Determinants of 14-3-3σ Protein Dimerization and Function in Drug and Radiation Resistance*

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Many proteins exist and function as homodimers. Understanding the detailed mechanism driving the homodimerization is important and will impact future studies targeting the "undruggable" oncogenic protein dimers. In this study, we used 14-3-3σ as a model homodimeric protein and performed a systematic investigation of the potential roles of amino acid residues in the interface for homodimerization. Unlike other members of the conserved 14-3-3 protein family, 14-3-3σ prefers to form a homodimer with two subareas in the dimeric interface that has 180° symmetry. We found that both subareas of the dimeric interface are required to maintain full dimerization activity. Although the interfacial hydrophobic core residues Leu12 and Tyr84 play important roles in 14-3-3σ dimerization, the non-core residue Phe25 appears to be more important in controlling 14-3-3σ dimerization activity. Interestingly, a similar non-core residue (Val81) is less important than Phe25 in contributing to 14-3-3σ dimerization. Furthermore, dissociating dimeric 14-3-3σ into monomers by mutating the Leu12, Phe25, or Tyr84 dimerization residue individually diminished the function of 14-3-3σ in resisting drug-induced apoptosis and in arresting cells at G2/M phase in response to DNA-damaging treatment. Thus, dimerization appears to be required for the function of 14-3-3σ.

14-3-3σ belongs to the highly conserved mammalian 14-3-3 protein family, the members of which function as chaperones (1–3) and bind to a variety of proteins important for various cellular processes (4), including cytokinesis (5), cell cycle regulation (6–9), and apoptosis (8, 10–12). Because of its roles in multiple cellular processes, 14-3-3σ has been considered as a double-edged sword of cancers (1). Silencing the expression of 14-3-3σ may contribute to tumorigenesis (13, 14), whereas its up-regulation in cancers causes resistance to chemotherapy (8, 15, 16).

The atomic structure of 14-3-3σ has been determined (4, 17) to have an overall dimeric structure that resembles a flattened horseshoe (1). Each subunit of the dimer consists of nine α-helices. Unlike most other 14-3-3 proteins that can form both homo- and heterodimers among the 14-3-3 family proteins, 14-3-3σ prefers to form only homodimers (17). It has also been found that mutation of Ser5, Gln20, and Gln40 promotes heterodimerization of 14-3-3σ with other 14-3-3 isoforms, whereas mutations of Phe25 and Gln55 promotes little heterodimerization, although they decrease the ability of 14-3-3σ to form homodimers (18). Interestingly, the combined mutations of all five residues result in a mutant that can no longer form homodimers but can form heterodimers with all of the other six 14-3-3 isoforms.

In a recent study analyzing the mechanisms of 14-3-3σ dimerization using molecular dynamics (MD) simulations and site-directed mutagenesis (19), we found that Phe25 is critical in packing and stabilizing hydrophobic core residues that are important for dimerization by organizing cooperativity of core and other residues for favorable hydrophobic and electrostatic interactions. This organizing activity of Phe25 for 14-3-3σ homodimerization is provided by its unique physical location, rigidity, size, and hydrophobicity.

In this study, we performed a systematic study of the amino acid residues in the hydrophobic core of the 14-3-3σ dimeric interface along with residues not located in the hydrophobic core for their potential role in 14-3-3σ dimerization and inves-

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**14-3-3σ Dimerization and Function**

**TABLE 1**

| Primers used for engineering 14-3-3σ constructs |
|-----------------------------------------------|
| **Tag/mutation** | **Primers** |
| **FLAG** Forward | 5'-GGAAATTGAGAGCTTGGAGAACCAGTTGTCG-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **HA** Forward | 5'-GGAAATTGAGAGCTTGGAGAACCAGTTGTCG-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **Myc** Forward | 5'-GGAAATTGAGAGCTTGGAGAACCAGTTGTCG-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **HAMP** Forward | 5'-GGAAATTGAGAGCTTGGAGAACCAGTTGTCG-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **Q8G** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **L12G** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **F25E** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **F25E** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **V81G** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |
| **Y84G** Forward | 5'-ATGAGAGAGCTGAGGAGGAGGGGAGGTGGAGAC-3' |
| **Reverse** | 5'-AGCTCGAGTCAGATCACTCCCTGATGTTAATAGCTGAGAAGCCGACC-3' |

The potential effect of mutations of these residues on 14-3-3σ function. We have identified amino acid residues that are important to maintain the dimerization activity of 14-3-3σ and shown that dimerization is required for the function of 14-3-3σ in contributing to drug and radiation resistance.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (BioWhittaker). MIA PaCa-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. PaCa-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2.5% equine serum (HyClone) and 1% penicillin/streptomycin (BioWhittaker). MIA PaCa-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. The protein levels of 14-3-3σ in stable clones were continuously monitored by Western blotting.

**Construct Engineering**—Wild-type and mutant 14-3-3σ constructs with different tags (FLAG, HA, Myc, and HA with two Myc epitopes (HAMP)) were engineered using PCR and QuikChange® site-directed mutagenesis kits as we described previously (8, 15, 19–21). The primer pairs used for each construct are shown in Table 1.

**In Vitro Transcription and Translation**—In vitro transcription and translation were performed as described previously (22, 23). Briefly, 14-3-3σ constructs were linearized using XhoI, followed by in vitro transcription using T7 RNA polymerase. The in vitro transcripts were then purified and used to program cell-free translation in rabbit reticulocyte lysate. The reactions were stopped by incubation at 65 °C for 5 min and subjected to co-immunoprecipitation and Western blot analysis.

**Immunoprecipitation and Western Blot Analysis**—Immunoprecipitation and Western blot analysis were performed as described previously (19, 24, 25). Briefly, cell lysates or in vitro translation products were precleared with protein A beads plus normal mouse IgG, followed by incubation with anti-HA (Covance), anti-Myc (Cell Signaling), or anti-FLAG (Sigma) primary antibody and protein A beads. The precipitated materials were washed extensively with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 20 mM EDTA, 5 mM NaF, 1 mM Na3VO3, 1 mM PMSF, and 1 mM DT. The final precipitated materials were pelleted by centrifugation and solubilized for separation by SDS-PAGE, followed by Western blot analysis and probing using anti-HA, anti-Myc, anti-FLAG, or anti-actin (Santa Cruz Biotechnology) antibody. The images were captured with a FluorChem HD2 system (Alpha Innotech Corp.) after incubated with enhanced chemiluminescence reagent (GE Healthcare).

**Survival Assay**—Survival assay was performed as described previously using sulforhodamine B or colony formation assay (20, 26). Briefly, for sulforhodamine B assay, 5000 cells/well were seeded in 96-well plates and cultured overnight. The cells were then treated with various concentrations of mitoxantrone for 3 days, followed by incubation with 0.4% (w/v) sulforhodamine B in 1% (v/v) acetic acid for 30 min at room temperature. Unbound sulforhodamine B was removed by washing three times with 1% acetic acid, and plates were air-dried. Finally,
bound sulforhodamine B was solubilized in 10 mM Tris base, and $A_{570\text{ nm}}$ was measured. For colony formation assay, 100 cells/well were seeded in 6-well plates and cultured overnight. The cells were then treated with 2 grays of γ-irradiation, followed by continuous culture for 2 weeks with media changes every 2–3 days. Cell colonies were stained with crystal violet and counted manually. Survival data were analyzed with GraphPad Prism 4.

Cell Cycle Analyses—Cell cycle analysis was performed with propidium iodide staining and FACS as described previously (27). Briefly, $3 \times 10^6$ cells were seeded in a 6-cm dish, followed by treatment with $10 \text{ mM}$ mitoxantrone or $\text{Me}_2\text{SO}$ for 3 days. Cells were then collected and fixed in cold 75% ethanol and stained with $100 \mu\text{g/mL}$ propidium iodide, followed by analysis with FACS. The data were analyzed using CellQuest and ModFit programs.

Molecular Modeling and MD Simulation—The Protein Data Bank 1YWT structure was used as the template structure for 14-3-3σ, and missing loops were modeled by MOE with the homology modeling module. Mutations of specific residues were introduced by the UCSF Chimera swapaa function. MD simulations of mutant 14-3-3σ dimers were carried out using the AMBER 9 package. FF03 parameters and hydrogen atoms were assigned to the protein by the leap module of AMBER 9. All dimers were solvated in a $84 \times 103 \times 67$-Å rectangular box. An appropriate number of counterions were added to neutralize each system. The particle mesh Ewald method was employed to calculate the long-range electrostatic interactions, and the non-bonded cutoff was set to 8.0 Å.

Each system was equilibrated by a four-step protocol prior to production MD simulation as described previously (19). The same conditions of the final equilibration step were used for 20-ns production MD simulations.

To determine how each mutation affects 14-3-3σ dimerization, binding free energies between the two associating subunits were calculated using the generalized Born and surface area solvation (GBSA) method (28). The binding free energy is computed by taking the difference between the molecular mechanics (MM)/GBSA free energy of the complex and that of the ligand and receptor: \[
\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}},
\]
where $G_{\text{sol}}$ and $G_{\text{solvent}}$ represent an average of the solvent-accessible surface area of the molecule that becomes buried upon dimerization (solvent-accessible area in monomeric protein that becomes buried upon dimerization) (Table 2). The top two most buried core residues, Tyr84 and Leu12, have 99% and 96% of their solvent-accessible area buried in the dimeric interface (solvent-accessible area in monomeric protein that becomes buried upon dimerization) (Table 2). The top two buried surface areas (120 and 88 Å², respectively) are related by the pseudo-2-fold symmetry of the protein (19). In each subarea, Lys9, Leu13, Ala13, and Ala16 of one subunit interact with helices C and D of the opposing subunit, respectively, and vice versa. The 980-Å⁴ total buried interface can be divided into two structurally similar subareas, which are related by the pseudo-2-fold symmetry of the protein (19).

RESULTS

Analysis of the Dimeric Interface of 14-3-3σ—To investigate the determinant residues of 14-3-3σ dimerization, we first examined the dimeric interface of 14-3-3σ (Protein Data Bank codes 1YZ5 and 1YWT). The overall structure of the 14-3-3σ dimer is composed of nine helices (A–I) from each monomer (4, 5). Helices A and B of one subunit interact with helices C and D of the opposing subunit, respectively, and vice versa. The 980-Å⁴ total buried interface can be divided into two structurally similar subareas, which are related by the pseudo-2-fold symmetry of the protein (19). In each subarea, Lys9, Leu13, Ala13, and Ala16 of one subunit and Ala58, Leu62, Tyr84, and Val88 of the opposing subunit form an interfaceal core as defined previously (19), and all have >80% buried surface area in the dimeric interface (solvent-accessible area in monomeric protein that becomes buried upon dimerization) (Table 2). The top two most buried core residues, Tyr84 and Leu12, have 99% and 96% of their solvent-accessible area buried in the dimeric interface, respectively, and provide the highest level of buried surface areas (120 and 88 Å², respectively).

Contribution of Leu12, Phe25, Val88, and Tyr84 to 14-3-3σ Dimerization—To investigate the potentially important residues in 14-3-3σ dimerization, we chose to further investigate the top two core residues, Tyr84 and Leu12. We also selected two other hydrophobic residues, Phe25 and Val81, which have 40–50% of their solvent-accessible area buried in the dimeric interface (Table 2) but are not located in the interfacial core.
Gln8, which does not appear to contribute to dimerization from our analysis, was chosen as a negative control residue.

Next, all five residues were mutated to Gly because MD simulations have shown previously that the L12G, F25G, and Y84G mutations do not interrupt the stability of the helices they reside on even though Gly is a potential helix breaker (19). A 3-ns MD simulation of the V81G and Q8G mutants also showed that these mutations did not disrupt the stability of their respective helices (data not shown). Therefore, the secondary structure of α-helices in these mutants may be maintained as in the wild-type protein (see also below).

Because the dimeric 14-3-3-σ structure has a 180° symmetry and each interfacial residue occurs twice, each residue contributes to two interactions, with one from each subunit in two interfacial subareas (Fig. 1A). To determine the potential contribution of Leu12, Phe25, Val81, and Tyr84 to 14-3-3-σ dimerization, we performed an experiment to determine whether mutant 14-3-3-σ can dimerize with wild-type 14-3-3-σ using co-immunoprecipitation. In this case, only one of the two interfacial subareas carries a mutation in the mutant subunit. We first engineered wild-type 14-3-3-σ tagged with an HA epitope (Fig. 1A) and mutant 14-3-3-σ tagged with an HA epitope and two Myc epitopes (Fig. 1A) for convenience of detecting both wild-type and mutant proteins. The broad bands for the L12G, F25G, V81G, and Y84G mutant proteins shown in Fig. 1A, B, and C are likely due to degradation. IB immunoblot.

FIGURE 1. Interaction between mutant and wild-type 14-3-3-σ. A, schematic diagram of tagged 14-3-3-σ molecules in whole cell (B) and cell-free (C) systems. HEK293 cells with stable expression of HA-tagged wild-type 14-3-3-σ were transiently transfected with HAM2-tagged wild-type or mutant 14-3-3-σ (B), or both HA- and HAM2-tagged wild-type and mutant 14-3-3-σ transcripts were co-translated in rabbit reticulocyte lysates (C) as indicated, followed by co-immunoprecipitation (IP) with anti-Myc antibody. The precipitates were then subjected to Western blot analysis and probed with anti-HA antibody to detect both HAM2- and HA-tagged proteins. The asterisks indicate the coprecipitated HAM2-tagged wild-type 14-3-3-σ proteins. The broad bands for the L12G, F25G, V81G, and Y84G mutant proteins shown in B are likely due to degradation. IB, immunoblot.
HA-tagged wild-type 14-3-3σ proteins generated heterodimers (HAM2/HAM2) between the two differentially tagged 14-3-3σ molecules with a mobility between those of the HAM2/HAM2 and HA/HA homodimers (lane 5). However, the F25G and F25E mutants alone could not form any detectable dimer by themselves (lanes 2 and 3). Furthermore, co-translation of wild-type and mutant 14-3-3σ generated only dimers of the HA-tagged wild-type protein, whereas the HAM2-tagged mutant 14-3-3σ proteins stayed as monomers (lanes 6 and 7). Thus, we conclude that wild-type 14-3-3σ likely prefers to bind to wild-type 14-3-3σ, that Phe25 mutant 14-3-3σ does not have affinity for the wild-type protein or itself, and that the mutation effect on dimerization is unlikely due to Gly used as a substitute residue.

Mutation V81G Does Not Cause Changes to the Hydrophobic Core Residues in the Dimeric Interface of 14-3-3σ—Previously, we have shown that the important role of Phe25 in 14-3-3σ dimerization is due to its physical location, rigidity, size, and hydrophobicity, although it is not located in the hydrophobic core (19). Mutation of Phe25 in both subunits results in loosely packed cores, which lead to lost affinity between the two opposing subunits. Because both Val81 and Phe25 have similar total/percentage surface areas that become buried upon dimerization (see also Table 2), we investigated the structural basis for the difference between Phe25 and Val81 in contributing to dimerization. For this purpose, we performed 20-ns MD simulations of both WT/V81G and WT/F25G dimers as well as the WT/WT dimer, followed by calculation of the total binding free energies. Because the purpose of the study was for comparison between wild-type and mutant proteins, we omitted the entropy term. The calculated total binding free energy represents only the enthalpy term. Although WT/V81G has a total calculated binding free energy of −35.49 kcal/mol, which is comparable to the total binding free energy of WT/WT at −39.77 kcal/mol, the calculated binding free energy of WT/F25G is much higher at −27.81 kcal/mol (19).

To investigate the effect of mutations on the stability of the core residues, the RMSFs of each core residue, including atoms on both the main and side chains, were calculated from the simulations. As shown in Fig. 3, although the F25G mutation was introduced into only subunit II, which directly affected core II, consisting of Ala58, Leu62, Tyr84, and Val88 of subunit I and Lys9, Leu12, Ala13, and Ala16 of subunit II, the stability of residues in core I was also affected. As a result, the average RMSFs of core residues in WT/F25G is 0.66. In contrast, the core residues of the WT/V81G and WT/WT dimers are stable, and both have the same lower average RMSF of 0.56.

Dimerization of 14-3-3σ with Mutations in Both Dimeric Interfaces—In the above studies using co-immunoprecipitation of two differentially tagged proteins, we found that the L12G, F25G, and Y84G mutants could not dimerize with wild-type 14-3-3σ. However, in our computational analyses of the mutant 14-3-3σ proteins, we found that both the L12G and Y84G mutants appeared to have some affinity to form L12G/L12G and Y84G/Y84G homodimers (19). Thus, we next tested if these mutant 14-3-3σ proteins can form homodimers or dimerize with each other and other mutants to form heterodimers. These possible combinations of dimers carry the same or different mutations in one or both interfacial cores (Fig. 4A). For this purpose, the Myc-tagged L12G, F25G, and Y84G mutants were transiently transfected into the corresponding cells with stable expression of the same mutant tagged with the FLAG epitope, followed by co-immunoprecipitation with anti-FLAG antibody and Western blot analysis with anti-Myc and anti-FLAG antibodies. As shown in Fig. 4B, the non-core mutant (F25G) did not form any mutant homodimer, consistent with the finding obtained by nondenaturing PAGE analysis shown in Fig. 3. However, the mutants of the hydrophobic core residues (L12G and Y84G) formed L12G/L12G and Y84G/Y84G mutant homodimers, although formation of the mutant homodimers appeared to be less compared with the wild-type homodimer (Fig. 4B).

We next determined dimerization between different mutants using the same approach as described above. As shown in Fig. 4C, the L12G/Y84G heterodimer was detected, albeit at a
lower level compared with the wild-type protein. However, no F25G/L12G or F25G/Y84G heterodimers were detected. Together with the data shown in Fig. 4B, these observations suggest that the F25G mutation completely eliminated its dimerization activity, whereas the L12G and Y84G mutants retained some of their activities to dimerize with themselves or with each other but not with wild-type or F25G mutant 14-3-3σ. The lack of interaction between the wild-type protein and the L12G or Y84G mutant may be due to the reduced dimerization activity of the mutants, which causes unfavorable kinetics in dimerization with and competing for the wild-type molecule, which have higher affinity for itself (see “Discussion”).

Monomeric 14-3-3σ May Not Be Functional—Previously, we found that overexpression of 14-3-3σ causes cellular resistance to anticancer drugs such as mitoxantrone and to γ-irradiation (8, 15, 20). The above studies showed that although wild-type 14-3-3σ could form strong homodimers, the hydrophobic core mutants L12G and Y84G had weaker interactions and formed less homodimers compared with the wild-type protein. On the other hand, the non-core F25G mutant could not form any detectable homodimers. We next wanted to determine whether monomeric 14-3-3σ is functional. For this purpose, we first established stable MIA PaCa-2 cell lines transfected with wild-type or mutant 14-3-3σ with similar expression levels (Fig. 5A). These cell lines were then subjected to sulforhodamine B assay to determine their relative resistance to mitoxantrone. As shown in Fig. 5 (B and C), expression of wild-type 14-3-3σ caused a 3-fold increase in resistance compared with the vector-transfected control cells, which did not express any detectable 14-3-3σ, consistent with our previous findings (8, 15, 20). However, ectopic overexpression of the F25G and Y84G mutants had no significant effect on cellular resistance to mitoxantrone. Interestingly, the stable cell line with the L12G mutant had a low but significant resistance to mitoxantrone compared with the vector-transfected control cells. This obser-

FIGURE 4. Homo- and heterodimerization between the mutant 14-3-3σ proteins. A, schematic diagram of the relative positions of mutations in the two subareas of the interface between mutant 14-3-3σ homo- and heterodimers. B and C, homodimerization (B) and heterodimerization (C) of mutant 14-3-3σ. MIA PaCa-2 cells with stable expression of FLAG-tagged wild-type or mutant 14-3-3σ were transiently transfected with or without Myc-tagged wild-type or mutant 14-3-3σ as indicated, followed by immunoprecipitation (IP) with anti-FLAG antibody. The precipitates were then subjected to Western blot analysis and probed with anti-FLAG or anti-Myc antibody. IB, immunoblot.

FIGURE 5. Dimerization is required for 14-3-3σ-mediated drug resistance. A, Western blot analysis of stable MIA PaCa-2 cell lines transfected with the vector (Vec) control, wild-type 14-3-3σ, or mutant 14-3-3σ. Actin was used as a loading control. B–D, survival analysis. Stable cell lines as shown in A were treated with different concentrations of mitoxantrone (MXT; B and C) or with or without 2 grays of γ-irradiation (D), followed by sulforhodamine B assay (B and C) or colony formation assay (D). B represents a typical experiment with survival assay. C and D are summaries of three to four experiments. RRF, relative resistance factor = IC50(MXT)/IC50(Vec). *p < 0.05; **p < 0.01.
vation is consistent with the fact that L12G had the most remaining dimerization activity among all three mutants. Together, these findings indicate that monomeric mutant 14-3-3σ may not be functional in causing cellular resistance to anticancer drugs.

To further determine whether monomeric 14-3-3σ is functional, we chose to analyze F25G as a representative in more detail in the following experiments. In a previous report (20), we showed that overexpression of 14-3-3σ contributes to resistance to γ-irradiation. To determine whether 14-3-3σ-mediated radiation resistance also requires dimeric 14-3-3σ, we performed clonogenic assay following γ-irradiation using MIA PaCa-2 cells with stable expression of wild-type and F25G mutant 14-3-3σ. As shown in Fig. 5D, MIA PaCa-2 cells with wild-type 14-3-3σ were significantly more resistant to γ-irradiation compared with the vector-transfected control cells. However, MIA PaCa-2 cells expressing the F25G mutant had similar sensitivity to γ-irradiation as the vector-transfected control cells. Thus, dimerization is likely also required for the function of 14-3-3σ in resistance to γ-irradiation.

Reduced Function of Monomeric 14-3-3σ in Resisting Drug-induced Apoptosis and G2/M Arrest—Previously, it has been shown that the molecular basis of 14-3-3σ function in drug and radiation resistance is due to its role in regulating DNA damage-induced apoptosis and cell cycle arrest (1). To determine whether monomeric mutant 14-3-3σ retains any of its function in cellular response to drug-induced apoptosis and G2/M arrest, we treated MIA PaCa-2 cells with stable expression of wild-type and mutant 14-3-3σ and vector-transfected control cells with mitoxantrone and then determined cleavage of poly-(ADP-ribose) polymerase (PARP), a target substrate of activated caspases. Fig. 6 shows a time course of production of the (ADP-ribose) polymerase (PARP), a target substrate of activated caspases. Fig. 6 shows a time course of production of the intact 115 kDa and cleaved 85 kDa PARPs shown in A were quantified using a gel densitometer, and the 85 kDa/total ratio (85 kDa/115 kDa) was calculated to serve as an indicator of apoptosis.

Monomeric mutant 14-3-3σ has reduced activity to resist drug-induced apoptosis. The L12G mutant retains more activity in resisting drug-induced apoptosis than the other mutants, consistent with the observation that it maintains more dimerization activity than the other mutant molecules.

Next, we tested the activity of monomeric mutant 14-3-3σ in regulating G2/M arrest in response to DNA damages. Following mitoxantrone treatment, MIA PaCa-2 cell lines with stable expression of wild-type and mutant 14-3-3σ and vector-transfected control cells were tested for their cell cycle distribution. As shown in Fig. 7 (A and B), all cells had a similar cell cycle distribution under the control growth conditions. However, following mitoxantrone treatment, ~51% of the cells expressing wild-type 14-3-3σ were arrested in G2/M phase compared with ~24% of vector-transfected control cells. Approximately 36–42% of the cells expressing mutant 14-3-3σ were in G2/M phase, with the L12G mutant having the highest G2/M population among all mutant molecules. Thus, monomeric mutant 14-3-3σ is less efficient than dimeric wild-type 14-3-3σ in arresting cells in G2/M phase in response to DNA-damaging treatment.

It was shown previously that 14-3-3σ arrests cells in G2/M phase possibly by retaining Cdc2 in the cytoplasm (8). To determine whether mutant 14-3-3σ possibly lost this activity, we performed an indirect immunofluorescence staining of MIA PaCa-2 cells with stable expression of wild-type and F25G mutant 14-3-3σ. Fig. 7C shows that Cdc2 was located in the cytoplasm in both cells under control conditions. However, Cdc2 moved into the nucleus in cells with F25G mutant 14-3-3σ but remained in the cytoplasm in the cells with wild-type 14-3-3σ. This observation suggests that the F25G mutant likely lost its ability to bind and retain Cdc2 in the cytoplasm in response to DNA-damaging treatment.

X-ray Scattering Analysis—The observation that the L12G mutant has residual activity in forming homodimers and contributing to drug resistance prompted us to further analyze the distribution of monomers versus dimers using the SAXS approach, which can be used to quantify the molecular weight for a single-component system and the concentration of individual components in a mixture. For a single-component system, the forward scattering relates to the molecular weight of a protein by $I_0/C_{\text{mass}} = k \times M_p$, where $C_{\text{mass}}$ is the mass concen-
tration (e.g. in mg/ml) and $k$ is a constant (31). If a system has more than one component, the apparent forward scattering will be the summation of that of individual components, i.e. $I_{a\text{ (app)}} = \sum I_{a\gamma}$. For a monomer/dimer mixture, the apparent forward scattering relates to the percentage ($x$) of dimer in mass concentration as a function of $I_{a\text{ (app)}} = 0.5k M_r(dimer)(1 + x)$, which is used to estimate the percentage of dimers in solution.

For SAXS determination, we engineered the mutations for production and purification of recombinant proteins (Fig. 8A). Because the F25G mutant did not form any homodimers and

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure.png}
\caption{Effect of dimerization deficiency on drug-induced G2/M arrest. A, FACS analysis of cell cycle distribution of stable MIA PaCa-2 cell lines transfected with the vector (Vec.) control, wild-type 14-3-3, or mutant 14-3-3 following treatment with 10 nm mitoxantrone (MXT) or dimethyl sulfoxide (DMSO; control) for 3 days. B, quantitative analysis of cell cycle distribution as shown in A. C, immunofluorescence staining of Cdc2. MIA PaCa-2 cells with stable expression of wild-type or mutant (F25G) 14-3-3 were treated with dimethyl sulfoxide (control) or 10 nm mitoxantrone for 3 days, followed by immunofluorescence staining of Cdc2.}
\end{figure}
had no detectable function (see above), we performed SAXS analysis on only purified L25G and Y84G mutants in comparison with the wild-type 14-3-3/H9268 protein (Fig. 8B). Assuming that all molecules of the wild-type 14-3-3 protein are dimeric, L12G and Y84G were estimated to have 76 and 58% molecules in dimeric form, respectively, using the above equation. The less dimeric form for Y84G compared with L12G in solution is also supported by its smaller apparent \( R_g \) (Fig. 8C) and shorter distance-shifted pair distance distribution function compared with L12G (Fig. 8D).

X-ray scattering (small- and wide-angle) measures the protein structural details at various levels (32). The small-angle region, i.e. \( q < 0.3 \text{ Å}^{-1} \), delineates the global shape of the protein, whereas the wide-angle data reflect the tertiary structure (\( 0.3 < q < 1.0 \text{ Å}^{-1} \)) and features within secondary structure motifs (\( 1.0 < q < 2.0 \text{ Å}^{-1} \)). The changes in the small-angle region for the mutants are likely due to the existence of monomers of the mutant proteins (Fig. 8B). However, in the wide-angle region, the scattering profiles of mutants are almost identical to that of the wild-type protein, with both resembling the simulated curves for MD structure models, indicating that the mutations do not alter the three-dimensional structures of the protein.

**DISCUSSION**

In this study, we investigated the role of four interfacial amino acid residues (Leu\(^{12}\), Phe\(^{25}\), Val\(^{81}\), and Tyr\(^{84}\)) in 14-3-3\(\sigma\) dimerization and if 14-3-3\(\sigma\) function requires the dimerization status. We found that although the interfacial hydrophobic core residues Leu\(^{12}\) and Tyr\(^{84}\) played important roles in
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14-3-3σ dimerization, the non-core residue Phe25 appeared to be more important in controlling 14-3-3σ dimerization activity. Interestingly, a similar non-core residue (Val81) was less important than Phe25 in contributing to 14-3-3σ dimerization. Furthermore, dissociating dimeric 14-3-3σ into monomers by mutating the important dimerization residue Leu12, Phe25, or Tyr84 significantly diminished the function of 14-3-3σ in resisting drug-induced apoptosis and in arresting cells at G2/M phase in response to DNA-damaging treatments and thus in drug and radiation resistance.

Although both the L12G and Y84G mutants retained some affinity for themselves, neither could potentially form detectable dimers with wild-type or F25G mutant 14-3-3σ. These two mutants could also dimerize with each other and form L12G/Y84G heterodimers. It appears that the dimerization affinity of 14-3-3σ molecules occurs in the following order: WT/WT > L12G/L12G > Y84G/Y84G ≈ L12G/Y84G > F25G/F25G ≈ L12G/F25G ≈ Y84G/F25G ≈ WT/mutants. This order is interesting and may be due to the fact that the wild-type protein has a much higher dimerization activity compared with the mutants, which is thus kinetically more favorable for forming dimers with wild-type molecules than with mutant molecules when both wild-type and mutant molecules are present. On the other hand, mutant molecules have low dimerization activity and thus form fewer mutant/mutant dimers compared with WT/WT. Consistent with this observation, the calculated binding energies from MD simulation for WT/WT and mutant/mutant dimers are also in the same order (19). The F25G mutant appears to have lost all of its ability to dimerize with itself or with wild-type and other mutant 14-3-3σ molecules. Thus, Phe25 is very critical to 14-3-3σ dimerization. These findings also suggest that disrupting the function of Phe25 by small molecules may be able to completely dissociate 14-3-3σ dimerization and that it may be targeted for drug discovery.

It is noteworthy that the V81G mutant behaved very differently from the F25G mutant, although both had similar buried surface areas upon dimerization and were not localized in the interfacial core (Table 2). Although the F25G mutant completely lost its dimerization activity with itself, other mutants, or the wild-type protein, the V81G mutant retained activity to heterodimerize with wild-type 14-3-3σ. Previously, we found that mutation of Phe25 causes disruption of the dimeric interface and shifting of side chains of Lys9. However, mutation of Val81 does not appear to affect hydrophobic core residues in the dimeric interface as revealed by structural analysis using MD simulation. It is also noteworthy that the two subareas of the dimeric interface are both important, and one intact subarea does not appear to be sufficient to maintain the full dimerization activity. In fact, the mutant proteins carrying mutations in the same subarea (e.g. L12G/Y84G heterodimer) did not form more dimers than the ones with mutations in both subareas (e.g. L12G/L12G) (Fig. 4A). Thus, we conclude that both subareas of the dimeric interface are required to maintain a full dimerization activity of 14-3-3σ and that maintaining one intact subarea has no advantage over mutations in both subareas.

In functional studies, we found that the L12G mutant had detectable activity in causing resistance, change in apoptosis, and G2/M arrest. This finding is interesting in that the L12G mutant had the highest activity in formation of L12G/L12G homodimers among the three mutants tested, confirming that dimerization status is required for the function of 14-3-3σ. It is noteworthy that the drug resistance activity of the L12G mutant was much less than that of the wild-type protein, although 76% of purified L12G was estimated to exist as homodimers. This discrepancy may be due to overestimation of homodimers for the mutant molecule because it was calculated with an assumption that 100% of wild-type 14-3-3σ exists as homodimers, which is unlikely the case (Fig. 2). Potential non-specific interactions at high concentrations of purified proteins for SAXS determination may also contribute to the higher estimation of homodimeric mutant molecules. Nevertheless, a lower level (58%) of homodimers for the Y84G mutant was detected, consistent with the observation using co-immunoprecipitation and its lack of drug resistance activity. Alternatively, the above discrepancy may also be due to the possibility that the mutations directly affect the functionality of the protein in addition to their effect on dimerization. However, because 14-3-3σ exerts its activity by functioning as a chaperone to bind to its ligand proteins and these mutations are far away from the ligand-binding site, it is unlikely that these mutations have any direct effect on the ligand-binding activity of 14-3-3σ. Indeed, MD simulation analysis showed that these mutations do not affect the conformation of the ligand-binding domain of 14-3-3σ (19), although these observations of computational analyses have not yet been validated experimentally. However, the data from wide-angle x-ray scattering analyses of the purified recombinant proteins suggest that these mutations do not cause major conformational changes to 14-3-3σ.

Previously, it was found that the monomeric mutant of Droso- phila 14-3-3ζ appears to be functional and may still bind to its Droso phila ligand Slob (33). However, in another study of mammalian 14-3-3ζ, it was found that dimerization of 14-3-3ζ is dependent on the phosphorylation of Ser58 (34), which is absent in 14-3-3σ, and dimerization is required for its activity in binding phosphorylated peptides and protein ligands (35). Interestingly, dimerization-deficient 14-3-3ζ is able to bind to unphosphorylated proteins. In our study, we also observed that F25G mutant 14-3-3σ could still bind to a group of proteins5 despite the fact that it could not form homodimers. It is, thus, possible that dimerization-deficient mutant 14-3-3σ is unable to bind to its natural phosphorylated protein ligands to exert its normal functions in contributing to drug and radiation resistance due to binding to other proteins. This is supported by the observation that the F25G mutant lost its ability to bind and arrest Cdc2 in the cytoplasm in response to DNA damage. Clearly, further work is needed to test this possibility.

In summary, both intact subareas of the dimeric interface appear to be required to maintain the full dimerization activity of 14-3-3σ, and mutation of hydrophobic core residues in any one of these subareas diminishes the dimerization activity. In addition to the core residues, the non-core residue Phe25 also plays an important role in driving 14-3-3σ dimerization by organizing other hydrophobic core residues. However, another

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similar non-core residue (Val81) is much less important, and its mutation does not affect the dimerization activity of 14-3-3σ. The dimerization-deficient mutant 14-3-3σ proteins also lose their function in causing drug and radiation resistance. Based on these findings, it is tempting to speculate that targeting any one of these important residues may help identify inhibitors that can disrupt 14-3-3σ dimerization for drug discovery to sensitize 14-3-3σ-mediated resistance in cancer treatments. The outcome of this study may help drug the “undruggable” oncogenic protein dimers in general.

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