Programmed cell death or apoptosis is a critical mechanism for the controlled removal of damaged or infected cells, and proteins of the Bcl-2 family are important arbiters of this process. Viruses have been shown to encode functional and structural homologs of Bcl-2 to counter premature host-cell apoptosis and ensure viral proliferation or survival. Grouper iridovirus (GIV) is a large DNA virus belonging to the Iridoviridae family and harbors GIV66, a putative Bcl-2–like protein and mitochondrially localized apoptosis inhibitor. However, the molecular and structural basis of GIV66-mediated apoptosis inhibition is currently not understood. To gain insight into GIV66’s mechanism of action, we systematically evaluated its ability to bind peptides spanning the BH3 domain of pro-apoptotic Bcl-2 family members. Our results revealed that GIV66 harbors an unusually high level of specificity for pro-apoptotic Bcl-2 and displays affinity only for Bcl-2–like 11 (Bcl2L11 or Bim). Using crystal structures of both apo-GIV66 and GIV66 bound to the BH3 domain from Bim, we unexpectedly found that GIV66 forms dimers via an interface that results in occluded access to the canonical Bcl-2 ligand–binding groove, which breaks apart upon Bim binding. This observation suggests that GIV66 dimerization may affect GIV66’s ability to bind host pro-death Bcl-2 proteins and enables highly targeted virus-directed suppression of host apoptosis signaling. Our findings provide a mechanistic understanding for the potent anti-apoptotic activity of GIV66 by identifying it as the first single-specificity, pro-survival Bcl-2 protein and identifying a pivotal role of Bim in GIV-mediated inhibition of apoptosis.

Programmed cell death or apoptosis is a crucial mechanism to remove damaged, unwanted, or infected cells and to maintain tissue homeostasis (1). The B-cell lymphoma 2 (Bcl-2)2 protein family is a critical regulator of the intrinsic or mitochondrial mediated apoptotic pathway (2). The family is characterized by the presence of 1–4 conserved Bcl-2 homology (BH) domains (BH1–4), and is subdivided into pro-apoptotic or pro-survival proteins (3). Pro-survival proteins harbor all four BH domains and in mammals comprise Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and Bcl-b. The pro-apoptotic family members are further subdivided into multidomain proteins, including Bax, Bak, and Bok, and family members that only feature the BH3 domain and are consequently referred to as the BH3-only proteins. Pro-apoptotic Bak and Bak are critical for apoptosis to proceed (4) and trigger the release of pro-apoptogenic factors, including cytochrome c from mitochondria, by forming oligomeric pores to perforate the mitochondrial outer membrane. Bak is found predominantly in the cytosol and translocates to the outer mitochondrial membrane after an apoptotic stimulus, whereas Bak is constitutively anchored to the outer mitochondrial membrane via a C-terminal transmembrane anchor.

Pro-apoptotic BH3-only proteins include Bim, Bid, Puma, Noxa, Bmf, Bik, Bad, and Hrk in mammals and induce apoptosis either indirectly by neutralizing pro-survival Bcl-2 or directly by interacting with Bax and Bak (5). This activity is mediated by the helical BH3 domain, which is able to bind to a canonical hydrophobic ligand-binding groove on both pro-survival and pro-apoptotic multidomain Bcl-2 proteins (6). In healthy cells, BH3-only proteins act as sentinels of cellular well-being and are up-regulated in response to cellular insults, including growth factor deprivation, exposure to cytotoxic drugs, or viral infections, leading to the activation of cell death mechanisms (2).

Whereas the intrinsic pathway of apoptosis is highly conserved from the worm to mammals (7), certain differences exist in the apoptotic machinery between mammals and fish. Zebrafish lack the pro-survival Bcl-2 proteins Bcl-w and A1 as well as pro-apoptotic Bak and Hrk; however, the interplay between the different Bcl-2 members and the mechanisms underlying control of apoptosis appear to be conserved (8).

The ability of multicellular organisms to restrict viral infections by triggering host-cell apoptosis led to the acquisition by viruses of numerous molecular strategies to circumvent premature host-cell apoptosis (9). These include the expression of sequence and/or structural mimics of cellular pro-survival Bcl-2 to hijack host-cell intrinsic apoptosis signaling (3, 10). Adenovirus encodes E1B19K (11), whereas among the Herpesviridae, Epstein–Barr virus (EBV) harbors two viral Bcl-2–like
proteins, BHRF1 (12) and BALF1 (13), and Kaposi's sarcoma-associated herpesvirus features KS-Bcl-2 (14). Among the Asfarviridae, African swine fever virus encodes for A179L (15). Whereas all of the aforementioned virus-encoded proteins are readily identified as Bcl-2 proteins by their primary sequence, among the poxviruses, a number of apoptosis-inhibitory proteins were only identified as Bcl-2 members after their structure determination. These include vaccinia virus– and variola virus–encoded F1L (16, 17) as well as myxoma virus–encoded M11L (18). In addition to these distant orthologs, the Poxviridae feature a number of other Bcl-2–like proteins, including fowl poxvirus FPV039 (19), canary poxvirus CNP058 (20), deer poxvirus DPV022 (21, 22), sheep poxvirus SPPV14 (23), and ORF virus ORF125 (24).

The Iridoviridae represent another family of large DNA viruses and are subdivided into four genera, iridovirus, choriiri-dovirus, ranavirus, and lymphocystivirus (25). Iridoviruses and chloririodiviruses commonly infect invertebrates, whereas ranaviruses and lymphocystivirus target vertebrates, including amphibians, fish, and reptiles. The sequencing of the grouper iridovirus genome revealed the presence of a putative Bcl-2–like protein (26), GIV66, and functional studies established that GIV66 locates to the outer mitochondrial membrane and is able to inhibit UV-induced apoptosis in grouper kidney cells (27). However, the molecular and structural basis of apoptosis inhibition by GIV66 remains unclear.

Results

It was shown previously that GIV66 is localized at the outer mitochondrial membrane and is able to inhibit Bcl-2–mediated apoptosis in UV-irradiated GK cells (27); however, no detailed mechanism was proposed to rationalize these findings. To understand the molecular mechanism of action and structural basis of GIV66-mediated inhibition of apoptosis, we recombantly expressed and purified GIV66 to perform isothermal titration calorimetry (ITC) with peptides spanning the BH3 motif of pro-apoptotic Bcl-2 proteins. Peptides were selected from pro-apoptotic Bcl-2 proteins identified in grouper fish (Epinephelus coioides) as well as from zebrafish (Danio rerio) where grouper homologs were unavailable (Fig. 1E). Unexpectedly, GIV66 only displayed measurable affinity for zebrafish encoded Bcl-2–like 11 (Bcl2L11 or DR_Bim, K_D = 887 nm), whereas all other BH3 motif peptides tested showed no detectable affinity (Fig. 1). Furthermore, GIV66 did not show any detectable affinity for peptides from human pro-apoptotic Bcl-2 family members (data not shown). These findings suggest that GIV66 is the first single-specificity pro-survival Bcl-2 protein.

Considering the unusual ligand-binding profile of GIV66, which is reminiscent of the restricted pro-apoptotic Bcl-2 ligand–binding profiles observed for homodimeric viral Bcl-2 (vBcl-2) homologs (21), we examined the oligomeric state of GIV66 in solution using size-exclusion chromatography (SEC) (Fig. 2). This revealed that GIV66 exists as a dimer in solution. SEC analysis at GIV66 concentrations of 1.5 and 30 mg/ml did not reveal a difference in elution volume, indicating that, within this concentration range, dimerization is not concentration-dependent (data not shown). Similarly, ITC analysis where GIV was titrated into buffer revealed no significant heat changes, suggesting that even at low concentrations, the GIV66 dimer does not dissociate (Fig. 1C).

We next examined the structural basis of GIV66–mediated apoptosis by determining the crystal structure of GIV66 (Table 1). GIV66 adopts the conserved Bcl-2 fold comprising eight α-helices that form a globular α-helical bundle (Figs. 3A and 4A). Helices 2–5 form the canonical hydrophobic ligand-binding groove found in all pro-survival Bcl-2 proteins that is used to engage BH3-motif sequences of pro-apoptotic Bcl-2 family members. A DALI analysis (28) revealed that Mcl-1 bound to Noxa BH3 (PDB code 4G35) (29) is most similar to GIV66, with an r.m.s.d. value of 2.0 Å over 129 Ca atoms and a sequence identity of 19%. The closest vBcl-2 structural homolog to GIV66 is murine γ herpesvirus M11 bound to Beclin-1 (PDB code 3BL2) (30), with an r.m.s.d. value of 2.3 Å over 131 Ca atoms and sequence identity of 12%. In the asymmetric unit, only a single chain of GIV66 was found, despite the observation that GIV66 is dimeric in solution (Fig. 2). A PISA analysis (31) did not identify a potential interface and suggested that GIV66 is monomeric. However, examination of the crystal packing revealed a conspicuous interface, where helix 3 from a neighboring symmetry-related molecule packs into the canonical hydrophobic ligand-binding groove formed by helices α3 and α4, burying 720 Å² of solvent-accessible surface in the process (Fig. 3B). An alternative configuration where the back faces of two GIV66 chains meet appears to be a less likely dimeric configuration. Interestingly, the groove-to-groove dimeric arrangement orients the C termini of both chains in the same direction and thus allows insertion of the putative transmembrane regions into the outer mitochondrial membrane. Considering the observation that GIV66 is able to bind DR_Bim BH3, the dimeric configuration would have implications for the ability of GIV66 to engage DR_Bim BH3. Utilization of the canonical ligand-binding groove would require dissociation of the dimer to unblock the groove for DR_Bim binding. Alternatively, GIV66 could remain dimeric and utilize an alternative non-canonical ligand-binding site, such as the one previously observed for Bax (32). To address this, we performed analytical SEC with the GIV66–DR_Bim BH3 complex. The retention volume of GIV66–DR_Bim BH3 compared with GIV66 on its own indicates that the complex is smaller in solution compared with the GIV66 dimer and corresponds to a 1:1 complex of GIV66 and DR_Bim BH3 (Fig. 5).

To understand the structural basis of DR_Bim binding to GIV66, we determined the crystal structure of the GIV66–Bim complex (Figs. 3C and 4B). A single 1:1 GIV66–DR_Bim complex was present in the asymmetric unit. In the complex structure, the DR_Bim BH3 motif is bound to the conserved hydrophobic ligand-binding groove in an overall configuration similar to that previously observed for other mammalian and viral pro-survival proteins, such as Mcl-1 (Fig. 3D) and BHRF1 (Fig. 3E). The Bim BH3 motif from zebrafish is bound to the canonical GIV66 hydrophobic groove formed by a helices 2–5, burying 851 Å² of surface-accessible area in the process. DR_Bim engages four hydrophobic pockets in the GIV66 ligand-binding groove using four conserved hydrophobic residues (Val-124, Leu-128, Ile-131, and Phe-135) (Fig. 6). Furthermore, a con-
Structural and biochemical characterization of GIV66

GIV66:DR_Bim BH3  GIV66:DR_Noxa BH3  GIV66:Buffer

| Peptide   | $K_D$ (nM) |
|-----------|------------|
| DR_Bim    | 887±64     |
| HS_Bim    | DMRPEIWIAQELRRIGDEENLYCEA |
| DR_Bid    | NB         |
| HS_Bad    | ALWAAKKYQQQLRRMSDEFDKQMKR |
| DR_Noxa   | NB         |
| HS_Noxa   | NWLAAQRYGRELRRMSDEFDVDSFKKG |
| DR_Bmf    | NB         |
| HS_Bik    | ERAAREMAAEELRIADLEEQVLSQ |
| DR_Puma   | NB         |
| HS_Bik    | QEDIIRNIARHQLAQVGVDSMRIPPG |
| DR_Bik    | NB         |
| HS_Bik    | NMRVVTITITIGQALAOIGDEMDOZKWEPE |
| DR_Bmf    | NB         |
| HS_Bmf    | AQSVETLIGKLOLIGDOFYEHEHIMH |
| DR_Bad    | NB         |
| HS_Bad    | EQTAVVECAGQLRNIGDLNWKRL |
| DR_Noxa   | NB         |
| HS_Noxa   | PAELEVECATQLFRFDKMGKFRQKLL |
| EC_Bax    | NB         |
| EC_Bax    | EEQAVEVARVAVLRTIGDEMNAVFLQR |
| DR_Puma   | NB         |
| HS_Bax    | EEQWAREIGAQERRMADINQAQYERR |
| DR_Bok    | NB         |
| EC_Bok    | CDPNTRRCLARCLIQIGDELDGNEVLQ |
| EC_Bok    | QDASTKL5SECRLRIGDELDVSMELQ |
| DR_Bec    | DGGTMENLSRRKLVTNSLFDIMSGQT |
| HS_Bec    | DGGTMENLSRRKLVTGDLDIMSGQT |
served ionic interaction between Arg-65GIV66 from the GIV66 BH1 motif and Asp-133Bim is present. Additional hydrogen bonds are formed by Asn-54GIV66 and Arg-129Bim as well as Asn-47GIV66 and Glu-121Bim. A comparison of the ligand-bound and ligand-free structures of GIV66 reveals that no significant rearrangements of helices are required to accommodate DR_Bim. GIV66 superimposes with GIV66–DR_Bim with an r.m.s.d. value of 1.3 Å over 115 Cα atoms. The bulk of the structural changes are located in the a3 helix, which shifts by 1.6 Å to allow the opening of the hydrophobic ligand-binding groove and the connecting loop between a2 and a3, which becomes shortened and results in a longer a2 helix.

Figure 2. GIV66 is a dimer in solution. Dimeric DPV022 (predicted molecular mass for dimer = 36,182 Da), monomeric BHRF1 (18,327 Da), and GIV66 (14,931 Da) were subjected to size-exclusion chromatography on a Superdex 75 3.2/300 column equilibrated with 20 mM Hepes, pH 7.5, and 150 mM NaCl. GIV66 elutes at a retention volume commensurate with a dimeric state in solution. Molecular weight standards are denoted by arrows. mAU, milliabsorbance units.

Table 1
Crystallographic data collection and refinement statistics

|                      | Native GIV66 | Hg GIV66 | Native GIV66–DR_Bim BH3 |
|----------------------|--------------|----------|-------------------------|
| **Data collection**  |              |          |                         |
| Space group          | P4₁2₁2       | P4₁2₁2   | P6₅                     |
| a, b, c (Å)          | 68.03, 68.03, 85.82 | 68.87, 68.87, 85.00 | 71.8, 71.8, 56.55       |
| α, β, γ (degrees)    | 90, 90, 90   | 90, 90, 90 | 90, 90, 120             |
| Resolution (Å)       | 42.91–1.65 (1.68–1.65) | 42.50–2.34 (2.42–2.34) | 41.83–1.70 (1.73–1.70) |
| Rmerge or Rcryst      | 0.057 (2.392) | 0.153 (2.212) | 0.101 (1.575)          |
| Completeness (%)     | 99.9 (100.0) | 100.0 (100.0) | 100.0 (99.2)           |
| Redundancy           | 10.0 (10.1)  | 27.8 (28.1)  | 19.9 (9.3)             |
| Anomalous redundancy | 15.4 (15.1)  | 0.999 (0.395) | 0.999 (0.480)          |
| **Refinement**       |              |          |                         |
| Resolution (Å)       | 41.77–1.70 (1.76–1.70) | 31.09–1.70 (1.76–1.70) | 0.183/0.214         |
| No. of reflections   | 22,470 (2195) | 18,316 (901) | 0.160/0.200            |
| Rwork/Rfree          | 0.138/0.214  | 0.101 (1.575) | 0.101 (1.575)          |
| No. of atoms         | Protein      | 992      | 1127                    |
|                      | Ligand/ion   | 28       | 44                      |
|                      | Water        | 106      | 119                     |
|                      | Protein      | 34.93    | 25.95                   |
|                      | Ligand/ion   | 47.72    | 44.64                   |
| B-factors            | Water        | 45.64    | 39.66                   |
|                      | r.m.s.d.     | 1.01     | 0.018                   |
| Bond lengths (Å)     | 1.02         |          |                         |
| Bond angles (degrees)|              |          |                         |

* Values in parentheses are for the highest-resolution shell.

Figure 1. GIV66 interactions with peptides from pro-apoptotic Bcl-2 family members. The affinity of recombinant GIV66 for BH3-motif peptides (26-mers, except for a Bax 28-mer and a Bid 34-mer) obtained from E. coioïdes (EC) or D. rerio (DR) was assessed using ITC. Shown are raw heats of titration for GIV66–DR_Bim (A), GIV66–DR_Noxa BH3 (B), and GIV66 (C) titration into buffer. D. summary of interaction measurements. NB, no binding. E. sequence alignment of BH3-motif sequences from E. coioïdes or D. rerio that were used in this study with their human counterparts. Shaded residues are the BH3 motif defining four conserved hydrophobic residues as well as the strictly conserved aspartic acid and the conserved small amino acid preceding the aspartic acid.
We then performed SEC in-line with small-angle X-ray scattering (SAXS) to examine the oligomeric state of GIV66 in the presence and absence of DR_Bim, as well as the topology of dimers of GIV66 (Table 2). GIV66 on its own as well as in complex with DR_Bim were measured. GIV66 was injected at a concentration of 5 mg/ml (Fig. 7(A and B) and Fig. S1), and SAXS data were collected while GIV66 was fractionated on the column. The chromatogram revealed that in solution, GIV66 displayed a small amount of aggregation, as shown by a small peak at the void volume. However, the scattering profile extracted from the well-resolved main peak (Fig. 7A) conforms to a straight line in the low q region on a Guinier plot (Fig. 7C). The molecular mass calculated from the forward scattering intensity (I(0)) (33) for this peak corresponds to a species of 27 kDa, indicating that in solution, GIV66 is a dimer (Fig. 7D). We then injected GIV66–DR_Bim at a concentration of 5 mg/ml (Fig. 7, A–B). The size-exclusion chromatogram revealed one major peak preceded by a smaller shoulder of higher molecular mass (Fig. 7A). The calculated molecular mass for the main peak corresponds to a species of ~19 kDa, indicating that although in solution the GIV66–DR_Bim complex exists as a mixture of GIV66–DR_Bim heterodimers and GIV66 dimers that is not fully resolved by size-exclusion chromatography, the main peak corresponds to a GIV66–DR_Bim heterodimer (Fig. 7, A–D). Because crystal structures of both GIV66 on its own as well as of GIV66–DR_Bim are available, we compared the experimentally obtained scattering data with the available models using CRYSOL. From those analyses, it is clear that GIV66 scatters commensurate with a dimeric configuration (Fig. 7E), with a $\chi^2$ of 5.76 and 1.26 for a back-to-back and groove-to-groove dimer arrangement, respectively, and not as a monomer ($\chi^2$ of 27.60). In contrast, the main-peak scattering data collected for GIV66–DR_Bim fits a GIV66–DR_Bim heterodimer model comprising a single chain of GIV66 and a single chain of Bim (Fig. 7F) with a $\chi^2$ of 1.60, compared with a $\chi^2$ of 17.47 and 12.71 for back-to-back and groove-to-groove dimers, respectively. We next attempted to identify which of the GIV66 crystallography dimers was present in solution using a rigid-body modeling approach. Modeling was carried out with CORAL using the monomeric GIV66 structure and generating a groove-to-groove as well as a back-to-back dimer observed previously in the GIV66 crystal packing. A model for a GIV66 back-to-back dimer fits the experimental scattering data poorly (Fig. 8 and Fig. S1), with a $\chi^2$ of 4.70. In contrast, a groove-to-groove dimer model resulted in an excellent fit of the scattering curves (Fig. 8 and Fig. S1) with

![Figure 3. Crystal structures of GIV66 and GIV66–DR_Bim BH3 complex and comparison with other BH3 complexes of Bcl-2 proteins.](image-url)
Having established a putative dimerization mechanism, we next attempted to disrupt the dimer via structure-guided mutagenesis to verify that GIV66 dimers form via the proposed interface. Inspection of the GIV dimer interface suggested that four residues, Thr-38, Ala-41, Phe-42, and Asn-54, are involved in the dimerization (Fig. 9A and B). Whereas single mutants of these residues displayed elution volumes on SEC identical to wildtype GIV66 (data not shown), a T38Y/A41Y/F42E triple mutant eluted at a volume commensurate with a monomeric state (Fig. 9C). Furthermore, the GIV66 T38Y/A41Y/F42E triple mutant retained the ability to bind Bim, suggesting that despite the mutations, the Bcl-2 fold was maintained (Fig. 9D).

Discussion

Numerous large DNA viruses have been shown to subvert host-cell apoptotic defenses by utilizing virus-encoded homologs of pro-survival Bcl-2 proteins (3). We now show that grouper iridovirus encodes for a pro-survival Bcl-2 protein, GIV66, that is unusually specific and only shows detectable affinity to DR_Bim. These findings suggest that GIV66 is the first single-specificity pro-survival Bcl-2 protein identified to date (34). In contrast to GIV66, mammalian Bcl-2 proteins display considerable promiscuity and bind the vast majority of BH3-only proteins as well as Bak and Bak. Bcl-b is the sole mammalian pro-survival Bcl-2 protein with a restricted ligand-binding profile and only engages Bim and Bak (35). Other highly specific pro-survival Bcl-2 proteins include the worm CED-9, which only binds the BH3-only proteins EGL-1 and CED-13, and the executor CED-4 (36), whereas the sponge BHP2 has only been shown to engage the BH3 motif of a related sponge Bak-like molecule (37). However, in both cases, the underlying apoptosis regulatory machineries are substantially less complex and involve only a limited number of pro-apoptotic effector proteins.

Among viruses, considerable diversity exists among vBcl-2 proteins regarding mechanism of action and ability to engage pro-apoptotic Bcl-2 proteins. Myxoma virus M11L was shown to inhibit apoptosis by Bak and Bak sequestration (18), whereas vaccinia virus F1L only requires Bim sequestration (17). EBV BHRF1 employs a hybrid mode where both Bim (38) and Bak (39) are targeted. The diversity of mechanisms of action is also reflected in the differing abilities of vBcl-2 homologs to directly bind pro-apoptotic host Bcl-2 proteins. African swine fever virus A179L is ultra-promiscuous and able to bind to all major pro-apoptotic Bcl-2 (15). Similarly, fowlpox virus FPV039 binds all pro-apoptotic Bcl-2 proteins except Bok, whereas sheeppox virus SPPV14 engages all BH3-only proteins except Bad, Bik, and Noxa (23). GIV66 is notable for its unique single-ligand specificity, which is in marked contrast to all other vBcl-2 homologs examined to date. The only vBcl-2 homologs with highly restricted ligand-binding profiles are vaccinia virus F1L (17, 40) and deerpox virus DPV022 (21), which bind Bim, Bak, and Bak, and variola virus F1L, which binds Bid, Bak, and Bak (16). All three proteins adopt a domain-swapped homodimeric configuration that can engage two BH3 domain ligands simultaneously, whereas GIV66 utilizes the canonical ligand-binding groove for dimerization and consequently requires breaking of

\[ a \text{ value of } \chi^2 \text{ of 1.16, suggesting that in solution, the canonical ligand-binding groove of GIV66 is buried and thus inaccessible.} \]

Figure 4. 2Fo – Fo electron density maps of GIV66 and GIV66–DR Bim BH3 complex. A, electron density map encompassing the hydrophobic binding groove of GIV66 (shown as yellow sticks). B, electron density map encompassing the hydrophobic binding groove of CNP058 in complex with Bim BH3. GIV66 is shown as yellow sticks, whereas DR_Bim BH3 is shown as cyan sticks. The electron density maps are shown as a blue mesh contoured at 1σ.
the dimer to form a 1:1 heterodimeric complex with a BH3 domain ligand.

Mechanistically, it is highly likely that GIV66 inhibits host-cell apoptosis by targeting the pro-apoptotic BH3-only protein Bim. This unusually high level of specificity suggests that Bim plays a key role during the initial host response to viral infection and is in accord with the observation that Bim is the only universal BH3-only pro-survival Bcl-2 antagonist (35). Whereas little is known about the regulation of intrinsic apoptosis in grouper fish, zebrafish have been used extensively to study apoptosis regulation (41). In zebrafish, intrinsic apoptosis appears to be regulated in an analogous manner to mammals, despite the absence of a small number of Bcl-2 family members (8). Overexpression of Bim is a potent trigger for apoptosis (42), and this ability is lost after targeted mutations in the Bim BH3 domain.

Interestingly, given the high level of specificity of GIV66 for DR_Bim, a comparison between the DR_Bim-bound and free form of GIV revealed minimal movement within the hydrophobic ligand-binding groove, with H9251 moving by 1.6 Å to accommodate DR_Bim in the binding site. This is reminiscent of Bak
BH3 binding to the M11L binding groove, which resulted in a modest shift of ~2 Å in the α4 helix. In both GIV66 and M11L, the binding groove appears to be more optimized for binding of a smaller set of pro-apoptotic ligands and thus requires fewer and subtler structural changes to accommodate a ligand, whereas the more promiscuous mammalian and worm pro-
survival Bcl-2 proteins undergo pronounced changes, including in the α3 and/or α4 helices (43–45).

Although the majority of proteins with a Bcl-2 fold appear to be monomeric, homodimerization has been observed in several instances. Mammalian pro-survival Bcl-2 proteins have been shown to form domain-swapped dimers after exposure to heat or high pH (46) involving helices α5 and α6, as well as truncation of the loop connecting helices α1 and α2 (47), leading to a domain swap via α1. Similarly, mammalian pro-apoptotic Bcl-2, including Bax and Bak, appears to require domain-swap homodimerization via α5 and α6 as a prelude to forming large oligomeric complexes that perforate the outer mitochondrial membrane (48, 49). vBcl-2 proteins that form dimers via a domain swap of the α1 helix include pro-survival F1L from...
Structural and biochemical characterization of GIV66

A

GIV66 dimer interface

B

C

\[ K_D = 1383 \pm 278 \text{ nM} \]
vaccinia and variola virus as well as poxvirus DPV022. Interestingly, whereas vaccinia virus—encoded NF-κB modulators with a Bcl-2 fold, such as N1 (50), B14, and A52, also form dimers (51), they do not adopt a domain-swapping topology and instead utilize an interface formed by helices α1 and α6. The dimerization mode observed for GIV66 has not been observed previously and reveals an unusual mechanism for Bcl-2–mediated homodimerization. Interestingly, our data suggest that the observed dimerization mode of GIV66 impacts BH3 domain binding via the canonical ligand-binding groove, a feature not observed in any of the other Bcl-2 family dimers to date. Indeed, engagement of DR_Bim by GIV66 leads to loss of the dimer, with a resultant 1:1 GIV66–DR_Bim heterodimeric complex, whereas other dimers, such as F1L (16, 17) and DPV022 (21), as well as Bax (49) and Bak (48) remain dimeric upon ligand binding and are able to form 2:2 heterotetrameric complexes with BH3 ligands. Such a ligand-induced dissociation of a Bcl-2 family protein dimer has not been observed previously and raises the question of whether the oligomeric state of GIV66 may impact the efficacy of GIV-mediated inhibition of apoptosis during infection. Importantly, we were able to confirm the observed dimerization mode of GIV66 by structure-guided mutagenesis, with a GIV66 T38Y/A41Y/F42E triple mutant appearing as a monomer rather than a dimer. However, although GIV66 T38Y/A41Y/F42E bound DR_Bim, the affinity measured was lower than what we observed for wild-type GIV66. Furthermore, no affinity for all other BH3-motif peptides was observed.

A detailed structural analysis of the GIV66–DR_Bim complex reveals that in addition to the canonical salt bridge and use of four hydrophobic residues, DR_Bim also forms additional interactions using Asn-54GIV66 and Arg-129Bim as well as Asn-47GIV66 and Glu-121Bim. A comparison of the complexes of Mcl-1 with Bim reveals that Mcl-1 uses the canonical salt bridge and four hydrophobic interactions as well as two additional salt bridges (Asp-256Mcl-1–Arg-63Bim and Arg-248Mcl-1–Glu-55Bim) and seven hydrogen bonds (Val-253Mcl-1–Arg-63Bim, Ser-255Mcl-1–Arg-63Bim, His-252Mcl-1–Arg-63Bim, Ser-245Mcl-1–Glu-55Bim, Arg-265Mcl-1–Gly-66Bim, Asn-260Mcl-1–Asp-67Bim, Trp-261Mcl-1–Asn-70Bim, and Gly-262Mcl-1–Asn-70Bim). In the complex of BHFR1 with Bim BH3, a second ionic interaction between Glu-89BHFR1 and Arg-63Bim is found, as well as hydrogen bonds between Ser-9BHFR1 and Asn-70Bim and between Gly-99BHFR1 and Asn-70Bim. The abundance of additional interactions in the Mcl-1 and BHFR1 complexes with Bim and the presence of ionic interactions rather than hydrogen bonds are reflected in the substantially higher affinities of both pro-survival Bcl-2 proteins for Bim compared with GIV66.

Interestingly, the highest ranked vBcl-2 protein in a DALI search with GIV66 was the complex of M11 bound to Beclin-1 (30). However, despite the overall structural similarity with M11, GIV66 does not bind Beclin-1 and appears to not affect autophagy signaling. Consequently, it appears that Beclin-1 binding by vBcl-2 homologs is restricted to the Herpesviridae and Asfarviridae.

In summary, our study reveals that GIV66 is a dimeric Bcl-2–like protein that suppresses host-cell apoptosis by sequestering DR_Bim. Binding of DR_Bim to the canonical GIV66-binding groove triggers the disassociation of a groove-to-groove GIV66 dimer, raising the question of whether the GIV66 oligomeric state impacts the efficacy of GIV66-mediated inhibition of apoptosis. Our data provide a mechanistic basis for GIV66-mediated inhibition of apoptosis and will form the platform for understanding how grouper iridovirus subverts host-cell death defenses in an in vivo setting.

**Experimental procedures**

**Protein expression and purification**

Codon-optimized cDNA of wildtype GIV66 (GenBank accession number AAV91093.1) lacking the C-terminal 25 residues (Genscript) as well as wildtype GIV66 T38Y/A41Y/F42E were cloned into the pGEX-6P-3 vector (Invitrogen) and expressed in the *Escherichia coli* BL21 Star cell line using the autoinduction method (52) in 2YT medium at 25 °C. Overnight cultures were harvested by centrifugation at 6000 rpm (JLA 9.1000 rotor, Beckman Coulter Avanti J-E) for 20 min and resuspended in lysis buffer (50 mM Tris, pH 8.5, 150 mM NaCl, and 1 mM EDTA) supplemented with lysozyme (Sigma–Aldrich) and DNase I (deoxyribonuclease I from bovine pancreas, Sigma–Aldrich). A cell disrupter (Constant Systems Ltd.) was used to lyse the cells at a pressure of 35 kilopascals at 4 °C for a single cycle. Cellular debris was removed by centrifuging the lysate at 16,000 rpm (JLA 25.50 rotor, Beckman Coulter Avanti J-E) for 20 min, and the supernatant was further filtered using a 0.22-μm syringe filter (Millipore). Affinity chromatography was performed using 5 ml of glutathione-Sepharose 4B resin in a gravity flow column (GE Healthcare) using lysis buffer. On-column cleavage was performed overnight in lysis buffer at 4 °C using HRV 3C protease to remove the GST fusion tag. Target protein was collected in the flow-through, concentrated to a volume of 1 ml using a centrifugal concentrator with a 3000 molecular weight cutoff (Amicon Pure system, GE Healthcare) equilibrated with 25 mM HEPES at pH 7.5 and 150 mM NaCl, where it eluted as a single peak. The peak fractions were pooled, concentrated using a centrifugal concentrator with 3000 molecular weight cutoff (Amicon Ultra 15) to a final concentration of 31.5 mg/ml, flash-cooled under liquid nitrogen, and stored at −80 °C.

**Figure 9. Structure-guided mutagenesis to disrupt the GIV66 dimer interface.** A, the GIV66 surface is shown in gray, and the floor of the canonical ligand-binding groove is shown in magenta, with the location of three key mutated residues, Thr-38, Ala-41, and Phe-42, shown in green, blue, and cyan, respectively, with the second GIV monomer shown as an orange ribbon. B, wildtype GIV66 (red), GIV66 bound to DR_Bim (black), and GIV66 T38Y/A41Y/F42E (green) were subjected to size-exclusion chromatography on a Superdex 75 3.2/300 column equilibrated with 20 mM Hepes, pH 7.5, and 150 mM NaCl. GIV66 elutes at a retention volume commensurate with a dimeric state in solution, whereas both GIV66–DR_Bim and GIV66 T38Y/A41Y/F42E elute at a higher retention volume indicative of a monomeric state in solution. C, raw heats of titration for GIV66 T38Y/A41Y/F42E binding to DR_Bim as obtained from isothermal titration calorimetry.
Analytical size-exclusion chromatography

Analytical size-exclusion chromatography was performed using a Superdex 75 5/150 column equilibrated with 50 mM sodium phosphate, pH 7.6, 50 mM NaCl at a flow rate of 0.2 ml/min. During elution, SAXS analysis was conducted in-line via a coupled coflow sample sheath flow environment run at a fractional sample flow rate of 0.5 (61). SAXS data were acquired on the SAXS/WAXS beamline at the Australian Synchrotron (62), which has been optimized for low instrument background. Data were acquired using a camera length of 1.430 m, providing q-ranges of 0.012–0.602 Å⁻¹ at 12 keV at a flux of 2.5 × 10^{12} photons/s, using continuous exposures with a 1-s integration time using a Pilatus 1M detector. Images were inspected, averaged, and subtracted using PRIMUS from the ATSAS suite of SAXS data analysis tools (63). Data analysis was carried out as described by the 2017 publication guidelines, using the R_{sv} and the I(0) normalized over concentration scattering data selection over a given peak (64). Initial data analysis based on
Structural and biochemical characterization of GIV66

Reduced data in the form of data files using Guinier plots to calculate the radius of gyration was conducted using AUTORG from the ATSAS suite of SAXS data analysis tools (63). Uncertainties from Guinier fits are 2 S.E. values of the slope of fitted linear regressions of ln(I) versus q^2. GIV66 and GIV66−DR_Bim scattering curves of the main peaks were compared with the available PDB models using CRYSOL (65). Rigid-body modeling of GIV66 scattering data with two possible dimeric configurations observed in the crystal structure was carried out using CORAL (63). All data collection and analysis statistics are summarized in Table 2.

Author contributions—S.B. data curation; S.B., S.C., and M.K. formal analysis; S.B., J.M., T.M.R., and S.C. investigation; S.B., T.M.R., S.C., and M.K. methodology; S.B., T.M.R., S.C., and M.K. writing-original draft; S.B., T.M.R., S.C., and M.K. writing-review and editing; T.M.R., S.C., and M.K. conceptualization; S.C. and M.K. supervision; M.K. funding acquisition; M.K. project administration.

Acknowledgments—We thank Bevan Marshall and Linda Ward for helpful discussion and experimental support. We thank staff at the MX beamlines at the Australian Synchrotron for help with X-ray data collection, the CSIRO C3 Collaborative Crystalization Centre for assistance with crystallization, and the Comprehensive Proteomics Platform at La Trobe University for core instrument support.

References
1. Fuchs, Y., and Steller, H. (2015) Live to die another way: modes of programmed cell death and the signals emanating from dying cells. Nat. Rev. Mol. Cell Biol. 16, 329–344 CrossRef Medline
2. Luna-Vargas, M. P., and Chipuk, J. E. (2016) The deadly landscape of pro-apoptotic BCL-2 proteins in the outer mitochondrial membrane. FEBS J. 283, 2676–2689 CrossRef Medline
3. Kvansakul, M., and Hinds, M. G. (2013) Structural biology of the Bcl-2 family and its mimicry by viral proteins. Cell Death Dis. 4, e909 CrossRef Medline
4. Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Fauwirth, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell 6, 1389–1399 CrossRef Medline
5. Shamas-Din, A., Kale, J., Leber, B., and Andrews, D. W. (2013) Mechanisms of action of Bcl-2 family proteins. Cold Spring Harb. Perspect. Biol. 5, a008174 Medline
6. Kvansakul, M., and Hinds, M. G. (2014) The structural biology of BH3-only proteins. Methods Enzymol. 544, 49–74 CrossRef Medline
7. Vaux, D. L., Haeccker, G., and Strasser, A. (1994) An evolutionary perspective on apoptosis. Cell 76, 777–779 CrossRef Medline
8. Eimon, P. M., and Ashkenazi, A. (2010) The zebrafish as a model organism for the study of apoptosis. Apoptosis 15, 331–349 CrossRef Medline
9. Jorgensen, K. Rayamjahi, M., and Miao, E. A. (2017) Programmed cell death as a defence against infection. Nat. Rev. Immunol. 17, 151–164 CrossRef Medline
10. Kvansakul, M., Caria, S., and Hinds, M. G. (2017) The Bcl-2 family in host-virus interactions. Viruses 9, E290 Medline
11. Chiou, S. K., Tseng, C. C., Rao, L., and White, E. (1994) Functional complementation of the adenovirus E1B 19-kilodalton protein with Bcl-2 in the inhibition of apoptosis in infected cells. J. Virol. 68, 6553–6566 CrossRef Medline
12. Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993) Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci. U.S.A. 90, 8479–8483 CrossRef Medline
13. Bellows, D. S., Howell, M., Pearson, C., Hazlewood, S. A., and Hardwick, J. M. (2002) Epstein-Barr virus BALF1 is a BCL-2-like antagonist of the herpesvirus antiapoptotic BCL-2 proteins. J. Virol. 76, 2469–2479 CrossRef Medline
14. Cheng, E. H., Nicholas, J., Bellows, D. S., Hayward, G. S., Guo, H. G., Reitz, M. S., and Hardwick, J. M. (1997) A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. Proc. Natl. Acad. Sci. U.S.A. 94, 690–694 CrossRef Medline
15. Banjara, S., Caria, S., Dixon, L. K., Hinds, M. G., and Kvansakul, M. (2017) Structural insight into African swine fever virus A179L-mediated inhibition of apoptosis. J. Virol. 91, e02228–e02216 Medline
16. Marshall, B., Puthalakath, H., Caria, S., Chugh, S., Doerflinger, M., Colman, P. M., and Kvansakul, M. (2015) Variola virus FI1 is a Bcl-2-like protein that unlike its vaccinia virus counterpart inhibits apoptosis independent of Bim. Cell Death Dis. 6, e1680 CrossRef Medline
17. Campbell, S., Thibault, J., Mehta, N., Colman, P. M., Barry, M., and Kvansakul, M. (2014) Structural insight into BH3 domain binding of vaccinia virus antiapoptotic FI1. J. Virol. 88, 8667–8677 CrossRef Medline
18. Kvansakul, M., van Delft, M. F., Lee, E. F., Gilbis, J. M., Fairlie, W. D., Huang, D. C., and Colman, P. M. (2007) A structural viral mimic of prosurvival Bcl-2: a pivotal role for queuestering proapoptotic Bax and Bak. Mol. Cell 25, 933–942 CrossRef Medline
19. Anasir, M. I., Baxter, A. A., Poon, I. K. H., Hulett, M. D., and Kvansakul, M. (2017) Structural basis of apoptosis inhibition by the poxvirus protein FPV039. J. Biol. Chem. 292, 9010–9021 CrossRef Medline
20. Burton, D. R., Caria, S., Marshall, B., Barry, M., and Kvansakul, M. (2015) Structural basis of Deerpoor virus-mediated inhibition of apoptosis. Acta Crystallogr. D Biol. Crystallogr. 71, 1593–1603 CrossRef Medline
21. Anasir, M. I., Baxter, A. A., Poon, I. K. H., Hulett, M. D., and Kvansakul, M. (2017) Structural basis of apoptosis inhibition by the poxvirus protein FPV039 mediated regulation of apoptosis. Viruses 9, E305 Medline
22. Banadyga, L., Lam, S. C., Okamoto, T., Kvansakul, M., Huang, D. C., and Barry, M. (2011) Deerpoor virus encodes an inhibitor of apoptosis that regulates Bak and Bax. J. Virol. 85, 1922–1934 CrossRef Medline
23. Okamoto, T., Campbell, S., Mehta, N., Thibault, J., Colman, P. M., Barry, M., Huang, D. C., and Kvansakul, M. (2012) Sheep pox virus SPPV14 encodes a Bcl-2-like cell death inhibitor that counters a distinct set of mammalian proapoptotic proteins. J. Virol. 86, 11501–11511 CrossRef Medline
24. Sippl, W. M., Ledergerber, E. C., Hibma, M. H., Fleming, S. B., Whelan, E. M., and Mercer, A. A. (2007) A novel Bcl-2-like inhibitor of apoptosis is encoded by the parapoxvirus ORF virus. J. Virol. 81, 7178–7188 CrossRef Medline
25. Van Regenmortel, M. H. V. (2000) Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses, Academic Press, San Diego, CA
26. Tsi, C. T., Ting, J. W., Wu, M. F., Guo, I. C., and Chang, C. Y. (2005) Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses. J. Virol. 79, 2010–2023 CrossRef Medline
27. Lin, P. W., Huang, Y. J., John, A. J., Chang, Y. N., Yuan, C. H., Chen, W. Y., Yeh, C. H., Shen, S. T., Lin, F. P., Tsui, W. H., and Chang, C. Y. (2008) Iridovirus Bcl-2 protein inhibits apoptosis in the early stage of viral infection. Apoptosis 13, 165–176 CrossRef Medline
28. Holm, L., and Rosenström, P. (2010) Dali server: conservation mapping in 3D. Nucleic Acids Res. 38, W545–W549 CrossRef Medline
29. Rasmussen, K., Aoki, K., Bird, G. H., Katz, S. G., Tur, H. C., Kim, H., Cheng, E. H., Tjandra, N., and Walensky, L. D. (2008)
