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Report

Characterization of 14-3-3sigma Dimerization Determinants

Requirement of Homodimerization for Inhibition of Cell Proliferation

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ABSTRACT

The seven highly conserved 14-3-3 proteins expressed in mammalian cells form a complex pattern of homo- and hetero-dimers, which is poorly characterized. Among the 14-3-3 proteins 14-3-3σ is unique as it has tumor suppressive properties. Expression of *14-3-3σ* is induced by DNA damage in a p53-dependent manner and mediates a cell cycle arrest. Here we show that the 14-3-3σ protein exclusively forms homodimers when it is ectopically expressed at high levels, whereas ectopic 14-3-3ζ formed heterodimers with the five other 14-3-3 isoforms. The x-ray structure of 14-3-3σ revealed five residues (Ser^{5}, Glu^{20}, Phe^{25}, Q^{55}, Glu^{80}) as candidate determinants of dimerization specificity. Here we converted these amino-acids to residues present in 14-3-3ζ at the analogous positions. Thereby, Ser^{5}, Glu^{20} and Glu^{80} were identified as key residues responsible for the selective homodimerization of 14-3-3σ. Conversion of all five candidate residues was sufficient to switch the dimerization pattern of 14-3-3σ to a pattern which is very similar to that of 14-3-3ζ. In contrast to wildtype 14-3-3σ this 14-3-3σ variant and 14-3-3ζ were unable to mediate inhibition of cell proliferation. Therefore, homodimerization by 14-3-3σ is required for its unique functions among the seven mammalian 14-3-3 proteins. As inactivation of 14-3-3σ sensitizes to DNA-damaging drugs, substances designed to interfere with 14-3-3σ homodimerization may be used to inactivate 14-3-3σ function for cancer therapeutic purposes.

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INTRODUCTION

The *14-3-3σ* gene has been linked to cancer formation as it is regulated by the p53 tumor suppressor gene product[1] and commonly silenced by CpG-methylation.[2] *14-3-3σ* is a member of a gene family which encodes seven highly homologous mammalian 14-3-3 isoforms β, γ, ε, η, σ, τ and ζ (reviewed in refs. 3–5). 14-3-3 proteins form cup-shaped homo- and hetero-dimers and bind their substrates through an amphipathic binding cleft that preferentially recognizes the phosphorylated motifs RSXpSXP or RXXXpSXP.[6-8] 14-3-3 proteins were the first protein motifs shown to interact with posphorylated residues in other proteins (reviewed in ref. 9). Recently, the number of proteins potentially regulated by 14-3-3 association increased to several hundreds as the result of proteomic approaches using various 14-3-3 isoforms as baits.[10-13] In most cases association with 14-3-3 proteins occurs after phosphorylation of the ligand by a specific kinase. Binding of 14-3-3 proteins may result in multiple different effects, which are dictated by the respective protein ligand. The association may lead to activation or repression of enzymatic activity or function, prevention of degradation, cytoplasmic sequestration, nuclear retention or facilitation/prevention of protein modifications (reviewed in ref. 14–16).

Although it has been established that distinct functions exist for the different isoforms, it is largely unknown how functional specificity is generated among the seven 14-3-3 isoforms. One mediator of functional specificity may be the selective binding to distinct ligand proteins. A prerequisite for ligand selectivity is presumably the formation of specific heterodimers and homodimers among the different 14-3-3 isoforms, as random heterodimerization would presumably allow binding to any given ligand. 14-3-3 proteins form homo- and hetero-dimers via interactions among the four N-terminal α-helices.[7,17,18] However, the description of the 14-3-3 dimerization patterns and the understanding of its molecular basis is far from complete. Here we have characterized the dimerization determinants of 14-3-3σ by a structure-based mutational analyses. Furthermore, we show that homodimerization of 14-3-3σ is required for inhibition of cell proliferation.

MATERIALS AND METHODS

Cell culture and cell lines. HEK293T cells were cultured in Dulbecco's modified eagle medium (DMEM) with high glucose (Invitrogen) supplemented with 5% fetal bovine serum (FBS) at 37°C and 5% CO_2. DLD-1 derived cell lines were cultivated in McCoy's 5A medium (Invitrogen), supplemented with 10% FBS. All media were supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin. For generating cell lines with conditional expression of 14-3-3 proteins, DLD-tTA cells[19] were transfected with pBI constructs encoding 14-3-3σ-WT, 14-3-3σ-5xmut or 14-3-3ζ-WT in combination with a pTMK-Hyg vector conferring hygromycin resistance using lipofectamine 2000 (Invitrogen). Cells were selected for 10 days with 100 μg/ml hygromycin (Invitrogen) before selection by limiting dilution.

Expression plasmids. N-terminally HA-tagged 14-3-3σ was obtained by PCR with the primers 5'-ATCGAATTC CCACCATGTACCCATATGACGTTCCAGACTACCAT GAGAGAGCCAGTCTGATC-3' and 5'-ACTGGTACC TGTACATCAGCTCTGGGGCTCCTG-3' using pHRC-MV-14-3-3σ[1] as a template. The resulting fragment was cut with *EcoR* I and *Kpn* I and ligated into pCMV, a vector obtained by religation of pEYFP-N1 (Clontech) after a *BamH* I / *Not* I digest. For HA-tagged 14-3-3ζ the primers 5'-ATCGAATTCCCACCATGTACCCATATGACGTTCC-AGACTACCATGATAAAAATGAGCTGGTTCAG-3' and 5'-ACTGGTACCTTAATTTTCCCCTCCTTCTCC-3' were used. Site-specific mutations in the 14-3-3σ reading frame were introduced by PCR. For conditional expression of 14-3-3 proteins the respective inserts were transferred to a pBI vector (Clontech). The sequences of the respective PCR primers are available upon request. All constructs were confirmed by sequence analysis.

Coimmunoprecipitation. HEK293T cells were transiently transfected in 14 cm plates with plasmids using calcium-phosphate precipitation with 18 μg of DNA per 14 cm plate. Twenty-four hours after transfection, cells were washed with PBS and lysed on ice for 15 min with lysis buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP-40) supplemented with protease (Complete Mini EDTA-free, Roche) and phosphatase inhibitors (2 mM sodium-orthovanadate, 100 nM okadaic acid). Lysates were centrifuged at 13.000 rpm for 20 min. Three milligrams of lysate were used for incubation with mouse anti-VSV antibody or mouse anti-HA-antibody (Covence). After 2 hours of incubation with antibodies, 25 μl of Protein-G Sepharose beads (Amersham) were added and incubated for an additional hour. After washing 3 times with washing buffer (10 mM Tris/HCl pH7.5, 150 mM NaCl, 1% NP-40, 2 mM sodium-orthovanadate), the bound proteins were eluted by boiling for 5 min in 40 μl of Laemmli-buffer.

Western blot analysis. The eluted Co-IP proteins and 80 μg of total cell lysates were separated by 12% SDS-PAGE and transferred onto Immobilon-P membrane (Millipore). Since VSV-tagged and HA-tagged 14-3-3 proteins have almost the same size, for each Co-IP experiment two gels were loaded with half of each eluate. The membrane was blocked for 1 h in wash buffer (TBS with 0.05% Tween 20) containing 10% nonfat dry milk, followed by incubation with the primary antibody. The membranes were incubated over night at 4°C with the following antibodies: mouse anti-VSV, mouse anti-HA (Covance) and rabbit anti-β-actin (Sigma). After extensive washing, the blot was incubated with the appropriate α-mouse (Promega) or α-rabbit secondary antibody (Sigma) conjugated with horseradish peroxidase for 45 min, washed. Signals were detected using the ECL⁺ Western Blotting Detection System (Perkin Elmer) and a CF440 imager (Kodak).

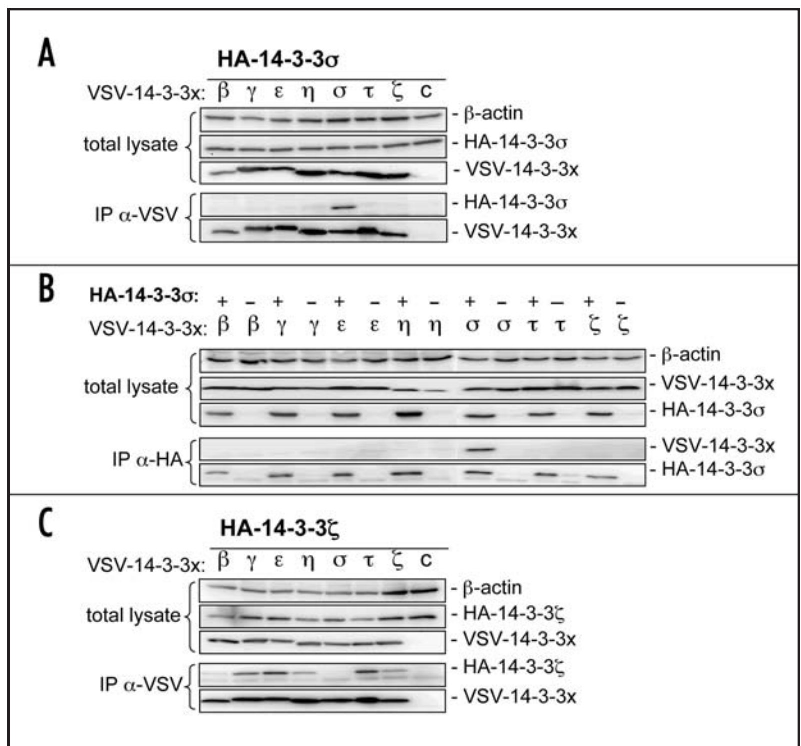

Figure 1. Dimerization specificity of 14-3-3σ and 14-3-3ζ (A) HEK293T cells were transfected with plasmids encoding HA-tagged 14-3-3σ and VSV-tagged 14-3-3 isoforms. 24 hours later cell lysates were subjected to immunoprecipitation (IP) with VSV-specific antibodies. After gel electrophoretic separation coprecipitated, ectopic 14-3-3 isoforms were detected by Western blot analysis. "c" cotransfection with empty vector. "total lysate" shows expression of the indicated proteins before IP. β-actin served as a loading control. "IP" shows coprecipitated 14-3-3 isoforms. B) The analysis was performed as in (Fig. 1A) but with inverted order of the respective antibodies: HA-specific antibodies were used for immunoprecipitation and VSV-specific antibodies for subsequent Western blot analysis. In addition, vectors encoding VSV-tagged 14-3-3 proteins were transfected alone for control purposes. C) As in (B) but performed with HA-14-3-3ζ instead of HA-14-3-3σ.

RESULTS AND DISCUSSION

14-3-3σ exclusively forms homodimers. To further substantiate previous observations indicating that 14-3-3σ may preferentially form homodimers in U2OS cells,[20] we coexpressed an HA-tagged 14-3-3σ with each of seven human 14-3-3 isoforms tagged with a VSV-epitope at high levels in human HEK293T cells. The different 14-3-3 isoforms were precipitated using a VSV-specific antibody and the amount of coprecipitated HA-14-3-3σ was determined by Western blot analysis (Fig. 1A). In this assay we found that 14-3-3σ forms homodimers and is not able to interact with other isoforms, although all isoforms were expressed at high levels and effectively precipitated with the VSV-specific antibody (Fig. 1A). This assay was repeated using the respective antibodies in a reversed order. The results were identical and ruled out any antibody-specific effects (Fig. 1B). In addition, VSV-14-3-3σ was only detected in

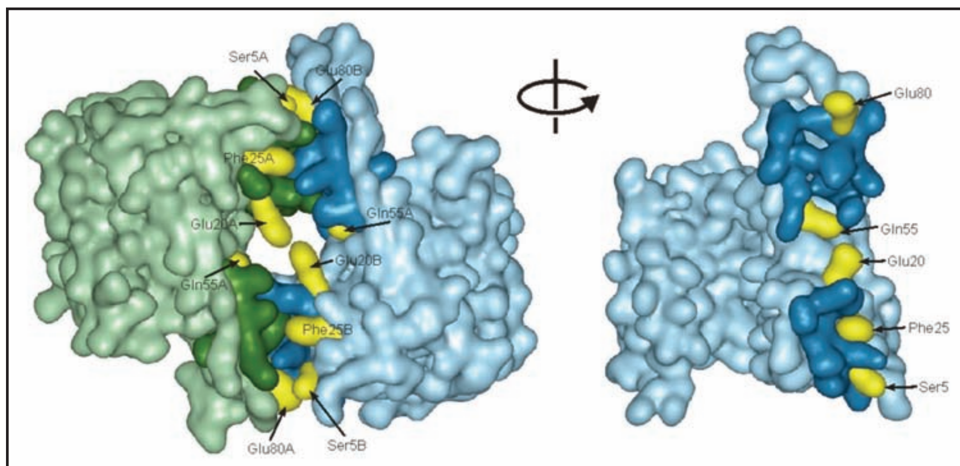

Figure 2. Overview plot of the 14-3-3σ surface. The monomers are presented in green and blue. Residues directly involved in dimer formation are colored in dark green and dark blue. Candidate residues responsible for dimerization specificity are colored yellow and labeled.

the HA-immunoprecipitation when HA-14-3-3σ was coexpressed, confirming the requirement for dimerization between HA- and VSV-14-3-3σ proteins. However, when a HA-tagged 14-3-3ζ protein was employed in this assay 14-3-3ζ formed homo- and hetero-dimers with all isoforms except 14-3-3σ (Fig. 1C). Therefore, 14-3-3σ is characterized by a uniquely restricted dimerization pattern.

Candidate residues mediating dimerization specificity of 14-3-3σ. We assumed that structural determinants in the dimerization interface mediate the specific dimerization pattern of 14-3-3σ. Comparison of the structures of 14-3-3σ and 14-3-3ζ revealed that five amino acids in the N-terminus could potentially provide a basis for homodimerization of 14-3-3σ through stabilizing homodimeric and destabilizing heterodimeric interactions.[20,21] These residues are Ser^5, Glu^{20}, Phe^{25}, Gln^{55} and Glu^{80} and are positioned at the interface between the two molecules of a 14-3-3σ dimer (Fig. 2). We converted these amino-acids into the corresponding residues of the 14-3-3ζ isoform by using site-directed, PCR-mediated mutagenesis. A more detailed view of these exchanges and their presumable consequences is shown in Figure 3A–E. Subsequently, we determined the impact of these alterations on the dimerization pattern of 14-3-3σ by coimmunoprecipitation analyses.

The 14-3-3σ variant Ser^5 to Glu. Substitution of Ser^5 of 14-3-3σ with Glu, which is present in 14-3-3ζ (and also in β and τ), reduced homodimerization of 14-3-3σ (Fig. 4A). This 14-3-3σ mutant formed heterodimers with all other isoforms except 14-3-3ε. In the structure of the 14-3-3σ homodimer the Ser^5 side chain is close to the side chains of Glu^{80} and Glu^{83} of the second 14-3-3σ molecule (Fig. 3A). Although the distance between the OG atom of Ser^5 and OE1,2 atoms of Glu^{80} and Glu^{83} does not allow for direct hydrogen bonding, a water molecule is located between these residues in both known structures of 14-3-3σ,[20,21] which may contribute to the stabilization of dimerization. Mutation of Ser^5 to Glu introduces repulsive negative charges to Glu^{80} and Glu^{83}. Also, there is no hydrogen donor (Ser^5) for creation of a hydrogen bond in this area. Therefore, formation of the homodimer is impaired. However, the interaction with other 14-3-3 isoforms seems to be possible, as they do not contain a negatively charged residue at the position of the 14-3-3σ Glu^{80}. In a heterodimer, Ser^5 with its short, polar side-chain would be situated unfavorably close to Met^{78} of the 14-3-3ζ isoform, while when mutated, the Glu^5 side-chain points outward, leaving the vicinity of Met^{78} completely hydrophobic. Taken together, mutation of Ser^5 to glutamic acid reduces homodimerization and increases the heterodimerization capability of 14-3-3σ.

The 14-3-3σ variant Glu^{20} to Asp. A mutant of 14-3-3σ, with a replacement of Glu^{20} by Asp (present in all other isoforms at this position), was still able to form dimers with HA-tagged 14-3-3σ, but could also form heterodimers with all other 14-3-3 isoforms (Fig. 4B). The strongest interaction was detected with 14-3-3ε. Glu^{20} forms an interesting bifurcated hydrogen bond (where the hydrogen atom interacts with two acceptors) with Glu^{20} of the second molecule in the dimer (Fig. 3B). The distance between OE atoms is 3.35 Å. It is the only fully symmetrical interaction in the dimer and is unique to the 14-3-3σ isoform. Mutation to Asp should cause an increase of the distance between OE atoms and would not allow for a hydrogen bond, therefore impairing homodimerization. Instead, Asp at position 20 is involved in the inter-monomer hydrogen bonding (as seen in the 14-3-3ζ isoform). This change of the side-chain position most probably shifts the main chain of Asp^{20} by about 0.8 Å. The shift affects also the neighboring Arg^{18} (which has a semi-flexible side-chain in 14-3-3σ) allowing it to form a salt bridge with 14-3-3σ Glu^{91} of the second monomer and it also influences Asp^{21}, which is involved in hydrogen bonding to Tyr^{84}. The interaction of Arg^{18} with Glu^{91} (Glu^{89} in all other isoforms) is characteristic for all 14-3-3 isoforms except 14-3-3σ and is therefore assumed to promote the formation of heterodimers.

The 14-3-3σ variant Phe25 to Cys. Conversion of the 14-3-3σ Phe^{25} into Cys, which is present in τ and ζ, still allowed the interaction with HA-tagged 14-3-3σ (Fig. 4C), but the binding seemed to be weaker than for the previously detected 14-3-3σ homodimerization (Fig. 1A). Heterodimerization with other 14-3-3 isoforms was not detected. Phe^{25} is a part of a hydrophobic region of the dimer interface together with Leu^{12} and, at the other side, with Val^{61}, Leu^{62}, Ile^{65} and Tyr^{84} (Fig. 3C). There is also a possibility for an aromatic interaction with Tyr^{84}, which itself is involved in a hydrogen bond pattern around the central part of the dimer. Mutation to cysteine should therefore weaken this part of the interface (due to the smaller size of the cysteine side chain and a possible solvent penetration). However, as cysteine is a hydrophobic residue, the effect of this mutation might not be significant. Indeed, the F25C mutation weakened the interaction of the 14-3-3σ homodimers while it had no detectable effect on the heterodimer formation.

The 14-3-3σ variant Gln^{55} to Arg. Conversion of Gln^{55} to Arg, which is present in all other isoforms at this position, reduced the homodimerization with HA-tagged 14-3-3σ (Fig. 4D). Very weak binding to 14-3-3γ and η was detected. Gln^{55} is a donor of hydrogen for Glu^{91} of the same monomer molecule (Fig. 3D). Glu^{91} is important because it interacts with Arg^{18} in the second molecule of the dimer. Change of the negatively charged Glu^{55} to the positively charged Arg might result in the formation of a strong salt bridge with Glu^{91}.

The 14-3-3σ variant Glu^{80} to Met. Substitution of Glu^{80} for Met, which is present in β, γ, ε and ζ, led to heterodimerization with all 14-3-3 isoforms except β (Fig. 4E). However, the interaction was

only strong with 14-3-3σ and γ, whereas weak binding was detected for ε, η, τ and ζ. These results could also be explained by structural characteristics of 14-3-3σ: mutation of Glu^{80} to Met introduces a hydrophobic residue, which mimics the hydrophobic interaction of other isoforms (Fig. 3E). Again, there is no possibility of hydrogen bond formation. Instead Met^{80} extends the hydrophobic part of the interface. A hydrophobic residue at position 80 does not introduce strong repulsive interactions. They are weaker than those caused by Glu^{80} or when Ser^{5} is mutated to Glu. Therefore, the homodimer formation in the 14-3-3σ isoform is impaired, but not as strongly as in the Ser^{5}Glu variant of 14-3-3σ.

Taken together, the single substitutions Ser^{5}Glu, Glu^{20}Asp, or Glu^{80}Met promote heterodimerization, whereas Phe^{25}Cys and Gln^{55}Arg reduce homodimerization of 14-3-3σ but have minor effects on heterodimerization.

Effects of combined mutations in the dimerization domain. We combined the different variants of 14-3-3σ. The double mutant Glu^{20}Asp/Phe^{25}Cys had a similar effect on dimerization as the exchange of Gln^{55} to Arg (Fig. 4F). The Glu^{20}Asp and Gln^{55}Arg changes are assumed to weaken the homodimer formation. The Phe^{25}Cys mutation apparently affects the Tyr^{84} side-chain position, therefore inflicting its hydrogen bonding.

The triple 14-3-3σ mutant Ser^{5}Glu/Glu^{20}Asp/Phe^{25}Cys formed heterodimers with all VSV-tagged 14-3-3 isoforms and also formed homodimers with 14-3-3σ although at reduced levels (Fig. 4G). Presumably, the Ser^{5}Glu mutation allowed formation of heterodimers while homodimerization was impaired by Glu^{20}Asp/Phe^{25}Cys mutations.

An interesting pattern of dimerization was formed by a 14-3-3σ mutant with four amino acids exchanged: Ser^{5}Glu/Glu^{20}Asp/Phe^{25}Cys/Gln^{55}Arg. This mutant hardly interacted with any of the VSV-tagged 14-3-3 isoforms; a weak interaction could be found with 14-3-3γ and η (Fig. 4H). Clearly, mutation of Gln^{55} to Arg is promoting the interaction with γ and η while the other mutations negatively affect dimerization in general (except Ser^{5}Glu which reduces dimerization selectivity). In this mutant Arg^{55} is able to form a salt bridge with Asp^{20} as seen in the ζ isoform. The mutation Gln^{55}Arg presumably introduces a drastic change in the pattern of salt bridges and hydrogen bonds localized around an "empty" region between monomers perpendicular to the dimer symmetry axis apparently promoting interactions with 14-3-3γ and η.

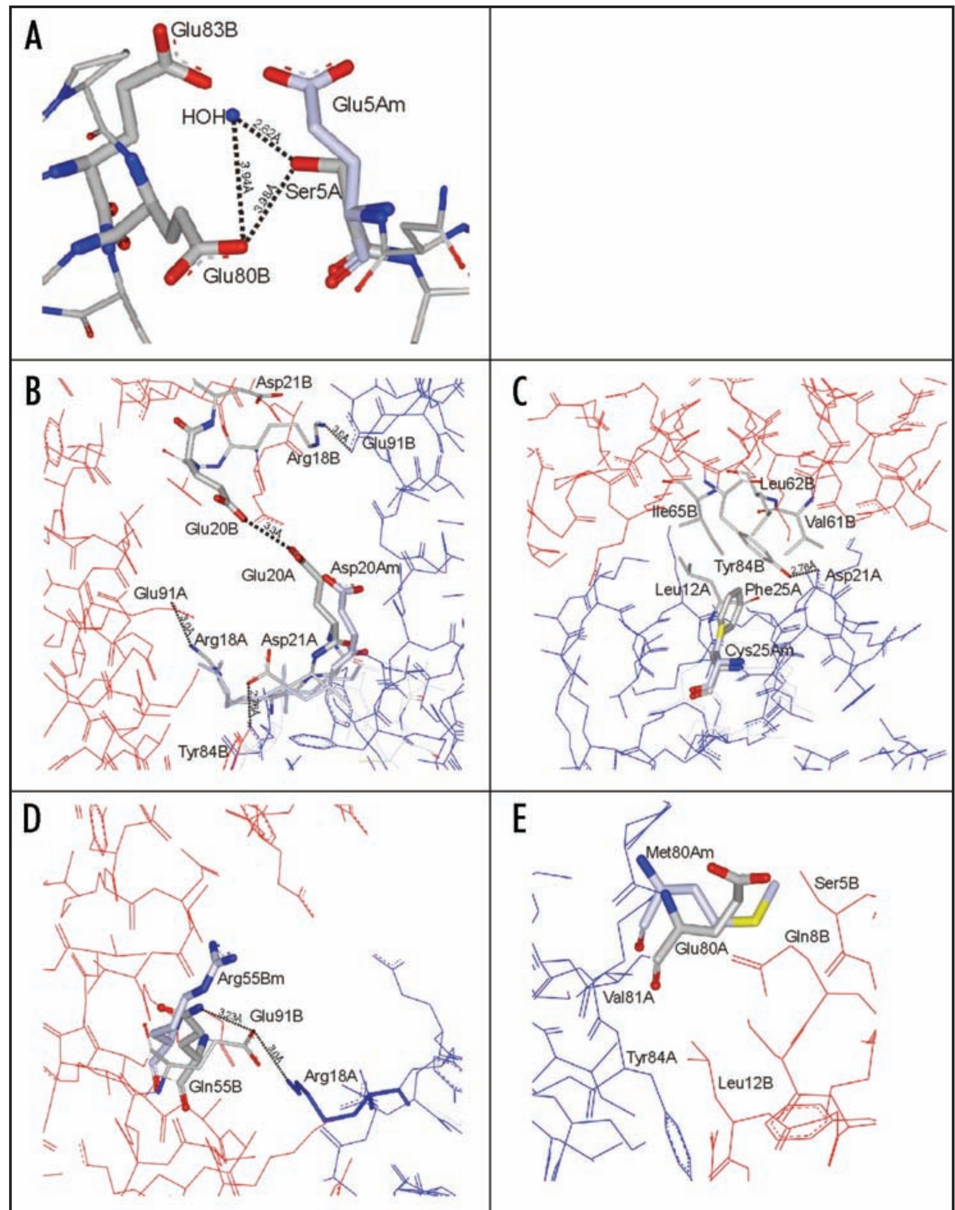

Figure 3. Structural effects of amino acid exchanges presumably affecting 14-3-3σ dimerization. The two monomers are shown in red and blue. Residues belonging to each monomer are marked by capital letters at the end of the name. Mutated residues are shown as thick sticks; other residues affected by mutations as thinner sticks. The introduced amino-acids are shown in light blue. Carbon atoms are shown in gray, oxygen in red, and nitrogen in dark blue. (A) Glu^{80} and Glu^{83} form a negative charge at a one side of dimerization interface. Ser^{5} forms a hydrogen bond with a water molecule. Although there is no direct hydrogen bonding between monomers, substitution of Ser for Glu creates repulsive forces (by introducing negative charges close to the side-chains of Glu^{80} and Glu^{83}. (B) The Glu^{20}Asp mutation breaks a bifurcated hydrogen bond between monomers. Backbone positions of Asp^{20}, Asp^{21} and Arg^{18} are affected. (C) In the Phe^{25}Cys mutation aromatic ring stacking may affect the position of Tyr^{84} and its hydrogen bonding; a nonpolar environment is not significantly affected. (D) The Gln^{55}Arg mutation breaks a hydrogen bond between Gln^{55} and Glu^{91} affecting the Glu^{91} interaction with Arg^{18}. (E) The Glu^{20}Met mutation extends the hydrophobicity of the interface important for heterodimerization.

A 14-3-3σ mutant incorporating all five residue exchanges (14-3-3σ-5x-mut.) could not form homodimers with wildtype 14-3-3σ, but engaged in heterodimers with the six 14-3-3 other isoforms (Fig. 4I). Therefore, conversion of these five residues in 14-3-3σ is sufficient to confer a dimerization pattern very similar to the pattern of 14-3-3ζ (compare Figs. 1C and 4I). This effect is

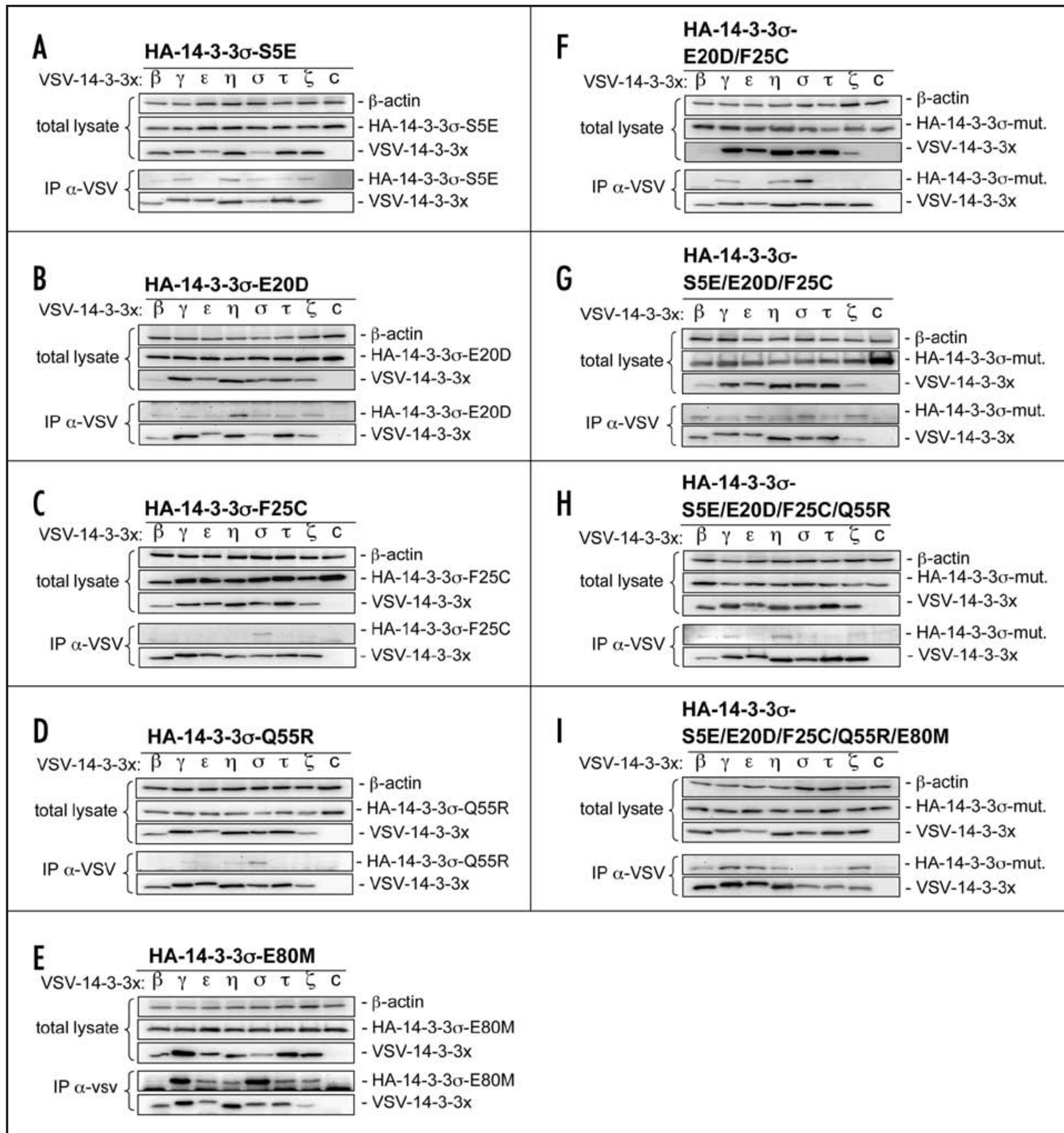

Figure 4. Effects of mutations on the dimerization specificity of 14-3-3σ. HEK293T cells were cotransfected with constructs for HA-tagged 14-3-3σ-mutants Ser^5Glu (A), $Glu^{20}Asp$ (B), $Phe^{25}Cys$ (C), $Gln^{55}Arg$ (D), $Glu^{80}Met$ (E), $Glu^{20}Asp/Phe^{25}Cys$ (F), $Ser^5Glu/Glu^{20}Asp/Phe^{25}Cys$ (G), $Ser^5Glu/Glu^{20}Asp/Phe^{25}Cys/Gln^{55}Arg$ (H) and $Ser^5Glu/Glu^{20}Asp/Phe^{25}Cys/Gln^{55}Arg/Glu^{80}Met$ (I) in combination with the seven VSV-tagged 14-3-3 isoforms. The subsequent analysis was performed as in Figure 1A.

presumably due to the summation of homodimer abolishing and heterodimer promoting interactions discussed above. Therefore, the five residues identified here represent the critical determinants of dimerization specificity of 14-3-3σ. A summary of the immunoprecipitation analyses is provided in Table 1.

The results shown here indicate that only a few amino acids determine whether 14-3-3σ forms homodimers. The residues Ser^5, Glu^{20} and Glu^{80} are required for prevention of heterodimerization since mutation of one of these amino acids is sufficient for heterodimerization of 14-3-3σ with other 14-3-3 isoforms. On the contrary, homodimerization seems to involve the combined interactions mediated by multiple residues as the simultaneous exchange of five selected amino acids was necessary to abolished homodimerization of 14-3-3σ.

Requirement of 14-3-3σ homodimerization for inhibition of cell proliferation. To analyze the biological role of 14-3-3σ homodimerization we generated DLD-1 cell lines conditionally expressing 14-3-3σ, 14-3-3ζ and 14-3-3σ-5xmut under control of a tet-operon. After transfection of the respective plasmids, multiple single cell clones were isolated and characterized for each 14-3-3 variant. Upon removal of DOX, induced expression of the ectopic 14-3-3 proteins was detected using the attached HA-epitope (Fig. 5). This indicated that all cell lines expressed similar levels of the different 14-3-3 variants. Next we determined the effect of the

Table 1. **Summary of the dimerization analyses.**

14-3-3 Isoform	Interacts with 14-3-3 Isoform						
	β	γ	ε	η	σ	τ	ζ
σ	-	-	-	-	++	-	-
ζ	+	++	++	+	-	++	+
σS5E	+	++	-	++	+	+	+
σE20D	+	+	+	++	+	+	+
σF25C	-	-	-	-	+	-	-
σQ55R	-	+	-	+	++	-	-
σE80M	-	++	+	+	++	+	+
σE20D_F25C	-	+	-	+	++	-	-
σS5E_E20D_F25C	+	+	+	+	+	+	+
σS5E_E20D_F25C_Q55R	(+)	+	-	+	(+)	-	(+)
σS5E_E20D_F25C_Q55R_E80M	+	++	++	+	-	+	++

"-" = no signal, "(+)" = very weak signal, "+" = weak signal, "++" = strong signal after combined immunoprecipitation and Western blot analysis.

ectopic expression of the 14-3-3 variants on cellular proliferation over a period of 6 days. Enhanced expression of wildtype 14-3-3σ had a clear antiproliferative effect as reported previously,[1] whereas 14-3-3ζ induced a minimal elevation of proliferation. Interestingly, expression of 14-3-3σ-5xmut was unable to suppress cellular proliferation, but rather had a weak stimulatory effect on proliferation similar to the effect observed after ectopic expression of the 14-3-3ζ isoform. Identical results were obtained with sister clones conditionally expressing the 14-3-3 variants (data not shown). In summary these results show that homodimerization of 14-3-3σ is required for its antiproliferative effects.

Whether homodimerization of 14-3-3σ is a prerequisite for interacting with specific proteins can not be answered at this point as exclusive 14-3-3σ ligands have not been identified yet. However, the unique phenotypes observed after expression of 14-3-3σ strongly suggest the existence of protein ligands which only bind to this 14-3-3 isoform. We are currently using proteomic approaches to identify protein ligands which interact with 14-3-3σ but not with other 14-3-3 isoforms. In addition common ligands with varying affinities for the different 14-3-3 isoforms may exist. Certain ligands may have a higher affinity for 14-3-3σ homodimers. This would allow replacement of other 14-3-3 isoforms by 14-3-3σ homodimers after DNA damage, which leads to the p53-mediated induction of 14-3-3σ expression, or in other situations of increased 14-3-3σ expression.

This study shows that the formation of homodimers is required for the biological effects of 14-3-3σ. Our results further suggest, that the exclusive homodimerisation of 14-3-3σ and the subsequent interaction with specific ligands is essential for its tumor suppressive functions. Experimental inactivation of 14-3-3σ by homologous recombination or RNA interference sensitizes cancer cells to DNA damaging agents.[22,23,24] In the future substances, which interfere with the critical interactions identified here, may allow to inactivate 14-3-3σ function on the protein level and thereby sensitize cancer cells to DNA damaging agents.

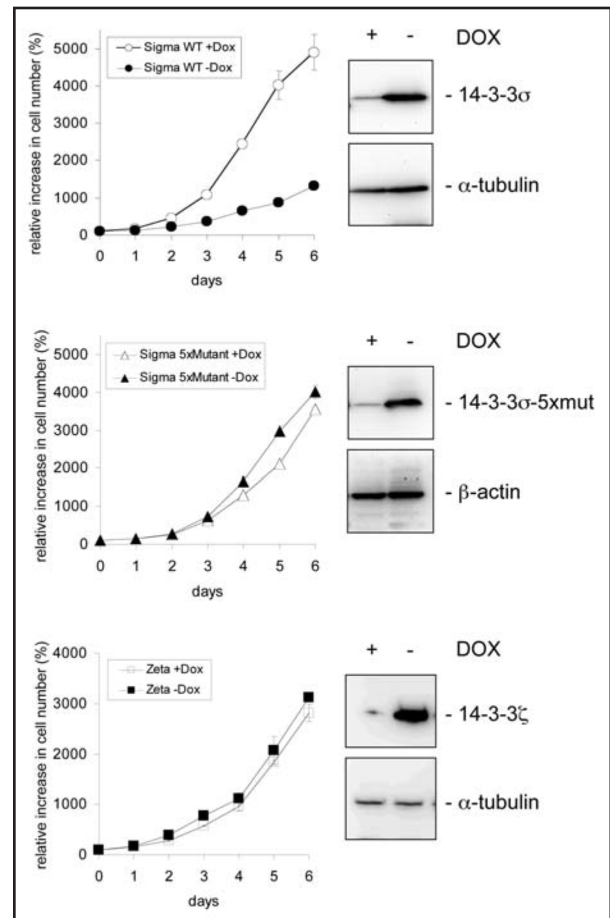

Figure 5. 14-3-3σ homodimerisation is required for inhibition of cell proliferation. Analysis of DLD-1 cell lines harboring three different 14-3-3 alleles under control of the tet-operon. After removal of DOX for 48 hours cell lysates were subjected to Western blot analysis with HA-specific antibodies. Detection of β-actin or α-tubulin served as a loading control. For determination of cell proleration cells were seeded at 50,000 per well in 6 well plates in triplicate with or without 500 ng/ml doxycycline to repress the exogenous 14-3-3 expression. Cells were harvested by trypsinisation and counted using a Coulter counter (Beckmann-Coulter). Bars represent standard deviations.

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