Overexpression of the dynein light chain km23-1 in human ovarian carcinoma cells inhibits tumor formation in vivo and causes mitotic delay at prometaphase/metaphase

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km23-1 is a dynein light chain that was identified as a TGFβ receptor-interacting protein. To investigate whether km23-1 controls human ovarian carcinoma cell (HOCC) growth, we established a tet-off inducible expression system in SKOV-3 cells in which the expression of km23-1 is induced upon doxycycline removal. We found that forced expression of km23-1 inhibited both anchorage-dependent and anchorage-independent growth of SKOV-3 cells. More importantly, induction of km23-1 expression substantially reduced the tumorigenicity of SKOV-3 cells in a xenograft model in vivo. Fluorescence-activated cell sorting analysis of SKOV-3 and IGROV-1 HOCCs demonstrated that the cells were accumulating at G2/M. Phospho-MEK, phospho-ERK and cyclin B1 were elevated, as was the mitotic index, suggesting that km23-1 suppresses HOCCs growth by inducing a mitotic delay. Immunofluorescence analyses demonstrated that the cells were accumulating at prometaphase/metaphase with increases in multipolar and multinucleated cells. Further, although the mitotic spindle assembly checkpoint protein BubR1 was present at the prometaphase kinetochore in Dox+/- cells, it was inappropriately retained at the metaphase kinetochore in Dox-- cells. Thus, the mechanism by which high levels of km23-1 suppress ovarian carcinoma growth in vitro and inhibit ovary tumor formation in vivo appears to involve a BubR1-related mitotic delay.

Epithelial ovarian cancer is one of the most common forms of ovarian cancer and the most lethal gynecologic malignancy among women in the United States, with 21,880 new cases and 13,850 deaths estimated in 2010.1 Although microtubule (MT)-binding agents, such as paclitaxel (taxol), are being used in the treatment of ovarian cancer, acquired and intrinsic drug resistance has significantly limited their efficacy. Thus, it is critical to develop novel, targeted therapeutics for ovarian cancer. More recently, the motors that move along the spindle MTs have been targeted for the development of anticancer therapeutics, although the minus-end-directed motor protein dynein has not received similar attention in terms of its targeting potential. Although dynein’s important functions in inactivating the spindle MTs have been targeted for the development of new therapies. For example, the kinetochore-associated mitotic motor kinesin, termed centromere-associated protein-E (CENP-E), has been targeted because of its key role in the mitotic spindle assembly checkpoint (SAC).2 Thus, motor protein regulatory agents may offer promise for providing improved efficacies with reduced side effects in the treatment of ovarian cancer and other human malignancies.3

Although the plus-end-directed kinesin MT motor has been targeted for the development of anticancer therapeutics, the minus-end-directed motor protein dynein has not been studied as widely in terms of its targeting potential. Although dynein’s important functions in inactivating the SAC have been widely studied,4,5 little is known about how regulation of dynein functions can contribute to ovarian cancer development or progression. We have identified a novel TGFβ receptor-interacting protein, termed km23-1,6 which is also a light chain of the motor protein dynein (also called DYNLRB1/LC7-1/robl-1/DNLC2A/DYRB1).7–11 Further, we have reported that km23-1 plays a critical role in normal TGFβ signaling.6,10,12–14 However, many ovarian carcinoma cells are resistant to the growth inhibitory effects of TGFβ.15,16 Accordingly, to define the role of the km23-1 dynein light chain (DLC) in ovarian carcinogenesis, we investigated whether forced expression of wild-type km23-1 could regulate cell growth and tumor progression of TGFβ-resistant human ovarian carcinoma cells (HOCCs).

Here, we demonstrate that overexpression of km23-1 in the highly aggressive SKOV-3 HOCCs inhibited both mono-layer proliferation and anchorage-independent growth of the cells, causing an accumulation of cells in mitosis at prometaphase/metaphase. Further, BubR1 was abnormally retained at the kinetochore in metaphase cells with high levels of km23-1, suggesting that the SAC had remained active. More
importantly, induction of km23-1 expression reduced the tumorigenicity of the HOCCs in a xenograft model. Thus, overexpression of the DLC km23-1 can function to decrease HOCC growth in vitro and tumor formation in vivo through a BubR1-related role in metaphase cells.

Material and Methods

Reagents

The antibodies used were as follows: polyclonal anti-Flag (F7425, Sigma-Aldrich, St. Louis, MO), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245, Abcam, Cambridge, MA), anti-dynein intermediate chain (DIC) (MAB 1685, Chemicon, Temecula, CA), human CREST (Immunovision, Springdale, AR), Mouse BubR1 (BD Bioscience, San Jose, CA) or mouse BubR1 (Millipore, Billerica, MA) and antibodies against cyclin B1 (41385), phospho-histone H3 (9706S), phospho-MEK1/2 (9121S), MEK1/2 (9122), phospho-ERK1/2 (4370S) and ERK1/2 (4780) were from Cell Signaling Technology (Danvers, MA). Annexin V-Fluos staining kit was from Roche Applied Science (Indianapolis, IN). Other chemicals were obtained from Sigma unless otherwise indicated.

Cell culture

SKOV-3 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as described thereby. The IGROV-1 cell line was a gift from D. MacLaughlin (Massachusetts General Hospital, Boston, MA). OVCA433 cells were obtained from R. Bast (M.D. Anderson Cancer Center, Houston, TX). IGROV-1 and OVCA433 cells were cultured as described. Cells were routinely screened for mycoplasma using Hoechst 33258 staining.

DNA constructs

Flag-tagged km23-1 constructs were generated by inserting the corresponding PCR products into pFlag-CMV5a or pBIG2r. Generation of km23-1 Tet-off expression system in SKOV-3 cells

To generate inducible cells that overexpress wild-type km23-1, SKOV-3 cells were transfected with the pBIG2r empty vector (EV) or pBIG2r-km23-1-Flag using Fugene 6 according to the manufacturer’s instructions. After transfection, cells were selected with hygromycin B (400 µg/ml) and either pooled or cloned by limiting dilution. Clones and pools were screened for km23-1-Flag expression after Dox removal by immunoblotting and were maintained in medium containing hygromycin B (400 µg/ml) and Dox (2 µg/ml).

Immunoblot analyses

Immunoblot analyses were performed as described.6,10,18

Cell proliferation assays

SKOV-3-pBIG2r EV pool (EV pool), km23-1 pool and km23-1 clones cells were plated at 6.3 × 10³ cells per centimeter square, treated +/-Dox and were analyzed using a crystal violet assay.6 Routine cell growth assays were also performed using the Vi-CELL Series Cell Viability Analyzer (Beckman Coulter, Brea, CA).

Soft agar growth assays

Soft agar growth assays were performed as described19 except that 5.2 × 10³ cells per centimeter square were plated in 0.3% agar on top of the 0.8% agar bottom layer +/-Dox. Colonies were scored by microscopy after 21 days.

FACS analysis

FACS analysis was used to determine cell cycle distribution as described.12

Immunofluorescence analyses

Immunofluorescence analyses were performed in cells grown in chamber slides +/-Dox for 9–14 days when mitotic delay was detectable in Flag-positive cells. For mitotic index, cells were fixed, permeabilized10 and incubated with mouse monoclonal anti-α-tubulin (1:1,200), mouse monoclonal anti-phospho-histone H3 (1:1,200) or rabbit polyclonal anti-Flag (1:600). Phospho-histone H3-positive cells, Flag-positive cells and total cell numbers were determined for each experiment from between 500 and 1,000 of each of +/-Dox cells. For kinetochore studies, immunofluorescence analyses were performed as described.20 Primary antibodies used were mouse BubR1 (1:100), human CREST (1:1,000) and rabbit polyclonal anti-Flag (1:500). For secondary fluorescence staining, cy3-conjugated goat anti-rabbit IgG (1:500), Alexa 568 goat anti-mouse IgG (1:500), Alexa 488 goat anti-mouse IgG, Alexa 488 goat anti-rabbit IgG or Alexa 488 goat anti-human IgG (1:200, Invitrogen) were used. Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). Images were captured on the same day using identical parameters on an Olympus IX81 microscope coupled with MetaMorph software.

Quantification of multipolar spindles and multinucleated cells

For multipolar cells, a minimum of 20 fields and a total of 100 cells were counted for each of three independent experiments as described previously.21 For multinucleated cells, an average of 450 cells from a minimum of 25 fields were counted, and the percentages of multinucleated cells (>2N and >4N) were scored as described22 in a cumulative fashion during the period when the Flag-positive cells were undergoing mitotic delay.

Annexin V assay

km23-1 clone #14 cells were grown in 35-mm dishes +/-Dox for 9–14 days, and the Annexin V-Fluos kit was used as described by the manufacturer. FACS analyses were performed every other day, values for early (annexin-positive only) and late (annexin and PI positive) apoptotic cells were
combined and cumulative apoptotic populations were normalized by number of days.

In vivo tumorigenicity studies
Athymic female Balb/c nude mice were obtained from Charles River Laboratories (Wilmington, MA). EV pool, km23-1 pool and km23-1 clone #14 cells (3.5 \times 10^6) were inoculated subcutaneously into the right flank of mice (eight mice per group) and fed continuously with Dox +/− chow (20 mg/kg) (Bio-Serv, Frenchtown, NJ) or regular chow (Dox −). Tumorigenicities were determined as described.23,24

Statistical analyses
Student’s t-tests were performed with Prism 4 software.

Results
Generation of a km23-1 Tet-off-inducible expression system in SKOV-3 cells
To explore novel functions of km23-1 in TGFβ-resistant HOCCs, we established a km23-1 tet-off-inducible expression system in the highly aggressive and TGFβ-resistant SKOV-3 HOCCs. Western blot analyses in Figure 1a demonstrate induction of km23-1-Flag expression in km23-1 pool cells and in two of the representative clones (#14 and #12) upon Dox removal. In contrast, EV pool cells did not express km23-1-Flag in +/−Dox. We also calculated the levels of induction of km23-1-Flag expression over endogenous levels. Quantitation of densitometric scans of Western blot analyses revealed that relative levels of km23-1-Flag expression after growth in −Dox for 6–14 days were ~9- to 17-fold higher than endogenous km23-1 protein expression levels (data not shown). Thus, the high levels of km23-1 induced in this model were used to perturb the normal functions of the km23-1 DLC and do not reflect physiological levels of km23-1.

km23-1 inhibits the monolayer cell proliferation and anchorage-independent growth of SKOV-3 cells
To test whether induction of km23-1 expression in SKOV-3 cells causes growth inhibition, EV pool, km23-1 pool and clone #14 cells were cultured in +/−Dox, and cell growth was monitored daily by crystal violet assays. The results in Figure 1b (top panel) demonstrated that the proliferation of SKOV-3 EV pool cells was not changed in +/−Dox. However, in km23-1 pool and km23-1 clone #14 cells, cell growth was significantly decreased upon Dox removal (middle and
Flag-positive cells began displaying a mitotic delay. To further examine whether km23-1 overexpression would affect the malignant potential of SKOV-3 cells, we examined the anchorage-independent growth of EV pool, km23-1 pool and km23-1 clone #14 cells in soft agar growth assays. As shown in Figure 1c, the number of colonies formed in soft agar was significantly reduced in km23-1 clone #14 and pool Dox− cells in comparison to Dox+ cells. Therefore, forced expression of km23-1 in SKOV-3 cells inhibited both anchor-age-dependent and -independent growth of the cells.

**Induction of km23-1 expression results in G2/M delay in HOCCs**

To address the mechanism by which km23-1 inhibited SKOV-3 cell growth, we performed FACS analyses on EV pool and km23-1 clone #14 cells cultured in +/−Dox. No significant change in the cell cycle distribution was found in the EV pool cells after Dox removal (Fig. 2a, top panel), whereas the km23-1 clone #14 cells exhibited an increase in the percentage of cells in G2/M from 8.4 to 21.3% at 12 days after Dox removal (Fig. 2a, middle panel). Similarly, Dox removal in km23-1 pool cells resulted in a 1.7-fold increase in the percentage of cells in G2/M (data not shown). FACS analyses of two additional km23-1 clones, clone #10 (Fig. 2a, boxed panel, bottom) and clone #12 (data not shown, approximately twofold increase), further confirmed the effect of Dox removal on the percentage of cells in G2/M. The level of km23-1-Flag induction upon Dox removal in clones #10 and #14 is shown in the Western blot analyses (Fig. 2a, lower panel).

To assess the generality of our findings, two other TGFβ-resistant HOCCs, IGROV-1 and OVC4A33, were transiently transfected with km23-1-Flag, and cell cycle analysis was performed. As shown in Figure 2b, wild-type km23-1-Flag expression was highly induced, and this induction of km23-1-Flag expression resulted in a 2.5-fold increase in the percentage of cells in G2/M in IGROV-1 cells (Fig. 2b, bottom and top panels). Similarly, when km23-1-Flag expression was highly induced in OVC4A33 cells, a twofold increase in the percentage of cells in G2/M was observed (data not shown), confirming the results observed for SKOV-3 cells.

Further analyses of the mitotic index in SKOV-3 Dox+/− cells, using the mitotic-specific marker phospho-histone H3, demonstrated that overexpression of km23-1 in clone #14−Dox cells significantly increased the number of mitotic cells (Fig. 2c, photos). The percentage of cells in mitosis was increased by ninefold when comparing the +/−Dox cells, the difference being statistically significant with a value of p < 0.05 (Fig. 2c, right panel). Together, these results demonstrate that km23-1 exerts its growth regulatory function in TGFβ-resistant HOCCs by delaying cells in mitosis. Thereafter, experiments were performed after 7 days −Dox, when the Flag-positive cells began displaying a mitotic delay.

**Induction of km23-1 expression in HOCCs results in mitotic accumulation of cells before the anaphase transition in prometaphase/metaphase**

As we have shown that km23-1 overexpression causes a mitotic delay in the HOCCs, it was of interest to determine at which phase of mitosis the cells were accumulating. Therefore, we examined markers (such as cyclin B1, phospho-ERK and phospho-MEK) known to be increased in mitosis, but which are degraded or reduced in their activity before the anaphase transition can proceed. As shown in Figure 3a, cyclin B1, phospho-MEK and phospho-ERK remained significantly elevated in SKOV-3 cells upon km23-1 induction, indicating that anaphase onset had not yet occurred in these cells.

Further, to more accurately assess the percentage of cells in the various phases of mitosis in +/−Dox, we performed immunofluorescence analyses of MTs using tubulin staining. As depicted in Figure 3b, the majority of cells with over-expressed km23-1-Flag (+Dox) were in prometaphase/metaphase (65–75%). Altogether, the data suggest that km23-1 overexpression causes cells to accumulate at prometaphase/metaphase.

**Overexpression of km23-1 results in aberrant metaphase cells with altered BubR1 localization and the formation of multipolar spindles and multinucleated cells**

Thus far, our results have shown that forced expression of km23-1 in HOCCs causes a delay in mitosis at prometaphase/metaphase. As there is a critical mammalian checkpoint at this point in the cell cycle, it was of interest to determine whether regulation of this SAC would be altered in the −Dox SKOV-3 HOCCs. Accordingly, we analyzed the subcellular distribution of the SAC protein BubR1 by immunofluorescence analyses of MTs using α-tubulin staining. As shown in Figure 4a, in these −Dox cells at prometaphase, both BubR1 and km23-1 were colocalized at kinetochores (first row). The white staining in the merge shows that some km23-1-Flag and BubR1 are colocalized at the kinetochore at this point. The CREST antiserum stains the inner kinetochores, and the merge (white) demonstrates that some km23-1 is also colocalized with CREST at the kinetochore (second row). The kinetochore localization of BubR1 was also confirmed by costaining of BubR1 and CREST (third row). In contrast, in metaphase cells, multiple spots of BubR1 were detected abnormally localized in both kinetochores and cytoplasm, whereas km23-1 was localized along spindle fibers with little BubR1 colocalization (first row). As expected, km23-1 was detected at the kinetochore (second row) as well as along the spindle fibers (first row), indicating that km23-1 is involved in the transport of cargos such as SAC proteins from the kinetochore to the spindle pole. The aberrant localization of BubR1 was apparent from the photos showing costaining of BubR1 with CREST (third row). The chromatin organization at the metaphase plate was also altered, as depicted by the DAPI staining (blue).
The situation in Dox+ cells, with only endogenous levels of km23-1, is quite different. As shown in Figure 4b, in prometaphase, BubR1 was still localized at kinetochores as expected. As these SKOV-3 cells are not overexpressing km23-1-Flag, there is no Flag staining. However, in these +Dox cells at metaphase, there is no detectable BubR1 at the kinetochore. We have used two different BubR1 antibodies from two different companies, and both of these reveal no detectable BubR1 staining in the Dox+ metaphase cells. To confirm that a decrease in overall BubR1 expression did not account for the absence of kinetochore BubR1 at metaphase in the +Dox cells, we performed Western blot analysis

Figure 2. Overexpression of km23-1 in HOCCs causes a mitotic delay. (a) Upper panel, cells were grown in +/-Dox for 9–14 days, and cell cycle distribution was determined by FACS analysis. Lower panel, Western blotting analysis of Flag expression. The same membrane was reblotted with an anti-GAPDH antibody. (b) Total cell lysates, collected from IGROV-1 cells that had been transfected with km23-1-Flag or EV for 72 hr, were analyzed by Western blot analyses using an anti-Flag antibody. The same membrane was reblotted with an anti-DIC antibody to assess equal loading (top panel). The transfected cells were also fixed and subjected to FACS analysis (bottom panel). (c) Left panels, km23-1 clone #14 cells were fixed, permeabilized and stained as described in “Material and Methods.” Bar = 100 μm. Right panel, the percentages of mitotic cells were quantitated as the number of phospho-histone H3-positive cells per the number of Flag-expressing cells in mitosis. The bars represent the mean ± SE, n = 3; *p < 0.05 relative to +Dox controls.
during the 9–14 days experimental period, and BubR1 levels were not significantly different between the +/−Dox cells (data not shown). Moreover, clear CREST staining indicated that the kinetochore itself was not aberrant in the Dox+ cells, suggesting that the BubR1-related checkpoint has been inactivated.30,31 Overall, our results suggest that the prolongation of prometaphase/metaphase induced by overexpression of km23-1 is associated with the sustained presence of BubR1 at the kinetochore, consistent with a prolonged activation of the BubR1-related SAC.

As a disrupted SAC would not only cause a delay before anaphase but could also lead to the formation of multipolar spindles and multinucleated cells, we investigated the spindle polarities of the HOCCs in +/−Dox. Representative photos of α-tubulin staining of MT spindles are shown in Figure 4c (red), in relation to chromatin staining (DAPI) and Flag staining in the −Dox cells (green). As indicated in Figure 4c (right panel), induction of km23-1 expression in the −Dox SKOV-3 cells showed a statistically significant increase in the percentage of multipolar spindles (threefold compared to +Dox). Similarly, km23-1-overexpressing SKOV-3 cells showed a higher percentage of multinuclear cells (threefold compared to control, Fig. 4d, right panel). Overall, these cellular changes suggest that km23-1 induction affected a mitotic delay associated with multinucleation/multipolarity, phenotypes often observed in mitotic catastrophe.32,33

As a result of our findings suggesting mitotic catastrophe, it was of interest to assess whether the cells were undergoing apoptosis. Accordingly, we measured the annexin V-positive cells in a cumulative fashion during the 9–14 days +/−Dox, when the Flag-positive cells began displaying a mitotic delay. As shown in Figure 4e, we observed an increased number of

![Figure 3. Induction of km23-1 expression in SKOV-3 cells results in accumulation of cells before anaphase at prometaphase/metaphase. (a) Western blot analysis was performed using the indicated antibodies. All data are representative of at least two independent experiments. (b) Upper panels, cells were grown and treated as for Figure 2 and were stained for DAPI, Flag and α-tubulin. Bar = 25 µm. Lower panel, mitotic cells were counted using Flag and α-tubulin staining.]
apoptotic cells in Dox+/Flag-positive cells, compared to the Dox+ cells. Overall, these cellular changes suggest that the high levels of km23-1 may be inducing a gradual apoptotic death process occurring after mitotic delay.32,33

**km23-1 inhibits the in vivo tumorigenicity of SKOV-3 cells**

As our results thus far suggested that the prometaphase/metaphase delay from km23-1 overexpression might be associated with cell death as a result of mitotic delay,32,33 we further assessed the tumorigenicity of SKOV-3 cells overexpressing km23-1. Figure 4 shows the effects of km23-1 overexpression on the SAC and spindle polarity. The images in Figure 4a and 4b illustrate the localization of BubR1 and km23-1 at the kinetochore in prometaphase and metaphase, respectively. The data presented show that km23-1 expression results in a higher percentage of multinuclear cells (Figure 4c) and apoptotic cells (Figure 4e) compared to control cells. The quantification of multipolar spindles (Figure 4d) confirms the mitotic delay induced by km23-1 overexpression.
catastrophe, it was of interest to determine whether over-expression of km23-1 in HOCCs would block tumor formation in vivo. In vivo tumorigenicity studies were performed as described in “Material and Methods.” Tumor volumes for mice inoculated with EV pool cells and fed with Dox+/− chow were not significantly different throughout the experimental period (Fig. 5a). In contrast, tumors from mice inoculated with km23-1 pool or km23-1 clone #14 cells (−Dox chow) were significantly smaller when compared to controls (+Dox chow, Figs. 5b and 5c). Western blot analyses of km23-1 expression in the resected tumor tissues confirmed that km23-1 expression was still induced in the Dox− group at the termination of the experiment (Fig. 5d). Thus, the in vivo tumorigenicities of SKOV-3 cells were substantially inhibited by the sustained high levels of km23-1 expression.

Figure 4. (Continued)
Discussion

Our results demonstrate that forced expression of km23-1 causes a growth-suppressive phenotype in HOCCs. First, induction of km23-1 expression inhibited both the monolayer and anchorage-independent growth of HOCCs. In addition, FACS analyses in SKOV-3, IGROV-1 and OVCA433 HOCCs...
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acting protein and was shown to be required for TGF
dependent Smad2 signaling.6,10,12–14 Further, in cells expressing
Overall then, our results demonstrate that km23-1 suppresses
at G2/M. Furthermore, the phospho-histone H3 and α-tubulin
staining data indicate that the overexpression of km23-1
caused a mitotic delay at prometaphase/metaphase. This was
associated with high levels of phospho-MEK, phospho-ERK
and cyclin B1, indicating that the cells had not yet progressed
to anaphase. Most importantly, km23-1 overexpression
reduced the tumorigenic potential of SKOV-3 cells in a xenograft model. Overall, these results demonstrate that km23-1 suppresses HOCC growth by causing a delay in prometaphase/metaphase and suggest that km23-1 is a critical growth regulator for HOCCs.

km23-1 was originally identified as a TGFβ receptor-interacting protein and was shown to be required for TGFβ-dependent Smad2 signaling.6,10,12–14 Further, in cells expressing normal levels of km23-1, knockdown of its expression was associated with a decrease in the percentage of cells in G2/M and a blockade of dynein-mediated transport.10,12 In contrast, here, we reveal a novel role for this DLC in mitosis. This novel role is distinct from km23-1’s role in TGFβ-mediated growth inhibition that occurs by blocking the cell cycle in late G1 in untransformed epithelial cells.12 The focus of our report is on km23-1’s ability to alter events in mitosis in TGFβ-resistant HOCCs.

Our data demonstrate that km23-1 inhibited HOCC growth by prolonging mitosis with upregulated cyclin B1 protein expression levels and elevated phospho-MEK and phospho-ERK. As the destruction of cyclin B1 starts at the end of prometaphase, with most of the cyclin B1 being degraded by the end of metaphase,34,35 our data suggest that the mitotic delay occurred before anaphase. Further, it has been reported that the poleward transport of MEK1/2 at the SAC in prometaphase/metaphase is dynein mediated.36 Thus, the finding that MEK and ERK remain activated in the km23-1-overexpressing cells suggests that the high levels of km23-1 may be disrupting the normal functions of km23-1 in the dynein-mediated streaming of mitotic checkpoint protein complexes away from the kinetochore to inactivate the SAC.

As mentioned above, the high levels of km23-1 appeared to regulate the HOCCs in a manner consistent with an effect in mitosis at the SAC. In km23-1-overexpressing SKOV-3 cells at prometaphase, km23-1 was colocalized with the SAC protein BubR1 at the kinetochore. In contrast, by metaphase, BubR1 was no longer colocalized with km23-1, but it abnormally remained at the kinetochore. This persistent localization of BubR1 at the kinetochore in metaphase suggests that the SAC remains active in km23-1-overexpressing cells. Persistent localization of BubR1 at the kinetochore with an activated SAC has been reported by others as well.30,31 In contrast, in the HOCCs not overexpressing km23-1, BubR1 was no longer visible at the kinetochore in metaphase, suggesting that it had been removed by the normal dynein-mediated streaming events that occur during inactivation of the SAC.5,5 These cells would then continue on to anaphase. Overall then, our results demonstrate that km23-1 suppresses HOCC growth by causing a delay in metaphase through a BubR1-related mechanism.

In relation to dynein functions at the SAC, to date, most studies have focused on cytoplasmic dynein subunits other than the regulatory/adaptor DLCs (i.e., on the dynein heavy chain and dynein light intermediate chain (DLIC), DIC). For example, the DLIC has been shown to remove the Mad1 and Mad2 checkpoint proteins from the kinetochore during SAC silencing, but not BubR1.32 Perhaps, the km23-1 DLC is normally responsible for stripping BubR1 from the kinetochore, but when it is overexpressed, this is aberrant or lost. There is one report describing the involvement of a member of another class of DLCs (DYNLT3) at the SAC through its interaction with the mitotic checkpoint protein Bub3.38 However, this report did not address a role for DLCs in tumor suppression as we have done herein.

Although overexpression of km23-1 may be affecting BubR1 transport away from the kinetochore to inactivate the SAC, there are other possible interpretations for our findings, because dynein is known to have functions in many aspects of spindle MT dynamics.39–42 For example, the high levels of km23-1 would be expected to alter dynein motor functions in stable MT attachment and maintenance of kinetochore orientation during metaphase chromosome alignment.39,43 Thus, an effect of km23-1 on MT–kinetochore interactions and/or dynein availability at the kinetochore would be consistent with a persistent localization of BubR1 at the kinetochore, which would ultimately result in prolonged SAC activation.

The phenotypic changes we have observed upon overexpression of the km23-1 DLC are consistent with the HOCCs undergoing a process referred to as “mitotic catastrophe” or “cell death preceded by multinucleation/multipolarity.”33,44 For example, we have shown that overexpression of km23-1 in HOCCs causes altered localization of the SAC protein BubR1 at metaphase, in association with the formation of multinucleated and enlarged cells.35,46 In our system, it appears that successive rounds of cell division after mitotic delay eventually lead to cell death, resulting in cell growth inhibition in vitro in monolayer culture and in semisolid medium as well as tumor suppression in vivo.

Overall, our results suggest that km23-1 is a novel mitotic tumor inhibitor for ovarian cancer, functioning at metaphase to alter the localization of the SAC protein BubR1, associated with mitotic catastrophe and eventual cell death. Along these lines, it has recently been reported that targeting the SAC and chromosome alignment simultaneously may selectively kill tumor cells.47 Thus, km23-1-based therapeutics designed to mimic the effects of overexpressed km23-1 may have potential for the treatment of ovarian cancer. Similar approaches have been used for the tumor suppressor p53.48 Alternatively, km23-1-based therapeutics may sensitize tumor cells to clinically relevant doses of taxol, as has been described for other therapeutic approaches.47 Additionally, a combined approach may be required rather than using a single therapeutic modality. This is often necessary because of
the higher complexity of in vivo conditions\textsuperscript{49,50} compared to in vitro responses, as we have seen here. Future studies will address these potential therapeutic approaches.

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Amyloid Fibrillation of Human Apaf-1 CARD†

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ABSTRACT: The idea of establishing the amyloid-like fibrillation tendency of pro- and antisurvival proteins of human apoptotic pathways is relevant for delineating the conditions that lead to aberrant differentiation, development, and tissue homeostasis. As the first step in this direction, we report here that the caspase recruitment domain (CARD) of recombinant human apoptotic protease activating factor-1 (Apaf-1) can be induced to undergo amyloid-like fibrillation. The study was initiated with a set of biophysical investigations into the possibility and in vitro conditions for fibril growth. By scanning the pH-induced conformational transitions, protein stability, and stopped-flow folding–unfolding kinetics, we detected a molten globule (MG) transition of the CARD at pH < 4. In a bid to reduce the surface-accessible hydrophobic patches in the MG state, the CARD monomer undergoes self-association to produce soluble oligomers that serve as precursor aggregates for protofibril formation. The monomer-to-oligomer self-association process is akin to the well-known homophilic CARD–CARD interaction by which CARDS of the same or different apoptotic proteins associate to transduce and regulate the apoptotic signal. The fibrillation reaction of the Apaf-1 CARD was conducted at pH 2.1 and 60 °C, because reduction of exposed hydrophobic surfaces in the MG state is more favored under the moderated solution condition. The Gaussian distributions of diameters of the fibril population suggest values of 2.1 and 2.7 nm for the mean diameter of precursor aggregates and protofibrils or elongated fibrils, respectively.

The supramolecular assembly of a sizable number of diverse proteins and polypeptides into amyloid and amyloid-like fibrils has attracted the attention of many for a number of reasons. First, the pathogenesis of a number of human conditions, including Alzheimer’s disease, Parkinson’s disease, spongiform encephalopathy, Huntington’s disease, senile amyloidosis, and type II diabetes (1–7), is fundamentally associated with amyloids. A detailed understanding of the processes leading to extracellular amyloid deposition, intracellular neurofibrillary tangles, and the development of amyloid toxicity is necessary in devising strategies for therapeutic intervention and management of these diseases. Such β-sheet-based structural assemblies are also promising for industrial application, and in material science and biotechnology (21–26). Second, the observation that a wide variety of non-disease-related proteins and polypeptides that presumably do not undergo amyloid-like transitions in vivo but can be induced to do so in test tubes via a change in the solution conditions (5, 8–13) has added newer dimensions to the multifarious response of proteins to solvent conditions. Given the number and variety of proteins from which amyloids have been formed, it is now generally accepted that amyloid aggregates are a generic structure for all proteins (14). Since the amino acid composition, sequence, and native-state structure are not determinants of amyloidogenesis (2, 14), amyloid fibrillation could originate from anywhere in the conformational landscape, enigmatically as low as the native or natively-like states to as high as unfolded states in a protein-specific manner (2, 15, 16), implying that some combination of the initial structure, number of intramolecular contacts, chain dynamics, and surface dielectric may be required to promote fibrillation and that these factors may also be related to the kinetic mechanism of the composite fibrillation reaction. Our understanding of such issues needs to be augmented. Atomic models of amyloid fibril structure based on various pieces of evidence, each limited by the extent amenable to the experiment, have been discussed (17–20). Although there is a consensus that amyloid and amyloid-like fibrils contain β-sheet conformations, achieving atom-level resolution of arrangements of β-sheet structures has been seriously hindered by the difficulties of handling fibrous supramolecular forms that are often insoluble, especially at concentrations required for structural work. Thus, the specific structural and chemical features of proteins and the solvent-dependent reactivity that promote amyloidogenesis are at the focus of current research.

With regard to the etiology of amyloid cytotoxicity at the molecular level, fewer studies conducted to date have indicated that the fibrils and the precursor β-oligomers trigger apoptosis in cells (27–35). These in vitro studies confined to cultured neurons have relied principally on the observation of cell morphology and biochemical characteristics of apoptosis in response to treatment with soluble fibrils and precursor aggregates (PA).1 The evidence that amyloid-induced activation of an apoptotic pathway is one of the reasons for neural cell death in neurodegenerative diseases is compelling.

1Abbreviations: Apaf-1, apoptotic protease activating factor-1; CARD, caspase recruitment domain; PA, precursor aggregate; GST, glutathione S-transferase; MG, molten globule; ANS, 8-aminonaphthalene-1-sulfonate; GdnHCl, guanidine hydrochloride; ΔG°, Gibbs energy of denaturation in the absence of denaturant; AFM, atomic force microscopy; fwhm, full width at half-maximum; PDB, Protein Data Bank.
We were contemplating the general possibility of an amyloid-like transition of the pro- and antisurvival proteins of mammalian apoptotic pathways, even though at present there is no known amyloidosis that results from aggregation of any of these proteins, wild-type or otherwise. Should any of them undergo amyloid fibrillation, the normal development, tissue differentiation, and homeostasis will be critically affected. To touch upon this possibility, this study reports on the in vitro transformation of the recombinant CARD of human Apaf-1 (Figure 1 into amyloid protofibrils. Apaf-1, a large antisurvival protein (~130 kDa), is the key molecule for activation of procaspase-9 in the mitochondrial pathway of apoptosis in neuronal and somatic cells alike (36, 37). Structurally, the Apaf-1 CARD consists of six tightly packed amphipathic α-helices [Figure 1 (38)]. The CARD is used because this domain is present as the N-terminal prodomain in a sizable set of apoptotic proteins, including some caspases, and CARDS are known to mediate apoptotic signaling through homophilic CARD–CARD interactions (38–42). To arrive at the in vitro conditions for transformation of the CARD protein to fibrils, we conducted a series of biophysical experiments as the prelude. The results of pH-dependent conformational transitions, protein stability, and folding–unfolding kinetics showed that the Apaf-1 CARD undergoes a MG-like transition under acidic conditions. This low-pH-denatured form of the CARD then undergoes further conformational transitions to produce soluble PA of amyloid-like protofibrils.

MATERIALS AND METHODS

Cloning and Generation of the CARD Expression Construct. Total RNA isolated from HeLa cells was used for the cDNA amplification of the 300 bp gene fragment of Apaf-1. Forward and reverse CARD primers were designed with pGEX4T-1 compatible restriction sites at their 5’ ends. The forward primer has a BamHI site and the reverse a XhoI site: CARD-F, 5’ CCGGATCCATGGATGCAAAAGCTCGAA 3’ (BamHI site underlined); CARD-R, 5’ CCTCGAGCTAAGAGAGACACAGGAATG 3’ (XhoI site underlined). The cDNA was synthesized by reverse transcriptase, and the 300 bp CARD fragment was amplified by Taq DNA polymerase. Both steps were achieved by using the “one-step RT-PCR kit” from ABGene Technologies. The PCR-amplified fragment was isolated by the standard procedure, ligated into the TA cloning vector, and transformed into DH5α bacterial cells by the standard CaCl2 procedure. The transformed cells were plated onto LB agar plates with 100 μg/mL ampicillin and 1 mM IPTG. The positive clones, selected by blue-white screening and colony PCR, were used for isolation of plasmid DNA by standard protocols.

The PCR fragment cloned into the TA cloning vector was restricted using BamHI and XhoI and analyzed on a 1% agarose gel. The pGEX4T-1 expression vector was also restricted in the same manner. The CARD gene fragment was then ligated into digested pGEX4T-1 by using T4 DNA ligase and transformed into DH5α cells. Cell colonies positive with the recombinant plasmid were screened by colony PCR. That the recombinant plasmids contained the expression vector carrying the insert was once again checked by restriction digestion. The sequence and orientation of the CARD fragment were confirmed by sequencing.

The restriction-positive plasmids were transformed into Escherichia coli BL21 cells for expression of the CARD protein.

Protein Expression and Purification. LB broth (500 mL) with 100 μg/mL ampicillin was inoculated with 25 mL of a culture grown overnight (1:20 ratio) that contained the recombinant positive clone. Protein expression was induced at an OD600 of 0.5 with 1 mM IPTG. Cells were grown for 5 h at 37 °C. Cells were pelleted, resuspended in 20 mL of PBS, and lysed by sonication for 3 min with repetitive 30 s on and off cycles. The lysate was centrifuged at 3000g for 30 min at 4 °C. The supernatant was collected, and DTT was added to a final concentration of 1 mM. This was loaded onto a 1 mL GSH Sepharose 4B column that was pregelatinized with 10 volumes of PBS. The column was washed with 20 volumes of PBS. The GST–CARD protein that was bound to the column was eluted with 10 volumes of elution buffer [50 mM Tris-HCl and 10 mM reduced glutathione (pH 8)]. The purified GST–CARD protein was dialyzed against 1× PBS to remove the glutathione and digested with recombinant thrombin (Amersham Biosciences) by standard protocols. Following digestion, thrombin was removed by benzamidine Sepharose, and the protein mixture was loaded onto a GSH-Sepharose 4B column. The 10 kDa CARD protein was eluted in the flow-through and was purified further by Sephadex G-75 chromatography.

Equilibrium Measurement of CARD Stability toward pH, NaCl, and GdnHCl. For these titrations, separate samples with identical protein concentrations (5–7 μM) were employed. Protein solutions held at different values of pH or NaCl concentration were incubated for ~6 h before fluorescence spectra were recorded. For GdnHCl unfolding, samples containing different concentrations of the denaturant were prepared by mixing two stock protein solutions, one with 4 M GdnHCl and the other without. Buffers for various pH ranges were as follows: 50 mM glycine for pH < 3, 50 mM sodium acetate for pH 3–5, HEPES, PIPES, and Tris (17 mM each) for pH 6–9, and glycine and CAPS (25 mM each) for pH 9–11. All experiments were conducted at 22 °C using a Fluoromax-4 (Horiba Jobin Yvon) instrument.

Stopped-Flow Kinetics of CARD Folding and Unfolding. These experiments involved two-syringe mixing and invariably employed 8-fold dilution of a 10 μM protein solution with the relevant buffer. For refolding, the protein initially unfolded in 5.4 M GdnHCl and after equilibration for ~1 h was mixed with the refolding buffer containing a variable amount of GdnHCl. Unfolding was initiated by diluting the native protein solution with the unfolding buffer containing desired concentrations of
RESULTS

Apaf-1 CARD Expression System and Recombinant Protein. Initially, we generated two gene constructs for *E. coli* expression of the human Apaf-1 CARD, one with an N-terminal His6 tag (pET28a vector) and the other with GST fused at the N-terminus (pGEX4T-1 vector), but the latter was chosen for the production of the recombinant protein because of the ease and convenience of purifying GST-fused small proteins. This expression vector was used in earlier studies of the NMR solution structure of the Apaf-1 CARD (38), although mutagenesis-based cloning and expression in the pET-3d vector have also been reported (43). As Figure 2 shows, the recovered CARD (∼6 mg/L of *E. coli* culture) is homogeneous and highly pure. Sephadex G-75 chromatography consistently show that the purified CARD is monomeric.

Different pH Forms of the CARD. The rationale for examining CARD conformational changes as a function of pH was that partly denatured proteins at acidic and alkaline pH can often reveal structural and functional regulation of proteins. A contextual example is low-pH dimerization of members of the apoptotic Bcl-2 family of proteins which possibly leads to ion channel formation in synthetic membranes (44). For the Apaf-1 CARD, Figure 3a shows the general trend of fluorescence decrease with an increasing pH. The primary structure of the CARD has no tryptophan, and the observed fluorescence with a λ_{max} of 309 nm, likely due to tyrosine, is weak. The absence of any shift in the fluorescence maximum across the pH range may be due to the very similar polarity of the environment of tyrosines under native and denaturing conditions. Closer examination of the pH dependence of the 309 nm fluorescence (Figure 3a) shows a pronounced dip at pH ∼4 on either side of which the fluorescence signal increases. In the pH range of 5–8, the fluorescence remains unchanged but decreases gradually for pH > 8. The data thus indicate three pH-induced transitions: an alkaline
The CARD monomers possibly oligomerize by hydrophobic interactions. Now, the shortage of hydrophobic surface results in fewer ANS molecules bound, and hence a decrease in fluorescence.

To further extend our understanding of the CARD conformational changes, we improved the pH-dependent fluorescence experiment by including the NaCl concentration as another variable (Figure 4a). Clearly, all three transitions seen in Figure 3b, the alkaline, the acidic, and the MG-like transitions, are reproduced in the presence of any concentration of NaCl used in range of 0–1 M (Figure 4a). In addition, NaCl sets the fluorescence amplitude in a pH-dependent manner (Figure 4b). At intermediate pH values, the fluorescence slightly increases with NaCl but decreases prominently at acidic and alkaline values most likely due to electrostatic screening of protein charges by Cl⁻ and Na⁺ ions, respectively. It means that the added Cl⁻ and Na⁺ ions render the acid or alkali-denatured expanded conformations compact by weakening the electrostatic charge—charge repulsion in the molten globule state (45), and a transition from the expanded to compact molecular conformation is expected to decrease the fluorescence intensity (see below). Thus, at pH ~2, the acid-denatured state is transformed to a molten globule state. In summary, the CARD at acidic pH (<4) undergoes a major denaturational transition accompanied by a substantial exposure of otherwise buried hydrophobic surfaces. Under strongly acidic conditions (pH <2), the denatured monomers interact with each other possibly by hydrophobic interactions to produce soluble oligomers or aggregates. We have called them precursor aggregates (PAs).

**Equilibrium and Kinetic Aspects of Folding of the Apaf-1 CARD at Acidic and Neutral pH.** To learn more about the influence of pH on the stability and structure, we examined the GdnHCl-induced folding behavior of the protein by equilibrium and stopped-flow kinetic methods. Figure 5a shows the equilibrium unfolding transition at pH 6 and 3.1. At pH 6, the initial increase of fluorescence in the pretransition region is followed by a relatively sharp drop in the unfolding transition region. The structural details associated with these changes are subject to scrutiny. For now though, a fit of the data to the two-state N ↔ U model (46), where N and U are native and unfolded states, respectively, by assuming a second-order polynomial dependence of the pretransition fluorescence with GdnHCl yields the protein stability (ΔG° = 12.5 ± 0.5 kcal/mol) and the transition midpoint (Cm ≈ 2.4 ± 0.1 M). In an earlier study of CARD unfolding in the presence of urea, a value of ~6 kcal/mol was reported for ΔG° (47). Generally, the ΔG° value determined by urea unfolding is considerably lower than that extracted from GdnHCl unfolding. Part of the discrepancy also arises from the large increase in the fluorescence in the pretransition baseline (Figure 5a) which the earlier authors did not notice in their study using urea (47). At pH 3.1, a clear unfolding transition of the CARD is not detected. The change in the fluorescence stretches out for >1.5 M GdnHCl, indicating less compactness and the lack of well-defined structural elements typical of an acid-denatured state in the absence of added anions (45). The monotony of the fluorescence change might simply reflect expansion of the chain. To show that the unfolded state in the presence of 4 M GdnHCl does not contain any considerable structure, the protein was titrated in the pH range of 1–7.5, holding the denaturant concentration constant at 4 M (Figure 5b). Within the error limit, the fluorescence hardly changes in the pH range of 2–7.5, suggesting that unfolding is complete at 4 M GdnHCl.
Figure 5: pH-dependent stability and folding kinetics of the Apaf-1 CARD. (a) GdnHCl-induced equilibrium unfolding at pH 6 (■) and 3.1 (■). At pH 6, the global unfolding transition is preceded by a pretransition zone characterized by a substantial increase in fluorescence. Since the details of CARD structural changes that occur in the pretransition region are unclear at the moment, the data were modeled with a two-state N ⇌ U transition by assuming that the pretransition changes are due to solvent-dependent baseline effects having a second-order polynomial dependence for fluorescence changes with GdnHCl. The fit of the data yields a ∆G° of 12.5 ± 0.5 kcal/mol and a Cm of ∼2.4 ± 0.1 M (see the text). At pH 3.1, no pronounced global transition is apparent, suggesting the absence of well-defined tertiary structure. (b) pH titration of the 4 M GdnHCl-unfolded protein. (c) Representative kinetic traces for refolding of the CARD in the presence of 1 M GdnHCl (pH 6) or 0.7 M GdnHCl (pH 3.1). For both experiments, the protein was initially unfolded in 5.4 M GdnHCl at the respective pH. The refolding at pH 6 is described by two rising exponentials: k_{fast} = 60 s⁻¹ and k_{slow} = 1.8 s⁻¹ with fractional observed amplitudes of 0.7 and 0.3, respectively. At pH 3.1, there is a fast decaying phase (k_{fast} = 262 s⁻¹) followed by a slow rising phase (k_{slow} = 2 s⁻¹), suggesting the possible formation of an aggregate or misfolded intermediate at early times of refolding. (d) Residuals of the two-exponential fits. (e) GdnHCl dependence of the apparent rates for the major phase (top chevron) and the minor phase (bottom chevron) at pH 6. In each chevron, the data forming the left (darker symbols) and right (lighter symbols) arms represent refolding and unfolding, respectively. The rate rollover in the folding arm of each chevron is classically taken as one of the indicators of the presence of folding intermediates. (f) At pH 3.1, the GdnHCl dependence of the apparent rate for the fast kinetic phase (■) shows an unfolding event the rate of which rolls over at >1 M GdnHCl. This phase may arise from protein aggregation or misfolding. The slow phase (bottom chevron) also barely shows protein refolding at this pH, suggesting the absence of well-defined tertiary structure and packing.

Figure 5c shows two representative kinetic traces for refolding of the CARD, one at pH 6 and the other at pH 3.1, both initially unfolded in 5.4 M GdnHCl at the respective pH and refolded in the presence of 1 M GdnHCl for pH 6, and 0.7 M GdnHCl for pH 3.1. Both traces are best fit by two exponentials, and the residuals are shown in Figure 5d. At pH 6, the two kinetic phases have the same sign for amplitudes and are associated with refolding. At pH 3.1, only the slow rising phase is indicative of refolding. The initial phase which is faster than the slow phase by at least 2 orders of magnitude and during which the fluorescence decays is associated with an unfolding event (Figure 5c). The GdnHCl distributions of the apparent rate constants (k_{obs}) for both kinetic phases of refolding and unfolding at pH 6 are shown in Figure 5e. We note that the GdnHCl concentrations corresponding to the rate minima for the fast and slow phases are ∼2.1 and ∼2.5 M, respectively. Except for the unfolding by the slow kinetic phase, the rates clearly roll over as strongly native-like and strongly unfolding conditions are approached. Classically, multiple chevrons indicate the existence of interconverting ensembles of unfolded conformations with ensemble-specific refolding rates producing parallel folding rates, and chevon rollover is thought to arise from accumulation of kinetic intermediates (48). For the Apaf-1 CARD, the two distinct chevrons with limb rollovers then suggest the occurrence of two ensembles of unfolded conformations, where one folds faster than the other. The two ensembles are unlikely to originate from proline cis ⇌ trans isomerization, because the rate constant for the observed slow folding–unfolding phase across the range of the denaturant concentration is much faster than the canonical rates for proline isomerization-related folding events (49, 50). The folding routes for both fast folding (U_F) and slow folding (U_S) ensembles involve kinetic intermediates, but the number of intermediates involved in the folding of U_F and U_S cannot be determined with the data at hand. Available results allow the description of Apaf-1 CARD folding by the following basic model

\[ I_{F,i} \rightarrow I_{F,i+1} \rightarrow \cdots \rightarrow I_{F,n} \rightarrow N \]

where I_{F,i} and I_{S,i} represent intermediates. Except for the kinetic intermediates invoked here, this model is consistent with the one proposed earlier on the basis of the kinetic study of Apaf-1 CARD folding (47). That work employed urea as the denaturant unlike GdnHCl used here and observed only one chevron with no rate rollover in the limbs. Part of the discrepancy in the results may arise from the use of two different chemical denaturants. Our study provides direct evidence of distinct
unfolded-state ensembles based on the finding of two distinct chevrons. Providing further details of the kinetic mechanism, including the appropriateness of the parallel folding routes depicted here, will require characterization of the $U_F \sim U_s$ equilibrium.

Figure 5f shows the rate-denaturant distribution for CARD folding and unfolding at pH 3.1. At all concentrations of GdnHCl, the observable fast phase in the stopped-flow kinetics is due to unfolding. This is also the case with the slow phase when the GdnHCl concentration is greater than $\sim 0.3$ M. Importantly, the GdnHCl dependences of both rates are associated with considerable positive slope or kinetic $m$ value (given by $m_u = 2.3RT \log k_u/[GdnHCl]$, where $k_u$ is the apparent rate constant of unfolding), indicating that the CARD at pH 3.1 is still substantially structured with a defined core. Also, as it happens at pH 6, the unfolding rates at pH 3.1 roll over under strongly unfolding conditions, suggesting the occurrence of two ensembles of unfolded conformations and an unknown number of kinetic intermediates as depicted in the folding model given above. It thus appears that the structure and topology of CARD at pH 3.1 resemble those of the pre-molten globule state characterized by fluctuating structural elements (45).

Kinetics of Protop fibril Formation for the Apaf-1 CARD. The indication provided by ANS fluorescence results (Figure 3d) that the acid-denatured and MG-like CARD can form soluble oligomers or PAs at pH $\sim 2$ led us to examine whether the precursors have the propensity to grow into protofibrils. Since the dye ThT is specifically used to probe amyloid fibrils (59), we incubated a 15 $\mu$M CARD solution containing 50 $\mu$M ThT at 60°C held at pH 2.1 in Gly-HCl buffer and periodically measured the time dependence of the dye fluorescence up to 100 h. As Figure 6 shows, following a lag time of $\sim 9$ h, the fluorescence increases in a single-exponential phase with an apparent rate constant of 0.04 h$^{-1}$, suggesting the formation of amyloid fibrils (53). Generally, the presence of the lag phase and the fibrillation rate both depend on the protein concentration as well as the incubation temperature and buffer conditions used. This should hold for the formation of CARD fibrils also, although we have not explored conditions that would reduce the lag time or increase the rate of fibril formation.

**Images and Dimensions of Apaf-1 CARD Fibrils.** The formation of fibrils was confirmed by direct images of CARD samples held at pH 2.1 and incubated at 60°C for varying periods of time (Figure 7). By using $\sim 10$ $\mu$M protein, we see signs of elementary combination of PA within $\sim 15$ min (Figure 7a, panel 1). The growth into protofibrils requires several hours, shown here at the end of 8 h (panel 2), and longer fibrillar structures begin to appear after incubation for $\sim 14$ h (panel 3). At $\sim 100$ $\mu$M protein, the fibrillation kinetics was very rapid (Figure 7b). The rich lattice of amyloid fibrils observed after incubation for 1 h (panel 1) becomes denser after $\sim 3$ h (panel 2) but appears diffused and dull at longer times, shown here for 9 and 20 h (panels 3 and 4, respectively).

To achieve a dimensional distinction of PA, protofibrils, and large fibrils, we determined the Gaussian distribution of diameter $d$ for each population. The diameters measured in 116, 44, and 68 readings for PA, protofibrils, and large fibrils, respectively, were arranged into groups of 0.25 nm increments, and the percent population falling in each diameter group was determined (Figure 8). The solid lines through the data are three-parameter Gaussian fits according to

$$P(d) = P(d_o) \exp \left[-0.5 \left(\frac{d-d_o}{b}\right)^2\right]$$

where $P(d_o)$ is the amplitude corresponding to the mean diameter $d_o$ and $b$ is the full width at half-maximum (fwhm). The value of $d_o$ for PA is 2.1 nm versus 2.7 and 2.63 nm for protofibrils and elongated protofibrils, respectively. This dimensional difference between the initial PA and the fibrils should arise from differences in the content of presumably $\beta$-sheet. The fwhm values (1.5, 1.6, and 1.25 nm for the distributions corresponding to PA, protofibrils, and elongated protofibrils, respectively) indicate population inhomogeneity, being largest for protofibrils and smallest for large fibrils. The differences in the inhomogeneity may partly arise from the fact that structurally PA and fully grown fibrils are characterized by $\alpha$-helical and $\beta$-sheet content, whereas the protofibrils at the initial formation stages contain both in a proportion different from one set of populations to another (large fwhm).

**DISCUSSION**

Examinations of the pH-induced conformational transitions and folding stability of the recombinant human Apaf-1 CARD have shown that the acid-denatured protein self-associates to form soluble precursor aggregates which can combine and undergo structural transitions to form amyloid protofibrils.

**Soluble Oligomers and Prototibrils of the Apaf-1 CARD.** Many pro- and antisurvival proteins of the apoptotic machinery are known to homodimerize, heterodimerize, and even homooligomerize to exert their survival and death effects (54–62). Some are constitutively oligomeric because of the ready accessibility of the interacting surfaces, and others are prevented from oligomerization by sequestration of the interacting surfaces until functional activation occurs. The former class is exemplified by the prosurvival protein Bcl-xl, for which a large fraction of the cellular population homodimerizes by homophilic interaction of the C-terminal hydrophobic tails (44, 62–64), and the latter is represented by quiescent Apaf-1 known to exist in the monomeric form (36, 65). It appears that the structural surface of the CARD of Apaf-1 required for homophilic interaction with the CARD of procaspase-9 (38) is partially buried in the inactive state of Apaf-1 due to intramolecular interactions, an observation based on the crystal structure of WD40-deleted Apaf-1 (66). When activated by binding with cytochrome c and dATP (65), Apaf-1 forms a wheel-shaped homoheptamer complex or apoptosome having 7-fold symmetry (67, 68). Although atomic details and packing interactions for the apoptosome are not known, the CARD can now interact with the CARD of procaspase-9. Thus, homoheptamerization of Apaf-1 seems to be crucial for sensitizing the CARD for binding interactions.
However, the Apaf-1 CARD alone in neutral-pH solutions exists in the monomeric state (Figures 3–5), consistent with the earlier report that no dimerization occurs even at concentrations as high as 1 mM (38). On the other hand, the isolated Apaf-1 CARD forms a complex with the isolated caspase-9 CARD in which the former deploys sets of acidic and hydrophobic residues in a manner that creates a contiguous binding surface, suggesting that intermolecular associations of the Apaf-1 CARD require both electrostatic and hydrophobic interactions (38). Should the Apaf-1 CARD alone undergo homo-oligomerization, the operative forces of interaction must be hydrophobic in nature, because surface charge complementarity is not available. The existence of the Apaf-1 CARD in the monomeric form in the neutral-pH region suggests that the hydrophobic interactions afforded by the native protein surface are insufficient for dimerization to occur. The situation is quite interesting under acidic conditions (pH 3–4) where the CARD undergoes a MG-like transition. Relative to that in the native state, the molecular surface in the MG state is more hydrophobic as evidenced by binding of small nonpolar molecules like ANS (Figure 3b,c). The increased surface hydrophobicity should favor CARD–CARD self-association, but electrostatic repulsion due to excess positive charge and large-scale fluctuations of the structural elements in the MG-like state (45) are the principal opposing forces. A dramatic increase in surface hydrophobicity can occur under strongly acidic conditions (pH <2.5) where the already weakened structural elements of the CARD MG break down, causing exposure of buried nonpolar residues. We believe the preponderance of hydrophobic surfaces in the acid-denatured proteins is now so overwhelmingly favorable toward CARD oligomerization that electrostatic repulsions are subdued, and a monomer equilibrium is readily established. The oligomerization event can be considered a transition to an alternative non-native global free energy minimum (69). We should also note that protein oligomerization by hydrophobic interactions at low pH very likely buries some positive charges in the protein–protein apolar interface, and because charge burial in the low-dielectric apolar environment is energetically expensive, the CARD oligomers are unlikely to be very highly stable.

These soluble oligomers are precursor aggregates (PAs) which grow in size to form elongated protofibrils (PA → protofibril). We have not investigated the mechanism and the events associated with the PA → protofibril condensation in this work, but the CARD fibrillation pathway may be depicted as

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**Figure 7:** AFM images of Apaf-1 CARD aggregates. (a) A 10 μM protein solution incubated at 60 °C and pH 2.1 shows the first signs of combination of the PA within ~15 min (panel 1). The growth and elongation of protofibrils are shown in panels 2 and 3 imaged after incubation for 8 and 14 h, respectively. Yellow, blue, green, and white arrows denote PA, elongated PA, nascent protofibrils, and elongated protofibrils, respectively. (b) At ~100 μM protein, fast and rich growth of fibrils is observed. Images shown in panels 1–4 were recorded with samples incubated for 1, 3, 9, and 20 h, respectively. An enlarged view of a small area is shown below each panel.
The CARD is a small domain of Apaf-1, and it independently restricted likelihood of Apaf-1 fibrillation in vivo. Even this like-undergo fibrillation, albeit under nonphysiological conditions, on proteins on one hand and the finding that the CARD couldrios (the absence of specific ev idence for amyloidosis of apoptotic to be empirically determined. Facing the two counteracting scena-

ons and stronger hydrophobic interactions between them, respectively. Such conditions are certainly not physiological, but one cannot exclude yet unknown intrinsic cellular factors, accidental biochemical insults, or pathological conditions that could promote formation of soluble oligomers of Apaf-1 required for protofibril growth. As mentioned earlier, fibril growth may require some combination of the initial molecular structure, packing density, bonding, intramolecular dynamics, and surface dielectric (5, 8—13). Since the underlying physicochemical principles of fibril growth are not fully understood, the conditions that might promote fibrillation in vivo need to be empirically determined. Facing the two counteracting scen-

mg fibrillation. The low pH and high temperature encourage higher dynamics, and surface dielectric (513). A comprehensive model for packing and hydration for amyloid fibrils of β2-microglobulin. J. Biol. Chem. 284, 2169—2175.

The low pH and high temperature encourage higher surface hydrophobicity for the monomers and stronger hydrophobic interactions between them, respectively. Such conditions are certainly not physiological, but one cannot exclude yet unknown intrinsic cellular factors, accidental biochemical insults, or pathological conditions that could promote formation of soluble oligomers of Apaf-1 required for protofibril growth. As mentioned earlier, fibril growth may require some combination of the initial molecular structure, packing density, bonding, intramolecular dynamics, and surface dielectric (5, 8—13). Since the underlying physicochemical principles of fibril growth are not fully understood, the conditions that might promote fibrillation in vivo need to be empirically determined. Facing the two counteracting scen-

MG → PA → protofibril → elongated protofibril. The inference that the MG-like conformation facilitates PA formation is consistent with the survey-based study which showed that the amyloidogenic conformation shares many structural and dynamic properties with the pre-molten globule state (2).

Relevance to in Vivo Fibrillation of Apaf-17. Although we obtained amyloid fibrils from the acid-denatured CARD at an elevated temperature, the question is the relevance this has for in vivo situations, given that none of the pro- and antisurvival proteins is known to undergo fibrillation. This study rests on a limited empirical search for an in vitro condition that facilitates fibrillation. The low pH and high temperature encourage higher surface hydrophobicity for the monomers and stronger hydrophobic interactions between them, respectively. Such conditions are certainly not physiological, but one cannot exclude yet unknown intrinsic cellular factors, accidental biochemical insults, or pathological conditions that could promote formation of soluble oligomers of Apaf-1 required for protofibril growth. As mentioned earlier, fibril growth may require some combination of the initial molecular structure, packing density, bonding, intramolecular dynamics, and surface dielectric (5, 8—13). Since the underlying physicochemical principles of fibril growth are not fully understood, the conditions that might promote fibrillation in vivo need to be empirically determined. Facing the two counteracting scen-


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Figure 8: Distribution of population diameters. The solid lines are three-parameter Gaussian fits to the measured data according to eq 1. (a) PA with a mean diameter d, of 2.1 nm. (b) Prototibrils and elongated prototibrils with d, values of 2.7 and 2.63 nm, respectively.


Bacterially expressed recombinant WD40 domain of human Apaf-1

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WD40 domain

A B S T R A C T

The apoptotic protease activating factor (Apaf-1) is a protein that binds to cytochrome c, and in the presence of dATP/ATP oligomerizes to assume the role of an adaptor platform for activating the caspase-9zymogen. In order to study the biochemical and structural details of Apaf-1 function, we have generated an expression construct from pcDNA 3-Apaf-1XL for production of the WD40 domain (WD40Apaf-1) in Escherichia coli. The WD40 domain expressed contains 825 amino acids in addition to an N-terminal His tag derived from the cloning vector. The expressed protein is invariably localized in the inclusion body fraction of E. coli. A simple protocol involving Sephadex G100 chromatography developed for purifying the protein starting from inclusion bodies has allowed protein recovery in highly pure form. Basic fluorescence and CD spectra indicate that the refolded protein has extensive secondary and tertiary structures. Immunoprecipitation studies have provided qualitative information about the binding interaction of [10–12]. A theoretical model for Apaf-1 heptamer assembly has also been reported [13]. However, there exists an acute dearth of atomic-level structural descriptions of Apaf-1 and its multimeric complex, although NMR solution structure of the CARD domain [14] and a 2.2 Å crystal structure of an ADP-bound CARD and CED-4 homologous domains together [15] have been reported.

The WD40 domain at the C-terminal part accounts for approximately 66% of Apaf-1 primary sequence. The finding of at least 12 WD40 repeats in this domain aroused interest right from the seminal stage of Apaf-1 discovery [8]. WD-repeat proteins invariably assume a β-propeller fold and are thus structurally related, but their functions remain poorly understood [16,17]. Nonetheless, the large content of Asp in the WD40 domain of Apaf-1 together with the finding that Apaf-1 binds to cytochrome c, a basic protein rich in Lys, points to the possibility that Asp–Lys charge–charge interactions form the basis for Apaf-1 binding to cytochrome c. There is no definite structural support for this possibility except for the demonstration that ionic strength has a profound effect on the interaction [18]. In view of the importance and interest in the structure, binding epitope, and functional regulation of Apaf-1, it is imperative that recombinant WD40 domain is made available. Proteins the size of the WD40 domain (825 amino acid residues for the present study) are often difficult to produce in a bacterial overexpression system.

As a part of our structural and functional studies of proteins involved in mitochondrial apoptosis, we have developed an expression construct for Escherichia coli expression of WD40Apaf-1.
The gene construct excludes the N-terminal contiguous CARD and CED-4 homologous domains whose cloning and *E. coli* expression was described recently from this laboratory [19]. Here, we report on the cloning, *E. coli* expression, refolding, and cytchrome c binding properties of the WD40-repeat domain of the human Apaf-1XL isoform, called $^{\text{WD40}}$Apaf-1 henceforth.

**Materials and methods**

**Cloning and generation of expression construct for $^{\text{WD40}}$Apaf-1**

The gene sequence encoding the 2.5 kb $^{\text{WD40}}$Apaf-1 (amino acid residues 424–1248) region was specifically amplified from pcDNA 3-Apaf-1XL, a generous gift from Gabriel Nunez’s laboratory, by standard PCR reaction. The forward and reverse primers flanking the WD40 region of Apaf-1XL were:

- **WD40-F:** 5′ CGGGAATCC TTTATCTGATCGGAAATGG 3′
- **WD40-R:** 5′ CCTCGAG GTTATTCGTAAGTCTGTAATATATA 3′

Restriction sites were included at the 5′ end of each primer to facilitate cloning into pRSETa vector (Invitrogen), and the region of ∼2.5 kb corresponding to $^{\text{WD40}}$Apaf-1 was amplified using MBI-Fermentas long PCR enzyme mix. The 50 μL reaction mixture contained 5.0 μL 10× long PCR buffer, 10 μM each of forward and reverse primers, 100 ng of pcDNA 3-Apaf-1XL as template, and 2.5 U/μL of long PCR enzyme mix. The PCR amplification involved initial denaturation at 94 °C for 2 min, followed by 30 cycles each consisting of a 30-s denaturation at 94 °C, a 30-s annealing at 55 °C, and a 2.5-min extension at 72 °C. To facilitate TA cloning, the final extension was allowed for 15 min at 72 °C. The amplified PCR product was analyzed by agarose gel electrophoresis. The 2.5 kb $^{\text{WD40}}$Apaf-1 fragment was sliced from gel, eluted by using Qiagen gel extraction column, ligated into the TA vector pTZ57R/C24, and transformed into DH5α *E. coli* cells.

The cells were plated onto agar plates containing 100 μg/mL ampicillin.

Positive recombinant clones of $^{\text{WD40}}$Apaf-1 were selected by colony PCR. Plasmids were isolated and digested with BamH1 and Xho1 restriction enzymes. The digested fragments were run on 1% agarose gel, and the digested 2.5 kb $^{\text{WD40}}$Apaf-1 was sliced from gel and eluted by gel extraction columns. This was ligated between the BamH1 and Xho1 sites of pRSETa expression vector (Invitrogen). The recombinant pRSETa–$^{\text{WD40}}$Apaf-1 clones were selected by colony PCR, and confirmed by restriction digestion.

**Protein expression**

The recombinant pRSETa–$^{\text{WD40}}$Apaf-1 plasmids were sequenced before transforming into *E. coli* BL21 DE3 cells. Many singly isolated colonies were selected and screened for soluble expression of $^{\text{WD40}}$Apaf-1. Unfortunately, we could not obtain any soluble expression of the protein. Use of different *E. coli* strains, and variable growth conditions, including temperature and IPTG concentration, did not ameliorate the situation. Facing this difficulty, we decided to proceed with inclusion bodies.

**Cell growth and harvest, cell lysis, and inclusion body washing**

These steps were carried out using procedures already described for $^{\text{AWD40}}$Apaf-1 [19] with minor modifications. Briefly, *E. coli* BL21 DE3 cells containing the pRSETa–$^{\text{WD40}}$Apaf-1 plasmid were grown in LB medium containing ampicillin (100 μg/mL). Typically, 10 mL of an overnight grown culture was added to a 1-L medium, and grown up OD$_{600}$ = 0.5 at 37 °C. Protein expression was then induced by adding IPTG to a final concentration of 1 mM, and growth was continued for 5 h. Cells were harvested by centrifugation at 4000g for 10 min at 4 °C, washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0), and frozen-stored at −80 °C. For lysis, 2 g of the cell pellet (wet weight) obtained from 1-L culture was suspended in 20 mL of the lysis buffer (20 mM Tris–HCl, 100 mM NaCl, 1 mM PMSF, pH 8.0, containing 10 μL of 1 mg/mL DNaseI), and sonicated at 4 °C with 10 cycles, each cycle consisting of 30 s on and 60 s off times. The lysate was treated with deoxycholic acid (4 mg per gram weight of *E. coli*), stirred for 30 min at room temperature, and centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was resuspended in −9 volumes of a buffer consisting of 20 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0, stirred at room temperature for 5 min and centrifuged at 10,000 rpm for 15 min at 4 °C. The step was repeated. Inclusion bodies were then washed with ∼10 volumes of 20 mM Tris–HCl containing 100 mM NaCl at pH 8.0 three times to remove the Triton X-100. In the effort to remove the unwanted materials as far as possible, a final wash was given with ∼10 volumes of 20 mM Tris–HCl containing 2.5 M GdnHCl at pH 8.0. Washed inclusion bodies are now ∼95% pure.

**Finding optimal pH for refolding**

To measure the optimal pH for refolding, the inclusion bodies were first unfolded in buffer A (50 mM Tris–HCl, 0.15 M NaCl, 5 mM EDTA, 5 mM DTT, 6.0 M GdnHCl, pH 8.0), incubated for 2 h at room temperature, and spun at 20,000g for 15 min. The supernatant was then diluted 10-fold by adding buffer B (refolding buffer: 0.5 M NaCl, 0.25 M arginine, 0.5 M urea, 1 mM EDTA, and 1 mM DTT in 50 mM Tris–HCl for the 7–10.5 range of pH values, and in 50 mM sodium acetate for the 4.0–6.5 range of pH values). In all of these refolding trials, the final protein concentration was ∼100 μg/mL. The refolded proteins samples were incubated for 2 h at 4 °C. The refolding efficacy was determined spectrophotometrically by turbidity measurement at 450 nm. By observing a dramatic decrease in turbidity at pH > 8.5, we decided to refold the protein at pH 8.8. It should be noted that attempts to refold the protein at lower pH values showed little success. However, in the steps followed for purification of the refolded protein, pH 8.0 was employed (see below). The multitude of refolding conditions checked by the turbidity measurement also suggested the inclusion of 400 mM arginine and 2 mM DTT in the refolding buffer. The buffer B mentioned above then would contain 0.4 M arginine, 2 mM DTT, and 0.1% glycerol.

**Preparative refolding and purification of $^{\text{WD40}}$Apaf-1**

Inclusion bodies containing ∼34 mg protein were solubilized in 2.5 mL of the unfolding buffer (buffer A as described above). Following 4 h of incubation at room temperature, the solution was centrifuged at 20,000g for 15 min at 4 °C. For refolding, the supernatant was diluted 10-fold by slow addition of the refolding buffer (buffer B: 50 mM Tris, 0.5 M NaCl, 0.5 M urea, 0.4 M arginine, 2 mM DTT, 0.1% glycerol, pH 8.8), and equilibrated overnight at 4 °C. The solution appeared a little turbid the following morning, but was clear when centrifuged at 20,000g for 15 min at 4 °C.

For purification of the refolded protein, two approaches were employed. In one, the supernatant obtained from the protein solution refolded at pH 8.8 was loaded onto a Ni–NTA column equilibrated in 20 mM Tris, 0.5 M NaCl, 0.5 M urea, 1 mM β-mercaptoethanol, pH 8.0, washed thoroughly with the same buffer, and eluted with 20 mM Tris containing 0.25 M imidazole at pH 8.0. Here, although the refolding was carried out at pH 8.8 outside the column, pH 8.0 was chosen to meet with the condition for Ni–NTA chromatography. Eluted fractions were pooled and dialyzed...
against 20 mM Tris containing 50 mM NaCl, pH 8.0. In the other
procedure, the refolding was carried out at pH 8.8, and the super-
natant obtained was simply chromatographed using a 200 mL
(100 × 1.6 cm) Sephadex G100 column equilibrated in 20 mM Tris
containing 50 mM NaCl, pH 8.0. Here again, we chose pH 8.0 for gel
filtration to avert undesirable exposure of the protein to the actual
refolding pH. As discussed later, the gel filtration method produces
not only higher purity protein, but is also more reliable and repro-
ducible. The eluted fractions were pooled and concentrated by cen-
trifugal filters (Amicon). Signs of aggregation however appear
whenever the protein concentration exceeds ~500 μg/mL.

Western blot

A mixture of 50 μg each of horse heart cytochrome c and WD40Apaf-1 in 0.5X PBS and incubated at 4 °C overnight with con-
stant shaking. To the mixture, 1 μg of anti-cytochrome c monoclo-
nal antibody (Novagen) was added and incubated for 3–4 h at 4 °C.
Then 100 μl of 50% protein A agarose beads were added and incu-
bated for 3 h at 4 °C with shaking. The beads were centrifuged at
2000 rpm for 1 min and the pellet was washed three times with
0.5× PBS taking 0.5 ml for each wash. To the washed beads, 60 μl of 2× sample-loading dye was added, boiled for 5 min to dis-
associate the immuno complex, and the beads were collected by cen-
trifugation. The protein-containing supernatant was separated by
12% SDS/PAGE, and transferred onto a PVDF membrane. One set
of membranes was probed with anti-His and the other with anti-
cytochrome c mouse monoclonal antibody. The membranes were
incubated with corresponding 2° antibodies (anti-mouse IgG-con-
jugated with alkaline phosphatase) and developed with BCIP-NBT
solution.

Mass spectrometry

The sample for mass spectrometric measurement was prepared by in-gel tryptic digestion. Briefly, the coomassie-stained SDS/
PAGE band containing pure WD40Apaf-1 was excised from the gel,
destained with a 1:1 (v/v) solution of ACN and 2 μg/μl NH4HCO3,
and dried with ACN. The protein still in the gel piece was reduced
with DTT in 25 mM L-Cys for 1 h at 50 °C, and alkylated for
45 min at room temperature using 55 mM L-iodoacetamide in 25 mM L-
NH4HCO3. The gel piece was then dehydrated with ACN, rehydrated with a minimal volume of 50 mM L-1 NH4HCO3
containing trypsin (10 ng ml−1), and incubated at 37 °C for 16 h.
The peptides were extracted twice with 50% (v/v) acetonitrile con-
taining 1% (v/v) TFA. The peptide mixture was concentrated under
vacuum.

The tryptic peptides were dissolved in 2 μl of 50% (v/v) ACN
containing 1% (v/v) TFA, and mixed with 2 μl of 1% cyano-4-
hydroxycinnamic acid (HCCA) dissolved in 50% ACN and 1% TFA.
About 1 μl of this solution was applied on MALDI target plates.
Spectra were taken using a MALDI TOF/TOF Autoflex spectrometer
(Bruker Daltonics) in reflectron mode. MS/MS of selected peptides
were performed by LIFT (a device integrated in the mass spectrom-
eter for raising the potential energy of the ions). The spectra were
calibrated by Pepmix (Bruker Daltonics).

CD and fluorescence measurement

Spectra of the purified protein in native and GdnHCl-unfolded
states were taken at 22 °C. The buffer was 20 mM Tris, 10 mM
NaCl, pH 7, and the final protein concentration in the samples
was ~3(±1) μM. To unfold the protein, the desired concentration of GdnHCl was incorporated in the buffer. For fluorescence, a pho-
ton counting instrument (FluoroMax-3, Jobin-Yvon) was used (excitation: 280 nm, 1.5 nm slits). CD spectra were taken in a JASCO
J715 spectropolarimeter with the protein solution contained in
2 mm pathlength cylindrical cell. Sixteen scans were averaged.

Determination of equilibrium constant for the binding of WD40Apaf-1
to cytochrome c

These experiments involved equilibrium titration of reduced
cytochrome c by WD40Apaf-1. A set of samples containing 0.8 μM
cytochrome c and variable concentration of WD40Apaf-1 in the 0–
1.4 μM range was prepared in 20 mM Tris, 10 mM NaCl, 1 mM
DTT, pH 7. This procedure for titration where separate samples
are used for each increment of the titrant avoids dilution effects.
Samples were incubated for ~4 h at 22 °C before recording base-
line-corrected optical absorption spectra in the 500–390 nm wave-
length region using a Cary 100 (Varian) spectrophotometer.

Absorbance at 415 nm, which is the Soret maximum for DTT-re-
duced cytochrome c, was used for binding analysis. The concentra-
tion of free WD40Apaf-1 in the titration mixture is given by

\[
|\text{WD40Apaf-1}|_{\text{free}} = \frac{[\text{cyt c}] \times \Delta A}{\Delta A_c}
\]

in which, \(\Delta A = A_{c} - A_{0}\) and \(\Delta A_{c} = A_{c} - A_{0}\), where \(A_{0}\), \(A_{c}\), and \(A_{c}\) are
415-nm absorbance of solutions containing cytochrome c alone, cytochrome c in the presence of x concentration of WD40Apaf-1, and
cytochrome c in the presence of saturating concentration of
WD40Apaf-1, respectively. The \(K_{\text{ass}}\) value for cytochrome c-WD40A-
pafl interaction is extracted from the x-intercept of the plot of
\(\log([\text{A}] / (A_{c} - A_{0}))\) vs \(\log|\text{WD40Apaf-1}|_{\text{free}}\) according to the equation

\[
\log \frac{\Delta A}{A_{c} - A_{c}} = \log K_{\text{ass}} + \log |\text{WD40Apaf-1}|_{\text{free}}
\]

Results and discussion

A 43-residue insert in the WD40 Apaf-1

Here, the 2.5 kb gene sequence used to create the expression
construct of WD40Apaf-1 has been amplified from pcDNA 3-Apaf-
1XL (Fig. 1a). The Apaf-1 protein has been found in multiple iso-
forms [3–5,9,19], and the occurrence of full-length isoforms in tum-
our cell lines is believed to arise from alternative splicing of the
gene that can generate an 11-residue insert between the CARD and
ATPase domains or a 43-residue insert almost in the middle
of the WD40 domain, or both [9]. The Apaf-1XL isoform carries
both inserts [4], and thus has 1248 amino acids instead of 1194
for the canonical Apaf-1 first identified in HeLa cells [8]. Hence,
the construct generated in this study codes for the entire WD40
domain (amino acid residues 424–1248) with the additional 43
residues inserted between residues 811 and 812. With reference to
the 12 WD40 repeats in the WD40 domain of the originally de-
scribed Apaf-1 from HeLa cells [8], the WD40Apaf-1 studied here contains the last three amino acids of a WD40 repeat followed by
another WD40 repeat (Fig. 1b), thus accounting for the addi-
tional 43 residues in the WD40 domain of Apaf-1XL [4]. Since the
additional WD40 repeat has been demonstrated essential for cyto-
chrome c binding function of Apaf-1 [9], obtaining the recombinant
WD40 domain containing all 13 WD40 repeats provides an oppor-
tunity to investigate if the CARD and ATPase domains are determi-
nants of the cytochrome c binding efficacy of Apaf-1.

Refolding and purification of WD40 Apaf-1 from the inclusion body

A recombinant protein the size of WD40Apaf-1 (825 residues) is
normally not expected to be localized in the E. coli soluble fraction.

The sample for mass spectrometric measurement was prepared by in-gel tryptic digestion. Briefly, the coomassie-stained SDS/PAGE band containing pure WD40Apaf-1 was excised from the gel, destained with a 1:1 (v/v) solution of ACN and 2 μg/μl NH4HCO3, and dried with ACN. The protein still in the gel piece was reduced with DTT in 25 mM L-Cys for 1 h at 50 °C, and alkylated for 45 min at room temperature using 55 mM L-1 iodoacetamide in 25 mM L-NH4HCO3. The gel piece was then dehydrated with ACN, rehydrated with a minimal volume of 50 mM L-1 NH4HCO3 containing trypsin (10 ng ml−1), and incubated at 37 °C for 16 h. The peptides were extracted twice with 50% (v/v) acetonitrile containing 1% (v/v) TFA. The peptide mixture was concentrated under vacuum. The tryptic peptides were dissolved in 2 μl of 50% (v/v) ACN containing 1% (v/v) TFA, and mixed with 2 μl of 1% cyano-4-hydroxycinnamic acid (HCCA) dissolved in 50% ACN and 1% TFA. About 1 μl of this solution was applied on MALDI target plates. Spectra were taken using a MALDI TOF/TOF Autoflex spectrometer (Bruker Daltonics) in reflectron mode. MS/MS of selected peptides were performed by LIFT (a device integrated in the mass spectrometer for raising the potential energy of the ions). The spectra were calibrated by Pepmix (Bruker Daltonics).
24 cysteines in for the p of the specific residue(s). The value of 8.4 distinctively stands out the data points), raises curiosity with regard to the involvement of the protein may deteriorate. But the pH midpoint of 8.4, was not chosen with the view that the structural and energetic sta-

(Fig. 2a), we set the refolding pH to 8.8. A higher pH value (>9) the observation of a sharp decrease in turbidity for pH > 7.5 is due to the reduction of the fraction of the dissociated cys-

We nonetheless tested several conditions of temperature, growth time, and IPTG concentration under which WD40 Apaf-1 could possibly be found in the soluble fraction, but the protein is invariably localized in the cytoplasmic inclusion body fraction. Therefore, to maximize the protein content in the inclusion bodies, we grew cells at 37 °C and induced protein production by using 1 mM final concentration of IPTG.

The details of buffer systems and the protein refolding procedure from the inclusion body fraction are described under Materials and methods. In search for optimal protein folding conditions, we refolded the protein at various final pH values and in the presence of variable concentration of DTT in the refolding buffer, and measured the turbidity of the refolded protein solutions. From the observation of a sharp decrease in turbidity for pH > 7.5 (Fig. 2a), we set the refolding pH to 8.8. A higher pH value (>9) was not chosen with the view that the structural and energetic stability of the protein may deteriorate. But the pH midpoint of 8.4, determined from the calculated transition (the solid line through the data points), raises curiosity with regard to the involvement of the specific residue(s). The value of 8.4 distinctively stands out for the pKa of the cysteine side chain, and since there are at least 24 cysteines in WD40 Apaf-1, it would appear that protonation of the cysteine side chains may be somehow associated with the observed turbidity or the yield of the refolded protein. In further study of the turbidity problem, we included DTT in the refolding buffer. For refolding at pH 7.5, the turbidity increased with increments of DTT up to ~4 mM and remained constant thereafter, but DTT had little effect on the turbidity when refolding was carried out at pH 9 (Fig. 2b). If the DTT-associated turbidity at pH 7.5 is due to the reduction of the fraction of the dissociated cysteine side chain at that pH, then it would appear that formation of the correct disulfides is necessary for functionally active refolding of WD40 Apaf-1. But DTT ensures complete reduction of dissociated cysteine side chains at pH 9 also, and yet no turbidity appears and the protein folds correctly (see below). These observations suggest that disulfide formation is not associated with folding, although no information on the structure and the content of disul-

Fig. 1. (a) Agarose gel electrophoresis of PCR amplified WD40 Apaf-1: 1000 bp DNA ladder (lane M), pcDNA 3-Apaf-1XL plasmid (lane 1), and the PCR product (lane 2). (b) Schematic of the Apaf-1 domains. The amino acid inserts in the Apaf-1XL isoform are also shown. The 43 amino acids insert in the WD40 domain of the latter introduces an additional WD40 repeat. In the present work, the expression construct for the WD40 domain of Apaf-1XL (called WD40 Apaf-1 in the text) is generated.

Another set of experiments (results not shown) also appeared to point to the absence of disulfides. We have generated expression construct for WD40 Apaf-1 with a GST (glutathione S-transferase) tag. After refolding the fused protein from the inclusion bodies at pH 7.5, the refolded protein solution was dialyzed against a buffer containing the GSH–GSSG redox couple that is known to facilitate generation of disulfides. The dialyzed protein was assayed for GST activity by using the synthetic substrate analog CDNB (1-chloro-2,4-dinitrobenzene). No GST activity was detected under any condition of redox potential generated by variable GSH:GSSG ratio. One of the reasons for the absence of GST activity is misfolding of the whole or parts of the fused protein, for which we do not have any direct experimental proof. If correct folding is assumed, then the lack of GST activity would mean that disulfides are redundant or absent in WD40 Apaf-1.

With this information, we chose to refold the protein using buffer B containing 0.4 M arginine and 1 mM DTT at pH 8.8. In the Ni–NTA column purification, the eluted protein fraction contained three additional proteins clearly visible in the silver-stained SDS/PAGE gel (Fig. 3). Imidazole gradient elution also did not eliminate these proteins from the fraction containing WD40 Apaf-1. Fortunately, the contaminating proteins are smaller enough (45–70 kDa) than WD40 Apaf-1 (Fig. 3), so we chromatographed the refolded protein solution on a Sephadex G100 column for which the equilibrating and running buffer was simply 20 mM Tris containing 50 mM NaCl, pH 8.0. The results shown in Fig. 4 clearly indicate virtually complete purification of WD40 Apaf-1 (Fig. 4c). Another major advantage of the gel filtration procedure at pH 8.0 is that the WD40 Apaf-1 solution obtained is devoid of anything other than 20 mM Tris and 50 mM NaCl. The eluted protein fraction can thus be used for structural and functional studies without any further processing. We should add a note of caution here that

We have synthesized three additional proteins which we have called CED-4 homolog WD40, CARD CED-4 homolog WD40, and WD40 Apaf-1. These proteins are localized in the cytoplasmic inclusion body fraction. Therefore, to be found in the soluble fraction, but the protein is invariably

Apaf-1 (or Apaf-1S)

11 amino acid insert

43 amino acid insert

Apaf-1XL

CARD CED-4 homolog WD40

WD40

1 98 811 1194

811 1194

1248

M         1         2

2.5 kb
WD40Apaf-1 is prone to aggregation in the buffer system (20 mM Tris, 50 mM NaCl, pH 8) used for gel filtration as well as for later studies. We suspect, this is part of the reason for the loss of some protein in the column matrix (Fig. 4a and Table 1). To avoid the aggregation problem, we recommend processing and handling of less than 500 μL protein (≈500 μg/mL). Also, concentrating the protein solution should be avoided. Hence, the gel filtration eluate was directly aliquotted and stored at −20 °C. The recovery and fractional purity of WD40Apaf-1 at various stages of purification are given in Table 1.

Mass spectrometric identification, and basic conformational characterization

Fig. 5 shows the mass spectrum for tryptic peptides of the purified protein. That the protein is the WD40 domain of human Apaf-1...
accompanied by a red shift of the emission maximum from there is nearly 3-fold enhancement of fluorescence intensity tive protein is unfolded by Apaf-1. Such studies are currently in progress, and the details will appears to suggest domain-independent folding-unfolding of protein is unfolded, which together with the present results tryptophan fluorescence is substantially quenched when the Apaf-1 isoform from HepG2 tumor cell line, we found that the in the unfolded state. In an earlier study with the WD40-deleted to tertiary packing and a large solvent exposure of the fluorophores to/C24
860 amino acid residues in our expressed WD40Apaf-1, 35 are
ues matched with a sequence coverage of 13%. Of the calculated
sequence database (NCBI) using the Mascot search engine (Matrix
was established by peptide mass fingerprint search in primary se-
sequence database (NCBI) using the Mascot search engine (Matrix Science). In the search, 61 tryptic peptide mass values were tested constraining the mass tolerance to 0.5 Da, and 20 peptide mass values
matched with a sequence coverage of 13%. Of the calculated 860 amino acid residues in our expressed WD40Apaf-1, 35 are extrinsic to the protein. These residues are: MRGSHHHHHHG MASMTGGQQMGRDLDDDDKDRWI; the six consecutive histi-
dines form the tag and the remaining residues are due to the pRS-
ETa vector. The calculated molecular mass of the cloned protein is 97.9 kDa, and the pI value is 5.84.
The folded conformation of the protein was checked by fluores-
cence and CD spectra taken with ~3 μM protein in 20 mM Tris, 10 mM NaCl, pH 7 at 25 °C. The tryptophan-excited fluorescence spectra of WD40Apaf-1 produced in Fig. 6a show that when the na-	ive protein is unfolded by ~4.5 M GdnHCl included in the buffer, there is nearly 3-fold enhancement of fluorescence intensity accompanied by a red shift of the emission maximum from ~325 to ~340 nm, suggesting quenched native-state fluorescence due to tertiary packing and a large solvent exposure of the fluorophores in the unfolded state. In an earlier study with the WD40-deleted Apaf-1 isomorf from HepG2 tumor cell line, we found that the tryptophan fluorescence is substantially quenched when the protein is unfolded, which together with the present results appears to suggest domain-independent folding-unfolding of Apaf-1. Such studies are currently in progress, and the details will be reported elsewhere. The far-UV CD spectrum of WD40Apaf-1 (Fig. 6b) indicates substantial content of secondary structure, suggesting that the protein we have purified is likely to be folded and functionally active. The content of secondary structure does not provide a full proof for correct folding of the protein, nor does it guarantee functional activity of the protein. However, demonstration of functional activity provides adequate proof for correct folding of the protein. The test for the functional activity of WD40Apaf-1 presented below indeed suggests that the protein is correctly folded.

Binding of cytochrome c to WD40Apaf-1

Central to the functioning of Apaf-1 is its ability to bind to cyto-
chrome c. The finding of WD40 repeats in the C-terminal domain of Apaf-1 [9] immediately led to the belief that it is the WD40 domain of Apaf-1 that interacts with cytochrome c [20,21]. WD40 repeats are generally known to be involved in protein–protein interactions [16,17], and given that cytochrome c is highly basic (19 lysines in the total of 104), it could be reasonably expected that a number of Asp–Lys interactions are generally known to be involved in protein interaction. Therefore, it was anticipated that a number of Asp–Lys interactions are involved in the binding of WD40Apaf-1 and cytochrome c. The interaction of cytochrome c and WD40Apaf-1 can of course be verified qualitatively by coimmunoprecipitation and immunoblot analyses (data not shown).

To determine the equilibrium binding affinity, we titrated 0.8 μM cytochrome c with WD40Apaf-1 in the 0–1.4 μM range. The buffer was 20 mM Tris containing 10 mM NaCl and 1 mM DTT at pH 7, and the titration was followed by the Soret optical absorption spectrum of the DTT-reduced cytochrome c (Fig. 7a).

![Table 1](https://example.com/table1.png)

Table 1

<table>
<thead>
<tr>
<th>Stage/sample</th>
<th>Amount of protein a (mg)</th>
<th>% purity b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>226</td>
<td>10–15</td>
</tr>
<tr>
<td>Insoluble pellet from the cell lysate</td>
<td>70</td>
<td>Not estimated</td>
</tr>
<tr>
<td>Washed inclusion bodies</td>
<td>34</td>
<td>–30–40</td>
</tr>
<tr>
<td>GdnHCl-solubilized inclusion bodies</td>
<td>–25</td>
<td>Not estimated</td>
</tr>
<tr>
<td>GdnHCl-unfolded solution → refolded</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>WD40Apaf-1 eluted from Sephadex G100 column</td>
<td>3–7</td>
<td>–100</td>
</tr>
</tbody>
</table>

a Starting with 4 gm of wet weight cells harvested from 2 L of culture.
b The figures in this column represent estimates only, and therefore may have large errors.
As described earlier [19,22], for quantitative in vitro binding analyses involving cytochrome c we rely on the spectrophotometric method because emission quenching due to the heme group of cytochrome c introduces large artifacts in the fluorometrically obtained titration results. Fig. 7b shows the binding analysis where the x-intercept of the linear fit of the data yields the association constant, \( K_{ass} = 2.56 \mu M^{-1} \) (binding affinity, \( K_{diss} = 1/K_{ass} = 390 \) nM). The data can be analyzed equally well by a nonlinear fit for single-site binding (inset, Fig. 7b), in which case the binding affinity is 122 nM. As discussed below, the linear analysis is more meaningful for extraction of binding stoichiometry. Thus, we should consider the linear analysis and take \( K_{diss} = 390 \) nM, which is at least four-orders of magnitude larger than the value of 0.01 nM reported in an earlier study of the interaction of full-length Apaf-1 with cytochrome c [9]. Since protein function is also known to be governed by motional dynamics, major difference in the flexibility of the full-length Apaf-1 and \( \text{WD40} \)Apaf-1 can affect cytochrome c binding parameters. Further studies will be required for a detailed understanding of these issues.

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It is interesting that the slope of the straight line in Fig. 7b is 2.8, indicating a 3:1 or at least 2:1 stoichiometry for the binding of cytochrome c to \( \text{WD40} \)Apaf-1. For the interaction of zinc substituted cytochrome c with baculovirally expressed full-length Apaf-1, a 2:1 binding stoichiometry was found [18]. Our results raise the possibility that each \( \text{WD40} \)Apaf-1 molecule may have provision for even three cytochrome c molecules under the conditions of ionic strength and pH employed here.

The value of 390 nM for the binding affinity does not reflect a rather tight binding. Such a complex could dissociate with a half time of seconds. As discussed elsewhere in the context of protein–protein interaction [24], weaker affinity often reflects broad binding specificity and floppy binding surfaces, suggesting that the binding epitope on Apaf-1 is dynamically flexible. The nature and the identity of the Apaf-1 surface involved in cytochrome c binding are unknown. Studies using mutant cytochrome c implicated not only lystines but also other residues of cytochrome c that are important for Apaf-1 binding [25]. Indeed, the cytochrome c epitope for Apaf-1 binding is suggested to be large and extensive [25,26], suggesting that the Apaf-1 epitope may contain other residues in addition to Asp. The atomic details of the binding epitopes for both proteins are unknown, and we hope to address these in future work.

**Summary and conclusion**

In *E. coli* expression, the recombinant WD40 domain of human Apaf-1 (\( \text{WD40} \)Apaf-1) is invariably localized in the inclusion body fraction. The protein, refolded at pH 8.8 by diluting the GdnHCl-solubilized inclusion bodies, can be purified to a very high homogeneity by Sephadex G100 gel filtration in 20 mM Tris, 50 mM NaCl, pH 8. The protein obtained thus can be used directly for structural and functional studies. Since the protein is prone to aggregation, concentrations exceeding \( \approx 5 \) μM should be avoided during gel filtration and all subsequent uses. The protein thus purified displays fluorescence and far-UV CD spectra typical of folded proteins. The binding of cytochrome c to \( \text{WD40} \)Apaf-1 is not rather tight. The value of 390 nM for the binding affinity is suggestive of a possibly floppy binding surface on Apaf-1. The binding stoichiometry suggests binding of 2 or 3 molecules of cytochrome c per molecule of \( \text{WD40} \)Apaf-1.

**Acknowledgments**

We are grateful to Gabriel Nunez for providing us with the pcDNA 3-Apaf-1XL plasmid. This work was supported by a Grant (4/1/2003-SF) from the Department of Science & Technology (DST), Government of India.

**References**


Cloning, *E. coli* expression, refolding, and ATP-binding properties of a WD40-deleted Apaf-1 isoform

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Abstract

The apoptotic protease activating factor (Apaf-1) is central to the regulatory mechanism by which procaspase-9 is activated in the cytochrome *c*-mediated pathway of apoptosis. For a detailed biochemical and structural investigation of Apaf-1 function, we have cloned and expressed in *Escherichia coli* inclusion bodies the WD40-deleted protein (D*WD*40*Apaf*1) from HepG2 cell. The construct contains an N-terminal His~6~ tag derived from the cloning vector so that the mass of the protein and the tag together is 51,594 Da, as determined by TOF/TOF mass spectrometric analysis. An optimized refolding protocol has allowed protein recovery in highly pure form. Basic fluorescence and CD probes indicate that the refolded protein retains secondary and tertiary structures, and unfolds in the presence of higher concentration of denaturant. The equilibrium ATP binding property of the protein has been measured by changes in fluorescence emission due to the fluorescent ATP analog, mant-ATP (2'(3')-O-((N-methylanthraniloyl) adenosine 5'-triphosphate). The results demonstrate a tight Apaf-1–ATP interaction, the binding affinity being 380 nM.

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Keywords: Apoptosis; Apoptosome; Apoptotic protease activating factor-1; Apaf-1

In the mitochondrial pathway of apoptosis [1], Apaf-1 is the central player in the upstream events of cytochrome *c* dependent activation of procaspase-9 [2,3]. Apaf-1 consists of a CED-4 homologous domain that straddles a Caspase Recruitment Domain (CARD) in the N-terminal region and a C-terminal WD-40 domain containing 12 or 13 WD-40 repeats [2,4]. The CED-4 domain is also called ATPase domain, owing to its apparent ATP hydrolysis activity [2,5–7]. The biochemical mechanisms by which Apaf-1 involves itself to assembling a functional apoptosome, a generic term for the initial platform provided by Apaf-1 and cytochrome *c* in the presence of ATP/dATP leading to the activation of caspases-9 and 3 [2,3,8], are astonishingly complex, and remains largely unknown. In the pursuit of this aspect at molecular and atomic level, Apaf-1 itself offers a major challenge by its large size and existence in isoforms [3,9]. Notwithstanding the largeness, electron cryomicroscopic methods have been applied to view the gross structural and topological features of the apoptosome, and the oligomerization of Apaf-1 [10–12]. More recently, a mathematical model for Apaf-1 heptamer assembly process has also been proposed [13]. Of the three domains of Apaf-1, atomic-level structural descriptions have been provided for the CARD domain alone by NMR [14], and together with the ATPase domain by X-ray crystallography [7].

Depending on the mode of mRNA splicing, multiple Apaf-1 splice variants can exist [3], but not all isoforms thus produced can activate procaspase-9 [9]. It has been demonstrated that the isoform with an 11-amino acid insert between the CARD and ATPase domain, and an extra WD-40 sequence in the WD-40 domain are essential for...
ATP hydrolysis-mediated cytochrome c binding, and regulation of pro-caspase-9. According to this model, the cytochrome c-binding region in the C-terminal WD-40 domain of functionally active Apaf-1 isoforms remains unavailable for binding until a conformational change possibly driven by ATP hydrolysis in the CED-4 domain unmasks the binding site [9]. However, Apaf-1 lacking the WD-40 domain can bind and hydrolyze ATP/dATP. Localization of ADP in the crystal structure of the CED-4 domain indicates that ATP → ADP + Pᵢ reaction is possibly driven by the domain itself [7]. Thus, several regulatory and mechanistic issues, including the regulation of nucleotide binding and hydrolysis, and the CARD’s role toward these, can be investigated using recombinant Apaf-1 lacking the WD-40 domain.

For a detailed understanding of the structural dynamics and mechanisms of involvement of Apaf-1 in the apoptosis formation, we have recently undertaken a series of studies. Here, we used RT-PCR to clone a gene corresponding to the N-terminal 1-419 amino acid sequence of Apaf-1 from HepG2 cell line. The gene construct excludes domain features a 4-residue deletion. The protein overexpressed in Escherichia coli is invariably localized in the cytoplasmic inclusion body. The protein was refolded, purified, and characterized in terms of quantification of its equilibrium interaction with ATP.

Materials and methods

Cloning and generation of expression construct for Apaf-1

Total RNA was isolated from ~5 million HepG2 cells by standard procedures that use TRI Reagent (Sigma), chloroform, and isopropanol. The isopropanol-pelleted RNA was washed with 75% ethanol, dissolved in nuclease-free water, and subjected to agarose gel electrophoresis to confirm the integrity of the isolated RNA. cDNA was prepared by using the SuperscriptII kit (Invitrogen). Approximately 5 μg of total RNA was added to the RT-PCR mix. Oligo dT primed cDNA synthesis was achieved by incubating the reaction mix at 47°C for 30 min. The first strand that was made using SuperscriptII was used for PCR amplification of Apaf-1. The primers were

BamH1
CARD-F: 5’GCGGATCCATGGATGCAAAAGCCTCG AA 3’
CED4-R: 5’CCCTCGAGCTAAAGAGACTTTTAC AAACTC 3’ Xho1

The 50 μL PCR mixture contained 5 μL 10X PCR buffer, 1 μL CARD-F primer (10 pmol), 1 μL CED4-R primer (10 pmol), 2 μL cDNA, 1 μL of 2.5 U/μL EasyA Taq DNA polymerase (Stratagene), and 40 μL water. PCR amplification involved the initial denaturation at 94°C for 2 min, followed by 30 cycles each consisting of a 30-s denaturation at 94°C, a 30-s annealing at 54°C, and a 1-min extension at 72°C. The final extension following the 30 cycles was allowed for 15 min at 72°C. The amplified PCR product, electrophoresed and eluted from agarose gel, was ligated into a TA vector, pTZ57 R/T (MBI-Fermentas). The positive clones, selected by Blue-white colony screening and colony PCR, were used to isolate plasmids employing QAprep spin miniprep kit (Qiagen). The plasmids were sequenced, and the clone containing the correct sequence was digested with BamH1 and Xho1. The digested fragment was then ligated into BamH1 and Xho1 sites of pRSETA vector (Invitrogen), and transformed into DH5α E. coli cells. Positive clones were selected by colony PCR, and confirmed by digestion.

Protein expression

The protein was expressed in LB medium containing ampicillin (100 μg/ml). To obtain the protein in the soluble fraction, several cell growth conditions generated by varying temperature and IPTG concentration were tried out. Typically, 20 ml of an overnight grown culture was added to a 2-L medium, and incubated with vigorous shaking at a temperature in the 5–37°C range. At OD₆₀₀ = 0.5 of the culture, protein expression was induced with IPTG in the 0.1–1 mM range. The growth was continued until the steady state was reached. Noting no trace protein in the soluble fraction under all conditions employed, we eventually chose to induce protein expression at 37°C with 1 mM IPTG, and let the cells grow for 5 h after induction. Cells were harvested by centrifugation at 4000g for 10 min at 4°C, washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0), and frozen stored at ~80°C.

Preparation of inclusion bodies

The cell pellet was resuspended in 30 ml Buffer A (20 mM Tris–HCl, 100 mM NaCl, 1 mM PMSF, pH8.0, containing 10 μL of 1 mg/ml DNaseI), and sonicated at 4°C with 10 cycles, each cycle consisting of 30 s on and 60 s off times. The lysate was treated with deoxycholic acid (4 mg per gram weight of E. coli), stirred for 30 min at room temperature, and centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was resuspended in ~9 volumes of Buffer B (20 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0), stirred at room temperature for 5 min and centrifuged at 10000 rpm for 15 min at 4°C. The step was repeated. Inclusion bodies were then washed with ~10 volumes of 20 mM Tris–HCl containing 100 mM NaCl at pH 8.0 three times to remove the Triton X-100. Washed inclusion bodies are now ~95% pure.
Refolding of $^{\text{AWD}}$40 Apaf-1

Three procedures were applied to refold the protein from inclusion body aggregates, and the compositions of unfolding and refolding buffers varied slightly from one procedure to another. The basic buffer for solubilization of inclusion body was Buffer C (6.0 M GdnHCl, 20 mM Tris–HCl, 500 mM NaCl, pH 7.5). For refolding by the use of classical gel filtration procedure, ~25 mg of the inclusion body preparation (by wet weight) was solubilized in 5 ml of Buffer C with 1 mM EDTA, 1 mM DTT and 50 mM L-arginine at pH 7.4, and incubated at 25 °C for ~90 min. The unfolded protein solution, cleared after centrifugation, was diluted five-fold by drop wise addition into 20 ml of the same buffer containing no GdnHCl. The refolded protein solution, now in ~1.2 M GdnHCl was faintly hazy, and was kept in cold for 90 min. The solution was then centrifuged at 15000 rpm for 15 min, and 20 ml of it was chromatographed in a 1.6 × 100 cm (diameter and height, respectively) Sephadex G75 column preequilibrated in the refolding buffer. Fractions each of 1.5 ml size were collected at a flow rate of 20 ml/h, and analyzed by SDS-electrophoresis and silver staining.

In the second refolding procedure, nickel affinity column was used to separate $^{\text{AWD}}$40 Apaf-1 from other proteins present in the refolded protein solution. Inclusion bodies were solubilized in Buffer C with 1 mM PMSF and 5 mM β-mercaptoethanol, pH 8.0, incubated for 2 h, and refolded by 20-fold dilution into Buffer C containing no GdnHCl but 0.4 M L-arginine, 0.5 M urea, and 1 mM β-mercaptoethanol, pH 7.5. The solution was kept in cold for 24 h, and then dialyzed extensively against the same refolding buffer (pH 8.0) that excluded L-arginine and β-mercaptoethanol. After discarding the precipitate by centrifugation, the solution was loaded onto a Ni-NTA-His bind column (Novagen) equilibrated with the dialysis buffer, and washed with the same buffer. The protein, eluted by passing 20 mM Tris, 150 mM NaCl, and 300 mM imidazole, pH 8, was dialyzed or centrifuged in Amicon 5 kDa cutoff filter devices to remove imidazole. Although nothing wrong in itself, this procedure for imidazole removal was time consuming under our laboratory conditions. We, therefore, used 20 mM Tris–HCl, 500 mM NaCl, pH 7.5 containing no EDTA. The protein was eluted with the same buffer containing no GdnHCl. The eluted protein solution was then dialyzed or centrifuge-filtered using the same buffer at pH 7.5 containing no EDTA.

Western blot

Purified $^{\text{AWD}}$40 Apaf-1 as well as the E. coli extract proteins were run on 10% SDS-PAGE, transferred onto a PVDF membrane (Amersham), incubated overnight at 4 °C with anti-Apaf-1 rabbit polyclonal IgG (Upstate), washed with TBS, incubated for 1 h at room temperature with 2° antibody (anti-rabbit conjugated with alkaline phosphatase), and developed with BCIP-NBT solution.

Mass spectrometry

Two microliters of the purified protein solution (~0.21 μg) was mixed with 2 μL of 2% TFA and 2 μL of the matrix solution (2.5 dihydroxyacetophenone with 10 mM di-ammonium citrate). Mass measurements were performed on a Autoflex III TOF/TOF spectrometer (Bruker) in the positive linear mode of operation. ~1000 single spectra were added. Spectra were processed using 10 Da Gauss filter smoothing and baseline subtraction.

Fluorescence and CD measurements

Fluorescence and CD spectra of $^{\text{AWD}}$40 Apaf-1 were recorded with 2 μM protein in 20 mM Tris, 100 mM NaCl, pH 7.5, 25 °C. For fluorescence, a photon counting instrument (FluoroMax-3, Jobin-Yvon) was used (excitation: 280 nm, 1.5 nm slit). CD spectra were taken in a JASCO J715 spectropolarimeter with the protein solution contained in 2 mm pathlength cylindrical cell. Sixteen scans were averaged.

Interaction of $^{\text{AWD}}$40 Apaf-1 and ATP measured by fluorescence

The titration of MANT-ATP (Invitrogen) with $^{\text{AWD}}$40 Apaf-1 was followed by the decrease in fluorescence of the former (excitation: 356 nm). A set of samples containing a fixed concentration of MANT-ATP (1 or 5 μM) and variable concentration of $^{\text{AWD}}$40 Apaf-1 was prepared in 50 mM Tris, 50 mM NaCl, 10 mM MgCl2, pH 7, and equilibrated at 25 °C for 1 h. With this procedure of equilibrium titration experiment, where different premixed samples are used instead of repeated addition of aliquots of the titrant to a single protein solution, no correction for dilution effects are required, and the data obtained are also more accurate. Fluorescence at 444 nm, which is the emission maximum for MANT-ATP, was used for binding analysis. The concentration of free $^{\text{AWD}}$40 Apaf-1 in the titration mixture is given by:

$$[^{\text{AWD}}\text{Apaf-1}]_{\text{free}} = \frac{[\text{MANT-ATP}] \times \Delta F}{\Delta F_{\infty}}$$

(1)
\[ \Delta F = F_e - F_o \text{ and } \Delta F_\infty = F_e - F_o, \text{ where } F_e, F_o, \text{ and } F_e \text{ are 444-nm fluorescence of solutions containing MANT-ATP alone, MANT-ATP in the presence of x concentration of } \text{AWD}^{40}\text{Apaf-1, and MANT-ATP in the presence of infinite or saturating concentration of } \text{AWD}^{40}\text{Apaf-1, respectively. The association constant, } K_{ass}, \text{ was extracted from the equation}
\]

\[
\log \left[ \frac{\Delta F}{F_\infty - F_e} \right] = \log K_{ass} + \log [\text{AWD}^{40}\text{Apaf-1}]_{\text{free}} \tag{2}
\]

The x-intercept of the plot of \(\log(\Delta F/(F_\infty - F_e))\) vs \(\log([\text{AWD}^{40}\text{Apaf-1}]_{\text{free}}\) gives the value of \(pK_{a}\) for the interaction between MANT-ATP and \(\text{AWD}^{40}\text{Apaf-1}.

**Results and discussion**

**4-residue deletion in the CARD domain of \(\text{AWD}^{40}\text{Apaf-1}**

To clone the \(\text{AWD}^{40}\text{Apaf-1} \text{ gene containing the N-terminal 1-419 amino acids, we used RNA from HepG2 cells (Fig. 1). The present gene sequence, aligned to the HeLa Apaf-1 sequence (also called Apaf-1S) originally described by Zou et al. [4] and also cloned later by others [9], indicated a deletion involving four consecutive amino acids (VRNE) in the CARD domain (Fig. 1b). In the past, several studies have reported Apaf-1 isoforms [2,3,6,9]. The occurrence of full-length Apaf-1 isoforms in tumor cell lines have been attributed to alternative splicing that can create an 11-residue insert straddled by the CARD and ATPase domains or an additional WD40 repeat between the fifth and sixth repeats of the WD40 domain, or both.

The 11-residue insert, not present in the Apaf-1S isoform, is found in the Apaf-1 gene that we have cloned (Fig. 1b). Accordingly, following the nomenclature of Benedict et al. [9], the \(\text{AWD}^{40}\text{Apaf-1} \text{ may be termed Apaf-1XL(1-419) or N+. A survey of all the isoforms conducted by the same authors has indicated that only Apaf-1 isoforms with the additional WD40 insert activate procaspase-9 in a cytochrome c/dATP-dependent manner. However, WD-deleted Apaf-1 should retain the dATP/ATP binding activity due to the presence of Walker’s A- and B-box consensus sequences in the ATPase domain [4]. Crystal structure of Apaf-1(1-591) does locate ADP at a binding pocket supported by both domains [7], indicating the essentiality of CARD domain for nucleotide binding. Any deletion or insertion in the sequence should not alter the functional activity significantly so long as the changes do not disorder the nucleotide binding pocket or the protein conformation critically. Indeed, the 4-residue deletion in the CARD domain of HepG2 cell Apaf-1 (\(\text{AWD}^{40}\text{Apaf-1}) \text{ studied here preserves both the conformational integrity and the ATP binding function (see below).}

**Refrolding of \(\text{AWD}^{40}\text{Apaf-1} \text{ from the inclusion body fraction**

In *E. coli* production of recombinant \(\text{AWD}^{40}\text{Apaf-1}, \text{ the protein is invariably localized in the cytoplasmic inclusion body fraction. We tested the expression under several growth conditions of temperature in the 5–37 °C range with variable use of IPTG concentration up to 1 mM, but could not localize the protein in the soluble fraction. Therefore, cells were grown at 37 °C, and protein produc-
tion was induced by adding IPTG to a final concentration of 1 mM. The insoluble aggregates of the inclusion body fraction were extensively washed, and solubilized in Buffer C that contained 6 M GdnHCl and other solvent additives (described under Materials and methods) depending on the refolding protocol considered.

Fig. 2 shows the isolation by gel filtration of \( \text{WD40} \) Apaf-1 from the refolded protein solution. Nearly complete homogeneity of the protein isolate, more surely from the left half of the elution peak, is seen in the silver-stained SDS gel (inset). Because smaller volume of sample is preferred for gel filtration work, the initial unfolded protein solution (in 6 M GdnHCl) was diluted only five-fold, so that the refolded protein solution had finally \( \sim 1.2 \text{ M GdnHCl} \). This is a subdenaturing condition, where the protein may or may not have actually refolded to the globally native state. In any case, when present in such subdenaturing amounts, GdnHCl can stabilize the folding structures by chain stiffening or entropic effect due to the cross-linking action of GdnH\(^+\) cations, and electrostatic effect due to the interaction of Cl\(^-\), and also possibly of GdnH\(^+\), with charged groups of the protein [15–17]. Use of \( \sim 1 \text{ M GdnHCl} \) in the refolding buffer is a common practice in the refolding of inclusion bodies [18, see also ref 19]. Indeed, we noticed more precipitation when the refolding was done by ten-fold dilution (\( \sim 0.6 \text{ M GdnHCl}, \) finally) of the unfolded inclusion body solution. Use of 50 mM arginine in the unfolding and refolding solutions did not alter the qualitative extent of precipitation during refolding. Gel filtration eventually removes the residual GdnHCl and arginine.

When refolded by 20-fold dilution of the unfolded protein solution so that the final concentration of GdnHCl in the refolded solution became 0.3 M, a sizable fraction of the protein precipitated, indicating that the protein-stabilizing action of the denaturant weakens as its concentration is lowered in the bid to shift the condition from subdenaturing to native-like [15]. We then worked on the solvent condition to minimize precipitation during refolding to a final GdnHCl of 0.3 M (20 mM Tris–HCl, 500 mM NaCl). This was done by checking light scattering at 500 nm as a function of pH and l-arginine content of the refolding buffer. For the experiment with l-arginine alone, the pH of the refolded protein solution was 7.5. Effects of arginine on protein refolding and purification have been extensively investigated by Arakawa and coworkers [20–22]. Fig. 3 indicates that precipitation is minimum when the pH is in the 7.5–8 range, and l-arginine concentration is 0.4 M or more. This concentration of l-arginine was previously found optimum in the refolding of recombinant mouse Bcl-2(1-203) also [23]. DTT in the 0-10 mM range does not influence protein precipitation (data not shown). Thus,

![Fig. 2. Sephade G-25 isolation of WD40 Apaf-1 from the protein mixture refolded at pH 7.5 by the first procedure (5-fold dilution of the protein solution initially unfolded in 6 M GdnHCl) described in the text. Lanes 1–3 in the SDS gel corresponds to the eluted fractions labeled 1–3 within circles.](image)

![Fig. 3. Effect of (a) pH, and (b) l-arginine concentrations on protein solubilization in the refolded protein solution, monitored by light scattering at 500 nm. In these experiments, the unfolded protein solution (6.0 M GdnHCl, 20 mM Tris–HCl, 500 mM NaCl, pH 7.5) was diluted 20-fold into the same buffer containing no GdnHCl and the pH adjusted to different values. For the variable l-arginine experiment, pH of the refolded protein solution was 7.5.](image)
for isolation of the refolded protein by nickel-column chromatography, we carried out refolding to a final GdnHCl of 0.3 M at pH 8 in the presence of 0.4 M L-arginine. Elution was achieved by using imidazole or EDTA as described under Materials and methods. Fig. 4a shows the EDTA elution result by silver-stained SDS gel electrophoresis. The identity and purity of \(^{\text{AWD40}}\)Apaf-1 is further confirmed by the Western blot developed with polyclonal anti-Apaf-1 (Fig. 4b). For removal of EDTA and Ni\(^{2+}\), the eluted protein solution was extensively dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.5.

We then looked at the quantitative aspect of this procedure by starting with 1 L culture that yielded 1.6 g wet weight cells. The details of steps and procedure involved were same as described above. Protein content was estimated at various stages of purification, although large errors were obtained in those steps that involved one or both GdnHCl and L-arginine. Excluding these, the amount of protein determined at different stages is listed in Table 1. As discussed above, \(^{\text{AWD40}}\)Apaf-1 is localized exclusively in the inclusion body fraction. The protein amount in this fraction is 13.2 mg, and that after Ni-NTA elution finally is 1.2 mg (Table 1), indicating \(\sim 9\%\) recovery. The percent purity at different stages was estimated by coomassie-stained SDS gel electrophoresis (Fig. 4c). The lane descriptions and other details are given in the legend to the figure. For each lane, the integrated density of the \(^{\text{AWD40}}\)Apaf-1 band was divided by the amount of protein loaded in that lane, because the volume and the amount of protein loaded in different lanes were not uniform. This yielded the fractional content of \(^{\text{AWD40}}\)Apaf-1 in the protein solution at various stages of purification. To normalize finally, the fractional contents of \(^{\text{AWD40}}\)Apaf-1 for all lanes were divided by the fractional content corresponding to the highest purity preparation. The error propagated through these estimations is in the 2–8% range. Table 1 provides the fractional purity at some of the stages of purification. The 0–1 scaling of purity should be considered relative to the purity of the protein eluted from Ni-NTA column (>95%).

We also considered on-column refolding, often called solid phase refolding [19], using the Ni-NTA resin column at pH 8. After loading the unfolded protein solution, the column was washed with 6 M urea, refolded with 20 mM Tris and 150 mM NaCl in the absence of L-arginine, and eluted with the same buffer containing 50 mM EDTA. The results are shown in Fig. 5.

Several other procedures and practices for protein refolding from inclusion bodies have been described by Tsumoto et al. [19]. Of the three procedures considered in this study, the on-column refolding strategy is preferred least because the unfolded \(^{\text{AWD40}}\)Apaf-1 often binds weakly to the Ni-resin. At times, the protein aggregates in the column, and eluted fractions are found to be visibly turbid. Both gel filtration and Ni-column approaches for the isolation of the refolded protein work well. Of these two, the

Table 1

<table>
<thead>
<tr>
<th>Stage/sample</th>
<th>Amount of protein (mg)(^a)</th>
<th>Fractional purity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed cell lysate pellet</td>
<td>69</td>
<td>0.13</td>
</tr>
<tr>
<td>Solubilized inclusion bodies</td>
<td>13.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Post refolding from inclusion bodies</td>
<td>7.9</td>
<td>Not estimated</td>
</tr>
<tr>
<td>The dialed refolded protein</td>
<td>4.15</td>
<td>0.6</td>
</tr>
<tr>
<td>Post Ni NTA chromatography</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) The experiment was started with 1.6 g of wet weight cells obtained from 1 L culture.

\(^b\) The fractional purity is relative to the purity of the protein (>95%) after Ni-NTA chromatography.
latter is preferred, because it permits processing relatively larger amount of protein solution, and the final elution volume is smaller too. The recovery and fractional purity of \( \text{WD40Apaf-1} \) at various stages of purification are listed in Table 1.

**Mass, and basic conformational characterization**

Fig. 6 shows the Gauss filter-smoothed mass spectrum (TOF/TOF) of \( \text{WD40Apaf-1} \). The singly charged \([\text{M}+\text{H}]^+\) fundamental peak corresponds to a mass of 51,594 Da. The molecular weight calculated for the 454-amino acid \( \text{WD40Apaf-1} \) is 51,534, fairly consistent with the experimental mass data. Part of the observed mass difference of 60 could be due to incorrectly determined side-chain charges used in calculations. The experimental mass should be more reliable. Of the 454 residues in the expressed protein, 35 residues in the N-terminus, including the six histidines (MRGSHHHHHHGMASTMGGQQ MGRDLYYDDDKDRW1), are due to the pRSETA vector. The calculated molecular weight of the actual 419-amino acid polypeptide is 47,436 Da at pH 7.

Fig. 7a shows the 280-nm excited tryptophan fluorescence spectra of the native and 6 M GdnHCl-unfolded proteins. The emission maximum of the native-state spectrum centered at 345 nm shifts to \( \sim 358 \) nm when the protein unfolds. The unfolding also accompanies a very significant quenching of fluorescence. Fig. 7b presents the far-UV CD spectrum of purified \( \text{WD40Apaf-1} \), clearly indicating substantial secondary structure content. These results suggest that the 4-residue deletion mutation (Fig. 1b) in the N-terminal part of the CARD domain of HepG2 Apaf-1 is unlikely to disorder the molecular conformation greatly.

**Binding of ATP to \( \text{WD40Apaf-1} \)**

To study the equilibrium binding of ATP to \( \text{WD40Apaf-1} \), we used a fluorescent analog of the nucleotide, mant-ATP, whose fluorescence emission is quenched detectably when bound to proteins. Earlier studies of nucleotide binding to Apaf-1 employed \([\alpha-32\text{P}]\text{dATP}\) for quantification by scintillation counting [5,7]. Because of high sensitivity, the fluorescence method provides more accurate data, and facilitates the measurement of rapid binding kinetics. The fluorescence emission due to a fixed concentration of mant-ATP is quenched when titrated by increments of the protein concentration as indicated in Fig. 8a. Fig. 8b plots the binding analysis as described under Materials and Methods. The \( x \)-intercept of the linear fit of the data yields the association constant, \( K_{\text{ass}} = 2.63 \mu\text{M}^{-1} \) (binding affinity), \( K_{\text{diss}} = 1/K_{\text{ass}} = 380 \text{ nM} \), indicating tight binding of ATP to \( \text{WD40Apaf-1} \). The slope of the plot (\( n = 0.6 \)) indicates a 1:1 interaction, and the Gibbs free energy for ATP–Apaf-1 interaction (\( \Delta G^\circ = -RT \ln K_{\text{ass}} \)) is \( \sim 8.7 \text{ kcal mol}^{-1} \) at 25 °C.

There appear certain ambiguities in past reports regarding nucleotide binding to Apaf-1. For example, the earlier

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Fig. 5. Silver stained SDS-PAGE analysis of Ni-resin on-column refolding. The unfolded protein (in 6 M GdnHCl) was allowed to bind to the column at pH 8, washed, and eluted as described in the text. Lane labels are: 1, marker; 2, uninduced cell lysate; 3, induced cell supernatant; 4, the inclusion body fraction from induced cells; 5, the 6 M GdnHCl-unfolded inclusion bodies; 6, flow-through from the column; 7, wash fraction; 8, the eluted fraction.

Fig. 6. Molecular mass of the His tagged \( \text{WD40Apaf-1} \) is 51,594 as indicated by mass spectrometry. The calculated mass is 51,534 (see text).
demonstration that ADP, dADP, ATP, and dATP bind to purified Apaf-1 [2] was somewhat inconsistent with a later study where Apaf-1 showed little nucleotide binding activity [5]. Similarly, it is not entirely clear whether cytochrome c enhances dATP binding [5] or not [2]. Our study demonstrates that ATP does bind to purified WD40Apaf-1 in the absence of any other cofactor, although the CD spectrum is a rather crude measure of folding. All spectra were taken in 20 mM Tris, 100 mM NaCl, pH 7.5, 25 °C.

Fig. 7. (a) Fluorescence spectra of the refolded native protein (N) with a peak wavelength of 345 nm. The N protein can be unfolded by the addition of 6 M GdnHCl. Fluorescence then (U) is quenched and the emission maximum red-shifts to 358 nm. (b) The far-UV CD spectrum of the refolded protein (N) appears to indicate the presence of native-state secondary structures, although the CD spectrum is a rather crude measure of folding. All spectra were taken in 20 mM Tris, 100 mM NaCl, pH 7.5, 25 °C.

addressed in detail in due course of the study. It is likely that slow oligomerization and/or aggregation of Apaf-1 is partly responsible for the inconsistencies and the disparate in vitro results. In our study, the purified WD40 Apaf-1 is monomeric, as reflected by its elution volume in the Sephadex G75 chromatogram (Fig. 2). Since the ATP binding experiments were performed with freshly prepared protein (without storage in between), it is likely that a large fraction of the protein was in monomeric form. At this stage, we cannot provide quantitative data for time dependent oligomerization.

Although the binding results presented here demonstrate that the 4-residue deletion in the CARD domain of HepG2 Apaf-1 does not abrogate ATP binding, it might affect other details—the rate constants for binding interaction, and hydrolysis to ADP and Pi, for example. As mentioned in the introduction, knowledge of such
mechanistic issues is central to understand the regulatory role of Apaf-1 in cell death. These studies should have applications in the induction of caspase activated apoptosis in therapeutics.

Summary and conclusion

Apaf-1XL isofrom from HepG2 cell line carries a 4-residue deletion in the CARD domain. The N-terminal His<sup>6</sup>-tagged protein, termed AWD<sup>D40</sup>Apaf-1, overexpressed in <i>E. coli</i> is localized exclusively in the cytoplasmic inclusion body fraction, unaffected by growth conditions in the 5–37 °C range of temperature and induction by IPTG concentrations from 0.1 to 1 mM. The protein is refolded best by 20-fold dilution of the initial 6 M GdnHCl-unfolded inclusion body preparation into a buffer system composed of 20 mM Tris–HCl, 500 mM NaCl, 0.5 M Urea, 0.4 M L-arginine, pH 7.5–8. Highly pure protein can be isolated by Ni<sup>2+</sup>-resin chromatography with 20 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 8.0 as the elution buffer. The protein thus purified displays fluorescence and far-Ultraviolet CD spectra characteristic of folded proteins. The interaction of AWD<sup>D40</sup>Apaf-1 with ATP has been studied by the use mant-ATP whose fluorescence decreases as it binds to the protein. The 1:1 ATP:Apaf-1 interaction is characterized by the equilibrium dissociation constant, <i>K<sub>diss</sub></i>, of 380 nM, and Δ<i>G</i>° = −<i>R</i>T ln <i>K<sub>diss</sub></i> = 8.7 kcal mol<sup>−1</sup> (25 °C), indicating tight binding.

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References

High affinity binding of Bcl-xL to cytochrome c: Possible relevance for interception of translocated cytochrome c in apoptosis

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Abstract

The release of cytochrome c from mitochondria and apoptosis relies on several preferential and selective interactions involving the Bcl-2 family of proteins. There is, however, no direct evidence for the interaction of cytochrome c with these proteins at any stage of apoptosis. To investigate if any pro-survival protein from the Bcl-2 family could intercept cytochrome c after its translocation from mitochondria, the interaction of cytochrome c with bacterially expressed human Bcl-xL was studied at pH 7. In size-exclusion chromatography, purified full-length His6-tagged Bcl-xL migrated as both dimer and monomer, of which the monomeric fractions were used for experiments. Coimmunoprecipitation studies show that cytochrome c interacts with Bcl-xL. The extent of caspase activity in cell lysate elicited by externally added cytochrome c is reduced when a preincubated mixture of Bcl-xL and cytochrome c is used instead. Equilibrium binding monitored by optical absorption of cytochrome c as a function of titrating concentrations of Bcl-xL yields the association constant, $K_{ass} = 8.4(\pm 4) \times 10^6 \text{ M}^{-1}$ (binding affinity, $K_{diss} = 1/K_{ass} \approx 120 \text{ nM}$) which decreases at high ionic strength. The rates for binding of Bcl-xL to cytochrome c, studied by stopped-flow kinetics at pH 7, show that the bimolecular rate constant for binding, $k_{bi} = 0.24 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Values of the thermodynamic and kinetic parameters for Bcl-xL–cytochrome c interaction are very similar to those known for regulatory protein–protein interactions in apoptosis.

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In the intrinsic apoptotic pathway, cytochrome c translocated from mitochondria assists in the assembly of an apoptosome required for activation of caspase-9 and subsequent activation of other caspases [1–3]. The rapid release of cytochrome c from mitochondria is believed to arise from effects of pro-apoptotic proteins of the Bcl-2 family, including Bax, Bak, and Bad, which form pores on the outer mitochondrial membrane to facilitate release of cytochrome c [4–9]. Alternatively, these proteins may function to cause a loss of mitochondrial membrane potential that can lead to membrane permeabilization [10–14].

The pro-survival members of the Bcl-2 family proteins, including Bcl-xL, Bcl-2, and Bcl-w, counter the release of cytochrome c from mitochondria by less understood mechanisms. Numerous studies have established that Bcl-xL participates in a number of protein–protein interactions to exert its pro-survival effect. In the intrinsic apoptotic pathway, Bcl-xL blocks cytochrome c release by preventing Bax from disrupting the integrity of the outer mitochondrial membrane [15,16], and in the extrinsic apoptotic pathway initiated by death ligands, Bcl-xL can prevent death by blocking Bid redistribution downstream of caspase-8 [17]. In hypoxia/reoxygenation-induced apoptosis, Bcl-xL has been found to interfere with the assembly of death-inducing signal complex (DISC), block the translocation of both Bax and Bid to mitochondria, and inhibit the activation of caspase-8 [18].

Thus, Bcl-xL appears to prevent cell death through diverse protein–protein interactions, seemingly in a case specific manner. However, there is no known mechanism by which cytochrome c translocated to cytosol can be intercepted, such that the apoptosome is not assembled. There have been reports that Bcl-xL can block the formation of the apoptosome by
associating itself with Apaf-1 and caspase-9 to produce an
antiapoptotic ternary complex [19,20]. In another earlier
immunoprecipitation study, cytochrome c was found to interact
specifically with Bcl-xL as a cellular response to ionizing
radiation and genotoxic stress [21]. The present study is
basically aimed at providing quantitative data on the interaction
between the two proteins. To characterize the affinity of this
interaction so that Bcl-xL could possibly be implicated for
arresting cell death by inhibiting the cytosolic cytochrome c, we
have investigated the interaction between bacterially over-
expressed full-length human Bcl-xL and horse cytochrome c
using coimmunoprecipitation and classical biochemical
methods. We report that cytochrome c displays very high binding
affinity for Bcl-xL, which closely matches the reported affinities
of BH3 peptides/domains for Bcl-xL. The bimolecular binding
rate of Bcl-xL to cytochrome c is also within the range set by
dimerization of Bcl-2 family proteins, and by BH3–Bcl-2
protein interactions. Possible significance and relevance of
these binding characteristics to the regulation of the intrinsic
pathway for apoptosis is discussed.

1. Materials and methods

1.1. Production and purification of Bcl-xL

A pET-16b (Novagen) vector coding for full length human Bcl-xL with
N-terminal His6 tag was kindly provided by Dr. Apurva Sarin, and the plasmid
was transformed into E. coli BL21(DE3) cells. The cells were grown in LB media
to a A600 of 0.5. Protein expression was induced with 0.8 mM IPTG at 30 °C and
180 rpm. Cells were harvested at 10000 rpm for 10 min. The cell pellet, washed
with PBS, was resuspended in 1:5 W/V buffer A (50 mM Tris, pH 8.0, 0.2 M
NaCl, 0.2 mM PMSF, 5 mM β-mercaptoethanol, 5 mM imidazole, and 1%
glycerol), and stirred for 15 min at 4 °C. The cells were disrupted by pulsed
sonication, and the lysate was spun at 15000 rpm for 30 min. The soluble fraction
was loaded onto a Ni-NTA-His bind column (Novagen) equilibrated with buffer A.
The column was washed with 45 mM imidazole in buffer A until the
protein was eluted with 200 mM imidazole in the same buffer.

The protein fractions were pooled, concentrated, and loaded onto a
Sephadex G-75 (Amersham) gel filtration column preequilibrated with buffer B
(50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT, 2 mM EDTA, and 0.2 mM
PMSF) at 4 °C. Of the two major peaks, the one corresponding to the monomeric
protein was collected by centrifuging the mixture at 31,000×g for 20 min.

The other peak was eluted with 500 mM NaCl gradient applied linearly. The protein-containing fractions were pooled and dialyzed
to 10 volumes of buffer B, and the protein was eluted with a 50–400 mM NaCl gradient
applied linearly. The protein-containing fractions were pooled and dialyzed
to 1× PBS at 4 °C. Protein purity was determined to be >99% by
SDS/PAGE silver staining. The protein concentration was determined
spectrophotometrically using ε280=41940 M−1 cm−1 in 6 M guanidine hydrochloride.

1.2. Coimmunoprecipitation

A mixture of 50 μg each of horse heart cytochrome c and the purified human
Bcl-xL was incubated with constant shaking. 1 μg monoclonal anti cytochrome
c(Novagen) was added to the mixture, and incubated for another 3 h at 4 °C.
To this mixture, 100 μl of 50% protein-A slurry was added, and incubated with
shaking at 4 °C for 3 h. The beads were separated by centrifuging the mixture at
2000 rpm for 1 min, and the pellet was washed 3 times with 0.5× PBS. To the
washed beads, 60 μl of 2× sample-loading dye was added, boiled for 5 min to
dissociate the immuno complex, and the beads were collected by centrifugation.

The protein-containing supernatant was separated by 15% SDS/PAGE, and
transferred onto a PVDF membrane. One set of membranes was probed with anti
cytochrome c from purified healthy mitochondria

Wistar rats (12 to 14 weeks old) were decapitated and the liver was taken in
ice cold 0.9% NaCl. The liver was washed thoroughly with 0.9% NaCl to
remove the blood, transferred to MSH buffer (220 mM mannitol, 70 mM
Sucrose, 2 mM HEPES, pH 7.4, 0.2 mM EDTA, 6 μM BSA), washed twice in
the same buffer, and cut into small cubes. The cubes were taken in MSH buffer
of which was ~10% of the body weight. The liver was homogenated using
dounce homogenizer and Teflon pestle, and centrifuged at 2000×g for 10 min at 4 °C.

The supernatant, collected after removing the white lipid layer using
cotton shrubs, was centrifuged again at 12000×g for 15 min. The pellet
was resuspended in 20 ml of a washing medium containing 220 mM mannitol,
70 mM sucrose, 2 mM HEPES, and 0.2 mM EDTA, pH 7.4, and centrifuged
again at 12000×g for 10 min at 4 °C. This pellet-washing step was repeated one
more time. 0.5 ml of the resuspended pellet was layered over pre-formed
discontinuous Percoll gradient consisting of a bottom layer of 2 ml of 40% Percol and
a top layer of 2 ml of 26% Percoll in the washing medium. The
gradient was spun at 31,000×g for 28 min in a Beckman SW-60 rotor. The
mitochondrial fraction appearing in the interface between the two Percoll layers
was transferred into a fresh tube, diluted 1:5 with the same (washing) medium,
and centrifuged at 31,000×g for 20 min. The pellet was then resuspended in
0.5 ml of 0.25 M sucrose and kept on ice. Mitochondrial protein was
determined by the Bradford method using BSA as a standard.

0.6 mg mitochondria contained in an endopod tube was incubated with
1.4 μmol Ca2+ per mg of protein for 2 h at 4 °C. Controls were generated by
incubating under identical conditions the same quantity of mitochondria with
125 mM KCl (in Buffer A) in one tube, and 250 mM sucrose (in Buffer B) in
another. After incubation, mitochondria were pelleted by centrifugation at
12,000×g for 10 min. 50 μg purified Bcl-xL was added to the supernatant,
and incubated for 2 h at room temperature by constant shaking. The mixture
was then divided into two halves; one half was treated with 1.0 μg of monoclonal
anti-cytochrome c (Novagen), and the other half with 1.0 μg monoclonal anti-
caspase-9 (Santa Cruz) as a negative control antibody. After incubation at room
temperature for 3 h, the mixture was combined with 50 μl of 50% protein-A
slurry, and incubated further for 1 h at room temperature. The beads were
separated by centrifuging the mixture at 2000 rpm for 1 min, and the pellet
was washed 3 times with 0.5× PBS. To the washed beads, 60 μl of 2×
sample-loading dye was added, boiled for 5 min to dissociate the immunocomplex,
and the beads were collected by centrifugation. The protein-containing supernatant
was separated by 15% SDS/PAGE, and transferred onto a PVDF membrane.
The first set of membranes was probed with anti bcl-xl (Promega), the second with
anti-His (Santa Cruz), and the third with anti cytochrome c. The bands
were detected with corresponding ALP conjugate 2° antibodies and NBT/BCIP.

1.3. Coimmunoprecipitation using HeLa cell stimulated to release
cytochrome c

Cells were maintained at 37 °C in Dulbecco’s modified eagle medium
(DMEM, Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 200 μg ml−1
penicillin, and 100 μg ml−1 streptomycin sulfate in a humidified atmosphere
(5% CO2/95% air). At the 70% confluent stage, apoptosis was induced with
10 ng ml−1 human TNF together with 10 μg ml−1 cycloheximide, and the cells
were incubated for 6 h at 37 °C. The media was then replaced by 0.1% trypsin-
EDTA (TE), and the flask was incubated at 37 °C for 30 min. The detached cells
were rinsed out with 5 ml of the fresh medium, pelleted at 1000 rpm for 5 min,
and washed with 1× PBS. Cytosolic extracts were prepared as described earlier
by Bossy-Wetzel et al. [22]. Cells (24×106) were incubated for 30 min on ice
in the lysis buffer (68 mM sucrose, 200 mM mannitol, 50 mM PIPES–KOH, pH
7.4, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, protease inhibitors,
ilysed with 80 strokes of a dounce eppendorf homogenizer, and centrifuged
at 14,000×g for 15 min at 4 °C. To the supernatant (cytosolic extract), 50 μg of Bcl-
xL was added, and incubated on a rocker for 3 h at room temperature. 10 μg of
monoclonal anti-cytochrome c was added to the mixture and incubated for 1 h.

The immunocomplex was precipitated with protein-A agarose beads, and
blots were developed using monoclonal anti-Bcl-xL, anti-cytochrome c, and anti-His
antibodies. The blots were probed with ALP conjugated 2° anti mouse and
detected by NBT/BCIP.
1.5. Induction of caspase-like activity by cytochrome c and the Bcl-xL–cytochrome c complex

These experiments were conducted using Spodoptera frugiperda (S/9) ovarian cells. Four million healthy cells pelleted by centrifugation at 3000 rpm for 5 min, were suspended in 1 M PBS, and centrifuged at 1000g for 15 s. The pellet was resuspended in a buffer consisting of 50 mM PIPES–KOH, pH 7.0, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10 μg ml⁻¹ pepstatin, 10 μg ml⁻¹ aprotinin, and 10 μg ml⁻¹ PMSF (400 μl for 10 ml culture), and the cells were allowed to swell on ice for 15 min. Cells were then disrupted with 0.01% NP40 detergent (optimized), vortexed gently, and spun at 1600 rpm for 30 min. The supernatant was spun again at 13000 rpm for 30 min. This final supernatant is the cytosolic extract (CE) required for this set of experiments. The pellet is the nuclear fraction containing the organelles including mitochondria, and was discarded. For measurement of caspase activity, 23 μl CE (~50 μg protein) was distributed on 20 μl of the dilution buffer (10 mM PIPES, pH 7.0, 5 mM EGTA, 2 mM MgCl₂, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase), 2 mM dATP, and one of the following: 1 μM DEVD-AFC substrate. AFC hydrolysis was measured by 490-nm emission for 4 h at 22 °C before recording baseline-corrected optical absorption spectra in the 600–400 nm wavelength region using a Cary 100 (Varian) spectrophotometer.

Absorbance at 415 nm, which is the Soret maximum for DTT-reduced cytochrome c, was titrated out by a fluorescein-labeled Bad BH3 peptide (Calbiochem). The titration was followed as described above, and k₅₀ was extracted from the equation

\[ \log \left( \frac{A}{A_\infty - A} \right) = \log K_{50} + \log([Bcl-xL]_{free}) \]

(2)

The x-intercept of the plot of \( \log \left( \frac{A}{A_\infty - A} \right) \) vs. \( \log [Bcl-xL] \) gives the value of pK₅₀ for cytochrome c–Bcl-xL interaction.

1.7. Equilibrium binding of the Bad BH3 domain to cytochrome c

In this set of experiments, 475 nM cytochrome c was titrated out by a fluorescein-labeled Bad BH3 peptide (Calbiochem). The titration was followed by both Soret optical absorbance due to cytochrome c as described above, and 535-nm fluorescence emission due to the BH3 peptide (excitation: 485 nm). Absorbance data were analyzed using Eqs. (1) and (2). The binding parameters from the fluorescence data were extracted from the equation

\[ y(x) = \frac{c}{1 + 10^{(x-x_{Kd})}} \]

(3)

where, n is the number of peptides bound to cytochrome c, K₅₀ is apparent dissociation constant, and c is the total change in fluorescence normalized to unity. In the concentration range of the peptide (0–7 μM) used for the titration experiment, there was no inner filter effect on the fluorescence emission, since the optical absorbance of the peptide at the excitation wavelength was less than 0.32 in this range of concentrations.

1.8. Stopped-flow kinetics of interaction of Bcl-xL with cytochrome c

All solutions for kinetic experiments were prepared in 50 mM phosphate, 4 mM DTT, and 50 mM NaCl, pH 7. In a two-syringe mixing procedure, a solution of 24 μM cytochrome c was mixed with Bcl-xL solutions of variable concentration in the 2–12 μM range. The mixing ratio was 1:7. These experiments were done in a Bio-Logic SFM 400 instrument. The temperature was regulated at 22 °C using an external circulating water bath. The traces were analyzed using a single-exponential function: \( y(t) = A_\infty + A e^{-k t} \), where \( A \) is the amplitude, \( A_\infty \) denotes the baseline signal at long times, and \( k \) is the observed rate constant. The bimolecular binding rate was calculated from the slope of the plot of the observed rate constant as a function of Bcl-xL concentration.

2. Results

Fig. 1a shows that the nickel column-eluted protein migrates as equal factions of dimer (D) and monomer (M) on the Sephadex G75 size-exclusion column, consistent with recent reports of homodimers of cytosolic Bcl-xL [23]. The result indicates that purified Bcl-xL exists in a slow dynamic equilibrium between the two forms. The monomeric fractions were further chromatographed on a DEAE anion-exchange
column, and more than 99% pure protein was obtained, as confirmed by silver-stained SDS/PAGE (Fig. 1b).

To check for the functional activity and binding specificity of the purified human Bcl-xL, we performed communoprecipitation and immunoblot analyses using anti-cytochrome c and anti-Bcl-xL antibodies. Mitochondria fractionated in a discontinuous Percoll gradient was stimulated to release cytochrome c by the use of Ca^{2+} in the wash buffer that contained 70 mM sucrose but no NaCl. We also generated two controls by simply incubating mitochondria in Buffer A containing 125 mM KCl, and in Buffer B containing 250 mM sucrose. Various buffer compositions are detailed under Materials and methods. As determined from the visible absorption spectrum of the supernatants (not shown), only Ca^{2+} induced the release of cytochrome c, and the released cytochrome c accounted for ~0.12% of the mitochondrial preparation (in terms of protein content) that was subjected to Ca^{2+} induction. We then carried out immunoprecipitation using all three supernatants, each with monoclonal anti-cytochrome c as the positive control, and monoclonal anti-capase-9 as the negative control, and developed each blot with anti-Bcl-xL, anti-His-tag, and anti-cytochrome c. Fig. 2 shows the Western blots. In each, lane 7 shows the pure protein blotted by the corresponding antibody. The supernatant obtained in buffer B that contained no calcium showed no trace of cytochrome c and Bcl-xL in both positive (lane 1) and negative (lane 2) immunoprecipitations. Similarly, buffer A did not produce any effect (lanes 3 and 4, corresponding to positive and negative controls, respectively). Lane 5 shows the Ca^{2+}-induced release of cytochrome c. Interestingly, this lane also exhibits a relatively faint band for Bcl-xL detected by anti-Bcl-xL, but not by anti-His-tag, suggesting that traces of endogenous Bcl-xL residing in mitochondrial membrane may have translocated and precipitated with cytochrome c. We notice that the proteins in this lane (lane 5) were immunoprecipitated by using anti-cytochrome c. Here, anti-His-tag does not blot Bcl-xL because the precipitated Bcl-xL is endogenous. The Ca^{2+}-induced supernatant immunoprecipitated by the use of anti-capase-9 (negative control) does not report any protein band (lane 6). These results suggest that cytochrome c does bind to Bcl-xL, whatever may be the mechanism by which the latter is made available in the medium. The His-tagged recombinant protein is detected by both anti-Bcl-xL and anti-His-tag, and the endogenous form is blotted only by the protein antibody.

To show that Bcl-xL indeed arrests cytosolic cytochrome c in apoptosed cells, we stimulated HeLa cells by using human TNF together with cycloheximide [3], and checked for precipitation of Bcl-xL with endogenous cytochrome c released from mitochondria of the induced cells. About 0.2 μg cytochrome c (determined from the visible region absorption spectrum; λ_{max} = 415 nm) was released per million of apoptosed cells. Blots were developed using all three antibodies: anti-cytochrome c, anti-Bcl-xL, and anti-His-tag (Fig. 3). In the extract of the apoptosed cells immunoprecipitated by anti-capase-9 (treated negative) none of the three antibodies detect a band (lane 1). But, as lane 2 shows, all three antibodies detect the corresponding proteins in the immunoprecipitate obtained from the apoptosed cell extract by the use of anti-cytochrome c (treated positive). Here also, both anti-His-tag and anti-Bcl-xL blot the band, because the cell extract was presented with exogenous recombinant Bcl-xL. Lanes 3 and 4 correspond to negative and positive controls, respectively, of the extract from healthy uninduced cells. Lane 5 presents the bands due to recombinant Bcl-xL and commercial horse cytochrome c; the former blotted by both anti-His-tag and anti-Bcl-xL, and the latter by anti-cytochrome c. These results again demonstrate that Bcl-xL specifically interacts with cytochrome c whenever the latter appears in the medium. Such immunological studies of Bcl-xL–cytochrome c interaction have already been reported for human leukemia cells challenged with γ-irradiation [21]. Our results elucidate the phenomenon with more examples by incorporating necessary controls. We conclude that the
cytochrome c translocated to cytosol from mitochondria in response to apoptotic stimuli is vulnerable to arrest by Bcl-xL.

Yet, the major objective of this study is to quantify the binding parameters for Bcl-xL–cytochrome c interaction. To study equilibrium binding in titration experiments, we initially attempted to use the intrinsic fluorescence of Bcl-xL. Full-length Bcl-xL is highly fluorescent due to the content of six tryptophan residues, but cytochrome c does not fluoresce due to intramolecular excitation energy transfer from its lone tryptophan (W59) to the heme. Therefore, the fluorescence of Bcl-xL can, in principle, be used to monitor its titration with cytochrome c. However, the concentration of cytochrome c in the solution (>1.5 μM) needed to achieve a complete titration titrate was found to quench the intrinsic fluorescence of Bcl-xL. The quenching of Bcl-xL fluorescence due to cytochrome c in the bulk solution obscures the actual change in fluorescence due to the interaction of the two proteins. A control experiment where a lysozyme solution was titrated with cytochrome c showed exactly the same behavior, suggesting that fluorescence is not preferred to probe the Bcl-xL–cytochrome c interaction.

We then turned to the measurement of optical absorption of a fixed concentration of cytochrome c in the Soret heme region as a function of variable concentration of Bcl-xL. Fig. 4a shows that the absorbance due to cytochrome c increases with increments of Bcl-xL; for the 3 μM solution of cytochrome c, the change in absorbance across the complete titration is 33 milliOD. The difference spectra generated by subtracting the spectrum of cytochrome c alone from spectra in the presence of Bcl-xL (Fig. 4a, inset) show that Bcl-xL binding causes the absorption peak to shift from 415 to 413.2 nm. These observations suggested that heme optical absorption of cytochrome c could be used as a reliable marker to follow its interaction with Bcl-xL. Fig. 4b shows the plot used for binding analysis as described under Materials and methods. The x-intercept of the linear fit of the data yields the association constant, $K_{ass} = 8.4(\pm 4) \times 10^6$ M$^{-1}$, indicating tight affinity of cytochrome c for Bcl-xL. The slope of the plot (n=0.6) indicates a 1:1 interaction. The Gibbs free energy for Bcl-xL–cytochrome c interaction ($\Delta G^0 = -RT \ln K_{ass}$) calculated by using this value of $K_{ass}$ is $\sim 9.3$ kcal mol$^{-1}$ at 22°C.

Cytochrome c is lysine-rich, and hence highly basic. It is therefore desirable to address as to how its interaction with Bcl-xL varies with ionic strength and pH. Fig. 4c shows that $K_{ass}$ is maximum at $\sim 80$ mM NaCl, and declines by nearly 12-fold in the presence of 600 mM NaCl, suggesting that the physiological ionic strength supports the tightest interaction between the two proteins. The dependence of $K_{ass}$ on pH could not be studied in detail due to alkaline isomerization of cytochrome c in basic medium (pH>8), and heme spin change in acid solutions (pH<5). In the accessible range of pH, $K_{ass}$ was found to change little.

The kinetics of protein–protein interactions are of special significance since rates of such interactions often play important regulatory roles. To determine the rate of binding of Bcl-xL to cytochrome c we performed stopped-flow experiments where the time dependence of the change in 415-nm absorbance due to cytochrome c was monitored after mixing the two protein solutions. The representative traces labeled 1, 2, and 3 in Fig. 5a show the time dependence of the change in absorbance after mixing 3 μM cytochrome c with 6, 4, and 2 μM Bcl-xL, respectively. The solid lines through the data are single-
exponential fits, showing clearly that the rate of interaction changes with the concentration of Bcl-xL. The dependence of the observed rate on the concentration of Bcl-xL is shown in Fig. 5b, the slope of the linear fit of which yields the bimolecular association rate constant,

\[ k_{bi} = 0.24 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}. \]

Incorporation of a control experiment is necessary to show that cytochrome c does not interact with other proteins indiscriminately. As a negative control, we checked for interaction of Bcl-xL with myoglobin (the latter was chosen because it resembles cytochrome c in containing a heme group and a fairly sizable number of positively charged amino acids), but did not detect any binding. As a positive control, we set to look into the interaction of cytochrome c with Bad BH3. Since the BH3 domain is involved in a number of interactions amongst the Bcl-2 family proteins and Bcl-xL is found to interact with cytochrome c, one naively expects binding of the BH3 peptide to cytochrome c. We used the optical absorbance of cytochrome c (\( \lambda_{\text{max}} \approx 415 \text{ nm} \)) to assess this interaction. We also took advantage of the fluorescence emission of the fluorescein-labeled Bad BH3 peptide (\( \lambda_{\text{max}} \approx 496 \text{ nm} \)) to monitor the titration of 475 nM cytochrome c with increments of the peptide up to 7 \( \mu \text{M} \). In this range of concentration of the peptide, no inner filter effect in the fluorescence of the peptide was detected. The analysis of the titration monitored by the optical absorbance of cytochrome c was performed (Eqs. (1) and (2)) yielding \( K_{\text{ass}} = 4.1 \times 10^6 \text{ M}^{-1} \), indicating rather tight affinity of the BH3 peptide for cytochrome c (Fig. 6a), and the slope of 0.91 suggests 1:1 interaction. Values of \( K_{\text{ass}} \) and \( n \) extracted from this analysis are 1.2(±0.2) \( \mu \text{M}^{-1} \) and 1.3, respectively.

In another control experiment, we studied the equilibrium binding of Bid with cytochrome c. The working rationale was the same as mentioned above in the context of interaction of Bad BH3 peptide with cytochrome c. Since the Bid protein Table 1

<table>
<thead>
<tr>
<th>Binding interactions</th>
<th>( K_{\text{ass}} ) (( \mu \text{M}^{-1} ))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-xL–cytochrome c</td>
<td>8.4 (±4)</td>
<td>This work</td>
</tr>
<tr>
<td>Bcl-xL–Bad BH3</td>
<td>46.5</td>
<td>[37]</td>
</tr>
<tr>
<td>Bad BH3–cytochrome c</td>
<td>2.65 (±1.45)</td>
<td>This work</td>
</tr>
</tbody>
</table>
The Bcl-2 family proteins are known to homodimerize, heterodimerize, and even homo-oligomerize to exert their survival and death effects [23–30]. Cellular Bcl-xL, irrespective of cytosolic or mitochondrial location, has been shown to form homodimers or oligomers [31] wherein monomers are able to sequester their C-terminal hydrophobic membrane anchors in the partner BH3-binding pockets [23]. Although the function associated with the homodimer (D) is less well understood, the pro-survival virtue of Bcl-xL is believed to be a consequence of its existence as homodimers. It is then worth considering as to what extent Bcl-xL may exist as monomer (M). In our experiments, the purified full length Bcl-xL shows a M = D equilibrium, where the equilibration between the two forms are very slow compared to the time of size-exclusion chromatography (Fig. 1b). We have observed this equilibrium consistently in all our preparations, as has also been reported previously [32]. To note is that Bcl-xL migrates as higher molecular weight large complexes, often with no trace of monomers, if nonionic detergents (Tween 20, for example) are included in the developer buffer [32,33]. The dimerization ability of Bcl-xL is entirely due to its C-terminal hydrophobic tail, a fact consistent with the observation that Bcl-xL ΔCT is recovered as a monomer [23]. Two additional observations: (1) the Western blots for Bcl-xL in healthy Cos-7 and HeLa cell cytosolic extracts show a small fraction of endogenous monomeric form, and (2) the equilibrium binding experiments for Bcl-xL–cytochrome c interaction show a 1:1 complex formation (Fig. 4b), indicate the presence of a monomeric fraction in both endogenous and purified Bcl-xL preparation. Even if there is no monomeric form, it might as well be that cytochrome c causes the dimers to dissociate, just as the proapoptotic Bad shifts the endogenous Bcl-xL homodimer formation, possibly by direct binding [34], causing translocation of Bcl-xL to mitochondrial membrane. It will be interesting to know if there are other factors that displace the cytosolic M = D equilibrium in favor of M that will expose the C-terminal membrane anchor tail and subsequent relocalization to mitochondria.

3.2. Binding of Bcl-xL to cytochrome c

The major finding of this study is a tight 1:1 binding of Bcl-xL and cytochrome c, and it is of interest as to how this affinity compares with those for known protein–protein or protein–peptide interactions involved in survival and death. The apparent binding affinities or dissociation constants \(K_{\text{diss}} = 1/K_{\text{ass}}\) for the interaction of Bcl-xL with other members of the Bcl-2 family have been reported in the 2–6 μM range at pH 7 [32]. In more recent studies, the apparent \(K_{\text{diss}}\) values for the
interactions of BH3 peptides from pro-apoptotic Bcl-2 family proteins with Bcl-xL ΔCx (truncated at the C-tail by x residues) have been found to fall in the 1–90 nM range [35], suggesting tighter binding, while the affinity of BH3 peptides from anti-apoptotic Bcl-2 family proteins with other survival or killer proteins is relatively low, into micromolar range [36]. Thus the tighter binding, while the affinity of BH3 peptides from anti-apoptotic Bcl-2 family proteins with Bcl-xL is relatively low, into micromolar range [36]. Thus the affinity of cytochrome c (K_diss ~ 120 nM at pH 7) is indeed tight, and is fairly comparable to its affinity for BH3 domains from the anti-survival proteins of the Bcl-2 family. Even the bimolecular/second order rate constants for the binding of Bcl-xL to cytochrome c (k_on = 0.24 μM^{-1} s^{-1}, Fig. 5b) and Bcl-xL to other Bcl-2 family proteins (k_on in the 0.27–0.34 μM^{-1} s^{-1} range [32]) are nearly identical, implying that these interprotein interactions closely match in terms of binding free energy and the activation energy barrier.

3.3. Cytochrome c–Bad BH3 and cytochrome c–Bid interactions

Another interesting finding is individual binding interactions of cytochrome c with Bad BH3 and Bcl-xL. The idea that such interactions could exist emanated as a corollary of the BH3-mediated interprotein interactions amongst the Bcl-2 family of proteins, and the high-affinity binding of Bcl-xL with cytochrome c observed in this study. The binding affinity (K_diss = 1/K_acs) of Bad BH3 for cytochrome c, averaged from optical absorption and fluorescence data (Fig. 6), is 538(±295) nm, which is numerically significantly higher than the value of 21.5 nm reported for the Bad BH3–Bcl-xL interaction [37,26]. The numerical value for the binding affinity of Bid for cytochrome c (373(±126) nM), on the other hand, is rather smaller than the reported value of 1.9 μM for Bcl-xL–Bid interaction at neutral pH [32]. This suggests that while the affinity of Bad BH3 for cytochrome c is considerably lower, the affinity of Bid for cytochrome c is higher, both compared with the affinity of cytochrome c for Bcl-xL (Table 1). Nonetheless, it does appear that the cytosolic pool of cytochrome c is capable of interacting with both pro- and antiapoptotic proteins with varying degrees of affinity. For a more definitive statement, we are in the process of a detailed characterization of the interactions of cytochrome c with several other members of the Bcl-2 family of proteins.

3.4. Possible structural factors for Bcl-xL and cytochrome c interaction

From parametric similarity of interactions between Bcl-xL and cytochrome c, and Bcl-xL and other Bcl-2 family proteins, one may naively assume that Bcl-xL deploys the same surface to bind cytochrome c as it does for binding with other Bcl-2 family members or the BH3 sequences derived from them. The availability of NMR and X-ray structures of Bcl-xL [23,38] augments deduction of the structural basis of these interprotein interactions. Muchmore et al. have proposed that an elongated hydrophobic cleft, the constituent residues of which are highly conserved in the Bcl-2 family of proteins, is the site of interaction of Bcl-xL with death-promoting proteins [38]. Structures of BH3 peptide-bound Bcl-xL show that the alignment of the BH3 helix along the hydrophobic cleft of Bcl-xL is stabilized by apolar interactions at the base and polar contacts along the sides of the cleft [39]. Cytochrome c is a highly charged protein with 19 lysine residues most of which are surface exposed. It is likely that the polar residues along the side of the same Bcl-xL cleft supports charged interactions with cytochrome c. The decrease of the binding affinity for the Bcl-xL–cytochrome c interaction at high NaCl concentration (Fig. 4c) lends support to this view. Strategic high-resolution surface mapping experiments are underway to test this conjecture.

3.5. How relevant is the Bcl-xL–cytochrome c interaction for upstream regulation of apoptosis?

From the results of this study together with earlier immunochemical results demonstrating that Bcl-xL interacts with cytochrome c as a part of the cellular response to ionizing radiation and other genotoxic agents [21], it might seem that this interaction is operative in vivo as well. If the interaction indeed exists under cellular conditions, then a regulatory role can be established on the basis of the current understanding of upstream interprotein interactions involving cytochrome c. Once translocated to cytosol in response to an apoptotic stimulus [1,10,15], binds Apaf-1 in the presence of dATP or dADP [10]. The binary complex in turn interacts with procaspase-9, and subsequently cleaves the CED-3-like prodomain of the zymogen form of caspase-9 [40]. The ternary cyt-Apaf-1–caspase-9 complex recruits procaspase-3 and possibly procaspase-7 in quick succession to form a functional apoptosome [2]. Thus, the initial ternary complex formed of cytochrome c, Apaf-1, and caspase-9 (the cyt-Apaf-1–caspase-9 complex) appears to be the hallmark of the initiation of the death cascade [41]. The quantitative and conclusive in vitro data presented here leads one to wonder if Bcl-xL competes with Apaf-1 in order to block the formation of the initial Apaf-1–cytochrome c complex, thereby inhibiting caspase activation.

Unfortunately, there is no conclusive evidence for this regulatory response in vivo. If a regulatory role of Bcl-xL–cytochrome c interaction is granted, the effectiveness of the regulation must be considered in the light of possible affinities of cytochrome c for other Bcl-2 family proteins. For example, some data here indicate variable affinity interactions of cytochrome c with Bid and with Bad BH3. In this perspective, the extent to which Bcl-xL can arrest cytochrome c to exert a regulatory role would depend on the balance of thermodynamics of various interactions involving the pro- and antisurvival proteins, provided these interactions operate in vivo. Clearly, more evidence must be obtained that the Bcl-xL–cytochrome c interaction is indeed relevant for regulation of apoptosis in vivo.

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