Application of Insect Virus Polyhedra to Protein Nanocontainers

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ABSTRACT

Cypoviruses (CPVs) are members of the family *Reoviridae* that infect insect larvae producing polyhedra in the cytoplasm of mid-gut epithelial cells [1, 2]. Polyhedra are remarkably stable protein crystals and function to protect the virus particles within the crystals from hostile environmental conditions. The polyhedron protein, which is called polyhedrin, is encoded by the virus genome. Recently we have determined the atomic structure of *Bombyx mori* CPV polyhedra using.a synchrotron microbeam to collect X-ray diffraction data [3]. Polyhedra are made from trimeric building blocks of the polyhedrin interlocked into a tight scaffold generated by the amino-terminal alpha-helix. BmCPV are known to be composed of five viral capsid proteins (VP1-5). We have previously demonstrated that the N-terminal 75 amino acids of VP3 can function as a polyhedrin recognition signal leading to the incorporation of foreign proteins into polyhedral [4, 5]. We also showed that the polyhedrin H1 helix can also function as a polyhedrin recognition signal [6]. I introduce a unique protein expression system in which the expressed target proteins are immobilized into the polyhedra.

Keywords: cypovirus, polyhedra, polyhedrin, immobilization.

1 INTRODUCION

CPVs are characterized by the presence of capsids made up of concentric icosahedrally symmetric layers of proteins organized in one, two or three shells containing 10 to 12 segments of linear, double-stranded RNA. Polyhedra function to facilitate the survival of the virus in the environment, stabilizing the virus particles and allowing them to remain viable for long periods. Polyhedra are remarkably stable under physiological conditions, but do dissolve at pH > 10.5. Infection occurs by the release of the virus particles in the alkaline larval midgut after ingestion. Only three virus families, all from insects, share this mode of propagation within infectious protein crystals. Besides cypovirus, DNA viruses in the baculovirus group (nucleopolyhedrovirus and granulovirus) and the entomopoxvirus group also produce polyhedral [7].

2 STRUCTURE OF POLYHEDRA

The atomic structure of the silkworm *Bombyx mori* CPV (BmCPV) polyhedra has been determined to a resolution of 2Å [3]. The crystals have body centered cubic symmetry with a 103Å unit cell. Each unit cell contains 24 identical polyhedrin molecules in different positions and orientations related by I23 space group symmetry. A typical 2-micron crystal contains about 200 million polyhedrin molecules, 8 million cubic unit cells and thousands of virus particles. The virus particles within polyhedra each displace approximately 200 unit cells from the crystalline polyhedrin lattice. The proportion of the volume occupied by virus particles in a 2-micron crystal containing 1000 particles is 2.5% (Fig. 1).

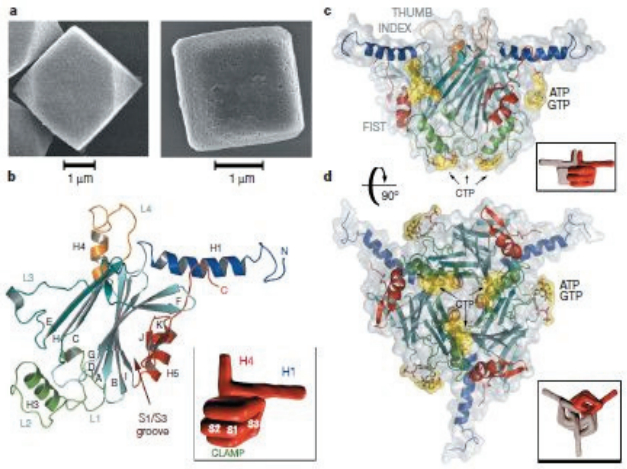

Fig. 1 Compact trimers are the building blocks of polyhedra. A, Scanning electron micrographs of recombinant (left) and infectious (right) polyhedra. We assume that leaching of CPV particles leaves the holes visible on the surface of virus-containing polyhedra. **B**, Cartoon representation of the polyhedrin coloured blue to red from the N- to the C-terminus. **C** and **D**, cartoon representation in a semi-transparent surface of a polyhedrin trimer in two orthogonal views. Nucleotides bound to the trimer are shown as sticks in a yellow surface. The diagrams represent trimers in the corresponding orientation with one of the monomers highlighted.

The atomic structure revealed that cypovirus polyhedra are extraordinarily intricate and densely packed, unlike laboratory-crystallized proteins where only a tiny fraction of the surface area of each molecule in the crystal interacts with other molecules. Analysis of all the contacts to other molecules in polyhedra revealed that the strongest crystalline interactions formed trimers of molecules within the crystal. For this reason, the structure of polyhedra is

conveniently described as being based on trimeric building blocks (although all the molecules in the crystal are identical). Individual polyhedrin molecules have a distinctive extended N-terminal α helix (the H1 helix) which projects from the main part of the molecule, a compact β sandwich domain with 3 layered β sheets and peripheral small α helices. The trimeric building blocks are linked to each other in polyhedra by multiple interactions, primarily involving these extended H1 N-terminal arms. The trimers themselves are formed by extensive hydrophobic interactions between monomers, particularly between the H4 helices which surround the three-fold axis. The trimeric building blocks may represent an assembly intermediate for polyhedra, but this is yet to be determined.

Polyhedra are stable in reagents that would normally be expected to denature proteins (concentrated urea, SDS, acid etc.). The structure is consistent with this, with numerous protein-protein contacts shielding over 70% of the monomer surface from solvent. Polyhedra do however dissolve readily above pH 10.5 and the deprotonation of a buried cluster of tyrosines (pKa ~10.1) may provide a clue for the disassembly mechanism. Larval alkaline proteinases degrade polyhedrin further contributing to the efficient release of virus from polyhedra in the mid-gut.

3 IMMOBILIZATION INTO POLYHEDRA

3.1 Polyhedrin

BmCPV polyhedrin gene was introduced into a baculovirus expression vector and the insect cell line *Spodoptera frugiperda* IPLB-Sf21AE (Sf21 cells) was infected with the recombinant baculovirus designated AcCP-H. The polyhedrin was massively produced and many cubic polyhedra were observed in Sf cells [8]. This result demonstrated that other cypovirus-encoded proteins were unnecessary for the crystallization of BmCPV polyhedrin.

3.2 VP3 signal

For cypovirus, similar specific interactions between CPV polyhedrin and a viral capsid protein may lead to occlusion of virus particles into polyhedra. Iodination of BmCPV virus particle and analysis of the labeled polypeptides by SDS-PAGE first indicated that VP1 and VP3 are outer components of the BmCPV particle [9, 10]. VP3 was first selected to investigate interaction with BmCPV polyhedrin in the occlusion of virus particles into the polyhedra [4]. Green fluorescent protein (GFP) was fused to the C-terminus of BmCPV VP3 and introduced into the baculovirus expression vector, resulting in AcVP3/GFP [4]. The fusion protein of VP3 and GFP was co-expressed with AcCP-H polyhedrin in Sf21 cells. The polyhedra were purified and green fluorescence observed under UV irradiation. The fluorescence indicated that the VP3-GFP chimera was incorporated into these polyhedra. The fluorescence disappeared when the polyhedra were suspended in acetate buffer at pH 4 and reappeared after the pH was increased. There was no change in the appearance of BmCPV polyhedra produced by AcCP-H below pH 10, however, when the pH increased above 10, the polyhedra dissolved rapidly and the green fluorescence was intensified [4]. This effect could be due to the release of the VP3-GFP chimera from the dissolving polyhedra. No fluorescence was detected in polyhedra obtained from cells infected with both AcGFP and AcCP-H, indicating that the chimeric protein occlusion was initiated by specific interactions between BmCPV polyhedrin and VP3 [4].

In order to map an immobilization signal to a fragment of the BmCPV VP3 protein, either the N-terminal or C-terminal half of VP3 was fused to the N-terminus of EGFP and recombinant baculoviruses encoding these two chimeric proteins were constructed [5]. One of these viruses, designated AcVP3(N)/GFP, encoded amino acids 1-448 of VP3 fused to EGFP and the other, designated AcGFP/VP3(C), encoded EGFP fused to amino acids 428-1057 of VP3. Each recombinant virus was used for double infections together with AcCP-H, which is a third recombinant that produces a cubic form of BmCPV polyhedra. Green fluorescence was observed, predominantly on the surface, with polyhedra isolated from cells that had been co-infected with AcVP3 (N)/GFP and AcCP-H [5]. In contrast, no green fluorescence was observed with polyhedra isolated from cells that had been co-infected with AcGFP/VP3(C) and AcCP-H.

A large number of recombinant baculoviruses encoding different VP3-GFP fusion proteins were subsequently constructed to identify the minimal region of VP3 required to target the GFP fusion construct to polyhedra. Sf21 cells were co-infected with these recombinant baculoviruses and AcCP-H, polyhedra were purified from each doubly-infected culture, and then the presence of each fusion protein in the polyhedra was determined by confocal microscopy. Based upon the results of these analyses, it was concluded that the occlusion of GFP into polyhedra required the region of VP3 between amino acids 1 and 79 and this amino acid sequence also was able to direct the immobilization of other foreign proteins into polyhedra [5]. The N-terminal 79 residues of VP3 form a separate domain on the outside of the turret [11]. The N-terminal 79 residues of VP3 were named the immobilization signal [5] or polyhedrin-binding domain (PBD) (Fig. 2), since these residues form a separate domain at the base of the turret, as seen in the 3.88Å resolution cryo-EM map [11].

3.3 H1 signal

The atomic structure of BmCPV polyhedra revealed trimeric building blocks connected by extensive interactions, many involving the polyhedrin H1 helix [3]. Each building block consists of a cluster of three identical polyhedrin molecules and the three corresponding N-terminal H1

helices project outwards from the centre of the trimer. The building blocks are interlocked with other identical building blocks to form a tight scaffold largely generated by H1-helix [3]. Because the H1 helix is at the N-terminus of the molecule and projects outwards from the structure it may form into a helix independently as the molecule folds. This possibility, together with its role in cross-linking polyhedra, led to the suggestion that the polyhedrin H1 helix might also prove to be a useful tag for incorporating foreign proteins into polyhedra, like the VP3 residue 1-79 immobilization signal (Fig. 2).

The H1-helix sequence was added to either the N-terminus or the C-terminus of enhanced green fluorescent protein (EGFP) [6]. Each recombinant EGFP (H1/EGFP and EGFP/H1) was co-expressed with BmCPV polyhedrin using the baculovirus expression vector system. Immobilization of EGFP fused with H1 signal was compared with VP3 signal. The emission of green fluorescence from H1/EGFP polyhedra displayed a greater intensity compared than either EGFP/H1 or EGFP/VP3 polyhedra. The VP3 signal and the H1signal were used together to produce polyhedra containing both EGFP and DsRed. EGFP and DsRed were fused with VP3 signal (EGFP/VP3) and H1 signal (H1/DsRed), respectively, and the reverse conformations (H1/EGFP and DsRed/VP3) were also constructed. The double-labeled polyhedra with EGFP and DsRed were isolated and imaged using dual wavelength confocal fluorescence microscopy, showing that a multiple proteins can be incorporated into single polyhedra using both VP3 and H1 immobilization signals (Fig. 3).

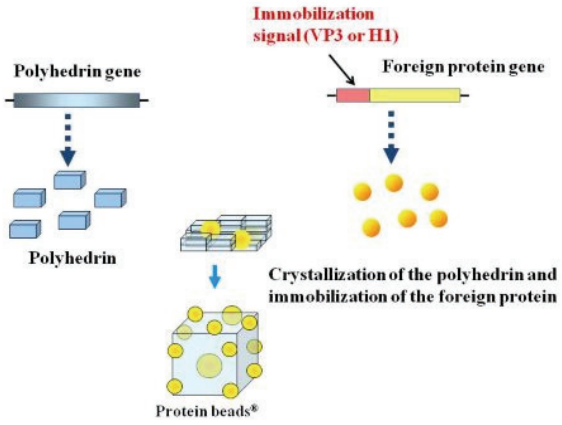

Fig. 2 Scheme of immobilization of foreign proteins into BmCPV polyhedra. Two recombinant baculoviruses are constructed. One recombinant baculovirus expresses BmCPV polyherin and other express a forein protein which is fused with an immobilization signal VP3 or H1. Sf cells are co-infected with these recombinant baculovirses and polyhedra are recovered from the infected cells.

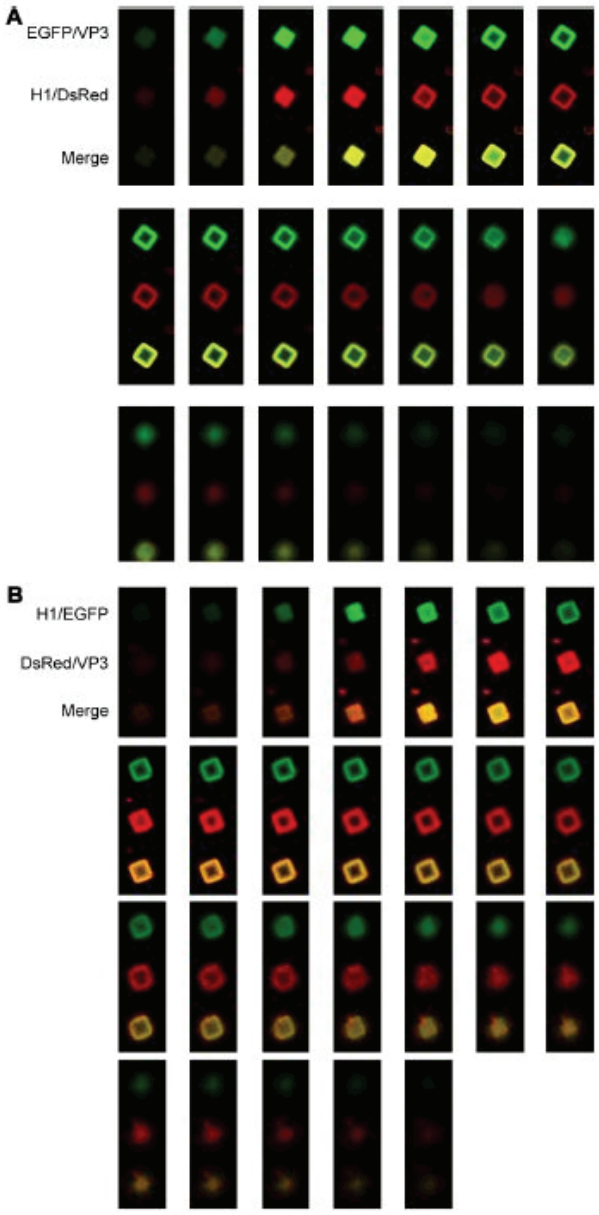

Fig. 3 Analysis of polyhedra containing two recombinant fluorescence proteins by confocal microscopy. A, Laser-scanning confocal images of one polyhedron containing EGFP/VP3 and H1/DsRed. **B,** Laser-scanning confocal images of one polyhedron containing H1/EGFP and DsRed/VP3. Polyhedra were prepared by triple-infection with AcCP-H and two recombinant viruses expressing EGFP and DsRed and optically sectioned using 0.2 μm intervals.

3.4 Immobilization of Fibroblast growth factor-2 (FGF-2)

FGF-2 was also fused with the VP3 signal at the C-terminal (FGF-2/VP3) or H1 signal at the N-terminal (H1/FGF-2) and the biological activity of FGF-2 polyhedra was assayed using mouse fibroblast NIH3T3 cells. In the presence of either FGF-2/VP3 polyhedra or H1/FGF-2 polyhedra, NIH3T3 cells proliferated in a dose-dependent manner to an extent that was comparable with the addition of recombinant human FGF-2 (rhFGF-2). When 2 x 10^4 H1/FGF-2 polyhedra were added to the culture, the NIH3T3 cell proliferation was twice that of non-stimulated cells. In contrast, 5 x 10^4 cubes of FGF-2/VP3 polyhedra were required for a similar two-fold proliferation. In the control experiment, empty-polyhedra did not induce NIH3T3 cell proliferation. The number of FGF-2 polyhedra required to obtain an equivalent proliferative response to 1 ng of rhFGF-2 was 1.5 x 10^4 for H1/FGF-2 polyhedra and 7.5 x 10^4 for FGF-2/VP3 polyhedra [6].

3.5 Immobilization of protein kinase C

Protein kinase C (PKC) was immobilized into polyhedra and enzymatic activity of the immobilized PKC was measured by using PepTag® assay for non-radioactive detection of PKC activity. PKC activity was detected from PKC/VP3 and H1/PKC polyhedra, and the phosphorylated C1 substrates were migrated toward the anode. However in the case of normal polyhedra, no activity was detected. The phosphorylated C1 substrates in agarose gel were measured by excitation at 568 nm and emission at 592 nm using a fluorescence spectrophotometer (Fig. 4). PKC activities of 1.0 x 10^6 polyhedra, in which PKC/VP3 and H1/PKC polyhedra were immobilized respectively, were estimated to be 0.94 units (nmol min^{-1} ml^{-1}) and 0.83 units of commercially used PKC. Therefore, we identified that the content of PKC of PKC/VP3 and H1/PKC polyhedra with 1.0 x 10^6 cubes corresponded to 19.2ng and 16.7ng, respectively [12].

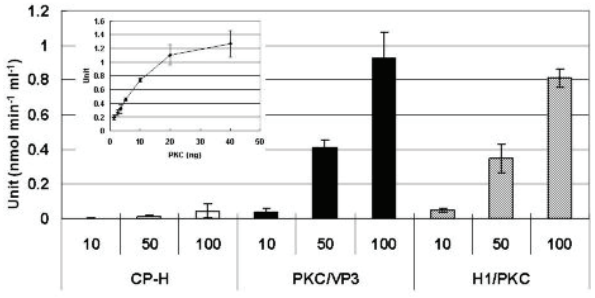

Fig. 4 Estimation of PKC activity from polyhedra. PKC activities from PKC/VP3 and H1/PKC polyhedra were quantitatively determined by a comparison with a standard curve according to the manufacturer's instructions (Promega). Each number of polyhedra with 1 x 10^5, 5 x 10^5, and 1 x 10^6 cubes was indicated as 10, 50, and 100. These activities were measured by excitation at 568 nm and emission at 592 nm using fluorescence spectrophotometer. The standard PKC activities were performed by using commercially used PKC, which purified from rat brain, with varying amounts (0 - 40ng). The insert shows standard curve of PKC activities. Each bar represents the mean SEM of three individual experiments.

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