Dimerization of Vaccinia Virus VH1 Is Essential for Dephosphorylation of STAT1 at Tyrosine 701*

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The gene product of *Vaccinia* virus gene H1, VH1, is the first identified dual specificity phosphatase (DSP). The human genome encodes 38 different VH1-like DSPs, which include major regulators of signaling pathways, highly dysregulated in disease states. VH1 down-regulates cellular antiviral response by dephosphorylating activated STAT1 in the IFN-γ/STAT1 signaling pathway. In this report, we have investigated the molecular basis for VH1 catalytic activity. Using small-angle x-ray scattering (SAXS), we determined that VH1 exists in solution as a boomerang-shaped dimer. Targeted alanine mutations in the dimerization domain (aa 1–27) decrease phosphatase activity while leaving the dimer intact. Deletion of the N-terminal dimer swapped helix (aa 1–20) completely abolishes dimerization and severely reduces phosphatase activity. An engineered chimera of VH1 that contains only one active site within the same dimer is essential for VH1 catalytic activity. Together with laforin, VH1 is the second DSP reported in literature for which dimerization via an N-terminal dimerization domain is necessary for optimal catalytic activity. We propose that dimerization may represent a common mechanism to regulate the activity and substrate recognition of DSPs, often assumed to function as monomers.

Like classical PTPs, VH1-like DSPs contain a catalytic triad consisting of a cysteine, an arginine, and an aspartic acid, usually arranged in the context of an extended consensus motif (7). DSPs employ a similar catalytic mechanism as PTPs, characterized by the formation of a transient enzyme-phosphosubstrate intermediate (1, 2). Similar to PTPs, the DSP catalytic core shows a great degree of substrate specificity. The substrate however, can be non-peptidic for a number of DSPs. For instance, PTEN-like phosphatases dephosphorylate D3-phosphorylated inositol phospholipids (8), or the DSPs PIR (also known as DUSP11) and laforin have been shown to dephosphorylate mRNA (9) and phosphoglucans (10), respectively.

The gene encoding VH1 is highly conserved among double-stranded DNA viruses of the *Poxviridae* family (11). Approximately 200 molecules of VH1 are packaged within the *Vaccinia* virion and are essential for the viability of *Vaccinia* virus (12). In *vivo*, VH1 is required for maturation of two-virion membrane-associated factors, namely A17 (13) and A14 (14). Upon infection, the DSP is released into the host cytoplasm, where *Vaccinia* virus establishes cell factories and replicates (15). Growing evidence indicates that VH1 blocks the host interferon-γ (IFN-γ) signal transduction pathways by dephosphorylating the transcription factor STAT1 at tyrosine 701 (Tyr-701) (16). This was demonstrated using both activated STAT1 immunoprecipitated from cells stimulated with IFN-γ (16), as well as recombinant STAT1 *in vitro* phosphorylated at Tyr-701 (17). Notably, VH1 phosphatase activity is specific to STAT1 and STAT2, but not STAT3 and STAT5 (18). Activated STAT1 is imported into the nucleus by the transport adaptors importin α5 bound to importin β (19). Binding of STAT1 to importin α5 requires tyrosine phosphorylation at position 701, which is the specific substrate of VH1. *In vitro*, importin α5 protects STAT1 from VH1-mediated dephosphorylation in a dose-dependent manner (17). Because importin α5 does not bind pTyr701 in STAT1 (20), the decreased susceptibility to VH1 in the presence of importin α5 is likely caused by the structural constraints imposed by importin α5 binding to STAT1. The competition with importin α5 together with the observation that VH1 is inactive with respect to DNA-bound STAT1 suggested that VH1 acts predominately on the cytoplasmic pool of activated STAT1 (17). Thus, VH1-mediated blockade of the IFN-γ/STAT1 signaling pathway has the likely function to prevent transcription of downstream STAT1 target genes, and thereby block or reduce an antiviral response.

The crystal structure of *Vaccinia* (17) and its closely related *Variola* (11) virus VH1 phosphatases were recently determined. In both cases, the structure revealed a conserved PTP-
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like fold similar to that previously reported for VHZ (21) and VHR (22). The VH1 active site consists of a shallow catalytic cleft only ~6 Å deep, which can accommodate both phosphotyrosine and phosphothreonine/serine residues. This active site structure contrasts from classical tyrosine phosphatases, where the catalytic cysteine is located at the bottom of a ~9 Å deep pocket, highly selective to recognize phosphotyrosine (22). Furthermore, in both Vaccinia and Variola DSPs, a putative dimeric quaternary structure was inferred by analysis of crystal for its catalytic activity and recognition of pSTAT1.

In vivo dimeric quaternary structure was inferred by analysis of crystal for its catalytic activity and recognition of pSTAT1. Furthermore, in both Vaccinia and Variola DSPs, a putative dimeric quaternary structure was inferred by analysis of crystal for its catalytic activity and recognition of pSTAT1. Intriguingly, the main cellular substrate of VH1, phosphorylated STAT1 (pSTAT1), is also dimeric upon phosphorylation at Tyr-701 (23). Therefore, it was proposed that the VH1 dimeric quaternary structure represents an evolutionary adaptation to pSTAT1 specific recognition (17).

In the present study, we have analyzed the mechanisms by which dimerization affects VH1 catalytic properties. We found that while there is no cross-talk between VH1 active sites, the integrity of the VH1 dimeric quaternary structure is essential for its catalytic activity and recognition of pSTAT1.

EXPERIMENTAL PROCEDURES

Biochemical Techniques—The gene-encoding Vaccinia virus VH1 was cloned in a pET-14b vector (Novagen) containing a PreScission Protease cleavage site between the His$_6$ tag and the first residue of VH1. VH1 was expressed in the Escherichia coli BL21(DE3) strain and purified as previously described (17). The His$_6$ tag was cleaved off by incubating with PreScission Protease, followed by gel filtration chromatography on a Superdex 75 column (GE Healthcare Life Sciences) in GF buffer (150 mM sodium chloride, 20 mM HEPES pH 7.0, 3 mM ß-mercaptoethanol, 2% glycerol, 0.1 mM PMSE). Monomeric VH1 lacking the N-terminal residues 1–20 was generated by deletion PCR. The protein was expressed and purified as wild-type (wt) VH1. Chimeric VH1 constructs were generated by introducing a 18 nucleotide linker (GGCGGAGGTGGCGGATCC) between two VH1 genes. Point mutations in the active sites were introduced by site-directed mutagenesis. All chimeras were expressed and purified as wt VH1. Human STAT1 was expressed and phosphorylated at position 701 in E. coli strain TKX-1 (Stratagene, La Jolla, CA), which harbors an inducible plasmid encoding a tyrosine kinase gene (pTK). Expression, in vivo phosphorylation and purification of STAT1 were performed as previously described (17).

SAXS Data Collection, Analysis, and ab Initio Shape Reconstruction—Small-angle x-ray scattering data were measured at beam line X9A at the National Synchrotron Light Source (Upton, NY). Data were collected by passing VH1 samples through a flow capillary at a rate of 10 μl/min. The samples were in the GF buffer previously mentioned, and at concentrations ranging from 1–5 mg/ml. They were centrifuged at 16,000 × g for 10 min before 20-s exposures were taken in triplicates. The GF buffer was used in blank experiments to subtract the solvent from VH1 sample scattering. Guinier analysis between the triplicate exposures was used to control for radiation damage and signs of aggregation. Data reduction was done by circular averaging of the images and scaling to obtain the scattering curve (scattering intensity (I) as a function of the momentum transfer vector q (q = 4π(sinθ)/λ). GNOM (24) was used to calculate P(r) plots from the scattering data. Ab initio model calculations to generate a scattering envelope were done using GASBOR (25). 14 solutions from GASBOR were used to check consistency and averaged to obtain the final model using the DAMAVER program suite (26). The final solution model was converted to a surface map using the SITUS program suite (27). The theoretical solution scattering of the crystal structure of VH1 was calculated using CRYSO (28). All figures in the report were prepared using the program Pymol (29).

Sedimentation Velocity and Thermal Stability—Analytical ultracentrifugation experiments for dimeric, monomeric, and chimeric VH1 were carried out in 20 mM HEPES pH 7.0, 150 mM sodium chloride in a Beckman XL-A Analytical Ultracentrifuge (AUC) under velocity sedimentation mode. 450 μl of sample and 400 μl of reference buffers were loaded into separate compartments of a 12 mm path-length Epon centerpiece cell. Runs were performed at 45,000 rpm and 10 °C. Absorbance values were collected at a wavelength of 276 nm using a protein concentration of 50 μM. The data were fit to a continuous sedimentation coefficient (c(s)) distribution model and an estimated molecular mass was obtained with the program SEDFIT (30, 31). Thermal stability assays were recorded using a Jasco J-810 spectropolarimeter equipped with a Neslab RTE7 refrigerated recirculator. VH1 samples at a final concentration of 7.0 μM in TM buffer (20 mM sodium phosphate (pH 7.4) and 100 mM NaCl) were measured using a 1 mm rectangular quartz cuvette (Starna Cells, Inc.). The variations in ellipticity at 220 nm as a function of temperature in 1 °C increments were measured over the range 30 °C-75 °C. Slow cooling to 25 °C followed by a CD scan for secondary structure demonstrated that the unfolding of all VH1 samples is irreversible.

Phosphatase Kinetic Assays—The assay was performed in dephosphorylation buffer (50 mM HEPES pH 6.8, 50 mM NaCl, and 10 mM ß-mercaptoethanol). The enzymatic activity of the various VH1 constructs was monitored using the colorimetric substrate 3-O-methylfluorescein phosphate (OMFP, Sigma-Aldrich) at an absorbance of 477 nm using varying concentrations of substrate ranging from 0.1 to 10 times the $K_m$ values. The reaction was initiated by addition of enzyme at a final dimer concentration of 1 μM to the reaction mix at 37 °C. All experiments were done in triplicate to calculate standard deviations. $K_m$, $V_{max}$, and $k_{cat}$ values were determined by non-linear regression using the software GraphPad (GraphPad Software, Inc).

STAT1 Dephosphorylation Assay—In vivo phosphorylated STAT1 was used at a concentration of 1 μg/ml. All VH1 samples were added at a final concentration of 24 μM. The final reaction conditions were 50 mM HEPES pH 7.0, 70 mM NaCl, 10 mM ß-mercaptoethanol in a volume of 50 μl at 37 °C. Time points were taken at 0, 1, 5, 30, 60, and 120 min. STAT1 dephosphorylation levels were measured by Western blot analysis using p-701-STAT1 (Cell Signaling Technology, Inc.) rabbit primary mAb and HRP-goat anti-rabbit secondary antibody (Invitrogen). All dephosphorylation reactions were repeated a minimum of three times. The software ImageJ (NIH) was used.
to quantify relative intensities of pTyr701 and plotted using the program SigmaPlot.

RESULTS

Dimeric Structure of VH1 in Solution Investigated by Small-Angle X-ray Scattering (SAXS) — The crystal structure of *Vaccinia* (17) and *Variola* (11) virus VH1 were recently reported. In both structures, the crystallographic asymmetric unit contains a single DSP-monomer. However, a dimeric quaternary structure was proposed based on analysis of crystal contacts. In both structures, applying 2-fold crystallographic symmetry generates a dimeric structure, which is stabilized by an N-terminal domain swap of the first ~27 amino acids (11, 17). Although *Vaccinia* virus VH1 dimerization was also confirmed in solution using analytical ultracentrifugation sedimentation velocity analysis (17), it is unclear if the quaternary structure proposed using crystallographic symmetry represents the actual dimer assembled in solution. To answer this question, we determined the structure of VH1 in solution using SAXS. We measured small and wide angle scattering data for VH1 at several concentrations and obtained similar results in each case (Fig. 1A). The gyration radius ($R_g$) and maximum dimension ($D_{max}$) calculated from the experimental scattering are 26.3 Å and 87 Å, respectively. This agrees well with the calculated $R_g = 26.1$ Å
and $D_{\text{max}} = 90$ Å for dimeric VH1 observed crystallographically (CRYSOL chi value $\sim 2.4$) (28). We generated a shape reconstruction from the scattering data using the program GASBOR (25), as described under “Experimental Procedures.” As shown in Fig. 1C, the averaged SAXS envelope for VH1 obtained has an elongated boomerang shape. This envelope fits well the crystallographic model of dimeric VH1, which has a slightly elongated shape of $\sim 85$ Å in length, $\sim 31$ Å in height and $\sim 28$ Å in width (Fig. 1D) (17). Ab initio reconstructions of 14 models from 14 independent GASBOR iterations all agreed exceptionally well with one another with a normalized spatial discrepancy value of $\sim 0.8$ (this value defines the similarity between two intrinsically distinct models, where a value smaller than 1.0 indicates a high degree of agreement between the two structures). Applying 2-fold symmetry to the SAXS envelope further improved the quality of the VH1 reconstruction, which best demonstrates the correctness of our solution (Fig. 1, C and D). Theoretical scattering profiles for the dimeric structure of VH1 were generated and are in good agreement with the experimental data (Fig. 1A). Likewise, the agreement between VH1 dimeric crystal structure and SAXS data is also mirrored by comparing experimental and calculated the $P(r)$ shape functions (Fig. 1B), which represent a distribution of interatomic distances present in the molecule. The match between calculated and experimental $P(r)$ functions for VH1 is remarkably good, which confirms the crystallographic dimer built using 2-fold crystallographic symmetry indeed corresponds to the conformation of VH1 adopted in solution. The shape function associated with the crystallographic model of VH1 is only slightly compressed toward an interatomic distance of $\sim 40$ Å, which corresponds to the distance between two VH1 active sites in the crystallographic dimer (Fig. 1D). This is likely explained by the breathing of VH1 dimer in solution, which is not seen in the crystal structure. With the only exception of this deviation, the dimeric crystal structure of VH1 previously reported fits exceptionally well with the SAXS envelope, suggesting that the VH1 domain-swapped dimerization interface is not an artifact of crystallization.

**Point Mutations at VH1 Dimerization Interface Reduce Catalytic Activity**—Having established that the VH1-DD is not conserved in other DSPs outside Poxviridae, we asked whether this domain is in fact required, or can modulate, VH1 phosphatase activity. Given that the DD is not part of the minimal catalytic core, we set out to answer this question by introducing alanine point mutations in key residues of the N-terminal swapped helix (Fig. 2A). First, we mutated Ser-14 and Thr-15 (mutant dVH1–2m) that mediate packing of the α1 helix against the DSP core (Fig. 2B). Then, we introduced three more mutations in residues protruding on the surface of swapped helix: Lys-8, Leu-11, and Leu-12 (mutant dVH1–5m) (Fig. 2A, B). The two mutants were expressed and purified under identical conditions as wild-type VH1 (henceforth referred to as dVH1). By sedimentation velocity analysis, both VH1 point mutants migrated as monodisperse dimers $\sim 40$ kDa in molecular mass, indistinguishable from dVH1 (Table 1). Therefore, even 5 point mutations in VH1-DD were insufficient to disrupt the phosphatase dimeric structure. To analyze the effect of these mutations on VH1 activity, we carried out an in vitro dephosphorylation assays using OMFP. Interestingly, both dVH1–2m and dVH1–5m hydrolyzed OMFP with reduced efficiency as compared with the wt enzyme. The $k_{\text{cat}}/K_{m}$ values for these two mutants were $\sim 1.7$- and 2.5-fold lower than that of dVH1 (Table 1). The observed loss in efficiency was mainly due to a decrease in affinity for the phosphosubstrate as witnessed by a 3- and 4-fold increased $K_{m}$ for dVH1–2m and dVH1–5m, respectively, as compared with dVH1 (Table 1). Because both mutants retain a dimeric structure, it is plausible that mutations in VH1-DD weaken the compactness of this binding interface that is likely required for optimal catalytic activity.

**Monomeric VH1 Is Poorly Active**—To determine the structural role of dimerization on catalytic activity, we investigated the activity of a monomeric VH1 (Fig. 2, A and C). Given that five point mutations in the DD were not sufficient to disrupt VH1 dimerization, we deleted the N-terminal helix α1 (aa 1–20), which is the main structural determinant bridging two VH1-DSP cores together in a functional dimer (Fig. 2B). This construct, mVH1, corresponds to VH1 minimal DSP core and is predicted to fold into a discrete VHZ-like core (21) $\sim 21$ kDa in molecular mass (Fig. 2C). mVH1 was expressed in E. coli and purified from a soluble fraction using an N-terminal His tag. To confirm mVH1 is monomeric and correctly folded, we carried out hydrodynamic and folding studies. By sedimentation velocity analysis, mVH1 migrated as a monodisperse species $\sim 22$ kDa (Fig. 3A, Table 1), in agreement with the expected mass of $\sim 21$ kDa. Furthermore, the normalized molar ellipticity for mVH1 showed minimal loss of α-helical signal as compared with the dimeric phosphatase (data not shown), consistent with a well folded DSP core.
To gain insight into mVH1 structural stability, we recorded temperature-mediated unfolding curves for mVH1 and dVH1, by measuring variations in ellipticity at 220 nm as a function of temperature (Fig. 3B). Interestingly, mVH1 displayed a broad unfolding transition with an apparent melting temperature \((T_m)\) of \(52^\circ\text{C}\). Although the \(T_m\) for dimeric and monomer VH1 are very similar \((55^\circ\text{C} \text{ versus } 52^\circ\text{C})\) (Fig. 3B), the two proteins denature in drastically different ways. dVH1 thermal unfolding is highly cooperative and can be interpreted as a dimer of folded VH1 unfolding to a monomer. In contrast, mVH1 unfolds non-cooperatively, as often seen for small globular monomeric proteins, characterized by formation of unfolding intermediates en route to the final unfolded state (32). Taken together, these data suggest that deleting the N-terminal swapped helix in VH1-DD disrupts the enzyme dimeric quaternary structure without affecting the DSP core tertiary structure.

To analyze mVH1 activity, we carried out an in vitro dephosphorylation assay using OMFP. We found that while dVH1 hydrolyzes OMFP efficiently \((K_m = 87.17 \mu\text{M}, k_{\text{cat}}/K_m = 2.05 \times 10^6\) (Table 1), mVH1 has dramatically lower affinity for OMFP \((K_m = 780.4 \mu\text{M})\) and ~120-fold reduced catalytic efficiency \((k_{\text{cat}}/K_m = 0.017 \times 10^6\), as compared with the dimeric enzyme (Table 1). The rate of OMFP dephosphorylation observed for mVH1 is higher than the spontaneous rate of OMFP hydrolysis in an aqueous environment and follows Michaelis-Menten saturation kinetics (Fig. 3C). The monomeric phosphatase is, however, only minimally active. Thus, a dimeric quaternary structure and not simply a folded DSP core is essential for VH1 optimal catalytic activity.

**FIGURE 2.** VH1 contains an N-terminal dimerization domain fused to a C-terminal DSP core. **A,** schematic diagram of a protomer of VH1 with DD domain and DSP core colored in red and green, respectively, illustrating the different constructs used in our study. Point mutations in the DD are shown in orange. **B,** ribbon diagram of dVH1 (PDB 3CM3) with the two protomers colored in green and cyan. The dimerization domain of one protomer is color coded as in panel A. The selected residues mutated in A were mapped onto the three-dimensional structure of dVH1 and are shown as sticks. **C,** DSP core of Vaccinia VH1 (green) is structurally superimposable to human VHZ (PDB 2IMG), considered the minimal essential core of DSPs (21) (brown).

**TABLE 1**

| VH1 construct    | Sedimentation coefficient | \(K_m\) \(\mu\text{M}\) | \(V_{\text{max}}\) | \(k_{\text{cat}}/K_m\) \(\mu\text{M}^{-1}\text{s}^{-1}\) |
|------------------|---------------------------|--------------------------|---------------------|-----------------------------------------------------|
| dVH1             | 3.24                      | 87.17 ± 12               | 349.5               | 2.05 \(\times 10^6\)                                 |
| dVH1-C110S       | 3.23                      | 263.4 ± 26               | 619.2               | 1.18 \(\times 10^6\)                                 |
| dVH1-2m           | 3.24                      | 351.1 ± 30               | 563.1               | 0.80 \(\times 10^6\)                                 |
| dVH1-5m           | 3.29                      | 780.4 ± 152              | 26.13               | 0.017 \(\times 10^6\)                                |
| mVH1             | 2.23                      | 108.2 ± 25               | 495.3               | 2.29 \(\times 10^6\)                                 |
| Chimeras         |                           |                         |                     |                                                     |
| chVH1-CC         | 3.17                      | 108.2 ± 25               | 495.3               | 2.29 \(\times 10^6\)                                 |
| chVH1-SS         | 3.18                      |                         |                     |                                                     |
| chVH1-CS         | 3.16                      | 73.63 ± 15               | 176.3               | 2.39 \(\times 10^6\)                                 |
| chVH1-SC         | 3.20                      | 48.95 ± 17               | 201.9               | 4.12 \(\times 10^6\)                                 |

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**FIGURE 3. Deleting residues 1–20 of VH1 results in a monomeric DSP core.** A, analytical ultracentrifugation sedimentation velocity analysis. The fitted distribution of the sedimentation coefficient calculated for dVH1 (2.27 S) and mVH1 (1.73 S) corresponds to an estimated molecular mass of ~39.6 and ~22.1 kDa, respectively. Given the predicted molecular mass of VH1 (~21 kDa), this indicates that in solution mVH1 exists as a monomer. B, thermal denaturation of mVH1 and dVH1 monitored by measuring changes in the ellipticity intensity at 220 nm as a function of temperature. Apparent T_m values for dVH1 and mVH1 are 55 and 52 °C, respectively. C, hydrolysis of OMFP (~400 μM) monitored at an absorbance of 477 nm as a function of time, in the presence of 1 μM of dVH1, mVH1 or dephosphorylation buffer. On the right, the blow up panel shows the Michaelis-Menten saturation kinetics of mVH1 as compared with the spontaneous rate of OMFP hydrolysis in an aqueous environment.

**VH1 Active Sites Are Independently Active in the Context of the Dimeric Phosphatase**—The discovery that as a monomer, VH1 has such a dramatic reduction in catalytic activity prompted us to investigate whether the two active sites within the dimeric phosphatase function cooperatively. Accordingly, loss of catalytic efficiency observed for mVH1 could be explained by loss of positive cooperativity. This was partially supported by a borderline Hill coefficient of n = 1.2 calculated for dephosphorylation of OMFP, which is potentially indicative of positive cooperativity. To test our hypothesis, we needed to inactivate one active site in dVH1 while leaving the second site active. To avoid unfolding the dimer and reconstituting it with mutated and wt monomers of VH1, we took a protein engineering approach. We generated a monocistronic chimera of VH1 that contains two VH1 genes fused by a five glycine residue linker (Fig. 4A). This linker bridges the C terminus of protomer
A (C₁) to the N terminus (N₂) of protomer B, which is located only 7 Å away in the crystal structure of dVH1 (17) (Fig. 4A). The monocistronic chimera of dimeric VH1 (chVH1) allows introducing point mutations in either protomer and thereby is a useful tool to study positive cooperativity in VH1.

As a proof of concept that engineering VH1 is compatible with the phosphatase folding, we expressed chVH1 in E. coli and purified it under identical condition as dVH1. We did not observe differences in expression level or solubility of chVH1 as compared with dVH1. We then introduced individual (C→S) mutations in the active sites that destroy phosphatase catalytic activity. In total, we generated four chimeras that contain either identical active sites (chVH1-CC and chVH1-SS), or just individually mutated active sites (chVH1-CS and chVH1-CS). On SDS-PAGE, all chVH1s migrated as a ~42kDa band, exactly twice the size of dVH1 (Fig. 4B). The oligomeric state of VH1 chimeras was further investigated by sedimentation velocity analysis (Table 1). In all cases, the sedimentation boundary exhibits monophasic behavior (data not shown) indicative of a single major (~99%) component in solution, migrating with a sedimentation coefficient varying between 2.18S and 2.22S (Table 1), which corresponds to a molecular mass between ~39 and 41 kDa ± 1.5 kDa. These values agree well to a molecular mass of ~40.5 kDa expected for dVH1. To demonstrate that chVH1s are correctly folded, we studied the temperature-induced equilibrium unfolding of purified VH1 chimeras. Variation in ellipticity at 220 nm, revealed a steep unfolding transition with an apparent temperature of melting (Tₘ) between ~55–68 °C (Fig. 4C). The same two-state highly cooperative unfolding transition observed for dVH1 was also seen for all chVH1s. For both dVH1 and chVH1, the catalytically inactive phosphatase was significantly more stable than their catalytically active counterparts; Tₘ measured for dVH1-C110S and chVH1-SS were 68 and 66 °C versus 55 and 56 °C of dVH1 and chVH1-CC (Fig. 4C). The enhanced reactivity and somewhat decreased structural stability of catalytically competent phosphatases is well documented in literature (7) and likely explains why most phosphatases have been crystallized as catalytically inactive. Accordingly, the mixed chimeras chVH1-SC and chVH1-CS had comparable stability (Tₘ ~62 °C), inbetween that of the fully inactive and active phosphatase. Thus, the chimeric model of VH1 reproduces faithfully the dimeric quaternary structure and stability of wt VH1, and can be used to study VH1 putative positive cooperativity.

We next tested if chVH1s are active toward the phosphatase substrate OMFP. As positive control, chVH1-CC displayed similar catalytic efficiency as wt dVH1 (kcat/Kₘ = 2.3 × 10⁶ versus 2.05 × 10⁶) (Table 1). As expected, chVH1-SS, like dVH1-C110S (the non-chimeric inactive dVH1) had no measurable activity (Table 1). Interestingly, both chVH1-CS and

FIGURE 4. Engineering monocistronic chimeras of dVH1 that carry mutations in the active sites. A, model of chVH1 (with one protomer in green and the other in cyan) with a 5-glycine linker (in red) bridging the C terminus of one protomer to the N terminus of the other. B, SDS-PAGE gel showing purified wt VH1 that runs as a ~20.5 kDa species and the different monocistronic VH1 chimeras constructed in this study, which run as two fused protomers of VH1 ~41 kDa in mass. C, stability of chimeric VH1s against thermal denaturation monitored by measuring changes in the ellipticity intensity at 220 nm as a function of temperature. Apparent Tₘ values for chVH1-CC, dVH1, chVH1-SC, chVH1-CS, chVH1-SS, and dVH1-C110S are 55, 54, 62, 63, 65, and 68 °C, respectively.
chVH1-SC were active toward OMFP, but to a different extent. Whereas chVH1-SC was twice more active than chVH1-CS \( (k_{\text{cat}}/K_m = 4.1 \times 10^6 \text{ versus } 2.4 \times 10^6) \) and equally as efficient as chVH1-CC, it displayed nearly half of the final \( V_{\text{max}} \) (Table 1). Remarkably, all three chVH1s containing at least one catalytic cysteine were over two orders of magnitude more efficient than mVH1. These data clearly rule out the idea of positive cooperativity in VH1 catalysis, and, instead, suggests that the VH1 active sites are non equivalent and function independently.

**Dephosphorylation of Activated STAT1 Requires an Intact Dimerization Interface**—All activity measurements presented so far employed the monomeric phosphosubstrate OMFP, which, although useful to recapitulate most monomeric substrates, does not reflect the quaternary structure of dimeric pSTAT1. Intuitively, the interaction between VH1 and dimeric pSTAT1 (~175 kDa) is likely much more complex than that with OMFP, as pSTAT1 also binds other parts of VH1 distinct from the active sites. To that end, we tested all VH1 constructs designed in this study (dVH1, mVH1, VH1 chimeras, dVH1–2m and dVH1–5m) for their ability to dephosphorylate purified pSTAT1 at position Tyr-701. Using an *in vitro* dephosphorylation assay, loss of a phosphate moiety at position 701 was determined by anti-pTyr Western blotting. In a time course of reaction, ~80% of full-length pSTAT1 was dephosphorylated by dVH1 over 120 min (Fig. 5, A and B). The phosphatase activity was specific, as the catalytically inactive dVH1–C110S showed no appreciable STAT1 dephosphorylation, even after prolonged incubation (Fig. 5, A and B). Indeed, the chimeras chVH1-CC, chVH1-SC and chVH1-CS all dephosphorylated pSTAT1 as much as 80% in 120 min, confirming comparable catalytic activities to that of dVH1 (Fig. 5, A and B). In agreement with its poor catalytic activity toward OMFP, mVH1 had considerably reduced activity compared with dVH1 and dephosphorylated only 20% of pSTAT1 over 120 min (Fig. 5, A and B). Surprisingly, the constructs of VH1 with point mutations in the dimerization domain had a stark reduction in phosphatase activity toward pSTAT1 comparable to that seen for mVH1 (Fig. 5, A and B). This was unexpected considering that
both mutants are significantly more active than mVH1 toward OMFP (Table 1) and retain a dimeric structure.

**DISCUSSION**

It is well documented that dimerization plays an important role in controlling and regulating catalytic activity and substrate specificity of classical PTPs (7). However, less is known about the role of dimerization in VH1-like DSPs, which are generally regarded as functional monomers. In this report, we provide compelling evidence that dimerization of the *Vaccinia* virus-encoded VH1 is necessary for its optimal catalytic activity. As suggested by a previous crystal structure (17), dimeric VH1 is held together by an N-terminal dimerization domain, that spans residues 1–27. SAXS analysis of purified VH1 confirmed the existence of a dimeric quaternary structure in solution. Likewise, mutations in VH1-DD reduce catalytic activity by decreasing the enzyme affinity for phosphosubstrate. This reduction in activity is much greater for the physiological substrate, STAT1 phosphorylated at Tyr-701 than the generic colorimetric substrate OMFP. This is likely explained by the fact that mutations in VH1-DD affect VH1 ability to recognize dimeric pSTAT1, thereby reducing the phosphatase catalytic efficiency toward this substrate. Intuitively, this loss of function was not detected when VH1 catalytic activity was probed with a small molecule compound like OMFP.

Deletion of VH1 swapped helix spanning residue 1–20, which builds much of the dimerization interface, results in a monomeric phosphatase that is ~120-fold less active than dVH1 and, at best, dephosphorylates 20% of pSTAT1 in a time course of 120 min. The dramatic difference in catalytic activity between monomeric and dimeric VH1 cannot be ascribed uniquely to a loss of structural stability of mVH1 as compared with dVH1. The difference in (apparent) $T_m$ between the two proteins is only 3 °C (~52 °C versus ~55 °C), although the two phosphatases unfold in a remarkably different way. Thus, the minimal DSP core (aa 28–171) of VH1 is only minimally active in the absence of its DD. This is in stark contrast to other VH1-like phosphatases like VHZ (also known as DUSP25), for which the minimal ~16 kDa DSP core carries all structural and functional determinants sufficient for catalytic activity (33). As a corollary, the minimal sets of secondary structural elements conserved in all known VH1-like DSPs (5 α-helices and 5 β-strands) as well as the presence of a catalytic triad (Cys/Arg/Asp) are not sufficient requirements for a catalytically active DSP core. mVH1 contains a folded and stable DSP core, but it is minimally active. Dimerization via the N-terminal swapped dimerization domain appears essential for VH1 optimal catalytic activity. Moreover, as demonstrated by our chimera model of dVH1, a “single active” active site within the same VH1 dimer is sufficient for catalytic activity toward both OMFP and pSTAT1. This rules out the idea of positive cooperativity between the two VH1 active sites and further emphasizes the importance of an intact dimerization interface. Accordingly, point mutations at the VH1 dimerization interface that alter the interface compactness without disrupting dimerization have a destructive effect on catalysis. It is important to note that while the point mutants dVH1–2m and dVH1–5m demonstrate much higher efficiencies than mVH1 when tested for OMFP dephosphorylation, the mutants (especially dVH1–5m) are just as ineffective as mVH1 at dephosphorylating pSTAT1 (Fig. 5B). Thus, even if these mutants contain wt catalytic sites, simple destabilization of the dimerization interface results in a dramatic reduction of pSTAT1 dephosphorylation. Having established that VH1-DD does not mediate cross-talk between active sites through positive cooperativity, the sensitivity of this dimerization interface to mutations likely reduces the phosphatase’s ability to recognize and bind pSTAT1. Furthermore, the observation that only one active site within VH1 dimeric structure is sufficient for catalysis indirectly suggests that this phosphatase functions as a distributive enzyme, which associates to the substrate, dephosphorylates at least one Tyr-701, followed by dissociation and a new round of catalysis. Dimerization likely affects the first step of this process, the recognition of pSTAT1, as well as it enhances VH1 intrinsic phosphatase activity, as demonstrated in this report.

**Analogies with Human Laforin—Liu et al. (34) reported that**
dimerization of the VH1-like DSP laforin is essential for its catalytic activity. Laforin is a ~331 amino acid phosphatase that contains an N-terminal carbohydrate binding module (CBM) fused to a C-terminal DSP core. In the diagram, the N-terminal dimerization domain is swapped both in VH1 and laforin, although it has not been demonstrated for the latter. Active sites are represented by orange circles.

![Schematic cartoon illustrating the basic domain organization of dimeric laforin and VH1](Image)

**FIGURE 6. Schematic cartoon illustrating the basic domain organization of dimeric laforin and VH1.** In both cases the two phosphatase protomers contain an N-terminal dimerization domain (DD and CBM in VH1 and laforin, respectively) fused to a C-terminal DSP core. In the diagram, the N-terminal dimerization domain is swapped both in VH1 and laforin, although it has not been demonstrated for the latter. Active sites are represented by orange circles.
is larger (~90 residues) and also binds carbohydrates (35). Deleting the laforin N-terminal CBM abolishes dimerization (34), suggesting that this domain, and not the DSP, is responsible for dimerization. Equally in VH1, removing the N-terminal swapped helix (aa 1–20) renders the phosphatase monomeric. Second, in both laforin and VH1, the DSP core is not sufficient per se for efficient catalysis, yet its activity is greatly stimulated by a dimeric quaternary structure, mediated by the N-terminal DD. It could be speculated that, like in VH1, laforin N-terminal CBMs are also swapped between two identical protomers (Fig. 6). Although not necessary for dimerization, domain swapping is a potentially convenient way to enhance enzyme specificity for substrate (36) and facilitate phosphosubstrate presentation to the active site. Third, and finally, both laforin and VH1 substrates present a modular and symmetric structure. Laforin dephosphorylates glycogen (10), which is formed by repetitive units of glucose, whereas VH1 dephosphorylates activated STAT1 phosphorylated at Tyr-701 (17, 20). A dimer of VH1 exposes two active sites spaced ~39 Å away from each other on the surface of the phosphatase (Fig. 2B), which is also the distance between two pTyr-701 in a dimer of STAT1 (37). We propose that the three-dimensional complementarity between a dimeric DSP and its dimeric substrate is a potential determinant for specificity.

In conclusion, dimerization of VH1-like DSPs emerges as a distinct and vital structural requirement for certain DSPs to adopt an active quaternary structure. Future studies will have to determine if dimerization plays a role in other DSPs that are commonly assumed to be monomeric. Furthermore, it will be interesting to determine if dimerization can modulate substrate specificity, in addition to be essential for optimal catalytic activity, as demonstrated in this report.

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