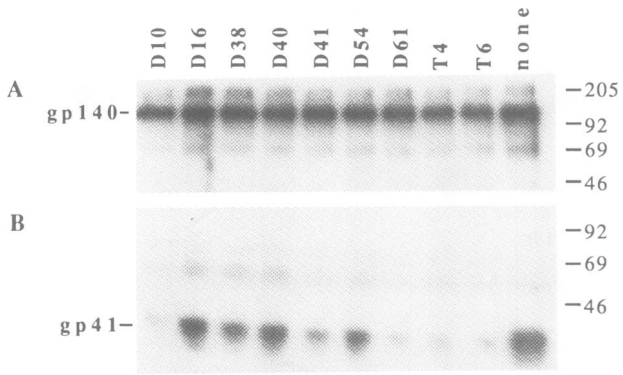

Fig. 5. Immunoprecipitation pattern of gp140 (**A**) and gp41(21–166) (**B**) with gp140-specific mAb. Molecular weight standards are shown. Bands were detected with a gp41-specific antiserum in a Western blot.

Disulfide bonds and oligomerization

In the absence of reducing agents, gp41(21–166) and gp41(39–166), either impure in cell culture supernatants (not shown) or purified and complexed with Fabs, exhibit oligomeric bands on SDS–PAGE (Figure 8A). The three bands and their molecular weights relative to marker proteins are similar to those observed by chemical cross-linking (Figure 2). To investigate whether these bands represent interchain disulfide-bonded oligomers or were due to SDS resistance of the oligomer, we denatured the gp41(39–166)–Fab complex in 8 M guanidine–HCl and separated the molecular species by size exclusion chromatography. CD measurements in 8 M guanidine–HCl revealed no secondary structure (data not shown).

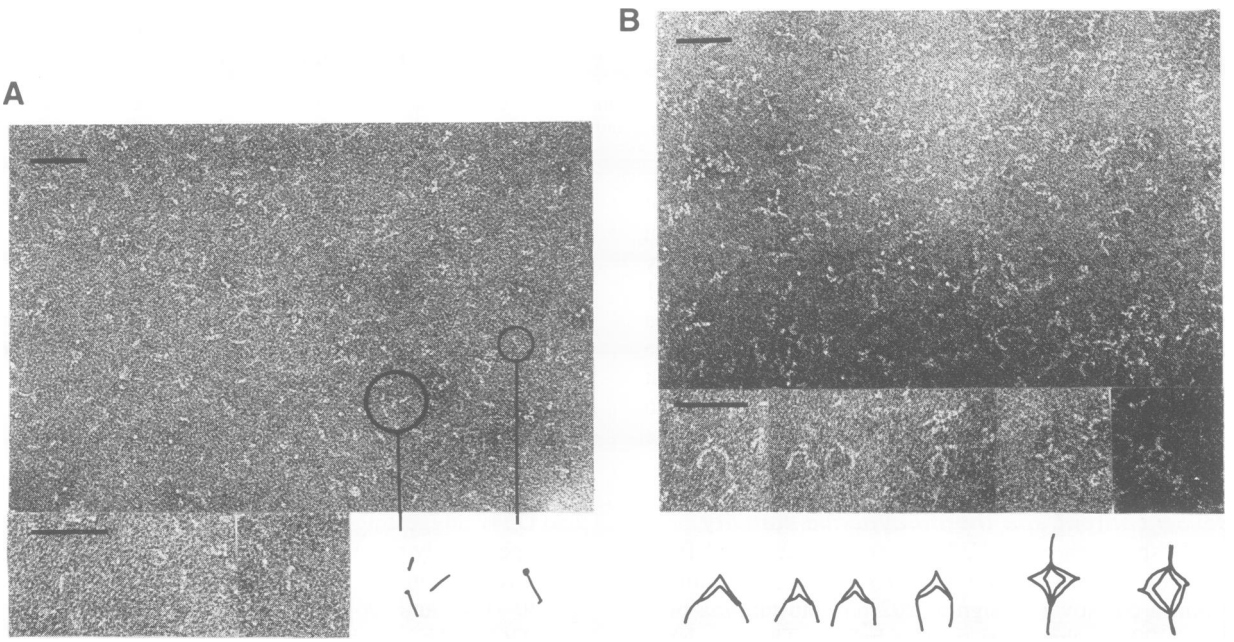

Fig. 6. Electron micrographs of gp41(21–166). (**A**) gp41(21–146) molecules alone. Diagrammatic representations of the molecules are shown below each micrograph. (**B**) gp41(21–146)–2A2 antibody complexes. The antibodies are shown as open shapes in the diagrams. Bar = 50 nm.

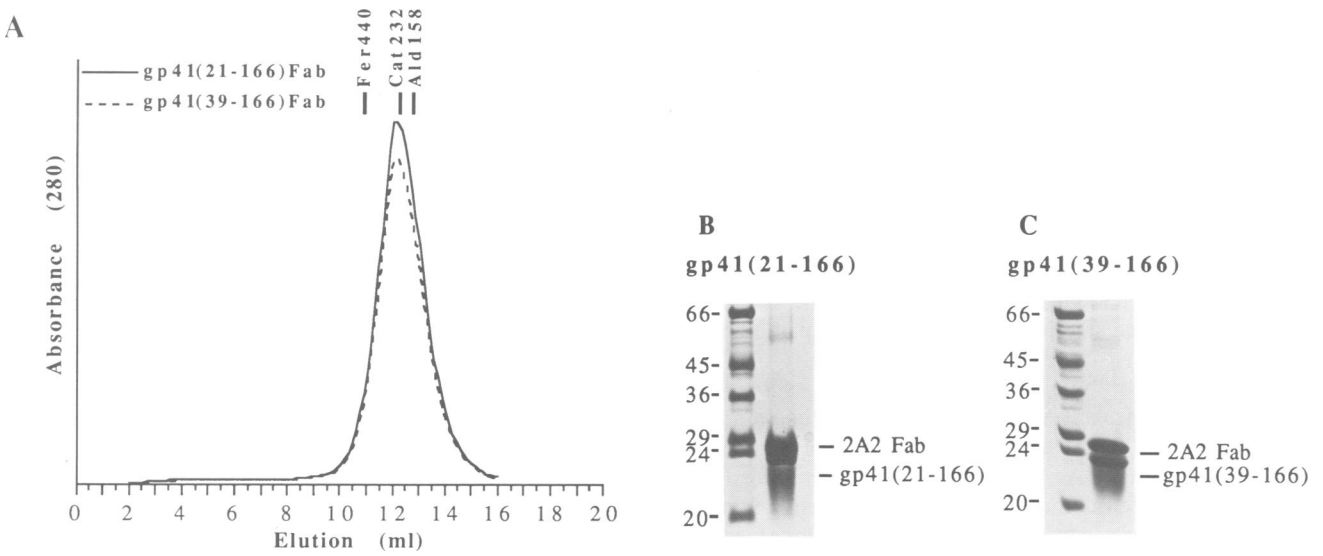

Fig. 7. (**A**) Gel filtration elution profile of complexes gp41(21–166)–Fab-2A2 and gp41(39–166)–Fab-2A2. The arrows mark the elution peak positions of standard proteins; ferritin 440 kDa, catalase 220 kDa and aldolase 158 kDa. SDS–PAGE analysis of gp41 and Fab-2A2 complexes; bands are stained with Coomassie Blue. (**B**) gp41(21–166)–Fab-2A2. (**C**) gp41(39–166)–Fab-2A2.

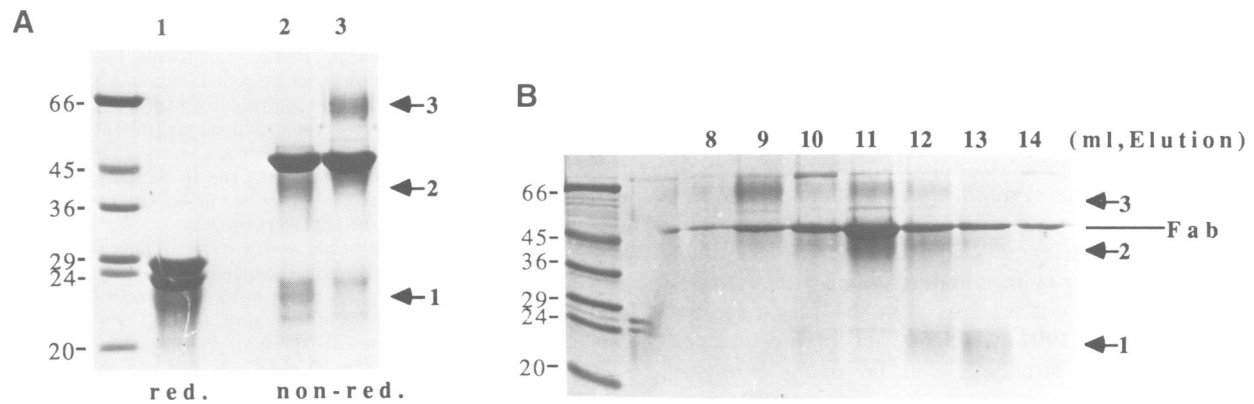

Fig. 8. (**A**) SDS–PAGE analysis of gp41(21–166)–Fab (lane 1) under reducing conditions and gp41(21–166)–Fab (lane 2) and gp41(39–166)–Fab (lane 3) under non-reducing conditions. The large band above band 2 is the Fab. (**B**) Gel filtration elution fractions of the gp41(39–166)–Fab complex as separated under denaturing conditions on a Superdex-200 gel filtration column. Samples of eluted fractions were separated under non-reducing SDS–PAGE conditions. The bands corresponding to monomeric, dimeric and probably trimeric gp41 are indicated with arrows. Molecular weight standards are shown. Bands have been detected by Coomassie Blue staining.

Analysis of fractions from the gel filtration column by SDS–PAGE in the absence of reducing agent (Figure 8B) showed that late eluting fractions (#13, Figure 8B) contain monomeric gp41 domain, while earlier fractions (#11, #9, Figure 8B) contain oligomeric gp41 domains corresponding to the second and third bands observed by chemical cross-linking and Fab fragments. This suggests that some interchain disulfide bonds exist in some of the oligomers. Using Ellman's reagent to measure free sulfydryl groups showed no free SH groups in either 2% SDS or 8 M guanidine–HCl, indicating that all cysteines are involved in either intra- or interchain disulfide bonds.

Discussion

Two soluble forms of the ectodomain of gp41 differing by 18 amino acids at their N-termini have been generated in insect cells by expressing molecules lacking their fusion peptides and C-terminal membrane anchors.

Characterization of these molecules lacking gp120 suggests that they are in the membrane fusion-active conformation, which may differ from the conformation of these sequences in the gp120–gp41 molecule. The molecules were only soluble to 0.4 mg/ml until complexed with Fab fragments of anti-gp41-specific antibodies, which increased the solubility to 15 mg/ml.

Chemical cross-linking and size exclusion chromatography indicate that the molecules are oligomeric. The observation of three cross-linked bands on SDS–PAGE coupled with the apparent molecular weight of the cross-linked products suggests that the gp41 domains form trimers (Figure 2). The apparent molecular weight estimated from size exclusion chromatography is consistent with that conclusion (Figure 3). The observation of two and three bands on SDS–PAGE in the absence of reducing agent also suggests trimers (Figure 8A). However, the possibility that the oligomers might migrate anomalously on SDS–PAGE or gel filtration columns precludes certainty in the assignment of a trimeric oligomeric state. The HIV-1 envelope glycoprotein has been described as a tetramer and a dimer of dimers (Pinter *et al.*, 1989; Schawaller *et al.*, 1989; Earl *et al.*, 1990), as well as a

trimer (Gelderbloom *et al.*, 1987; Weiss *et al.*, 1990). Our experiments provide evidence that gp41, at least in the absence of gp120, forms trimers. The width of the gel filtration peaks (Figure 3A) might suggest that different oligomeric forms co-exist, at least at the dilute concentrations during chromatography.

Both CD spectra and thermal stability measurements (Figure 4), as well as length measurements of molecules in electron micrographs (Figure 6), suggest that gp41(21–166) contains a long α-helical coiled-coil. The CD spectrum, thermal stability and length are comparable with that of $TBHA_2$ (Ruigrok *et al.*, 1988) and $EBHA_2$ (Chen *et al.*, 1995b), soluble versions of the HA_2 subunit of the influenza virus HA in the membrane fusion-active conformation; $TBHA_2$ was formed by proteolysis of low pH-treated viral HA, and $EBHA_2$ was produced by expressing the ectodomain of HA_2 with the fusion peptide deleted. The X-ray structure of $TBHA_2$ (Bullough *et al.*, 1994) shows a triple-stranded α-helical coiled-coil 105 Å long as the central element of the molecule. A triple-stranded α-helical coiled-coil has also been observed by X-ray crystallography in a soluble domain of the transmembrane subunit of the glycoprotein of the Moloney retrovirus (D.Fass and P.Kim, personal communication). Synthetic peptides corresponding to an N-terminal predicted coiled-coil region in gp41 adopt an α-helical secondary structure as measured by CD (Wild *et al.*, 1994b) and induce oligomerization (Bernstein *et al.*, 1995). These helical segment(s) might form the core of a triple-stranded coiled-coil.

Conformational differences between gp41(21–166) and the ectodomain of gp41 in complex with gp120 (gp140) were observed here with a series of mAbs, raised against gp140 (Earl *et al.*, 1994). At least one of the changes between these two structures can be mapped to the immunodominant region within the cysteine-containing segment of gp41 (Xu *et al.*, 1991) (Figure 1), because mAb D61, which shows differential reactivity (Figure 5), recognizes a peptide corresponding to this region (and blocks binding of mAb T4 and T6, which also show differential reactivity; P.L.Earl, unpublished results). The increased exposure of a cryptic epitope in gp41 has been observed upon CD4 receptor binding to gp120–gp41 (Sattentau and Moore, 1991).

The observation of interchain disulfide bonds in the recombinant gp41 ectodomains is also indirect evidence for a structural difference exhibited by gp41 expressed without gp120 (Figure 8A). Although the amount of one or two new bands (dimers and trimers) observed under non-reducing SDS–PAGE conditions seems to be dependent on the N-terminus of the expressed gp41 molecule (Figure 8A), chemical cross-linking showed the same oligomeric banding pattern for both of the constructs. Two earlier studies reported interchain disulfide bonding in gp160 (Owens and Compans, 1990; Willey *et al.*, 1991). Disulfide-bonded monomers, dimers, trimers and possibly tetramers were observed in cell lysates and were attributed to misfolding of the gp160 precursor (Owens and Compans, 1990), and a mutation at Asp226 resulted in the expression of monomeric and disulfide-linked dimeric env glycoprotein (Willey *et al.*, 1991). A possibility suggested by our data is that some of the gp160 precursors and the mutant protein may be folded into the fusion-active conformation. A change in the disulfide bonding from intra- to interchain [gp70 to p15(E)] has been observed in the glycoprotein of the murine leukemia viruses (Pinter and Fleissner, 1977; Pinter *et al.*, 1978).

The observation of interchain disulfide bonds in gp41(21–166) (Figure 8) and a difference in antibody reactivity of an mAb which recognizes the disulfide loop in gp41 (Figure 5, mAb D61) suggest that the region of gp41 including the two cysteines (Figure 1) may change conformation during the activation of the membrane fusion state of the HIV-1 glycoprotein.

Materials and methods

Expression of gp41 in insect cells

The regions in the *env* gene corresponding to residues 21–166 [gp41(21–166)] and residues 39–166 [gp41(39–166)] of the HIV-1 HXB2 strain (Fisher *et al.*, 1985) were amplified with synthetic oligonucleotides by standard PCR methods. The 5′-specific oligonucleotide contained the restriction site of *Mlu*I (adding amino acids Thr and Arg to the N-terminus of expressed gp41). The PCR fragments were restricted with *Mlu*I and *Pst*I. To direct secretion, the CD4-specific leader sequence was obtained as a *Bam*HI–*Mlu*I PCR DNA fragment from plasmid pCAS.ENV (Pollard *et al.*, 1992). Both fragments were subcloned into the transfer vector BlueBacIII (Invitrogen). The sequence was verified to be correct by DNA sequencing. Recombinant baculovirus clones were obtained by co-transfection of vector DNA and linearized AMNcp virus DNA. Recombinant viruses were purified by two rounds of plaque purification, and virus stocks were obtained by following standard procedures (O'Reilly *et al.*, 1992). SF9 cells were cultured in serum-free medium Excell401 (Bioscience Inc.) and Hi-5 cells were cultured in Excell405 (Bioscience Inc.) in 1 l spinner flasks. Gp41 expression of recombinant viruses was tested by Western blots.

Briefly, cell culture supernatants were harvested 3 days post-infection and analyzed on 12% SDS–PAGE (Laemmli, 1970). The protein was transferred to nitrocellulose. As a first mAb, either mAb Md-1 (AIDS Research and Reference Reagent Program Catalog, 1995) or complete serum, raised against bacterial expressed gp41, was applied. Gp41-specific bands were detected with α-IgG alkaline phosphatase-labeled mAb (Promega) and visualized with a mixture of BCIP and NBT (Promega).

Chemical cross-linking

SF9 cell culture supernatants containing gp41(21–166) and gp41(39–166) were concentrated 1:5 and dialyzed against phosphate-buffered saline (PBS) for 24 h. Twenty microlitres of protein solution was buffered with 100 mM HEPES pH 7.5. Chemical cross-linking reagent EGS (Pierce) was dissolved at 50 mM in DMSO. Dilutions of cross-linker were added to the protein solution as indicated. Reactions were incubated on ice for 1 h and subsequently quenched with 100 mM glycine for 30 min at room temperature. The samples were analyzed by SDS–PAGE under reducing conditions. Gp41-specific bands were detected by Western blot. Gp41 was stained with mAb Md-1 (AIDS Research and Reference Reagent Program Catalog, 1995).

Gp41 purification

Complete IgG was purified on a protein G affinity column from rabbit serum obtained by immunization of a rabbit with bacterial expressed gp41 (aa 41–123). Total IgG (100 mg) was coupled to 15 ml of cyanogen bromide-activated Sepharose (Pharmacia). Cell culture supernatant was concentrated 1:10 in an Amicon Concentrator and passed over the gp41 antibody affinity column. The column was first washed with PBS containing 1 M NaCl, then with 40 mM phosphate pH 3.5, 0.5 M NaCl. Gp41 was eluted with 100 mM glycine, pH 2.3. Fractions were collected and immediately buffered with 50 mM Tris–HCl, pH 7.0. Fractions containing gp41 were concentrated and buffer was changed to 20 mM NaOAC pH 5.0, 1 M NaCl. For complex formation with Fab 2A2, fractions were dialyzed against 20 mM Capso, pH 9.0.

Size exclusion chromatography

Gp41 was concentrated to 0.4 mg/ml and injected onto a Superdex-200 gel filtration column (Pharmacia). The separation was performed in 20 mM NaOAC pH 5.0, 1 M NaCl. The separation of the gp41–Fab

complexes was performed in 20 mM Capso, pH 9.0, 1 M NaCl. The separation of gp41(39–166)–Fab under denaturing conditions was performed in 8 M guanidine–HCl pH 7.0 on a Superdex-200 gel filtration column (Pharmacia). One ml fractions were collected and guanidine–HCl buffer was exchanged against H_2O. Aliquots were then separated on a 12% SDS–PAGE under non-reducing conditions.

2A2 Fab preparation and complex formation

2A2 mAb (10 mg/ml) was digested with papain (Boehringer Mannheim) at a 1:1000 (w/w) ratio for 1 h at room temperature. The reaction was stopped with E64 (Sigma). The buffer was changed to 70 mM Tris–CH_3COOH pH 9.3 and protein was loaded onto a Mono P column (Pharmacia). 2A2 Fab was eluted from the column with a pH gradient using Polybuffer 96 (Pharmacia) pH 6.0, CH_3COOH. A major peak of 2A2-specific Fab eluted at pH 8.0 and was used for complex formation. Gp41 fractions, eluted from the affinity column, were dialyzed against 20 mM Capso pH 9.0. 2A2 Fab were added in a 1:2 molar ratio. Complexes were concentrated to 500 µl (1–10 mg/ml) and excess 2A2 Fab fragments were separated from the complex on a Superdex-200 gel filtration column (Pharmacia) in 20 mM Capso pH 9.0, 1 M NaCl.

Circular dichroism

The CD spectrum of gp41(21–166) (0.19 mg/ml) in 100 mM NaF pH 7.5 was recorded at 20°C using a 1 mm cell on an AVIV 623DS spectropolarimeter with a thermoelectric controller. Five independent measurements were averaged. Thermodynamic stability was measured at 222 nm by monitoring the CD signal in the range 20–95°C with a scan rate of 1°C per min. Gp41(21–166) was in 10 mM phosphate pH 7.5 and 1 M NaCl at a concentration of 0.190 mg/ml. The concentration was determined by measuring the OD_{280}, with an extinction coefficient of 59 580/M/cm.

Immunoprecipitation of gp41(21–166)

One hundred ng of gp41 were incubated with gp140-specific mAbs in 50 µl of PBS at 4°C. Anti-mouse IgG coupled to agarose (Sigma) was used to precipitate the complexes, which were washed twice with buffer (100 mM Tris pH 8.0, 100 mM NaCl, 0.5% Triton). Immunocomplexes were separated on a 10% SDS–PAGE and blotted onto nitrocellulose. Gp41- and gp140-specific protein bands were detected by a polyclonal rabbit serum to gp41. ^{125}I-Labeled protein A (Amersham) was used to visualize the bands.

Electron microscopy

Samples were adsorbed onto carbon films and negatively stained with 1% sodium silicotungstate (pH 7.0). A JEOL 1200EX microscope was operated at 100 kV and magnification was calibrated regularly with catalase crystals. Micrographs were taken under minimum dose and accurate defocus conditions to preserve detail to 1.5 nm (Wrigley *et al.*, 1983).

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