Caspase Inhibition by Baculovirus P35 Requires Interaction between the Reactive Site Loop and the β-Sheet Core*

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Baculovirus P35 is a universal substrate-inhibitor of the death caspases. Stoichiometric inhibition by P35 is correlated with cleavage of its reactive site loop (RSL) and formation of a stable P35-caspase complex through a novel but undefined mechanism. The P35 crystal structure predicts that the RSL associates with the β-sheet core of P35 positioning the caspase cleavage site at the loop’s apex. Here we demonstrate that proper interaction between the RSL and the β-sheet core is critical for caspase inhibition, but not cleavage. Disruption of RSL interaction with the β-sheet by substituting hydrophobic residues of the RSL’s transverse helix α1 with destabilizing charged residues caused loss of caspase inhibition, without affecting P35 cleavage. Restabilization of the helix/sheet interaction by charge compensation from within the β-sheet partially restored anti-caspase potency. Mutational effects on P35 helix/sheet interactions were confirmed by measuring intermolecular helix/sheet association with the yeast two-hybrid system. Moreover, the identification of P35 oligomers in baculovirus-infected cells suggested that similar P35 interactions occur in vivo. These findings indicate that P35’s anti-caspase potency depends on a distinct conformation of the RSL which is required for events that promote stable, post-cleavage interactions and inhibition of the target caspase.

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EXPERIMENTAL PROCEDURES

p35 Mutagenesis—The HindIII-BamHI fragment of p35 from pPRM 35K-ORF (24) was inserted into p35KORF-hrf5'-NdeI-NotI (7) to generate p35KORF-NdeI-Stop. Mutations in p35 were generated in either plasmid p35KORF-hrf5'-NdeI-NotI by using the Kunkel method (25) or plasmid p35KORF-NdeI-Stop by using overlap extension polymerase chain reaction with complementary primers. Mutagenic oligonucleotides (the altered nucleotides underlined) used to generate each mutation included: I1SD, 5'-ACCTCGAACAATCTGGATCCTGCGGAACGC-3'; ca22, 5'-CTTCTGTGCTGTTTGGCACTGACTCTTGCTG-3'; ca41, 5'-AACATCATGAAACGGGCCGTAGATTGCTGTT-3'; 167K, 5'-GGTCGACCAGAAAGAATCTGGTTAATGTCG-3'; 167Y, 5'-ATTTGCGGACGATATACTTTAATCTTATGTCG-3'; AT-99, 5'-TCTTGACTGCGTGGATCTGCTGTTTGC-3'; 99AAC-99TTT, 5'-AGATAAAATCAGGGAGTAAATCATTGAT-3'; and 5'-ATCCAAAATGTCTTTTATTTATTTCT-3'. All nucleotide substitutions were verified by DNA sequencing. Other p35 mutations (ca17, ca26, ca46, ca70, ca79, D84A, D87A, ca90, ca112, ca126, ca143, in10, in52, in74, in83, in273, and in278) were described previously (7).

Cells and Viruses—Spodoptera frugiperda cell line IPLB-SF21 (26) was propagated at 35°C in TC100 growth medium that contained 2.6% tryptose broth and 10% fetal bovine serum. For infections, cell monolayers were inoculated with the indicated plaque-forming units per cell. After 1 h, the inoculum was replaced with fresh growth medium, and layers were inoculated with the indicated plaque-forming units per cell. Trypsinization was corrected by normalizing mutated P35 proteins to wild-type P35. Impurities that affected V71K-P35-His6 concentration determined by SDS, 10%–20% polyacrylamide gel analysis indicated that the proteins, except V71K-P35-His6, were overexpressed in strain BL21(DE3) by using the pET

P35K/lacZ (0.5 plaque forming units/cell). For inhibition assays, increasing concentrations of test compounds were added to cell lines infected 16 h later with vP35 and ruptured by repetitive freeze-thaw cycles. Clarified lysates

Immunoblot Analysis—Recombinant C-terminal, His-tagged human caspase 3 (23) and baculovirus P35-His (wild-type or mutated) were expressed in Saccharomyces cerevisiae strain Y190 (MATa, his3–200, trpl–901, leu2–3, 112, URA3::GAL-z, LYS2::GAL-HIS) was transformed with 2 μg of plasmid DNA using the lithium acetate-heat shock method (27) and grown on selective medium lacking Trp, Leu, His, or the appropriate combination thereof, His-deficient medium was supplemented with 45 mg 3-aminotriazole. β-Galactosidase production by transformed colonies was assayed using filter lift assays (28), in which replica colonies were transferred to filter circles (Whatman #1), permeabilized by submergence in liquid N2, and incubated overnight in Z-Buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) containing 0.35 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml. Liquid β-galactosidase activity was determined using the substrate o-nitrophenyl-β-D-galactopyranoside as described previously (29). Reported values are expressed in standard units (30) multiplied by 1000.

Affinity Purification of Intracellular P35—SF21 cells were harvested 43 h after infection with recombinant baculoviruses (25)-His and ∼1,000× ruptured by repetitive freeze-thaw cycles. Clarified lysates (16,000 g × 10 min) were mixed with Ni2+–conjugated agarose beads (Novagen) in binding buffer (20 mM Tris, pH 7.9, 5 mM imidazole, 0.5 mM NaCl) for 3 h at 16°C. After washing with binding buffer containing 30 mM imidazole, bound proteins were eluted with binding buffer containing 1 M imidazole and subjected to immunoblot analysis by using α-P35NF and α-HA sera.

RESULTS

Disruption of RSL Interactions Causes Loss of P35 Function—Stoichiometric inclusion of caspases by P35 causes cleavage of the RSL at Asp87, the P1 residue located at the apex of the loop. The transverse helix α1 (residues 63 to 75) is an essential component of the RSL, and may contribute to proper positioning of the cleavage site. The P35 crystal structure (23) suggests that α1 interacts with the β-sheet core (Fig. 1). The
nonpolar residues on the bottom of α1 are accommodated by the hydrophobic environment provided by nonpolar residues of the β-sheet. Conversely, the charged or polar residues comprising the top side of α1 are solvent exposed (Fig. 2A). To investigate the significance of the interaction between α1 and the β-sheet, we tested the effect of site-specific mutations within these domains on P35 anti-caspase activity.

To disrupt α1/β-sheet association, hydrophobic residues Ile67 and Val71 comprising the underlying face of α1 (Fig. 2A) were substituted with either lysine or tyrosine. The anti-apoptotic activity of I67K-, I67Y-, or V71K-mutated P35 was first determined by marker rescue. In this assay, anti-apoptotic function is measured by the capacity of the mutated P35 to block apoptosis and thereby restore replication of a deletion mutant virus in cultured, apoptosis-sensitive SF21 cells (6, 7). Neither I67K-P35 nor V71K-P35 exhibited anti-apoptotic activity in vivo (Fig. 2B). In contrast, the function of I67Y-P35 was comparable to that of wild-type P35. Thus, substitution of either Ile67 or Val71 with a positively charged residue disrupted P35 function, whereas substitution with an aromatic residue had no effect. These data suggested that the hydrophobic interaction between α1 and the β-sheet is required for P35 function. Consistent with this interpretation, charged-to-alanine substitutions (Fig. 2B) of α1 solvent-exposed residues had no effect on P55 function in vivo (7).

α1 Helix Mutations Disrupt Caspase Inhibition, Not P55 Cleavage—To determine whether loss of P35 function was due to loss of caspase inhibition, we measured the capacity of I67K-P35 and V71K-P35 to inhibit purified recombinant human caspase-3 (CPP32). Each mutated P35 was purified as a C-terminal His6-fusion protein overexpression in E. coli and tested in dose-dependent caspase inhibition assays that used the tetrapeptide DEVD-amc as substrate (Fig. 3A). As expected (7, 23), wild-type P35 stoichiometrically inhibited caspase-3, whereas D87A-P35 which lacks the requisite cleavage residue Asp87 failed to affect caspase-3 activity at all concentrations. V71K-P35 was as ineffective as D87A-P35 in inhibiting caspase-3 (Fig. 3A). Likewise, I67K-P35 had significantly reduced (>10-fold) anti-caspase activity when compared with wild-type P35. In contrast, caspase-3 inhibition by I67Y-P35 was comparable to that of wild-type P35, a finding consistent with its normal anti-apoptotic function in vivo. Thus, the loss-of-function phenotype of P35 carrying the α1 lysine substitutions was correlated with loss of anti-caspase activity.

To determine whether I67K-P35 and V71K-P35 were recognized as caspase substrates, we examined their susceptibility to cleavage by caspase-3. After mixing caspase-3 with a 4-fold molar excess of purified wild-type or mutated P35-His6, the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3B). With the exception of D87A-P35, all forms of P35 were cleaved efficiently. α1-Mutated I67K- and V71K-P35 were cleaved to completion, as judged by loss of full-length protein and accumulation of their 25- and 10-kDa cleavage fragments (Fig. 3B, lanes 5 and 9). In contrast, wild-type and I67Y-P35 cleavage was limited as a direct result of caspase inhibition (lanes 3 and 7). Time course analysis (Fig. 3C) indicated that I67K-P35 cleavage was rapid. I67K-P35 cleavage fragments were detected within seconds of caspase-3 addition; by 6 h >95% of the 4-fold excess I67K-P35 was cleaved. In comparison, only limited cleavage of wild-type P35 occurred during the same period (Fig. 3C). Cleavage site substitution D87A-P35 was a poor substrate since cleavage was not detected. The finding that both I67K-P35 and V71K-P35 were efficient substrates, but ineffective caspase inhibitors, suggested that the α1/β-sheet association is required for P35 anti-caspase activity, but not cleavage.

β-Sheet Charge Compensation Increases P35 Anti-caspase Potency—We predicted that electrostatic stabilization of the weakened interaction between mutated α1 and the β-sheet would increase the potency of I67K-P35. In the P35 crystal structure, β-sheet residue Ile15 is proximal to α1 residue Ile67 (Fig. 1). We therefore substituted Ile15 with Asp to neutralize the nearby positive charge of I67K. Caspase-3 inhibition by double-mutated I15D/I67K-P35 was then measured by using in vitro
vitro dose-dependent assays (Fig. 4A). I15D:I67K-P35 was a more potent inhibitor than I67K-P35 at all concentrations tested, but less potent than single-mutated I15D-P35. On the basis of multiple experiments, the IC50 of I15D:I67K-P35 ranged from 3.0 to 5.5 nM, compared with 9.5 to 13 nM for I67K-P35. Thus, with respect to caspase inhibition, the two mutations were compensatory, not additive, when present in the same P35 molecule. Extended incubations (Fig. 4B) demonstrated that inhibition was achieved in less than 30 min, since anti-caspase activity of I67K- and I15D:I67K-P35 was constant over a 6-h period. Thus, each mutated P35 did not exhibit delayed caspase inhibition. The increased anti-caspase potency of I15D:I67K-P35 compared with that of I67K-P35 suggested that the negative charge of substitution I15D stabilized interaction with I67K-mutated a1. These data confirmed the necessity of proper interaction between a1 and the b-sheet core for caspase inhibition.

P35 Oligomerizes in the Yeast Two-hybrid Assay—Previous studies have indicated that the yeast two-hybrid system (31) can detect intramolecular associations through bimolecular interaction of two independently synthesized protein domains (32–34). We predicted that the RSL of P35 could associate with the b-sheet of a different P35 molecule and that this intermolecular interaction could therefore be investigated using the two-hybrid system. To this end, we fused full-length P35 (residues 1 to 299) to the C terminus of the Gal4 DNA-binding domain (DBD) and the Gal4 activation domain (AD) to generate DBD-P35 and AD-P35, respectively (Fig. 5A). Co-transformation of S. cerevisiae with plasmids encoding DBD-P35 and AD-P35 induced strong Gal4-dependent lacZ expression as determined by filter lift assay and thus suggested stable interaction between both proteins (Fig. 5C). Neither plasmid alone induced lacZ expression in yeast (data not shown). The DBD-P35 and AD-P35 interaction was confirmed by growth of cotransformants on medium lacking histidine (see below, Fig. 7). The specificity of P35-P35 interaction in yeast was verified by mating strain Y190 (MATa) containing DBD-P35 with strain Y187 (MATα) containing unrelated proteins SNF4, lamin, CDK2, or p53 fused to the Gal4 activation domain. The resulting diploids failed to express lacZ as determined by filter lift....

**FIG. 3. Effects of a1 mutations on caspase inhibition by P35. A, in vitro assay of caspase-3 activity. Purified human caspase-3 (200 fmol) was incubated with increasing amounts of purified wild-type (wt) P35-His6 or P35-His6 containing the indicated mutations. After 30 min, residual protease activity was measured in fluorometric assays by using the tetrapeptide DEVD-amc as substrate. Plotted values are the averages ± S.D. of multiple determinations and are expressed as a percentage of uninhibited caspase activity. A representative experiment of three trials is shown.**
We generated a series of mutations (insertions and substitutions) in DBD-P35 and tested their effects on interaction with AD-P35. Immunoblot analysis of yeast lysates containing the DBD-P35 mutations confirmed that each fusion protein was stably synthesized (Fig. 5B) and ruled out the possibility that effects on P35 interaction were due to loss of protein stability. The capacity of each mutated P35 to interact in the two-hybrid system was subsequently compared with its anti-apoptotic activity in vivo (Fig. 5C).

Within the P35 RSL, only those loss-of-function mutations within the underlying nonpolar face of $\alpha_1$ caused loss of P35 interaction (Fig. 6). Mutations I67K and V71K failed to interact with AD-P35 (Fig. 5C). Similarly, Ala-Ser insertion in74, which disrupted the amphipathicity of $\alpha_1$, caused loss of interaction and loss of function. Conversely, I67Y-P35 which was fully functional for caspase inhibition interacted normally with AD-P35. In addition, charged to Ala substitutions of residues comprising the solvent-exposed portion of $\alpha_1$ had no effect on P35-P35 interaction and were fully functional for apoptotic suppression (Fig. 5C and Fig. 6). Although the $P_1$ and $P_1$ cleavage site substitutions D84A and D87A caused loss of anti-apoptotic function, both mutated forms of P35 interacted normally with AD-P35, as did all mutations of RSL residues other than those altering the hydrophobic face of $\alpha_1$.

Mutations within the $\beta$-sheet (I15D, ca17, ca26, in52, and in278) also disrupted P35 interaction and caused loss of function (Fig. 5C and Fig. 6). Conversely, mutations in loops that connected various $\beta$-strands (in10, ca22, ca41, ca143, and ca273) had no effect on P35 interaction. In addition, charged to Ala mutations (ca112 and ca126) within helix $\alpha_2$ and $\alpha_3$ had no effect. The pattern of P35 interaction was identical in reciprocal experiments where mutations were introduced into AD-P35 and tested for interaction with wild-type DBD-P35 (data not shown). Collectively, these data suggested that the residues involved in intramolecular $\alpha_1/\beta$-sheet association also participate in two-hybrid interactions.

Compensatory P35 Mutations I15D and I67K Interact—Since the $\beta$-sheet substitution I15D partially restored anti-caspase activity to $\alpha_1$-mutated I67K-P35 (Fig. 4), presumably by stabilizing $\alpha_1/\beta$-sheet interaction, we predicted that I15D would also restore intermolecular association with I67K-P35. To test
this possibility, we assayed for two-hybrid interaction between I15D-P35 and I67K-P35 by using filter lifts and growth of yeast transformants on histidine-deficient medium (Fig. 7A). Although I15D-mutated and I67K-mutated DDB-P35 failed to interact with wild-type AD-P35, interaction between I15D-mutated DDB-P35 and I67K-mutated AD-P35 was strong. This finding was confirmed in reciprocal experiments where interaction between I15D-mutated DDB-P35 and I67K-mutated AD-P35 was as strong as that between wild-type P35s (Fig. 7A). These data indicated that intermolecular α/β-sheet association can occur in which the RSL of one P35 molecule interacts with the β-sheet of another (Fig. 7B). Thus, intramolecular interactions of the RSL with the β-sheet core within a P35 monomer also mediate intermolecular association of separate P35 molecules.

P35 Oligomerizes in Vivo—The intermolecular interaction of P35 within the two-hybrid system suggested that P35 also multimerizes in vivo. To investigate this possibility, electrophoretically distinct forms of P35 were co-synthesized in cultured SF21 cells and tested for interaction. By using recombinant baculoviruses, wild-type (untagged) P35 and HA epitope-tagged P35 were synthesized separately or together in these cells. Immunoblot analysis of total cell lysates with P35- and HA-specific antisera demonstrated that both forms of P35 were produced and were readily distinguished (Fig. 8). Metal (Ni²⁺) affinity chromatography of cell extracts containing only P35-His readily isolated this single protein (lane 10). Affinity purification of extracts containing cosynthized P35-His and untagged P35 isolated both proteins (lane 12). Untagged P35 was not detected upon affinity purification of extracts containing untagged P35 alone (lane 11). Thus, co-purification of untagged P35 required the presence P35-His. Stained gels failed to detect proteins other than uncleaved P35-His and untagged P35, arguing against the presence of bridging molecules such as caspases (data not shown). Thus, the association of P35-His and untagged P35 was due to direct P35-P35 interaction.

To verify P35 oligomerization, metal affinity-purified P35-His from SF21 cells was cross-linked with glutaraldehyde. Immunoblot analysis demonstrated a loss of P35 with a mass of 35 kDa and an increase in P35 multimers (Fig. 9, lanes 1–4). Under these conditions, purified monomeric ovalbumin failed to cross-link as expected (Fig. 9, lanes 5–8). Thus, the ability to cross-link purified P35 complexes confirmed in vivo P35 oligomerization. In the absence of cross-linker, a P35-specific protein with a size (70 kDa) expected of dimeric P35 was routinely detected by immunoblot analysis (Fig. 9, lane 1). The stability of the dimer-like P35 was unaffected by reducing agents, including dithiothreitol or 2-mercaptoethanol (data not shown). Thus, the detection of oligomeric P35 even after detergent treatment (1% SDS) suggested that hydrophobic interactions contribute to P35 oligomerization, a finding consistent with the hydrophobic association of helix α1 with the β-sheet core of a different P35 molecule.

DISCUSSION

P35 stoichiometrically inhibits caspases through a multistep mechanism involving slow binding, cleavage, and stable association with the target protease (7, 19, 20, 23). Cleavage of the P35 RSL is correlated with formation of a stable complex with the target caspase, suggesting that cleavage is required for caspase inhibition. However, since cleavage is not sufficient for inhibition, additional undefined events are required (23). Our studies here demonstrate that regions or domains outside the P35 RSL cleavage site contribute to these events, either by direct participation or through the stabilization of required interactions between the RSL and the main core of P35.

Interactions between the RSL and P35 β-Sheet Core—The P35 crystal structure predicted that transverse helix α1, com-

**FIG. 7. Restoration of P35 α/β-sheet interactions.** A, two-hybrid assays. Plasmids containing the indicated α and β-sheet mutations in DDB-P35 and AD-P35, respectively, were co-transformed into *S. cerevisiae* strain Y190. After growth on Trp-Leu medium, recovered colonies were streaked onto His-Trp-Leu plates. Growth on histidine-deficient medium indicated P35-P35 interaction since his3 expression is Gal4 responsive. Interaction was independently assessed by filter lift assays of hundreds of colonies from three independent transformations. Positive (+) or negative (−) interaction was scored by unambiguous staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). B, model of the P35 swap-mer. Within the swap-mer (top), the RSL of one molecule associates with the β-sheet of another through the same interactions that occur intramolecularly between α1 and the β-sheet of a P35 monomer (bottom).
These findings suggested that the charged lysine destabilized the anti-caspase function from vP35HAHis6-infected cells, P35HA-His6 (4 μg) was treated with the indicated concentrations (percent) of glutaraldehyde (CHO). The reaction products were subjected to electrophoresis on an SDS-4 to 20% polyacrylamide gel and immunoblot analysis with P35 antisera (lanes 1–4). After identical treatment, purified ovalbumin (4 μg) was subjected to electrophoresis and stained with Coomassie Brilliant Blue (lanes 5–8).

Cross-linking of P35 oligomers. After affinity (Ni²⁺) purification from vP35HAHis6-infected cells, P35HA-His6 (4 μg) was used to demonstrate that intramolecular interactions between the RSL molecule increased anti-caspase efficacy, as demonstrated by using the yeast two-hybrid assay, which demonstrated that P35 oligomerization can occur through direct P35-P35 interaction in which the RSL of one molecule interacts with the β-sheet core of another. This RSL swapping generates a novel dimeric structure which we have designated the "swapper" (Fig. 7B). It is unclear what fraction of in vivo P35 oligomers consist of swap-mers. In insect cells, I67K- and V71K-mutated P35s were poorly synthesized, complicating efforts to test interaction with I15D-mutated P35 in swap-mers. It is noteworthy that when independently synthesized P35 molecules were mixed in vitro, hetero-oligomerization was not detected. Thus, dissociation and reassociation of P35 multimers may be unfavorable, a property consistent with strong hydrophobic interactions occurring within the swap-mer.

Oligomeric P35 may have multiple functions in vivo. Since caspases possess two catalytic sites that are independently inhibited by P35 (20, 37–40), oligomers may increase the local concentration of P35 and thereby accelerate inhibition at both active sites. Due to structural constraints, it seems unlikely that the P35 swap-mer or other dimeric forms could interact simultaneously with both active sites of a single caspase. Nonetheless, simultaneous interaction of a P35 swap-mer with the active site of separate caspases is possible, raising the intriguing possibility that P35 is a multivalent protease inhibitor. P35 multimerization may also contribute to in vivo protein stability. Consistent with this role, mutated P35s that failed to interact in the two-hybrid assay (I67K, V71K, V71P, I15D, Ile⁶⁷, Val⁷¹) were not detectable in an oligomeric complex that was readily cross-linked (Figs. 8 and 9). It is unlikely that this in vivo P35 multimerization was mediated by an interacting caspase since only full-length, uncleaved P35 was detected in these complexes (Fig. 8). Our analyses using the yeast two-hybrid system demonstrated that P35 oligomerization can occur through direct P35-P35 interaction in which the RSL of one molecule interacts with the β-sheet core of another. This RSL swapping generates a novel dimeric structure which we have designated the "swapper" (Fig. 7B). It is unclear what fraction of in vivo P35 oligomers consist of swap-mers. In insect cells, I67K- and V71K-mutated P35s were poorly synthesized, complicating efforts to test interaction with I15D-mutated P35 in swap-mers. It is noteworthy that when independently synthesized P35 molecules were mixed in vitro, hetero-oligomerization was not detected. Thus, dissociation and reassociation of P35 multimers may be unfavorable, a property consistent with strong hydrophobic interactions occurring within the swap-mer.

2 S. J. Zooq, unpublished data.
Truncation p35\textsuperscript{1–76} dominantly interferes with the capacity of wild-type p35 to block apoptosis (8, 41). It was originally speculated that hetero-oligomerization with P35 accounted for the observed dominant inhibition by p35\textsuperscript{1–76}. However, by using the yeast two-hybrid assay, we found no evidence for interaction of P35\textsuperscript{1–76} with wild-type P35 (data not shown). Moreover, mutations that caused loss of two-hybrid P35 interaction did not affect p35\textsuperscript{1–76}-mediated inhibition of wild-type p35 in vitro. Thus, despite the presence of α1 residues in P35\textsuperscript{1–76}, it is unlikely that dominant inhibition by p35\textsuperscript{1–76} is a consequence of interaction via the α1 helix. Nonetheless, investigation of other dominant-negative mutations of P35 may provide insight into the biological role of multimerization for P35 anti-apoptotic activity.

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REFERENCES

1. Green, D. R. (1998) Cell 94, 695–698
2. Nicholsen, D. W., and Thornberry, N. A. (1997) Trends Biochem. Sci. 22, 299–306
3. Cryns, V., and Yuan, J. (1998) Genes Dev. 12, 1551–1571
4. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
5. Clem, R. M., Flechheimer, M., and Miller, L. K. (1991) Science 254, 1388–1390
6. Herzogfferer, P. A., Dickson, J. A., and Friesen, P. D. (1992) J. Virol. 66, 5525–5533
7. Bertin, J., Mendrysa, S. M., LaCount, D. J., Gaur, S., Krebs, J. F., Armstrong, K. C., Tomaselii, K. J., and Friesen, P. D. (1996) J. Virol. 70, 6251–6259
8. LaCount, D. J., and Friesen, P. D. (1999) J. Virol. 71, 1550–1557
9. Hay, B. A., Wolff, T., and Rubin, G. M. (1994) Develop. 120, 2121–2129
10. Sugimoto, A., Friesen, P. D., and Rothman, J. H. (1994) EMBO J. 13, 2623–2628
11. Rahibazadeh, S., LaCount, D. J., Friesen, P. D., and Breiden, D. E. (1993) J. Neurochem. 61, 2318–2321
12. Beidler, D. R., Tewari, M., Friesen, P. D., Poizier, G., and Dixit, V. M. (1995) J. Biol. Chem. 270, 16552–16558
13. Xue, D., and Horvitz, H. R. (1995) Nature 377, 248–251
14. Martinou, I., Fernandez, P. A., Missotten, M., White, K., Allet, B., Sadow, R., and Martinou, J. C. (1995) J. Cell Biol. 128, 201–208
15. Robertson, N. M., Zangrilli, J., Fernandes-Alnemri, T., Friesen, P. D., Litwack, G., and Alnemri, E. S. (1997) Cancer Res. 57, 43–47
16. Izquierdo, M., Grandien, A., Criado, L. M., Robles, S., Leonardo, E., Albar, J. P., de Buitrago, G. G., and Martinez, A. C. (1999) EMBO J. 18, 156–166
17. Davidson, P. F., and Steller, H. (1998) Nature 391, 587–591
18. Jacobson, M. D. (1998) Curr. Biol. 8, 418–421
19. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Fondacaro, C., Franklin, S., and Friesen, P. D. (1996) Science 272, 17907–17911
20. Zhou, Q., Krebs, J. F., Snips, S. J., Price, A., Alnemri, E. S., Tomaselii, K. J., and Friesen, G. S. (1998) Biochemistry 37, 10757–10765
21. Ahmad, M., Sririvasalum, S. M., Wang, L., Litwack, G., Fernandes-Alnemri, T., and Alnemri, E. S. (1997) J. Biol. Chem. 272, 1421–1424
22. Thornberry, N. A., Ranon, T. A., Peterson, E. F., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) J. Biol. Chem. 272, 17907–17911
23. Fisher, A., dela Cruz, W., Zoog, S. J., Schneider, C. L., and Friesen, P. D. (1999) EMBO J. 18, 2031–2039
24. Herschberger, P. A., LaCount, D. J., and Friesen, P. D. (1994) J. Virol. 68, 3467–3477
25. Kinkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
26. Vaughan, J. L., Goodwin, R. H., Thompkins, G. L., and McCawley, P. (1977) In Vitro 13, 213–217
27. Schiestel, R. H., and Gietz, R. D. (1989)Curr. Genet. 16, 339–346
28. Breeden, L., and Nasmyth, K. (1985) Cold Spring Harbor Symp. Quant. Biol. 50, 643–650
29. Guarante, L. (1983) Methods Enzymol. 101, 181–188
30. Miller, J. H. (1972) CSH Laboratory Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Fields, S. A., and Og-Kyu, S. (1989) Nature 340, 245–246
32. Huang, Z., Curtis, K. D., and Rich, L. P. (1995) Science 267, 1169–1172
33. Huang, L., Ichimaru, E., Pestonjamasp, K., Cui, X., Nakamura, H., Lo, G. Y. H., Lin, F. I. K., Luna, E. J., and Fyrthmayr, H. (1988) Biochem. Biophys. Res. Commun. 248, 548–553
34. Bartel, P. L., Roeschlein, J. A., SenGupta, D., and Fields, S. (1996) Nat. Genet. 12, 72–77
35. Whistock, J., Skinner, R., and Lesk, A. M. (1998) Trends Biochem. Sci. 23, 63–67
36. Merrit, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D 50, 869–873
37. Mittl, P. E., R. Di Marco, S., Krebs, J. F., Bai, X., Karanewsky, D. S., Priestle, J. P., Tomaselii, K. J., and Grutter, M. G. (1997) J. Biol. Chem. 272, 6539–6547
38. Rotonda, J., Nicholson, D. W., Fazlz, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Rasper, D. M., Ruel, Y., Vaillancourt, J. P., Thornberry, N. A., and Becker, J. W. (1996) Nature Struct. Biol. 3, 613–625
39. Walker, N. P., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Rasket, M. C., Harn, L. D., et al. (1994) Cell 78, 343–352
40. Wilson, K. P., Black, J. A., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 279–275
41. Cartier, J. L., Herschberger, P. A., and Friesen, P. D. (1994) J. Virol. 68, 7728–7737
42. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
43. Bacon, D. J., and Anderson, W. F. (1988) J. Mol. Graphics 6, 219–220