BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 235–238 (1998)
ARTICLE NO. RC989447

High Level Expression and Structural Characterisation of Herpes Simplex Virus Type I Transcriptional Activator VP16 (α-*trans* Inducing Factor)

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Received August 17, 1998

We have developed a baculovirus expression system for the rapid and efficient production of large quantities (>10 mg/l) of VP16. The recombinant VP16 binds to a complex of host cell transcription factors and TAATGARAT motif. Secondary structure calculations from circular dichroism measurements indicate a content of 32.0 % α-helix and 17.5 % β-sheet. This is the first structural CD analysis of VP16 which will be very useful for high-throughput assay development and mechanistic studies.

Key Words: **herpes simplex virus; recombinant protein; VP16; circular dichroism; baculovirus expression system.**

Herpes simplex virus type 1 (HSV-1) and HSV-2 package a protein called VP16 (otherwise also known as Vmw65 or α-TIF), which activates transcription of the viral immediate early genes, and is essential for efficient viral replication. VP16 interacts with the cell proteins Oct-1 and HCF to form a multiprotein complex at DNA sites containing the sequence TAATGARAT (R is a purine nucleotide) within viral immediate early promoters [1]. VP16 is a major late phosphoprotein with an apparent molecular weight of 65 kDa [2]. It contains two functionally distinct regions: an N-terminal 340-amino-acid region (residues 49 to 388) is responsible for VP16 induced complex formation [3] and an acidic 80-amino-acid carboxyterminal region responsible for activating herpes simplex virus immediate early gene transcription [4].

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Abbreviations used: BEVS, Baculovirus expression vector system; HSV, herpes simplex virus; CD, circular dichroism.

The baculovirus expression vector system (BEVS) is frequently a method of choice for the expression of recombinant mammalian proteins (see O'Reilly *et al.* [5]). Apart from the simplicity and cost effectiveness of this method, the insect host cells possess many of the protein-processing and protein-folding mechanisms of mammalian cells; therefore functional and antigenic differences are rarely seen. In this paper we describe a BEVS for the efficient production of biologically active VP16 which was purified to homogeneity, and report for the first time its structural characterisation via circular dichroism measurement.

MATERIALS AND METHODS

Construction of transfer vector. The VP16 gene was amplified from the plasmid pGX158 (kindly provided by Dr. R. Everett, University of Glasgow). pGX158 consists of the vector pAT153 into which the EcoRI fragment F of HSV 1 strain 17 was inserted. VP16 was amplified by PCR using the Advantage-GC cDNA PCR Kit (Clontech Laboratories Inc, Palo Alto, CA, USA). The two oligonucleotide primers used for the amplification were 5′-GGAATTCATGGACCTCTTGGTCGACGAG-3′ (upstream) and 5′-GCTCTAGACTACCCACCGTACTCGTCAAT-3′ (downstream). The resulting 1488 bp fragment comprised the entire coding region for VP16 including the start codon, the termination codon and flanking artificial *EcoRI* and *XbaI* enzyme restriction sites for subcloning. This DNA fragment was subcloned into the baculovirus transfer plasmid pFastBac HTa (Life Technologies, Gaithersburg, MD, USA) and its DNA sequence was checked using an automated sequencer. The plasmid contains the powerful polyhedrin promoter to drive gene expression. Gene expression gives rise to a fusion protein which consists of a N-terminal 6 x histidine tag linked via a spacer region and a rTEV protease cleavage site to the protein of interest, adding an additional 28 amino acids to the N terminus in the case of VP16. The polyhedrin promoter and the 6 x histidine tag, as well as the multiple cloning site and a SV40 polyadenylation signal are inserted between the left and right arms of mini-Tn7 element.

Cell culture and production of recombinant virus. Recombinant baculovirus was generated using the Bac-To-Bac baculovirus expression system (Life Technologies), according to the manufacturer's instruction, using the *Spodoptera frugiperda* host insect cell line Sf9 (Invitrogen Corp, San Diego, CA, USA). Essentially the recombinant

0006-291X/98 $25.00

baculovirus DNA was constructed by transposing a mini-Tn7 element containing the VP16 gene from the pFastBac HTa donor plasmid to the mini-attTn7 attachment site on the bacmid (a bacmid is a form of the baculovirus genome that replicates in *E. coli*). The Tn7 transposition functions are provided in *trans* by a helper plasmid (pMON7124). Recombinant baculovirus is generated by relying on site-specific transposition in *E.coli*. Upon transformation of the *E.coli* strain DH10Bac (Life Technologies) with the recombinant pFastBac HTa vector, recombinant baculovirus particles are generated due to the transposition of pFastBac HTa and the bacmid, bMON14272 in the presence of the transposase expressed from the helper plasmid pMON7124 in DH10Bac. bMON14272 contains the alpha peptide of beta galactosidase and a kanamycin resistance gene. By plating DH10Bac on kanamycin, gentamycin and tetracyline plates and choosing white colonies, recombinant bacmids were selected. Recombinant baculovirus DNA was prepared from a miniprep of the transformed DH10Bac cells and was transfected into insect cells using cellfectin (Life Technologies). Recombinant virus was isolated, expanded on suspension cultures and titrated using standard methods [5]. Sf9 cell monolayer cultures were grown at 28°C in SF900 II serum-free medium (Life Technologies), supplemented with 1 mM glutamine, 100 units/ml streptomycin, 100 μg/ml penicillin. Suspension cultures included an additional supplement of 10 units/ml heparin (sodium salt; grade 1-A from porcine intestinal mucosa; Sigma-Aldrich Handels G.m.b.H., Vienna, Austria). These cultures were shaken in Erlenmeyer flasks at 80 rev./min at a density of (0.3-2.0)x10^6 cells/min.

Large scale protein expression. For large scale expression of protein in suspension culture High Five host cells (BTI TN 5B1-4; Invitrogen Corp.) were used. These cells were grown in Express Five (Life Technologies) serum free medium. Different volumes of cells were grown at 1x10^6 cells/ml in suspension culture and infected by adding corresponding volumes of a viral stock solution with 10^8 p.f.u./ml. to give a multiplicity of infection of 1. The infection was allowed to proceed for a further 72 h at 28°C and 80 rev./min. Cells were pelleted by centrifugation for 10 min at 1500 g.

Purification. Recombinant VP16 was purified from cells using Nickel-chelat affinity chromatography with Ni-NTA Agarose (Qiagen G.m.b.H., Hilden, Germany). All steps were performed at 4°C. The cell pellet was resuspended in 5 ml/g 50 mM Tris/HCl pH 8.5, 1% Nonidet P40, 10 mM β-mercaptoethanol, containing 1 x Complete protease inhibitor cocktail, EDTA-free (Boehringer Mannheim G.m.b.H., Mannheim, Germany). The cells were disrupted by sonication using a Branson Sonifier Model 250/450 (Branson Ultrasonics Corporation, Danbury, CT, USA) with three strokes, each at 40% output for 30 sec. The resulting suspension was centrifuged for 20 min at 10.000 g at 4°C and the supernatant was applied to 1 ml Ni-NTA Agarose per 50 ml of resuspended cells with a flow rate of apr. 0.5 ml/min. The agarose beads were washed with 10 column volumes 20 mM Tris-HCl pH 8.0, 500 mM KCl, 20 mM imidazol, 10% (v/v) glycerol, 10 mM β-mercaptoethanol (buffer A), five column volumes 20 mM Tris-HCl pH 8.5, 1 M KCl, 10% (v/v) glycerol (buffer B) and again two column volumes of buffer A. Protein was eluted by addition of 20 mM Tris-HCl pH 8.5, 100 mM KCl, 100 mM imidazol, 10 % (v/v) glycerol, 10 mm β-mercaptoethanol. Protein containing fractions (identified using Bradford assay) were collected and dialysed for a minimum of 1 h against PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.09 mM Na_2HPO_4, pH 7.2 As a final "polishing" step, the protein was chromatographed on a ResourceQ (Pharmacia, Uppsala, Sweden) anion-exchange column. Chromatography was performed using a Pharmacia Äkta Explorer System (Pharmacia, Uppsala, Sweden). Briefly, after loading, unbound material was washed from the column with PBS and pure VP16 was eluted in a gradient of PBS (A) and PBS + 0.5 M NaCl (B) at 72% B. VP16 containing fractions were pooled, dialysed against PBS and stored at –80°C. Protein concentration was determined by reading the adsorption of pure protein (as demonstrated by SDS gel electrophoresis) at 280 nm with a molar extinction coefficient ϵ=51000 $M^{-1}cm^{-1}$.

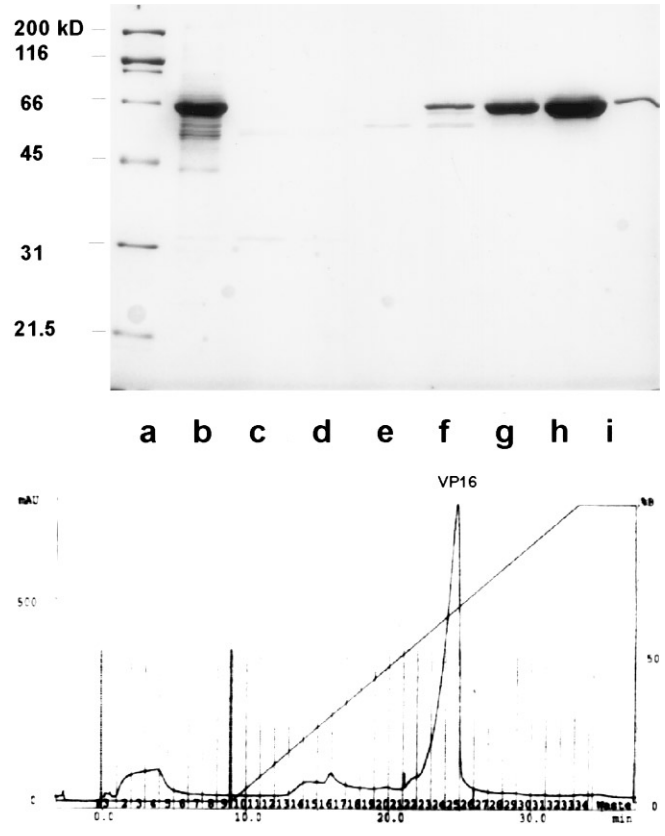

FIG. 1. Purification of recombinant VP16. Upper panel shows SDS-PAGE of colum fractions: Lane a, broad range molecular weight marker; b, eluate from Ni-column; c, fraction 17; d–i, elution profile for VP16 (fractions 22 to 26) Lower panel: elution profile of VP16 in a gradient of 0–100 % 0.5 M NaCl in PBS. VP16 elutes at apr. 500 mM NaCl.

SDS/PAGE analysis. Electrophoretic separation of proteins by SDS/PAGE was carried out in the presence of β-mercaptoethanol on 10% polyacrylamide gels [6]. Protein bands were visualised using Coomassie Brilliant Blue stain [7].

Western blot analysis. After electrophoretic separation, the proteins were transferred to nitrocellulose sheets. The sheets were blocked with PBS containing 1% Tween-20 and 5% low fat dry milk powder (Bio-Rad Laboratories, Hercules, CA, USA) and then incubated with rabbit anti VP16 polyclonal antibody (kindly provided by Dr. John Capone, McMaster University, Hamilton, Ontario, Canada) at a dilution of 1 in 5000. After washing off excess antibody, the bound antibody was detected using horseradish peroxidase conjugated to anti rabbit IgG at a dilution of 1 in 3000 with the ECL detection system (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England).

Electrophoretic mobility shift assay (EMSA). The EMSA was performed as described previously [8]. Briefly, recombinant VP16 was assessed for functional activity by testing if the protein was able to interact with cellular factors to form a protein/DNA complex with oligonucleotides containing a TAATGARAT element. Complementary oligonucleotides 5′-GATCCCGTGCATGCTAATGATATTCTTT-3′ and 5′-CTAGAAAGAATATCATTAGCATGCACGG-3′ were synthesised by Life Technologies and labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs). Briefly, reaction mixtures contained in a final volume of 20 μl 0.2–0.5 ng of ^{32}P-end-labelled probe, 10 mM Hepes pH 7.9, 60 mM KCl, 2 mM EDTA, 0.1 μg BSA, 4% (v/v)

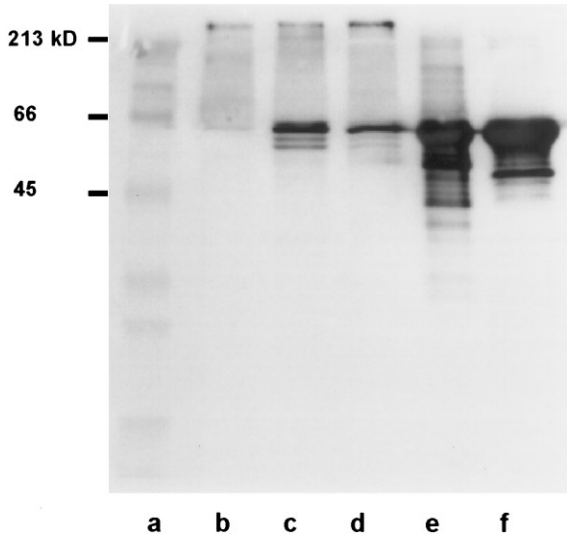

FIG. 2. Purification of recombinant VP16 using Western Blot. Lane a, prestained molecular weight marker; b, mock-infected cells; c, supernatant after cell lysis and centrifugation; d, pellet after centrifugation, resuspended in 8 M urea, pH 8.0; e, eluate from Ni-column; f, VP16 fraction after chromatography on a ResourceQ column.

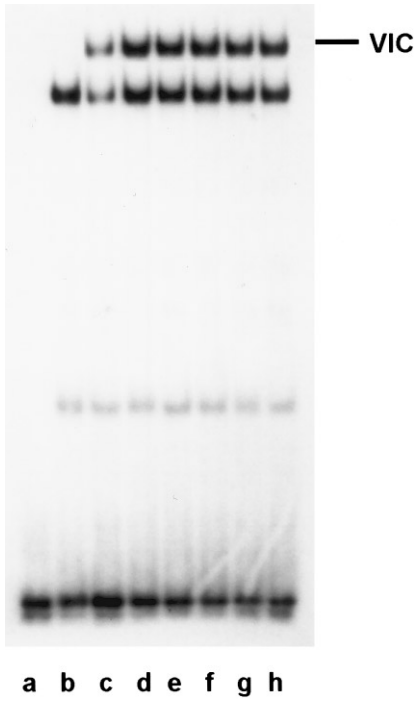

FIG. 3. EMSA data for VP16. A 5′-end labelled probe containing TAATGARAT motif was incubated with HeLa cell nuclear extract. Lane a, labelled probe without addition of nuclear extract and recombinant VP16; b, probe with addition of nuclear extract; c–h, probe with addition of nuclear extract and recombinant VP16. Reactions contained 0.05, 1.0, 2.0, 3.0, 4.0 and 5.0 μg of recombinant and purified VP16.

glycerol, 0.1 mM PMSF, 1 mM DTT, 2 μg poly(dI-dC) · poly(dI-dC) and 4 μg HeLa nuclear extract (Promega GmbH, Mannheim, Germany). Reactions contained 0, 0.05, 1.0, 2.0, 3.0, 4.0 and 5.0 μg of recombinant and purified VP16. Binding reactions containing nuclear extract and protein were preincubated at room temperature for 5 minutes prior to addition of probe and then incubated at 30°C for 20 minutes after probe addition. Reaction mixtures were loaded on a 3.5% polyacraylamide gel (30:1 acrylamide-methylenbisacrylamide) containing 0.5 x TBE (1 x TBE = 89 mM Tris, 89 mM boric acid, 20 mM EDTA). The gel was run at RT and 200 V for apr. 2-3 h. After that the gel was dried and exposed to X-ray film for autoradiography.

Documentation. Wet stained SDS gels, western blots and autoradiographs were scanned using the Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories) and printed with a Sony Color Video Printer, Model UP-5600 MDP (Sony Europa G.m.b.H., Koeln, Germany).

CD measurements. Far-UV circular dichroism (CD) spectra were measured in 0.1 cm path length cells from 190 nm to 280 nm with a JASCO J-710 spectropolarimeter. The spectra were recorded with the following experimental parameters; speed: 20 nm/min, step resolution: 0.2 nm, response: 0.5 sec, bandwidth: 2 nm, sensitivity: 50 mdeg, temperature 20°C. 7 scans were averaged to improve the signal to noise ratio and the spectra were corrected for buffer background. The VP16 sample was prepared in 10 mM potassium phosphate buffer (KPi), pH = 7.6, 5 mM potassium fluoride (KF) at a concentration of 4.52 μM. The concentration of the protein was determined from the absorption at 280 nm using an absorption coefficient of 51280 $M^{-1}\,cm^{-1}$ (theoretically calculated by the method of Pace *et al.* [9]). The absorption spectra were corrected for stray-light influence at 330 nm. The protein contains 6 cysteines. It currently not known how many of the cysteines form cystines, and thereby contribute to the absorption of the protein. Given the accuracy $<$ 5% of the Pace method for estimating the absorption coefficient it was justified to use the average of the cysteines-only- (50920 $M^{-1}\ cm^{-1}$) and the cystine-only absorption coefficient (51640 $M^{-1}cm^{-1}$). The mean residue ellipticity was calculated per amide bond. The percentage of secondary structural elements was performed using the program SELCON [10].

RESULTS AND DISCUSSION

Expression and purification. We have generated a recombinant baculovirus for the high yield production of VP16 in High Five insect cells. The virus was made by site specific transposition between genetically engineered baculovirus DNA ("bacmid" DNA) and a trans-

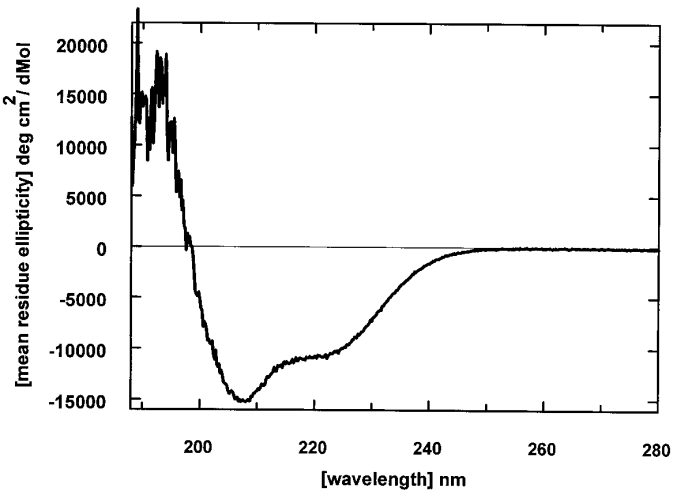

FIG. 4. Circular dichroism spectra of recombinant VP16 between 185 and 280 nm.

TABLE 1

Estimated Secondary Structure Content of Recombinant VP16

Method	α-Helix	β-Sheet	Turn	Random coil
CD	32.0[a]	17.5	26.0	25.4
GOR[b]	42.3	16.9	20.0	24.6

Note. The CD data for recombinant VP16 were recorded at 20°C according to the method of Sreerama and Woody [10].

[a] Numbers given in percent.

[b] Theoretical secondary structure prediction method in the GOR [13] prediction from the University of Southampton Bioinformatics Data Server (SBDS).

fer vector containing the VP16 gene [11]. The VP16 transcription unit codes for all 1224 amino acids of the mature protein, plus an additional N-terminal 28 amino acids including the 6 x histidine tag.

Recombinant virus was isolated and titrated by end-point dilution, as described previously [5]. Expansion of virus in suspension cultures generated large volume of high-titre stocks ($> 10^7$ p.f.u./ml). These viral supernatants were then used for large scale protein production. Expression cultures generated in this way gave rise to apr. 10 mg pure VP16 for a total of 10^9 cells in 1 l of medium.

Purification of the 6 x his-VP16 fusion protein was greatly simplified by using immobilised metal chelat chromatography (IMAC) giving rise to a relatively pure protein (>70%) in just a single step. Further purification of the protein was achieved using a ResourceQ ion exchanger and rendered protein higher than 95% purity (see figure 1). Western blot analysis clearly demonstrated the enrichment typically achieved through adsorption of protein from the cell supernatant to Ni-beads (see figure 2). The entire purification process is rapid and can easily be completed within one day. This method is an efficient alternative to established protocols that typically involve $(NH_4)_2SO_4$ precipitation, extensive dialysis and gelfiltration [8].

Protein characterisation. To analyse the biological activity of the recombinant VP16, the protein was incubated with double stranded DNA containing the TAATGARAT motif and nuclear extract from HeLa cells. The probe used for the binding reactions was a synthetic duplex which contains sequences corresponding to positions –170 to –143 of the IE110 gene of HSV-1 [8,12]. Addition of recombinant VP16 to a mixture of TAATGARAT DNA and HeLa nuclear extract leads to a VP16 induced super shift (VIC = "virus induced complex") indicating that the protein is functionally active (see figure 3).

In order to gain structural information on the protein CD analysis were performed. The CD spectrum of the VP16 protein in solution, at a concentration of 4.52 μM, is dominated by the negative maximum at 208 nm and a shoulder around 225 nm suggesting defined parts with alpha helical secondary structure within the 507 amino acids of the fusion protein (see figure 4). From a qualitative investigation of the spectrum the content of β-sheet conformation is lower than that of α-helix (and turn structure). Careful analysis of the fractions of the secondary structure from the self-consistent method of Sreerama and Woody (SELCON) using 18 crystal structures of proteins in the database revealed 32.0 % α-helix, 17.5 % β-sheet, 26.0 % in turns and approximately the same number of amino acids, 25.4%, in random conformation (see table 1).

These numbers correspond very well with the results of theoretical secondary structure prediction methods suggesting for example 42.3 % α-helix, 16.9 % β-sheet, 20.0 % turn and 24.6 % random coil structure in the GOR secondary prediction [13] from the university of Southampton bioinformatics data server (SBDS).

From the data presented here, we conclude that a BEVS using suspension-cultured High Five cells provides a very efficient and practical means of expressing VP16 that can easily be purified from infected cells. The CD spectrum presented in this work is the first structural report for the VP16 protein and clearly demonstrates that the preparation and purification method presented in this work results in a properly structured and folded protein which will be very useful in high-throughput assay development and mechanistic studies.

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