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Crystallization and preliminary diffraction studies of the ectodomain of the envelope glycoprotein D from herpes simplex virus 1 alone and in complex with the ectodomain of the human receptor HveA

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Gycoprotein D (gD) is a glycoprotein expressed on the surface of several human and animal alpha herpes viruses. Binding of gD to cell-surface receptors has been shown to be necessary for herpes simplex virus 1 and 2 (HSV-1 and HSV-2) cell entry. The gD ectodomain consists of 316 residues and has no sequence homology to any other proteins of known structure. Two fragments of the HSV-1 gD ectodomain ($gD_{22–260}$: residues 22–260 and gD_{285}: residues 1–285) have been crystallized in two crystal forms. The complex between gD_{285} and the ectodomain of HveA, a gD cellular receptor member of the tumor necrosis factor (TNFR) superfamily, has also been crystallized. Moreover, insect-cell-expressed selenomethionine-substituted gD_{285} has been purified and crystallized alone and in complex with HveA.

1. Introduction

Alpha herpes viruses are large double-stranded DNA viruses that infect humans [herpes simplex virus 1 and 2 (HSV-1 and HSV-2)] and animals [*e.g.* bovine herpes virus 1 (BHV-1) and porcine pseudorabies virus (PrV)]. HSV-1 and HSV-2 are widespread in the human population: HSV-1 is normally associated with oral and ocular lesions, whereas HSV-2 is isolated primarily from genital and oral lesions. After an initial infection in epithelial cells, the virus spreads to host neuronal cells where it can remain latent for life (Roizman & Sears, 1987). The mechanism of infection of this family of viruses is complex and not fully understood. At least five glycoproteins (gB, gC, gD and the gH/gL complex) on the virion envelope are involved in HSV cell entry (reviewed in Spear *et al.*, 2000). Among those, glycoprotein D (gD) has been shown to mediate binding to cellular receptors (reviewed in Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000).

HSV-1 gD is a 369-residue glycoprotein with an N-terminal ectodomain of 316 residues and three N-linked oligosaccharide-attachment sites (Watson *et al.*, 1982). The protein has six cysteine residues which form three disulfide bonds (Long *et al.*, 1992; HSV-1 and HSV-2 gD ectodomains have 85% sequence identity) and five methionines. In the past three years, several gD receptors have been identified (Campadelli-Fiume *et al.*, 2000; Spear *et al.*, 2000). The first receptor to be cloned was HveA, a member of the TNFR/NGFR superfamily expressed on the surface of activated T-lymphocytes, where it mediates HSV-1 and HSV-2 entry (Montgomery *et al.*, 1996).

HveA (also called HVEM or TNFRSF14) is a type I membrane protein with an ectodomain of 162 residues and two N-linked oligosaccharide-attachment sites. Sequence alignments show that the first N-terminal 100 residues have a significant sequence identity (close to 30%) with the TNFR family member TNFR-1, whose structure is known (Banner *et al.*, 1993; Naismith *et al.*, 1996).

gD is an important target for antiviral agents and soluble forms of gD are in clinical trials as vaccines against HSV-1 and HSV-2. To gain insights into the role of gD in HSV entry, we have started structural studies on gD (Krummenacher *et al.*, 1998; Nicola *et al.*, 1997; Rux *et al.*, 1998; Whitbeck *et al.*, 1997; Willis *et al.*, 1998) and on complexes of gD with cellular receptors. Here, we report the crystallization and preliminary diffraction data of a recombinant gD ectodomain alone and in complex with the ectodomain of HveA.

2. Material and methods

2.1. Protein purification, preparation of selenomethionine-substituted gD_{285} and gD_{285}–HveA complex reconstitution

Both the HveA ectodomain (residues 1–162 plus a C-terminal His_5 tag) and two soluble C-terminally truncated forms of gD (residues 1–306 and 1–285) were purified from Sf9 insect-cell culture supernatants (Sisk *et al.*, 1994). HveA was purified on an Ni^{2+}-resin followed by size-exclusion chromatography on a Pharmacia analytical Superdex-200 column. gD_{285} and gD_{306} were purified using DL6-antibody affinity chromatography with pH 11 elution (DL-6 recognizes a linear epitope,

residues 272–279, near the C- terminus of gD; Isola *et al.*, 1989) followed by ion exchange on a MonoQ column (Pharmacia) and size-exclusion chromatography on an analytical Superdex-200 column (Pharmacia). Selenomethionine-substituted gD_{285} was produced following published protocols (for a review, see Bellizzi *et al.*, 1999) that were slightly modified. Sf9 cells were grown to a density of 10^6 cells ml^{-1} in 1 l spinner flasks in methionine-containing Sf9-II00 media (GIBCO) (500 ml). 24 h post-infection, the cells were gently spun down and moved to methionine-free Grace's media (GIBCO) to remove the intracellular pull of methionine. 12–14 h later the cells were transferred to a fresh methionine-free Grace's media supplemented with 5% dialyzed fetal bovine serum (FBS; Sigma) and 75 mg ml^{-1} selenomethione (Sigma) for an additional 48 h. The protein was then purified from the Sf9 insect-cell culture supernatants by the same methods as the native protein. The yield for the selenomethionine protein was approximately 50–60% compared with that normally achieved in a native protein preparation. Amino-acid analysis showed that 85% selenomethionine incorporation had been obtained.

The gD_{285}–HveA complex was prepared by titration of gD_{285} with increasing amounts of HveA, observing the gel shift with native PAGE (without SDS or β-mercaptoethanol). The complex was then purified by size-exclusion chromatography on an analytical Superdex-200 column, which allowed separation of the complex from the excess unbound gD.

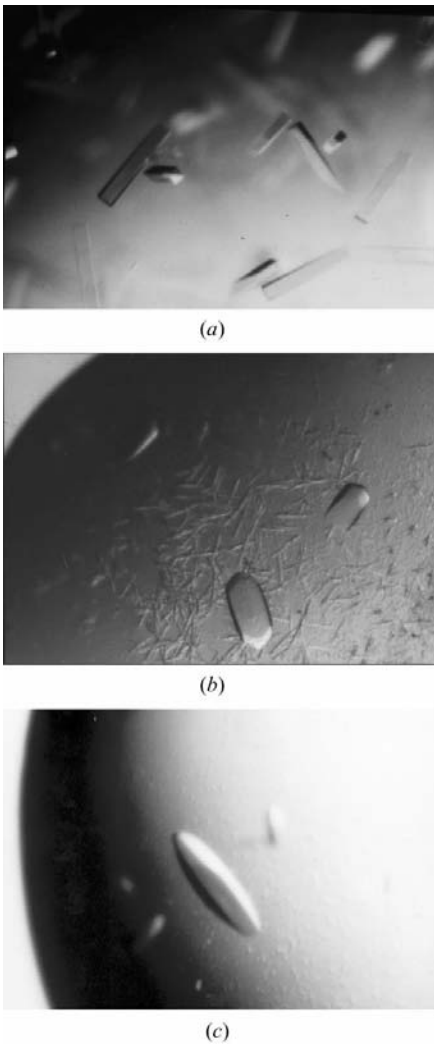

Figure 1
Crystals of (*a*) gD_{285}, (*b*) $gD_{22–260}$ and (*c*) gD_{285}–HveA

2.2. Crystallization

Preliminary crystallization trials were performed by the hanging-drop vapour-diffusion method. Typically, 1 µl of protein solution (5.5–12 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.0 and 100 m*M* NaCl) was mixed with an equal volume of well solution. Once crystals were obtained, crystal growth was optimized using the sitting-drop method with larger protein droplets.

We first tried to crystallize gD_{306}. Small crystals (approximately 0.15–0.2 mm per side; Fig. 1*b*) grow in about three months in droplets equilibrated with solutions containing 50 m*M* MES pH 6.0 as buffer and 1.6 *M* ammonium sulfate as precipitating agent. SDS gels of washed crystals showed that the protein had been partially proteolysed during the long crystallization period. N-terminal sequencing showed that the crystals contained a gD core starting at residue 21 of the mature protein and this information together with MALDI mass spectrometry indicates that the new C-terminus is in the proximity of residue 260.

Crystals of gD_{285} or Se-Met gD_{285} were obtained in the pH range 6.0–9.0 using 1.4 *M* ammonium sulfate as precipitating agent. The largest crystals (0.6 × 0.3 × 0.3 mm) (Fig. 1*a*) were grown by mixing 3 µl of purified complex solution with an equal volume of a reservoir solution containing 50 m*M* Bicine pH 9.0 (or 50 m*M* MES pH 6.0) as buffer and 1.6 *M* ammonium sulfate as precipitating agent in sitting-drop experiments. SDS gels and N-terminal sequencing on washed crystals confirmed the presence of the full protein (residues 1–285) in the crystals.

More recently, crystals of the gD_{285}–HveA and SeMet gD_{285}–HveA complexes have been obtained (Fig. 1*c*). The complex was prepared by titrating gD285 with increasing amounts of HveA162 and observing the gel shift with native PAGE and was further purified by size-exclusion chromatography on a Superdex-200 column. Large crystals (0.8 × 0.3 × 0.2 mm) were obtained in sitting-drop experiments by mixing 3 µl of purified complex solution with an equal volume of a reservoir solution containing 50 m*M* Tris–HCl pH 8.0 as buffer and 1.8 *M* ammonium sulfate and 5% PEG 400 as precipitating agents.

Table 1
Crystallographic statistics for gD_{285}–HveA, $gD_{22–260}$ and gD_{285} crystals.

Values in parentheses are for the highest resolution shell.

Crystal	gD_{285}–HveA162	$gD_{22–260}$	gD_{285}
Wavelength (Å)	0.91960	1.0	0.9754
Space group	$P3_121$	$P4_22_12$	$P422$
Unit-cell parameters (Å)	a = 129.15, b = 129.15, c = 80.94	a = 158.67, b = 158.67, c = 155.70	a = 131.43, b = 131.43, c = 83.27
Resolution (Å)	2.65	2.80	2.85
Beamline	A1, CHESS	14BMC, BIOCARS	X25, NSLS-BNL
Total observations	121389	194888	75650
Unique observations	22623	48333	17930
Redundancy	5.4	4.1	4.5
Completeness (%)	99.9 (99.9)	99.3 (99.8)	96.3 (76.8)
R_{sym} (%)	7.1 (36.9)	6.5 (31.1)	8.9 (26.4)

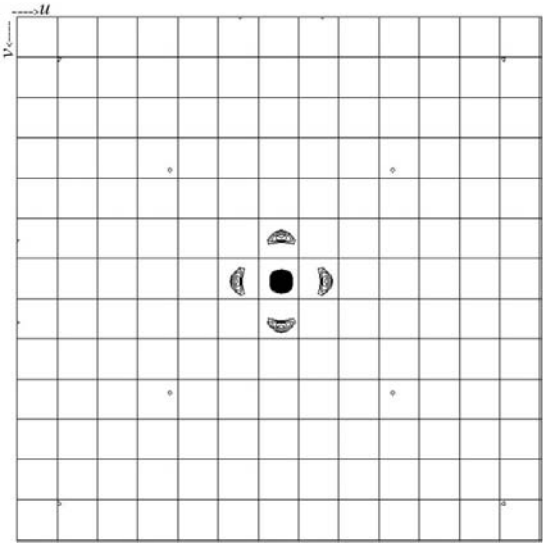

Figure 2
w = 0.4 section in the gD_{285} native Patterson map ($0 < u < 1$ and $0 < v < 1$). The peak at u = 0.5, v = 0.5 in this section has half the intensity of the origin peak.

3. Results and discussion

For X-ray data collection at cryogenic temperature, crystals were transferred stepwise to cryoprotectant solutions containing 20% glycerol (gD_{285} and $gD_{22–260}$) or 20% ethylene glycol (gD_{285}–HveA) in crystallization buffer and flash-cooled at 100 K in N_2 gas. Preliminary characterization of the crystals was carried out in the laboratory on a MAR 30 cm detector mounted on a GX-13 X-ray generator with Charles Supper double focusing mirrors. Owing to the small size of $gD_{22–260}$ crystals and to the weak diffraction proprieties of the gD_{285} and gD_{285}–HveA crystals, synchrotron X-ray sources had to be used for data collection (Table 1). The *HKL* suite was used for data integration and scaling (Otwinoski & Minor, 1997). The *CCP*4 suite was used for further data processing and analysis (Collaborative Computational Project, Number 4, 1994).

A 2.80 Å data set from $gD_{22–260}$ crystals was collected at the BIOCARS 14BMC beamline at the APS on a Q4-CCD detector and at 1.00 Å wavelength. The crystals belong to the space group $P4_22_12$ (Table 1), with unit-cell parameters $a = b = 158.67$, $c = 155.70$ Å and at least four molecules in the asymmetric unit (V_M = 3.8, corresponding to 67% solvent).

In the case of gD_{285}, several crystals were screened. A 2.85 Å native data set was collected at the X25 beamline at BNL-NSLS on a Brandeis B4-CCD detector at 0.9754 Å wavelength. The data were indexed and integrated in the tetragonal space group $P422$, with unit-cell parameters $a = b = 131.43$, $c = 83.27$ Å (Table 1). The diffraction images were characterized by the presence of streaked reflections in the b^* and a^* directions and also by the absence of many predicted reflections. A native Patterson map contained a peak with half the intensity of the origin peak at $u = 0.5$, $v = 0.5$ and $w = 0.4$ (Fig. 2), which indicates the presence of two molecules in the asymmetric unit (V_M = 2.75 Å^3 Da^{-1}) related by translational non-crystallographic symmetry. The presence of streaked reflections in the diffraction pattern might be indicative of static disorder in the crystals.

For the gD_{285}–HveA crystals, a 2.65 Å resolution native data has been collected at the A1 beamline at CHESS on a Q4 CCD detector (Table 1). The data were indexed and integrated in the $P3_121$ space group, with unit-cell parameters $a = b = 129.15$, $c = 80.94$ Å, and presumably contain one complex molecule per asymmetric unit (V_M = 3.7 Å^3 Da^{-1}, corresponding to 67% solvent if we assume a 1:1 complex).

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