The SH3 Domain of αII Spectrin Is a Target for the Fanconi Anemia Protein, FANCG[†]

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ABSTRACT: The structural protein nonerythroid α spectrin (αIISp) plays a role in the repair of DNA interstrand cross-links and is deficient in cells from patients with Fanconi anemia (FA), in which there is a defect in ability to repair such cross-links. We have proposed a model in which αIISp, whose stability is dependent on FA proteins, acts as a scaffold to aid in recruitment of repair proteins to sites of damage. In order to get a clearer understanding of the proposed role of FA proteins in maintaining stability of αIISp, yeast two-hybrid analysis was carried out to determine whether FA proteins directly interact with αIISp and, if so, to map the sites of interaction. Four overlapping regions of αIISp were constructed. FANCG interacted with one of these regions and specifically with the SH3 domain in this region of αIISp. The site of interaction in FANCG was mapped to a motif that binds to SH3 domains and contains a consensus sequence with preference for the SH3 domain of αIISp. This site of interaction was confirmed using site-directed mutagenesis. Two FA proteins that did not contain motifs that bind to SH3 domains, FANCC and FANCF, did not interact with the SH3 domain of αIISp. These results demonstrate that one of the FA proteins, FANCG, contains a motif that interacts directly with the SH3 domain of αIISp. We propose that this binding of FANCG to αIISp may be important for the stability of αIISp in cells and the role αIISp plays in the DNA repair process.

Fanconi anemia (FA)[1] is a genetic disorder characterized by bone marrow failure, diverse congenital abnormalities, a marked predisposition to development of cancer, and genomic instability (*1*−*4*). Cells from patients with FA have a marked cellular hypersensitivity to DNA interstrand cross-linking agents which correlates with a defect in ability to repair damage produced by these agents (*4*−*11*). We have demonstrated that the structural protein, nonerythroid α-spectrin (αIISp), is present in the nucleus of human cells and that it plays a role in the repair of DNA interstrand cross-links (*8, 12*−*15*). We have also shown that there are reduced levels of αIISp in cells from a number of FA complementation groups, which correlates with the reduced levels of DNA repair observed in these cells after cross-link damage (*8, 9, 11, 12*). The levels of αIISp mRNA in these FA cells, however, are similar to those in normal cells (*16*). We have therefore proposed that the reduced levels of αIISp in FA cells are due to reduced stability of this protein, rather than to reduced expression, and that this, in turn, is due to deficiencies in one or more of the FA proteins which are important in its stability (*11, 16*). In support of this, we have shown that in FA cells corrected with the appropriate FANC cDNA the levels of αIISp are increased to normal levels (*12*).

Though αIISp in the cytoplasm of nonerythroid mammalian cells is known to be involved in a number of different functions, its precise roles in the nucleus are less well understood (*17*−*22*). In the cytoplasm, it is a major structural component of the membrane cytoskeleton and plays an important role in providing mechanical support for the cell membrane; it is involved in protein sorting, organelle and vesicle trafficking, neurite outgrowth, and neurotransmitter release (*17*−*21*). It is also important in cell growth and differentiation (*17, 21*). We have shown that in the nucleus αIISp is involved in repair of DNA interstrand cross-links (*13, 14*). It binds to purified DNA containing an interstrand cross-link; it colocalizes with the cross-link repair protein, XPF, in damage-induced nuclear foci after treatment of cells with a DNA interstrand cross-linking agent, and it is needed for the production of incisions by XPF at the site of an interstrand cross-link (*13, 14*). On the basis of these findings, we have proposed a model for the role of αIISp in DNA repair (*12*−*14*). In this model, FA proteins are involved in the regulation of the stability of αIISp. After cells are damaged, αIISp binds to DNA at sites of damage, where it acts as a scaffold, aiding in the recruitment of FA and repair proteins to sites of damage, enhancing the efficiency of the repair process. In FA cells, deficiencies in FA proteins lead to decreased stability of αIISp and thus to decreased levels of αIISp in these cells. This in turn leads to reduced binding

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[1] Abbreviations: αIISp, nonerythroid α spectrin; FA, Fanconi anemia; SH3, src-homology 3; BD, binding domain; AD, activation domain; 8-MOP, 8-methoxypsoralen; PTP, low molecular weight phosphotyrosine phosphatase.

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of αIISp to damaged DNA and decreased recruitment of repair proteins to sites of damage, thus leading to decreased levels of DNA repair in these cells.

The stability of αIISp in the nucleus may be important not only for DNA repair but also for other cellular functions. We have shown that it interacts with a diverse number of proteins in the nucleus which include structural proteins, such as lamin and actin, proteins involved in additional DNA repair pathways, such nucleotide excision repair, homologous recombination and nonhomologous end joining, chromatin remodeling proteins, FA proteins, and transcription and RNA processing factors (*15*). Though it is not yet clear whether these interactions are direct or indirect, these studies demonstrate that αIISp may form an extended network with other proteins in the nucleus and function in additional nuclear processes. Thus a deficiency in αIISp, such as occurs in FA cells, could have far reaching consequences due to the number of different proteins with which it interacts and, thus, the number of different processes which could be affected by perturbations in these interactions.

In order to get a clearer understanding of the proposed role of FA proteins in maintaining the stability of αIISp in the cell, studies were carried out to determine whether there is a direct interaction between FA proteins and αIISp. Three FA proteins, FANCC, FANCF, and FANCG, which are components of the FA core complex, a group of FA proteins shown to be important in repair of DNA interstrand cross-links (*2*−*4*), and which we have shown to coimmunoprecipitate with αSpII (*12, 14, 15*), were examined for their ability to bind directly to αIISp using yeast two-hybrid analysis. The results demonstrated that one of these FA proteins, FANCG, has strong affinity for αIISp, specifically for the Src-homology 3 (SH3) domain. Analysis of FANCG showed that it contains two motifs with consensus sequences that could potentially bind to SH3 domains. Mapping the site of interaction of FANCG with αIISp demonstrated that one of these motifs in FANCG bound to the SH3 domain of αIISp. Two FA proteins that did not contain these motifs, FANCC and FANCF, did not bind to the SH3 domain of αIISp. These results thus indicate that FANCG contains a class of motifs that has specificity for binding to SH3 domains and binds to the SH3 domain of αIISp via this motif. We propose that this binding may be important for the stability of αIISp and for the role it plays in DNA interstrand cross-link repair.

EXPERIMENTAL PROCEDURES

Bacterial and Yeast Strains. Escherichia coli strain DH5α (Invitrogen) was used in the construction and propagation of all plasmid constructs. *E. coli* were grown in Luria broth at 37 °C. Plasmids were maintained in DH5α under the selection of ampicillin. *Saccharomyces cerevisiae* strains EGY48, EGY194, and EGY188 (Origene Technologies) were grown in liquid YPD (Q-Biogene) or YPD agar plates at 30 °C.

Yeast Vectors and Expression Constructs. All constructs were made in vectors supplied as part of the DupLexA yeast two-hybrid system (OriGene Technologies). LexA DNA-binding domain (BD) constructs were made in the pEG202 vector, and the B42 transcriptional activation domain (AD) constructs were made in the pJG4-5 vector. All fusion proteins had an HA tag on the N-terminus. Interactions between the BD and AD fusion proteins were detected using reporter plasmids pSH18-34 or pJK103, which had a *LacZ* reporter gene. Restriction endonuclease sites were added to the pEG202 and pJG4-5 vectors to aid in the process of subcloning.

FANCC, FANCF, and FANCG cDNAs were amplified by PCR from pREP4 (gift from Hans Joenje, Vrije Universiteit Medical Center, Amsterdam, The Netherlands) using Accuprime Pfx DNA polymerase supermix (Invitrogen), and restriction sites were added to the 5′ and 3′ ends of the cDNAs. The oligonucleotide primers used are given in Supporting Information Table S1. For FANCC cDNA, a forward primer added *Bam*HI and *Sac*II sites and a reverse primer added an *Xho*I site. For FANCF cDNA, forward and reverse primers added *Sac*II and *Xho*I sites to the 5′ and 3′ ends, respectively. For FANCG cDNA, the forward primer added an *Eco*RI site and a reverse primer added *Not*I and *Mlu*I restriction sites. An N-terminal FANCG sequence, corresponding to amino acids 1−293, was created using the above FANCG forward primer and a reverse primer containing a *Not*I site and an added stop codon. An N-terminal FANCG sequence, corresponding to amino acids 1−481, was created by excising FANCG from the pEG202 vector at the *Xho*I site in the FANCG cDNA. A C-terminal FANCG sequence (codons 266−622) was amplified using a forward primer containing an *Eco*RI site and the reverse primer described above. All of these cDNAs were subcloned into the pEG202 vector at the appropriate restriction sites. The entire FANCC, FANCF, and FANCG sequences and the FANCG fragments and linker sites were verified by sequencing (Molecular Resource Facility, New Jersey Medical School, UMDNJ, Newark, NJ).

Four overlapping regions of αIISp were constructed by PCR amplification of sequences from αIISp (GenBank accession number NM003127) in the pCI vector (gift from Dr. Jon S. Morrow, Yale University School of Medicine) using Accuprime Pfx DNA polymerase supermix. Each of the four regions was amplified with a forward primer, which added an *Eco*RI site to the 5′ end and a reverse primer which added an *Xho*I site to the 3′ end. Primers used to amplify these four regions are listed in Supporting Information Table S2. The constructs were then ligated into the pCR4-TOPO vector using the TOPO-TA cloning kit (Invitrogen). After sequence verification, the inserts were then subcloned into the pJG4-5 vector using the appropriate restriction sites. Since region 4 had an internal *Eco*RI site, it was excised from the pCR4-TOPO vector as two fragments. The *Eco*RI/*Xho*I fragment was subcloned into pJG4-5 followed by subcloning of the *Eco*RI/*Eco*RI fragment. A peptide containing the SH3 domain was produced by amplification of the SH3 domain sequence (residues 969−1026) in region 2 using the primers shown in Supporting Information Table S2. The SH3 domain cDNA was subcloned directly into the pJG4-5 vector at the appropriate restriction sites.

Site-Directed Mutagenesis. Specific point mutations or deletion mutations were created in FANCG and αIISp region 2 sequences. For these studies, FANCG and αIISp region 2 cDNAs were subclonned into the pENTR3C (Invitrogen) and pGEX4T-1 (Amersham) vectors, respectively. The majority of the mutations were created with a PCR mutagenesis strategy based on the protocol used in the ExSite PCR-based

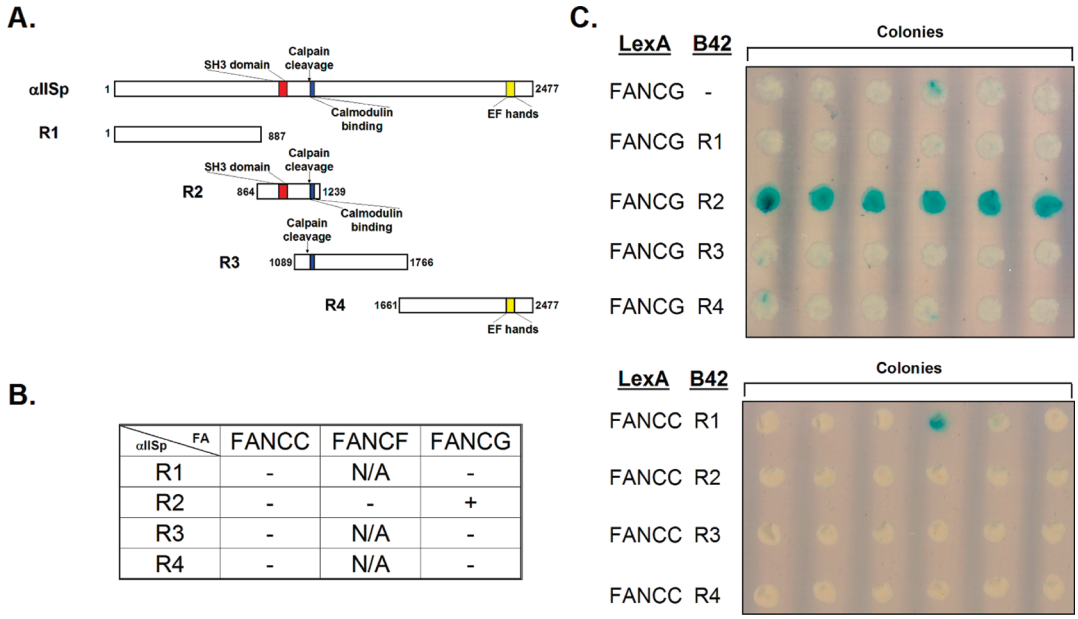


FIGURE 1: Yeast two-hybrid analysis of interaction of αIISp with FA proteins. (A) A diagram of human αIISp showing the SH3 domain, calpain cleavage and calmodulin binding sites, and EF hands. αIISp was divided into four overlapping regions, R1, R2, R3, and R4, which were subcloned into yeast two-hybrid vectors. Numbers refer to amino acid positions within αIISp. (B) Yeast two-hybrid analysis of the interaction of FA proteins with the four regions of αIISp: (+) good binding; (−) no binding. (C) Yeast two-hybrid analysis in which the LexA-FANCG or LexA-FANCC fusion proteins, in the pG202 vector, were coexpressed in yeast with either an empty vector (−) or B42-αIISp(R1), B42-αIISp(R2), B42-αIISp(R3), or B42-αIISp(R4) constructs in the pJG4-5 vector. Six randomly selected colonies from each of these yeast transformations were replica plated in a row to plates containing X-gal to test for activity of the reporter gene, β-galactosidase. Positive interaction between fusion proteins is seen by the presence of blue colonies, which indicates that the reporter gene has been activated. White colonies indicate no positive protein interactions.

Table 1: Primers Used To Create Mutations in FANCG, αIISp Region 2, and the αIISp SH3 Domain cDNAs

cDNA	primer sequences
αIISp region 2	
Del SH3	5′-AAGAAATTGGACCCCGCCCAG-3′
	5′-CAAGACCAGCTCCTTCCCAG-3′
E985A	5′-GCGGTCACCATGAAGAAGGG-3′
	5′-CCGGGGACTCTTCTCCTG-3′
WWSS	5′-CACCAACAAGGATTCGTCGAAAGTGGAAGTGAACG-3′
	5′-CGTTCACTTCCACTTTCGACGAATCCTTGTTGGTG-3′
FANCG	
G1	5′-GAGGTGTTTTTGGAGGCAGC-3′
	5′-CTCCGAGCTATCCAGCAACAG-3′
G2	5′-GGTCCCTGTATGCCTGAGG-3′
	5′-CTCCGAGCTATCCAGCAACAG-3′
G3	5′-GAGGTGTTTTTGGAGGCAGC-3′
	5′-TGGAGGGGAGGGGGGTGGG-3′
G4	5′-GCATGGCTGAGGTGTTTTTGG-3′
	5′-ATGCCCCTGGAGGGGAGG-3′
G5	5′-CCCGGGGCCCTGTATGCCTGAGGTG-3′
	5′-GGGGAGGCGGCTGGGGAGAACCTTGGC-3′
G6	5′-TCCCCACCCCCCTCCCCT-3′
	5′-CTCCGAGCTATCCAGCAACAG-3′
G7	5′-GATAGCTCGGAGCCAAGGTCCTCCCCACCC-3′
	5′-GGGTGGGGAGGACCTTGGCTCCGAGCTATC-3′
G8	5′-CTGCGCACTCTGGGTCTCTGCC-3′
	5′-TATGCCAGTTCCTTGGTTCC-3′

site-directed mutagenesis kit (Stratagene). In region 2 of αIISp, the SH3 domain was deleted. The primers used for this are listed in Table 1. A point mutation was also created in the SH3 domain of region 2 of αIISp in which a glutamine was mutated to alanine (E985A). A second mutation was additionally created in the SH3 domain in which two tryptophans were mutated to serines (W1004S and W1005S). The primers used to create these cDNAs are given in Table 1. These mutant SH3 domains of region 2 were used as templates for PCR amplification of the SH3 domain using the same forward and reverse primers as above. The mutant region 2 and SH3 domain cDNAs were subcloned into the pJG4-5 vector at the appropriate restriction sites. The vectors containing the inserts were then sequenced to ensure that the desired mutation was present.

Mutations were created in FANCG in the two motifs containing proline-rich class 1@ and class 1 consensus sequences, residues 380−394 and 443−449, respectively. The G1 mutation was a deletion of FANCG residues P380−P394 in the class 1@ consensus sequence. In the G2 mutation, residues P380−P389 were deleted in this sequence. In the G3 mutation, residues G390−P394 were deleted. In the G4 mutation, proline residues 391 and 394 were mutated to alanines (P391A and P394A). In the G5 mutation, proline residues 385 and 386 were changed to alanines (P385A and P386A). A mutation was also created that included both the G4 and G5 mutations. For this double mutant, the G5 mutant FANCG was used and the G4 mutation created in this mutant using the same forward primer utilized for the G4 mutation and a reverse primer 5′-ATGCCCCCGGGGGGGAGG-3′. In the G6 mutation, residues P380−F382 were deleted. The G7 mutation was a point mutation in which the phenylalanine was mutated to serine (F382S). A mutation was also created in the motif of FANCG containing the class 1 consensus sequence. In this G8 mutant, two proline residues were mutated to alanines (P446A, P449A). The primers used to create all of these mutations are shown in Table 1. All mutations were verified in the expression constructs by DNA sequencing. For those studies in which binding of the mutant FANCGs to αIISp was being examined, the mutant FANCG cDNAs were subcloned into the pEG202 vector at the appropriate restriction sites and the sequences verified by sequence analysis. For those studies in which binding of the

mutant FANCGs to wild-type FANCG was being examined, the mutant FANCG cDNAs were subcloned into the pJG4-5 vector.

Yeast Two-Hybrid Assay. The DupLEX-A yeast two-hybrid system (OriGene Technologies) was used for yeast two-hybrid analysis. Transformation of the yeast strains was performed with the AD vector (pEG202), BD vector (pJG4−5), and reporter vectors according to the manufacturer's protocol. Briefly, these vectors were transformed into the yeast strains using a PEG/ssDNA/lithium acetate procedure. Six yeast colonies were randomly selected and inoculated in rows onto master YNB (glucose-His-Trp-ura) plates which were incubated overnight. These master plates were then replica plated onto YNB X-gal plates and the colonies grown for 2−5 days. Positive interactions between the AD and BD fusion proteins were documented by the presence of blue colonies, which indicated that the reporter gene, β-galactosidase, had been activated. Each experiment was repeated four to eight times.

Coimmunoprecipitation of Proteins and Immunoblot Analysis. Normal human lymphoblastoid cells (GM 3299) (Coriell Institute for Medical Research, Camden, NJ) were grown in suspension culture in RMPI 1640 medium. Cells were treated with 3.5 μM 8-methoxypsoralen (8-MOP) plus two doses of long-wavelength UVA light (6 kJ m^{-2}) as previously described (*14*). Nuclear extracts were prepared according to the method of Yamashita et al. (*23*). Coimmunoprecipitation of FA proteins with αIISp from damaged and undamaged cells was carried out as previously described using non-erythroid α-spectrin antibody (Chemicon International Inc.) (*12, 15*). The proteins were separated by SDS−PAGE and analyzed by Western blot analysis as previously described (*12, 13, 15*). The primary antibodies used were anti-α-spectrin (Chemicon International Inc.) and anti-FANCC, anti-FANCF, and anti-FANCG (Bethyl Laboratories). Images were scanned using a Hewlett-Packard ScanJet 4c/T scanner. Band volumes were quantitated using Image Quant software.

RESULTS

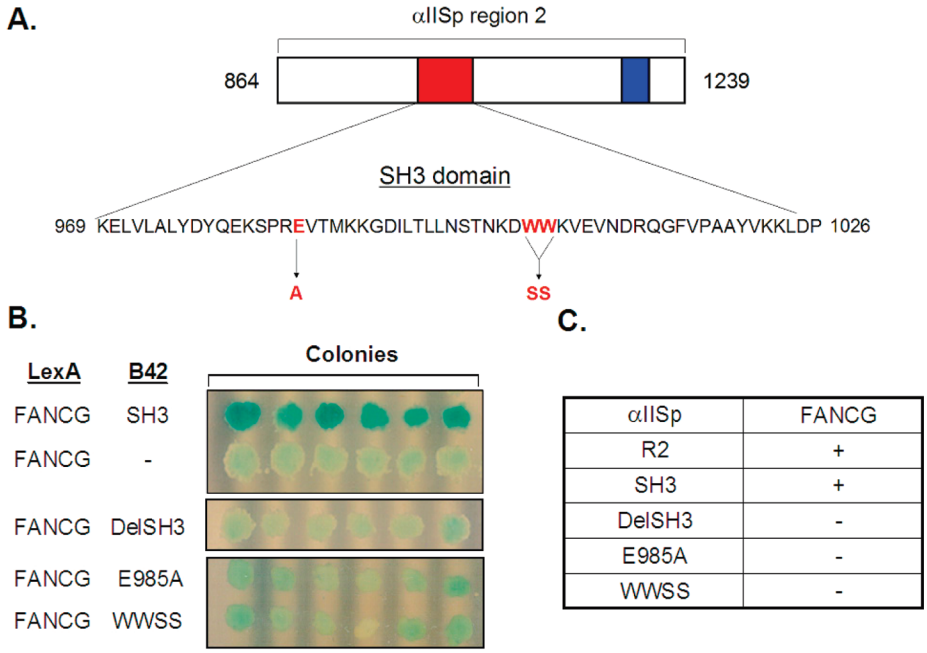
FANCG Interacts Directly with αIISp. In order to determine whether specific FA proteins directly interact with αIISp, a yeast two-hybrid screen was carried out. Because of the large size of αIISp, four overlapping regions of αIISp, R1 (residues 1−887), R2 (residues 864−1239), R3 (residues 1089−1766), and R4 (residues 1661−2477) (Figure 1A), were examined for interaction with FANCC, FANCF, and FANCG. Direct interaction was detected between FANCG and region 2 of αIISp as evidenced by the development of blue color in the colonies, which indicated activation of the β-galactosidase reporter gene (Figure 1B,C). Interaction was not detected between FANCG and regions 1, 3, and 4 of αIISp (Figure 1B,C). The results shown are from a single experiment in which six randomly chosen colonies were examined. Analysis of the binding reactions showed that FANCC and FANCF did not interact directly with any of the four regions of αIISp (Figure 1B,C). Each of these binding experiments was repeated four to eight times with similar results. Immunoblot analysis of protein extracts of the transformed yeast cells showed that FANCC and FANCF were expressed in the yeast cells as were regions 1, 2, 3, and 4 of αIISp, indicating that this lack of interaction was not due to lack of expression of the hybrid proteins in the experimental system (data not shown).

FANCG Specifically Interacts with the SH3 Domain of αIISp. The interaction between FANCG and region 2 of αIISp was examined further in order to map the site on αIISp that binds to FANCG. Region 2 of αIISp contains a SH3 domain (Figure 2A) that preferentially binds to ligands containing a class I@ proline-rich consensus sequence Px@xxPxxP, where P is proline and x is any amino acid, but tends to be a hydrophobic residue, and @ is an aromatic or aliphatic residue (*24*). FANCG contains a motif that has a class I@ proline-rich consensus sequence (residues 380−388) (Table 2), which has two overlapping C-terminal PxxP flanking sequences, residues 388−394. In addition, FANCG contains a second motif that binds to SH3 domains, residues 443−449. This motif contains a class I consensus sequence, +xx(x)PxxP, where P is proline, + is a positively charged amino acid, usually an arginine or a lysine, and x is as above. (x) refers to an x which may or not be present, and which happens not to be present in FANCG (Table 2) (*25−30*).

Studies were therefore carried out to determine whether FANCG was binding to the SH3 domain in region 2 of αIISp. Deletion of the SH3 domain of region 2 resulted in loss of interaction of this region of αIISp with FANCG (Figure 2B,C) as evidenced by loss of activation of the reporter gene, β-galactosidase. Involvement of the SH3 domain in this interaction was further examined by producing a peptide that contained the SH3 domain of αIISp (residues 969−1026) (Figure 2A). As shown in Figure 2B, a strong interaction was observed between FANCG and the SH3 domain of αIISp in this peptide. FANCG, by itself, exhibited a very low level of reporter gene activation; however, this level was considerably less than in yeast expressing FANCG and either region 2 or the SH3 domain of αIISp.

To help to confirm the specificity of interaction of FANCG with the SH3 domain of αIISp, a glutamic acid residue in the SH3 domain was mutated to an alanine (E985A) (Figure 2A). This mutation was designed to maintain the integrity of the SH3 domain structure while decreasing its binding affinity (*25, 31*). This E985A mutation disrupted interaction of the SH3 domain of αIISp with FANCG (Figure 2B). A second mutation in the SH3 domain was also examined where tryptophan residues 1004 and 1005 were both converted to serines (W1004S and W1005S) (Figure 2A). This mutation (WWSS) also resulted in loss of interaction of FANCG with the SH3 domain of αIISp (Figure 2B). These results, which are summarized in Figure 2C, indicate that there is specificity of binding of FANCG to the SH3 domain of αIISp.

A Specific Motif in FANCG That Interacts with the SH3 Domain of αIISp. In order to determine the region in FANCG that interacts with the SH3 domain of αIISp, deletion constructs of the FANCG cDNA were prepared. Two N-terminal constructs and one C-terminal construct of FANCG were expressed as fusion proteins in yeast two-hybrid experiments (Figure 3A). Both the N-terminal constructs of FANCG, residues 1−293 and 1−481, and the C-terminal construct, residues 261−622, failed to show positive interaction with the αIISp SH3 domain (Figure 3B). Only full-length FANCG was capable of binding the SH3 domain of αIISp (Figure 3B). This suggested that the overall



αIISp	FANCG
R2	+
SH3	+
DelSH3	-
E985A	-
WWSS	-

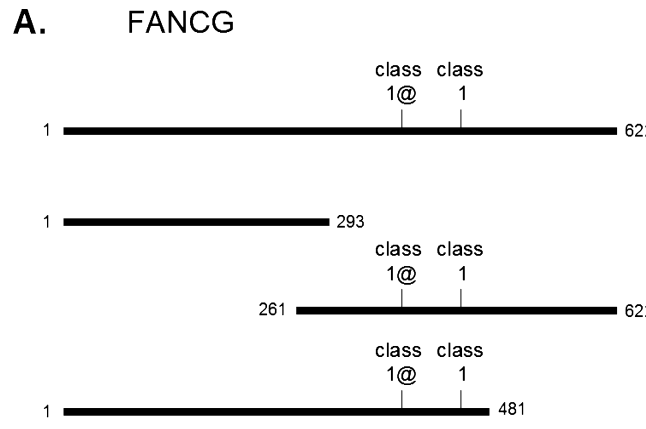
FIGURE 2: Interaction of FANCG with the SH3 domain of αIISp. (A) A diagram of region 2 (residues 864−1239) of αIISp showing the sequence of the SH3 domain. Using site-directed mutagenesis, point mutations were separately created in the amino acids highlighted: E985A, W1004S, and W1005S. (B) Yeast two-hybrid analysis in which the LexA-FANCG fusion protein, in the pG202 vector, was coexpressed in yeast with either an empty vector (−) or the pJG4-5 vector containing either the SH3 domain of αIISp (αIISpSH3), region 2 of αIISp (αIISpR2), region 2 of αIISp with the SH3 domain deleted (DelSH3), the SH3 domain of αIISp containing the E985A mutation, or the SH3 domain containing the W1004S and W1005S mutations (WWSS). Six colonies were selected and replica plated in rows as in Figure 1. Blue colonies indicate that the reporter gene has been activated and that there is a positive interaction between the fusion proteins. (C) Summary of the results of the interaction of FANCG with the various constructs of the αIISp SH3 domain shown in (B): (+) good binding; (−) no binding.

Table 2: Presence of Motifs in Fanconi Anemia Proteins That Contain Class I, Class I@, or Class II Consensus Sequences That Can Bind to SH3 Domains

protein	motif	sequence	location
FANCA	class II	PLVPGK	497−502
	class I	RVLPKVP	591−597
	class II	PRCPKK	1408−1413
FANCB	none		
FANCC	none		
FANCD1/BRCA2	class II	PYLPSR	2883−2888
FANCD2	class I	RRHPSYP	84−90
	class II	PSYPK	87−91
FANCE	none		
FANCF	none		
FANCG	class I@	PRFSPPPSP	380−388
	class I	KELPYCP	443−449
FANCI	class I	KLLEPSSP	994−1001
FANCJ	none		
FANCL	class II	PEKPPR	216−221
FANCM	class II	PEKPSR	649−654
FANCN	class I	KSELPDSP	203−210
	class I	KVSSPAGP	486−493
	class I	KSHPKRP	681−687

motif	consensus sequence[a]
class I	+xx(x)PxxP
class I@	Px@xxPxxP
class II	PxxP(x)+

[a] + is K or R. x is any amino acid. (x) is any amino acid, which may or may not be present. @ is F, Y, or W.

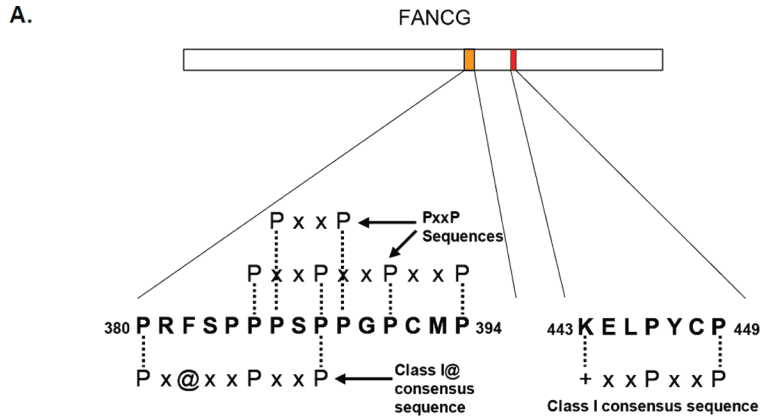


FANCG construct	Binding to SH3
full-length	+
residues 1-293	-
residues 261-622	-
residues 1-481	-

FIGURE 3: Full-length but not fragments of FANCG bind to the SH3 domain of αIISp. (A) Diagram showing the deletion constructs of FANCG that were created and subcloned into yeast two-hybrid vectors. Class I@ and class I motifs in FANCG are indicated. (B) Summarization of the results of yeast two-hybrid analysis of the binding of full-length and the deletion constructs of FANCG to the SH3 domain of αIISp: (+) good binding; (−) no binding.

structure of FANCG is critical for interaction with the SH3 domain of αIISp. This is in agreement with other studies which have shown that full-length FANCG is very important for its ability to bind to other proteins (*32*−*34*).

Therefore, in order to map the sites in FANCG responsible for its binding to the SH3 domain of αIISp, site-directed mutagenesis was performed. These studies focused on the proline-rich class I@ and class I motifs of FANCG (Figure 4A). When the entire class I@ motif with its flanking



B.

	Mutation	Sequence (Residues 380-394)	Binding to SH3
FANCG	None	PRFSPPPSPPGPCMP	+
G1	P380_P394del	~~PRFSPPPSPPGPCMP~~	-
G2	P380_P389del	~~PRFSPPPSPP~~GPCMP	+
G3	G390_P394del	PRFSPPPSPP~~GPCMP~~	+
G4	P391A; P394A	PRFSPPPSPPG**A**CM**A**	+
G5	P385A; P386A	PRFSP**AA**SPPGPCMP	+
G6	P380_F382del	~~PRF~~SPPPSPPGPCMP	+
G7	F382S	PR**S**SPPPSPPGPCMP	+
G4-G5	P391A; P394A; P385A; P386A	PRFSP**AA**SPPG**A**CM**A**	-
		(Residues 443-449)	
FANCG	None	KELPYCP	+
G8	P446A;P449A	KEL**A**YC**A**	+

C.

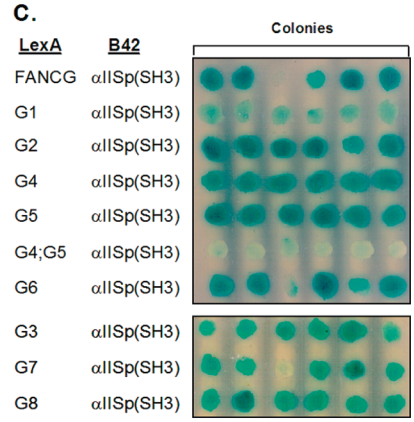


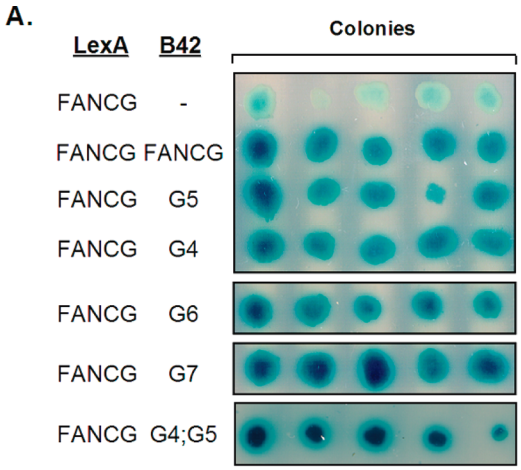
FIGURE 4: Mutations created in the motif in FANCG, which contains a class I@ consensus sequence, that affects its ability to bind to the SH3 domain of αIISp. (A) Diagram of FANCG showing the location of the class I@ and class I consensus sequences. Numbers refer to amino acid positions within αIISp. (B) Summary of the results of yeast two-hybrid analysis of the interaction of the motifs in FANCG containing mutations (G1−G8) in the class I@ consensus sequence with its flanking PxxP sequence (residues 380−394) or in the class I consensus sequence (residues 443−449) with the SH3 domain of αIISp: (+) good binding; (−) no binding. (C) Yeast two-hybrid analysis in which LexA-FANCG or a LexA mutant FANCG fusion protein is coexpressed with the B42 construct containing the SH3 domain of αIISp (αIISpSH3). Six colonies were selected and replica plated as in Figure 1. Blue colonies indicate that the reporter gene has been activated and that there is a positive interaction between the fusion proteins.

C-terminal PxxP sequences (P380−P394) was deleted (G1 mutation), FANCG did not bind to the SH3 domain of αIISp, indicating that the presence of this proline-rich motif of FANCG is important for its interaction with the SH3 domain of αIISp (Figure 4B,C). However, when just the I@ motif was deleted (G2 mutation) or just the two flanking PxxP sequences were deleted (G3 mutation), FANCG was able to bind to the SH3 domain of αIISp (Figure 4B,C). FANCG, in which two proline residues in the flanking PxxP and in the I@ motif sequences were separately replaced with alanines (P391A and P394A (G4 mutation) and P385A and P386A (G5 mutation), was also able to bind to the SH3 domain of αIISp (Figure 4B,C). However, when a double mutant was created in which both the G4 and G5 mutations were present, the interaction of FANCG with the SH3 region of αIISp was abolished (Figure 4B,C). Deletion of the first three amino acids, P380−F382, in the I@ sequence of FANCG (G6 mutation) and mutation of the aromatic phenylalanine residue to a serine (F382S) (G7 mutation) did not affect the interaction of FANCG with the SH3 domain of αIISp (Figure 4B,C). Mutations were also created in the class I motif of FANCG. In this mutant FANCG (G8 mutation), prolines at amino acids 446 and 449 were converted to alanines (P445A and P449A). This mutated FANCG also bound to the SH3 domain of αIISp (Figure 4B,C), indicating that this proline-rich region is not involved in the interaction of FANCG with the SH3 domain of αIISp. These studies thus indicate that a motif in FANCG that contains a class I@ consensus sequence is the site of interaction of FANCG with the SH3 domain of αIISp.

To further confirm that the SH3 domain of αIISp is the site of interaction with FANCG, two of the mutant FANCGs that bound to the SH3 domain of αIISp, G4 and G5, were examined for the ability to interact with the SH3 domain of αIISp containing the E985A mutation. These mutant FANCGs failed to interact with the SH3 domain of αIISp containing this mutation. This further shows that FANCG interacts with αIISp at the SH3 domain (Supporting Information Figure S1).

Mutations in these residues in FANCG had no effect on the ability of FANCG to interact with itself. As seen in Figure 5, FANCG shows good binding to itself. FANCG containing either the G4, G5, G6, or G7 mutation was able to bind to the wild-type FANCG (Figure 5). In addition, FANCG containing the combined G4-G5 mutations, which could not bind to the SH3 domain of αIISp, was able to bind to itself (Figure 5). Thus mutations in the motif in FANCG that inhibit its ability to bind to the SH3 domain of αIISp do not affect its ability to bind to itself.

FANCC and FANCF but Not FANCG Show Enhanced Binding to αIISp in Cells after Cross-Link Damage. Though yeast two-hybrid analysis demonstrated that FANCG but not



B.

	Mutation	Binding to FANCG
FANCG	None	+
G4	P391A; P394A	+
G5	P385A; P386A	+
G6	P380_F382del	+
G7	F382S	+
G4;G5	P391A; P394A; P385A; P386A	+

FIGURE 5: Mutations created in the motif in FANCG that contains a class I@ consensus sequence do not affect its ability to bind to itself. (A) Yeast two-hybrid analysis was carried out in which LexA-FANCG is coexpressed with the B42 construct containing a mutant FANCG fusion protein. Five colonies were selected and replica plated as in Figure 1. Blue colonies indicate that the reporter gene has been activated and that there is a positive interaction between the fusion proteins. (B) Summary of the results of yeast two-hybrid analysis of the interaction of FANCG containing mutations in the class I@ consensus sequence with its flanking PxxP sequence (residues 380−394) with wild-type FANCG: (+) good binding; (−) no binding.

FANCC or FANCF showed strong binding to αIISp, it is possible that, if FANCC and FANCF are involved in the repair process, they may bind to αIISp after cells are damaged with a cross-linking agent. Immunoprecipitation experiments were therefore carried out to examine whether any of these FA proteins showed enhanced association with αIISp after cells were damaged with a DNA interstrand cross-linking agent, 8-MOP, plus UVA light. As is shown in Figure 6A, there was greater binding of FANCC and FANCF to αIISp 15 h after cells were damaged compared to binding to αIISp from undamaged cells. We have previously shown that 15 h after cross-link damage is the optimum time for the localization of αIISp and repair proteins in damage-induced nuclear foci (*14*). In contrast, FANCG showed no significant enhancement in binding to αIISp from cells that had been damaged compared to αIISp from undamaged cells. Quantitation of these results shows that FANCC and FANCF have a 2−3-fold enhanced affinity for αIISp after cells are damaged with 8-MOP plus UVA light (Figure 6B). Thus, though FANCC and FANCF did not bind directly to αIISp, as ascertained by yeast two-hybrid analysis, they did bind to αIISp after cells are damaged with a cross-linking agent. Whether this binding is direct or indirect is not yet known.

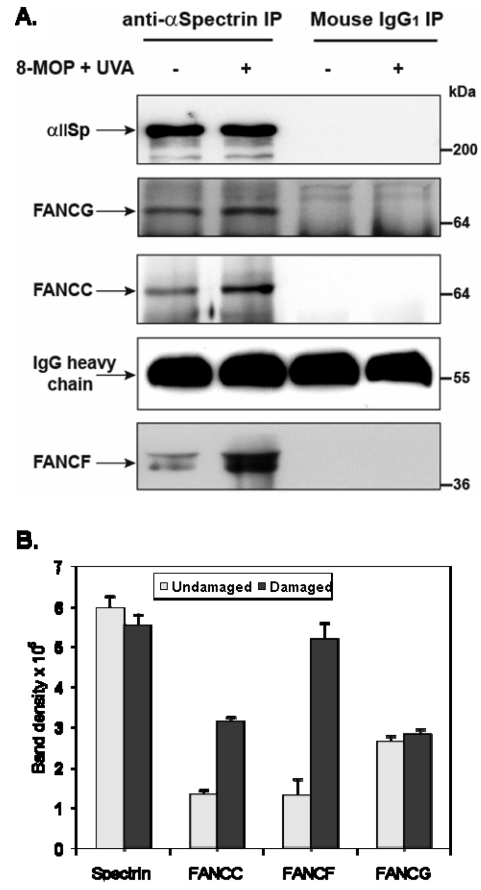


FIGURE 6: Binding of FANCC, FANCF, and FANCG to αIISp from cells damaged with 8-MOP plus UVA light. (A) Normal human lymphoblastoid cells in culture were either undamaged or damaged with 8-MOP plus UVA light. Fifteen hours after damage, nuclear extracts were prepared from the cells, and coimmunoprecipitations were carried out using anti-α spectrin. Interaction of the FA proteins with αIISp was examined by Western blot analysis using anti-FANCC,-FANCF, and -FANCG. (B) Quantitation of binding of FANCC, FANCF, and FANCG to αIISp in normal human lymphoblastoid cells 15 h after damage with 8-MOP plus UVA light. Coimmunoprecipitations were carried out as in (A). Band intensities were quantitated. Vertical lines represent ±SEM for three to five separate experiments.

DISCUSSION

Nonerythroid α-spectrin consists of 21 triple-helical repeats (*17, 18, 35, 36*). The 10th repeat contains a highly conserved SH3 domain (*17, 18, 21, 35−37*). SH3 domains are modular domains which play a role in protein−protein interactions and assembly of complex protein networks involved in mediating signal transduction, intracellular signaling, protein synthesis, cellular organization, and membrane traffic (*38−44*). These domains interact with ligand proteins containing proline-rich motifs with the minimal consensus sequence of PxxP (*26, 27, 43, 44*). There are two major classes of protein ligands that bind to SH3 domains. Class I ligands are characterized by a consensus sequence of +xxPxxP and are defined in the Results and in Table 2. Class II ligands, which bind in the reverse orientation from class I ligands, are characterized by a consequence sequence of xPxxPx+, as defined in Table 2 (*25−30*). There are also class I@ ligands, which are similar to class I but have an aromatic or sometimes aliphatic residue, @, instead of a positively charged one at position 3 preceded by Px, Px@xxPxxP (*24*). The SH3

domain of αIISp preferentially binds to class I@ ligands (*24*). The binding surface of a SH3 domain is formed by three shallow pockets or grooves where the ligands bind (*25, 27, 43*−*48*). Two of these pockets are hydrophobic and accommodate one each of the two proline dipeptides in the class I and class II motifs (*26, 27, 30, 39, 44, 49*). The third pocket is negatively charged and is flanked by two variable loops, the RT and n-Src loops, that can modulate ligand binding and determine the specificity and orientation of the ligand (*26, 27, 30, 39, 44, 49*).

In the present paper, the binding of the FA proteins to αIISp was examined by construction of four overlapping regions of αIISp (Figure 1A). The results of yeast two-hybrid analysis show that one of the FA proteins examined, FANCG, had high binding affinity for region 2 of αIISp, which contained the SH3 domain. It did not bind to any of the other three regions of αIISp. That within region 2 it was the SH3 domain to which FANCG bound was ascertained by creation of deletion mutations and point mutations in the SH3 domain of αIISp. Deletion of the SH3 domain in αIISp eliminated binding. Creation of a point mutation in the SH3 domain in which a glutamine was mutated to an alanine (E985A) eliminated binding of FANCG to the SH3 domain. This glutamine is positioned in the RT loop, which flanks the third binding pocket, and has been shown to be an important determinant in the specificity of binding of a ligand to a SH3 domain (*24, 25, 31*). Mutations in this residue in the SH3 domain have been shown to alter the binding affinity of a proline-rich ligand for the SH3 domain without altering the structure of the binding site within the SH3 domain (*25, 31*). A second mutation in the SH3 domain of αIISp, in which two tryptophans were mutated to serines (W1004S and W1005S), also eliminated binding of FANCG. These tryptophans are highly conserved and are located in one of the hydrophobic binding pockets of the SH3 domain of αIISp (*26, 31, 45, 49*). Thus both of the mutations created in the SH3 domain of αIISp were in regions important in ligand binding to this domain. Therefore, elimination of binding of FANCG to αIISp when these mutations were created further strengthens the view that FANCG binds to the SH3 domain of αIISp.

Analysis of the FANCG sequence, in turn, shows that it contains two motifs that can potentially bind to SH3 domains. One motif contains a class I@ consensus sequence and the other motif contains a class I consensus sequence (Table 2). The class I@ sequence has two flanking C-terminal PxxP sequences. Deletion of this entire region eliminated binding to the SH3 domain. Deletion of just the I@ region or the flanking PxxP sequences did not eliminate this binding nor did creation of separate mutations in highly conserved prolines in either of these two sequences in FANCG. However, when these mutations were created simultaneously in both the I@ sequence and the flanking PxxP sequences, binding of FANCG to the SH3 domain of αIISp was eliminated. It is thus possible that the flanking PxxP sequence acts as a "back up" sequence and only when key prolines in both sequences are mutated is binding inhibited. It is possible that this flanking PxxP sequence aids in the specificity of binding of the I@ motif in FANCG to the SH3 domain of αIISp since it has been found that flanking sequences outside of the class I or II motifs can provide extra SH3 domain contacting sites, which can significantly increase binding affinity and selectivity to the SH3 domain (*26*−*29, 39*). The mutations produced in the motif in FANCG that contains a class I@ consensus sequence specifically affect the binding of FANCG to the SH3 domain of αIISp and have no effect on the ability of FANCG to interact with itself. Our finding that FANCG can dimerize with itself confirms other reports of the ability of FANCG to interact with itself (*32, 50*).

Mutations of the two prolines in the motif in FANCG that contains the class I consensus sequence did not diminish binding of FANCG to the SH3 domain of αIISp. Thus these studies clearly show that there is specificity of binding of FANCG to the SH3 domain of αIISp via a motif that contains a class I@ consensus sequence. Binding of FANCG to αIISp is further corroborated by coimmunoprecipitation data in which we have previously shown that FANCG coimmunoprecipitates with αIISp (*15*), confirming that this interaction takes place in intact cells.

The other two FA proteins examined, FANCC and FANCF, neither of which have motifs that bind to SH3 domains (Table 2), did not bind to region 2 of αIISp upon yeast two-hybrid analysis. Thus, there is a correlation between the presence of a SH3 binding motif in one of the FA proteins, FANCG, with its ability to bind to the SH3 domain of αIISp.

An important question is whether patient-derived FANCG mutations affect binding of FANCG to αIISp. Examination of the Fanconi Anemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate/jumpg.php) shows that of the 48 unique DNA variants of the *FANCG* gene thus far reported in FA-G patients, at least 18 are mutations that result in truncated FANCG proteins which are missing the motif that binds to the SH3 domain of αIISp. One of these mutations, 1158delC, results in a frame shift mutation which affects the motif containing the class 1@ consensus sequence and its flanking PxxP sequences (P380−P394). This frame shift results in the changing of two prolines, P388 and P394, to leucines and a protein which terminates with residue 401. All of the truncated proteins resulting from these 18 mutations would presumably be unable to bind to the SH3 domain of αIISp. The database shows that there are also two mutations in *FANCG* in FA-G patients that result in C-terminal truncated FANCG proteins which retain the motif that binds to the SH3 domain of αIISp and which terminate with residue 398. Since our present studies show that a C-terminal truncated FANCG protein, which terminates with residue 481 and contains the motif that binds to SH3 domains, does not bind to the SH3 domain of αIISp, this suggests that the overall structure of FANCG could be critical for interaction of this motif in FANCG with the SH3 domain of αIISp. Other studies have also shown that the presence of full-length FANCG is very important for its ability to bind to specific proteins (*32*−*34*). These two patient-derived C-terminal truncated proteins could also potentially be defective in ability to bind to the SH3 domain of αIISp. Thus there are potentially a number of patient-derived mutations in FANCG which could affect the ability of this protein to bind to the SH3 domain of αIISp.

It is possible that the binding of FANCG to αIISp is constitutive. There is no enhanced interaction of FANCG with αIISp after cells are damaged with a DNA interstrand cross-linking agent, as ascertained by coimmunoprecipitation studies. There is, however, a severalfold increase in binding

of FANCC and FANCF to αIISp after cells are similarly damaged. This binding could be indirect through binding to FANCG or another protein or it could be direct. It is thus possible that FANCG binds to αIISp constitutively and this binding is not affected when cells are damaged. It is possible to speculate that one important role for this interaction is to aid in the stability of αIISp in the cell and in the role αIISp plays in the repair process. In FA-G cells, reduced stability and hence reduced levels of αIISp could be the result of a deficiency in FANCG. In support of this is our finding that corrected FA-G cells, which express FANCG, show normal levels of αIISp (unpublished experiments) as well as normal levels of incisions at sites of DNA interstrand cross-links (a process for which αIISp is required) (*11*). The enhanced binding of FANCC and FANCF to αIISp after normal human cells are damaged with a DNA interstrand cross-linking agent supports our model in which αIISp acts as a scaffold to aid in the recruitment of proteins to sites of damage (*13, 14*).

The integrity of αIISp in the cell is dependent upon whether or not it undergoes proteolytic cleavage at specific sites by μ-calpain and caspases (*51*−*57*). Proteolytic cleavage of αIISp by μ-calpain is controlled by phosphorylation of residue Y_{1176}, which is at the site of calpain cleavage in repeat 11 (*51, 56*−*58*). When this residue is phosphorylated by c-Src, a kinase that binds to the flanking SH3 domain, αIISp is resistant to calpain cleavage (*59*). When Y_{1176} is dephosphorylated, upon binding of low molecular weight phosphotyrosine phosphatase (PTP) to its SH3 domain, αIISp is cleaved by μ-calpain (*58*). Based on the present results, it is possible to speculate that FANCG binds to the SH3 domain of αIISp and inhibits binding of PTP to this domain and thus inhibits dephosphorylation of Y_{1176} and calpain cleavage of αIISp. Preliminary data show that in cells from one of the FA complementation groups, FA-A, there is increased calpain breakdown product compared to normal cells (unpublished experiments), which supports the theory that there is enhanced calpain cleavage of αIISp in FA cells. Some type of equilibrium in the binding of PTP, c-Scr, and FANCG to the SH3 domain of αIISp could exist which could be involved in the regulation of αSpII integrity. The affinity of ligands that bind to SH3 domains, in particular the αIISp SH3 domain, is quite low, which means that the on- and off-rates for binding can be fast and binding partners can exchange relatively rapidly (*30, 44, 60*). FANCG could thus be involved in one aspect of maintaining αIISp integrity, which could potentially represent a new and additional role for FANCG in the cell.

Of interest is the finding that sequence analysis of all the FA proteins shows that eight of these proteins contain motifs that can bind to SH3 domains. These motifs contain either class I, class I@, or class II consensus sequences: FANCA (class I and two class II), FANCD1 (class II), FANCD2 (class I and class II), FANCG (class I@ and class I), FANCI (class I), FANCL (class II), FANCM (class II), and FANCN (three class I) (Table 2). Of these FA proteins, only FANCG contains a class I@ consequence sequence with flanking PxxP sequences, which may be of importance in its binding to αIISp. Whether the motifs in the other FA proteins that bind to SH3 domains bind to αIISp will be determined in future studies. It is also possible that these motifs in these FA proteins could be involved in the interaction of these FA proteins with other proteins in the cell that contain SH3 domains, such as proteins involved in signal transduction and intracellular signaling. Thus, there is another class of protein−protein interaction motifs present in a number of the FA proteins, and in at least one of these proteins, FANCG, this motif binds to the SH3 domain of the structural protein, αIISp. We hypothesize that this binding of FANCG to αIISp may be important for maintance of the stability of αIISp in the cell and may be important for the role αIISp plays in the DNA interstrand cross-link repair process.

SUPPORTING INFORMATION AVAILABLE

Tables showing the primers used to amplify the FANCC, FANCF, and FANCG cDNAs and the cDNAs for the four regions of αIISp and the SH3 domain of αIISp and a figure showing the interaction of mutant FANCGs with the SH3 domain of αIISp which contains mutations. This material is available free of charge via the Internet at http://pubs.acs.org.

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