Resistance to Mitomycin C Requires Direct Interaction between the Fanconi Anemia Proteins FANCA and FANCG in the Nucleus through an Arginine-rich Domain*

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Fanconi anemia (FA) is a genetically heterogeneous disorder characterized by bone marrow failure, birth defects, and chromosomal instability. Because FA cells are sensitive to mitomycin C (MMC), FA gene products could be involved in cellular defense mechanisms. The FANCA and FANCG proteins deficient in FA groups A and G interact directly with each other. We have localized the mutual interaction domains of these proteins to amino acids 18–29 of FANCA and to two noncontiguous carboxyl-terminal domains of FANCG encompassing amino acids 400–475 and 585–622. Site-directed mutagenesis of FANCA residues 18–29 revealed a novel arginine-rich interaction domain (RRRAWAELLAG). By alanine mutagenesis, Arg1, Arg2, and Leu8 but not Arg3, Trp5, and Glu7 appeared to be critical for binding to FANCG. Similar immunolocalization for FANCA and FANCG. The complementation function of FANCA was abolished by mutations in its FANCG-binding domain. Conversely, stable expression of FANCA mutants encoding intact FANCG interaction domains induced hypersensitivity to MMC in HeLa cells. These results demonstrate that FANCA-FANCG complexes are required for cellular resistance to MMC. Because the FANCC protein deficient in FA group C works within the cytoplasm, we suggest that FANCC and the FANCA-FANCG complexes suppress MMC cytotoxicity within distinct cellular compartments.

Chromosomal instability is a feature of many different mendelian cancer susceptibility syndromes as well as acquired tumors (1) and may be induced by a variety of physical, chemical, and biological agents. Although chromosomal instability induced by ionizing radiation or ultraviolet radiation has been studied extensively, there is relatively little information on the molecular factors that protect cells against damage by other genotoxic agents. A number of such agents are known to induce apoptosis mediated by p53 (2) in collaboration with other cellular proteins (3); such protein-protein interactions determine the sensitivity of cells to genotoxic agents and antineoplastic drugs.

Fanconi anemia (FA) is a human disease model for chromosomal instability and hypersensitivity to genotoxic agents. Cells from FA patients have unstable chromosomes and are highly susceptible to apoptosis after exposure to specific clastogens, particularly bifunctional cross-linkers, such as mitomycin C (MMC) (4). The phenotype of FA is pleiotropic and includes birth defects, bone marrow failure, and cancer predisposition. Somatic cell fusion studies have identified eight distinct complementation groups, designated A–H, and the genes defective in FA complementation groups A (FANCA) (5, 6), C (FANCC) (7), and G (FANCG) (8) have been cloned. Although all three genes were cloned by virtue of their ability to suppress the toxicity of MMC in FA lymphoblastoid cells of appropriate complementation groups, the encoded proteins have no significant homology to each other or to other known proteins, save for limited homology between FA and heme peroxidases (9). Our previous data as well as data from other laboratories have shown that FANCC is predominantly cytoplasmic (10–12), but others have reported that a fraction of FANCC is also nuclear (12, 13). FANCA is both nuclear and cytoplasmic (13–15). Forced targeting of these proteins to particular subcellular compartments has revealed that FANCC requires cytoplasmic localization (16) and FANCA nuclear localization (14) to be able to suppress cross-linker-induced cytotoxicity. Furthermore, the function of FANCC has been partially elucidated by its interaction with the microsomal membrane protein NADPH cytochrome P-450 reductase, an enzyme involved in the metabolic activation of many xenobiotics, including MMC (17). The function of other FA proteins, however, remains elusive and may involve a variety of functions including DNA repair, detoxification, and regulation of apoptosis (4). We (18) and others (19) have recently described an interaction between FANCA and FANCG within protein complexes found in non-FA but not in FA cells from several complementation groups. However, FANCA-FANCG complexes were not universally absent in all FA subgroups, and in the latter case the complexes were presumed to be functionally deficient. Therefore, a direct link between the expression of FANCA-FANCG complexes and cellular resistance to MMC could not be established. Also, except for an amino-terminal domain of FANCA required for interaction with FANCG in vitro, the precise structural determinants of this interaction were not known. In this study, we have analyzed the amino acids that define this interaction domain.

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† The abbreviations used are: FA, Fanconi anemia; MMC, mitomycin C; NLS, nuclear localization signal; NES, nuclear export signal; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; PCR, polymerase chain reaction.
acid residues required for the integrity of these complexes in vitro. We have also modeled the FA defect in non-FA cells by deregulation of FANCA-FANCG complexes with dominant negative FANCA alleles. Our results demonstrate that the integrity of FANCA-FANCG complexes is essential for a novel cellular function that regulates resistance to MMC.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and MMC Sensitivity Assay**—COS-1 and HeLa cell were cultured in Dulbecco’s minimal essential medium (Life Technologies, Inc.) with 10% fetal calf serum. COS-1 cells were transfected with DEAE-dextran, and HeLa cells were transfected using Superfect (Qiagen, Valencia, CA), as described (14). Stably transfected HeLa cells were selected on medium supplemented with 400 μg/ml G418 (Life Technologies, Inc.) with 10% fetal calf serum. COS-1 and HeLa cell were cultured in Dulbecco’s minimal essential medium (Life Technologies, Inc.) supplemented with 200 μg/ml hygromycin B. The MMC growth inhibition assays were performed within 2–3 weeks of stable transfection by exposing cells (7.5 × 10⁴/ml) to various concentrations of MMC. For HeLa cells and derived stable transfected cell lines, cells were plated in 24-well dishes at a density of 10,000 cells/well followed by exposure to different MMC concentrations. Cell numbers were determined using a Coulter counter, as before (14).

**Constructs**—FANCA- and FANCG-derived deletion constructs were generated by using standard PCR-based cloning strategies. PCR products were subcloned into the mammalian expression vector pDNA3 (Invitrogen, San Diego, CA) or the Epstein-Barr virus-based episomal vector pDR2 (CLONTECH, Palo Alto, CA). For site-directed mutagenesis, specific PCR primers were designed, and the products were subcloned into either pDNA3 or pDR2, as indicated below. PCR-generated mutations were confirmed by sequencing. pDNA3HA-FANCG has been described before (18). Chimeric constructs encoding the SV40 large T antigen nuclear localization signal (NLS) or the nuclear export signal (NES) from the gene for the inhibitor of cAMP kinase as well as a mutant version were generated by appending these motifs to FANCA deletion mutants, as before (14, 16).

**Immunoprecipitation, Western Blotting, and in Vitro Translation**—Cellular lysates and immunoprecipitation conditions have been described previously (14). In vitro translated [35S]methionine-labeled proteins were generated using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to the suggestions of the manufacturer. Proteins were allowed to form complexes in 20 mM Tris- HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40 supplemented with protease and phosphatase inhibitors. After immunoprecipitation, samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. Antibodies used included the affinity-purified rabbit polyclonal antibody directed against the amino terminus of FANCA (14) and a mouse monoclonal antibody, 12CA5 (Roche Molecular Biochemicals), directed against the hemagglutinin (HA) epitope. The bands on these autoradiograms were quantified by densitometry.

**Immunofluorescence Microscopy**—HeLa cells were transiently transfected with FANCA or modified FANCA constructs together with HA-FANCG in pDNA3, as indicated. Cells were replated on glass coverslips and processed for immunofluorescence microscopy, as described previously (14). Secondary antibodies included fluorescein-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Nuclei were identified by Hoechst staining (Sigma).

**RESULTS**

**Mapping of the FANCG Interaction Domain on FANCA**—Using immunoprecipitation and Western blotting, we initially tested the interaction of FANCA and FANCG with each other in lysates of transfected COS-1 cells. We used both full-length FANCA and a panel of deletion mutants to map the binding
domains. HA-tagged FANCG co-precipitated with full-length and carboxyl-terminal truncated mutants of FANCA using anti-FANCA antibody in the immunoprecipitation step and the anti-HA 12CA5 antibody in the Western blotting step (Fig. 1A, top). In the reciprocal experiment, full-length FANCA and the same panel of carboxyl-terminal deletion mutants co-precipitated with HA-FANCG using anti-HA antibody in the immunoprecipitation step and the FANCA antibody in the Western blotting step (Fig. 1A, bottom). In both cases, FANCA mutants lacking amino acids 301–1455 remained capable of binding to FANCG. Under similar conditions, co-transfection of HA-FANCG with FANCC followed by immunoprecipitation and Western blotting experiments failed to reveal an interaction between FANCC and HA-FANCG (data not shown). These results extend our earlier studies that the amino-terminal region of FANCA mediates binding to FANCG in vitro (18). Because of the concordance between in vitro binding assays and those utilizing cell lysates, we used the former to map the boundaries of the FANCG-binding domain on FANCA more precisely. In these experiments, both binding partners were visualized simultaneously by labeling the in vitro-translated products with [35S]methionine. The amino terminus of FANCA contains a consensus site for a bipartite NLS motif encompassing amino acid residues 18–35 (8). To determine the position of the interaction domain relative to the NLS motif, we generated several mutants lacking the complete NLS (35–300), containing half of the NLS (29–300) or the entire NLS (18–300), and examined their ability to interact with HA-tagged FANCG (Fig. 1B). The residues between position 18 and 29 were essential for binding to FANCG. By contrast, deletion of an additional 100-amino acid segment from the carboxyl terminus (1–200) had no effect on binding. These results demonstrate that residues 18–29 of FANCA are required for interaction with FANCG in vitro.

Identification of FANCA Residues Critical for FANCG Binding—The 11-amino acid domain of FANCA required for interaction with FANCG has no similarity to other known protein interaction motifs. Using the Chou-Fasman algorithm for secondary structure prediction, we noted that this domain is positioned within an α-helical structure. We evaluated the role of specific residues within this domain for binding to FANCG by site-directed mutagenesis (Fig. 2A). First, we changed the charged amino acid residues to alanine. Each mutant was translated in vitro along with HA-FANCG and analyzed by...
immunoprecipitation. While the substitution of alanine for arginine at position 20 (R20A) had a relatively minor effect on binding, the substitution of two (Arg<sup>19</sup>-Arg<sup>20</sup>) or three adjacent arginine residues (Arg<sup>18</sup>-Arg<sup>19</sup>-Arg<sup>20</sup>) by alanine strongly reduced binding to FANCG (Fig. 2B). By contrast, the substitution of alanine for glutamic acid at position 24 (E24A) resulted in a modest increase in binding. These results indicate a requirement for either arginine or other basic hydrophilic amino acids at positions 18 and 19 of FANCA for binding to FANCG.

We then tested the roles of two nonpolar hydrophobic residues that are located in the vicinity of charged residues and the α-helix. The substitution of alanine for tryptophan at position 22 (W22A) resulted in a minor decrease in binding to FANCG (Fig. 2B). By contrast, the substitution of alanine for leucine at position 25 (L25A) profoundly reduced binding to FANCG. Similarly, substitution by proline (L25P) completely abolished binding. In every case, the level of expression of these mutant proteins was comparable (Fig. 2B, middle panel), ruling out the trivial possibility that the observed variations in binding are due to differences in protein input or stability. Because both proline, a residue known to disrupt α-helical structures, and alanine at position 25 compromised binding to FANCG, the requirement for an α-helix per se could not be determined. Taken together, these results demonstrate that positions 18, 19, and 25 of FANCA are critical for interaction with FANCG.

**Two Noncontiguous Domains on FANCG Mediate Binding to FANCA**—Next, we performed deletion analysis on FANCG to delineate the region(s) involved in binding to FANCA. Several HA-tagged carboxyl-terminal deletion mutants of FANCG were generated and tested for complex formation with FANCA. As before, FANCG mutants were co-translated and radiolabeled in vitro with wild type FANCA and tested for binding by immunoprecipitation with the 12C5 antibody (Fig. 3). Deletion of 37 residues from the carboxyl terminus of FANCG (HA-FANCG<sub>1–400</sub>) completely abolished binding to FANCA. However, although larger carboxyl-terminal deletions extending to the amino acid position 475 did not further compromise the binding, the deletion of a further 75-amino acid segment (HA-FANCG<sub>1–475</sub>) resulted in a strong reduction in binding to FANCA. Consistent with this observation, the binding of HA-FANCG<sub>1–475</sub> was reduced by a factor of 100 compared to full-length HA-FANCG (Fig. 2B, middle panel), ruling out the coincidental, or it may reflect a dynamic association of FANCA and FANCG in the cytoplasm.

**Co-localization of FANCA and FANCG**—The interaction between FANCA and FANCG in vivo was investigated by studying their subcellular localization. HeLa cells were transiently co-transfected with vectors encoding FANCA and HA-FANCG. The use of the rabbit polyclonal anti-FANCA antibody and the mouse monoclonal 12C5 antibody allowed us to detect simultaneously the localization of these proteins by immunofluorescence microscopy. The specificity of the anti-FANCA antibody has been demonstrated previously (14): in these experiments, it did not cross-react with HA-FANCG, and the 12C5 antibody did not react with HA-FANCG (data not shown). As before (18), FANCA and HA-FANCG were present in both the nucleus and the cytoplasm (Fig. 4). Although the proportion of nuclear to cytoplasmic FANCA and HA-FANCG differed among transfected cells, there was close concordance in the subcellular expression patterns of these proteins: cells with high levels of nuclear FANCA also had high levels of HA-FANCA in the nucleus, and those with high levels of FANCA in the cytoplasm also had high levels of HA-FANCG in the cytoplasm. These data suggest that FANCA and FANCG co-localize in both the nucleus and cytoplasm.

The static co-localization of FANCA and FANCG may be coincidental, or it may reflect a dynamic association in vivo. To distinguish between these possibilities, HeLa cells were cotransfected with HA-FANCG and either FANCA fused to the SV40 NLS (FANCA-NLS) or to the NLS of the inhibitor of cAMP kinase (FANCA-NES), which show, respectively, nearly exclusive nuclear and cytoplasmic localization of FANCA (11). Consistent with an in vivo association, HA-FANCG was found primarily in the nucleus when it was co-expressed with FANCA-NLS, and it was found primarily in the cytoplasm when it was co-expressed with FANCA-NES (Fig. 4). As before (14), no such concordance was observed between FANCA and FANCC by cotransfection of FANCA-NES or FANCA-NLS with FANCC (data not shown). These data support the model that FANCA and FANCG associate with each other in vivo.

**Functional Activity of Amino-terminal Truncated FANCA Mutants**—The complementing activity of several FANCA mutants was assessed after stable expression in the HSC72 lymphoblastoid cells that belong to FA complementation group A. Stable expression of the FANCA mutants was confirmed by Western blotting (data not shown), and cytotoxicity to MMC was assessed as before (14). Although FANCA<sub>18–1455</sub> was fully capable of correcting the MMC sensitivity of HSC72 cells, the FANCA<sub>29–1455</sub> construct remained functional when targeted to the nucleus with the SV40 NLS. However, cytoplasmic targeting with wild type NES abolished its complementation function. As a control, a mutant NES appended to FANCA<sub>29–1455</sub> with the SV40 NLS failed to rescue its complementation function (data not shown). We also tested the effect of cytoplasmic or nuclear enrichment on the complementation function of these mutants. Forced nuclear targeting of the FANCA<sub>29–1455</sub> with the SV40 NLS failed to complement this defect (Fig. 5A). We also tested the effect of cytoplasmic or nuclear enrichment on the complementation function of these mutants. Forced nuclear targeting of the FANCA<sub>29–1455</sub> with the SV40 NLS failed to rescue its complementation function (data not shown). By contrast, the FANCA<sub>18–1455</sub> construct remained functional when targeted to the nucleus with the SV40 NLS. However, cytoplasmic targeting with wild type NES abolished its complementation function. As a control, a mutant NES appended to FANCA<sub>18–1455</sub> that results in both nuclear and cytoplasmic localization described previously (14) remained active (data not shown). These result suggest that (a) the ability of FANCA to bind FANCG is essential for its complementation function and (b) the functionally relevant interaction occurs in the nucleus.

**Dominant Negative Effect of FANCA Mutants in HeLa Cells**—The notion that FANCA-FANCG interaction is required for complementation activity suggests that overexpression of mutant versions of these proteins that contain otherwise intact interaction domains should induce MMC sensitivity in cells with wild type patterns of resistance. To test this hypothesis, we generated stably transfected HeLa cells expressing...
FANCA1–1200, FANCA1–600, FANCA1–300, and FANCA29–1455. From each transfection, clonal cell lines were derived, and the expression of mutant proteins was confirmed by Western blotting (data not shown). Two independent clones from each mutant class were assessed for resistance to MMC. For each clone, the expression of the truncated protein was readily detectable and represented approximately a 3–6-fold increase compared with the expression of the full-length, endogenous FANCA protein in parental HeLa cells. The overexpression of all three carboxyl-terminal deletion mutants that are capable of interacting with FANCG induced hypersensitivity to MMC (Fig. 5B; data for the FANCA1–600 mutant is not shown for clarity). As before (14), full-length FANCA overexpression (also approximately 3–6-fold higher than in parental HeLa cells) did not induce hypersensitivity to MMC in HeLa cells. By contrast, similar overexpression of the FANCA29–1455 deletion mutant that lacks the FANCG-binding domain failed to induce MMC hypersensitivity. These results provide strong genetic evidence for the importance of FANCA-FANCG interaction in the regulation of cellular resistance to MMC.

DISCUSSION

In this report we have performed co-precipitation, co-localization, and functional assays to demonstrate that FANCA and FANCG interact with each other in the nucleus to protect cells from the cytotoxicity of MMC. Because MMC sensitivity is a surrogate marker of chromosomal instability in FA cells, we propose that the interaction of FANCA with FANCG is important in the regulation of chromosomal stability. This interaction appears to be direct without the need for accessory factors. However, it is possible that a residual protein or another macromolecule in the reticulocyte lysate facilitates this interaction. Immunofluorescence analysis showed that the localization of FANCA and FANCG is highly coordinated, and overexpression figures 4 and 5.
of mutant FANCA alleles that retain the ability to bind to FANCG resulted in a dominant negative effect by a mechanism that most likely involves the dislodging of FANCG from wild type FANCA-FANCG complexes. However, this observation does not exclude potentially important functions for other regions of the FANCA protein. Although FANCA-FANCG complex formation was shown previously to take place in both the cytoplasm and nucleus, a nuclear function was suspected (18, 19) but not demonstrated. The delineation of FANCA-FANCG binding domains allowed us to perform a genetic experiment using nuclear and cytoplasmic isoforms of FANCA. Our data demonstrate for the first time that the nuclear localization of FANCA-FANCG complexes is critical for cellular resistance to MMC. This is in contrast to the requirement for FANCC to remain in the cytoplasm to function: its major function is apparently carried out in the cytoplasm as nuclear targeting abolishes its complementation function (16). We have also demonstrated that FANCC interacts with NADPH cytochrome P-450 reductase and affects its activity (17). Therefore, we hypothesize that at least two spatially distinct protein complexes regulate the cellular resistance to MMC.

We have also mapped the interaction domains on both FANCA and FANCG. An unusual domain of 11 amino acids in the amino terminus of FANCA delineated by residues 18–29 was required for binding to FANCG. Two arginines (Arg18 and Arg19) in a row of three played dominant roles in contacting plex involving two noncontiguous regions. Although residues FANCA-binding domain on FANCG appeared to be more conserved, we hypothesize that it is likely indirect (12, 19, 20), involving a mechanism of this enhancement is unclear. One possible model to account for this finding is that the carboxyl terminus of FANCG forms a loop around the amino terminus of FANCA, and the two noncontiguous domains of FANCG interact with FANCA for optimal binding. However, it is also possible that there are two independent interaction sites with FANCA. The topology of this region may be clarified by structural studies of the FANCA-FANCG complex.

FANCG is the last identified member of the FA family of genes and was cloned originally by virtue of its ability to rescue mutant Chinese hamster ovary cells from the cytotoxicity of MMC (21). Thus, FANCG as well as FANCC (22) are functionally conserved in other mammals. However, no orthologues have been identified in more distant species. Although all known FA gene products are capable of attenuating the toxicity of bifunctional cross-linkers, their precise functions remain obscure. Both oxidative stress and defective interstrand DNA cross-linking have been considered. Cross-link repair in Saccharomyces cerevisiae may require the function of genes involved in DNA double-strand break repair (23). However, interstrand cross-link repair is not defective in FA group C cells, and the function of FANCC precedes cross-link repair (16).

Interestingly, the limited homology of FANCA to peroxidases (9) raises the possibility that FANCA also functions in a pre-repair step, albeit in the nucleus. Thus, FANCA-FANCG and FANCC-reductase complexes may have functionally similar roles in their respective cellular compartments, and cellular sensitivity to cross-linkers may depend on the integrity of both complexes. Conversely, the disruption of these complexes by dominant negative molecules or small drugs could present opportunities for the chemosensitization of neoplastic cells to bifunctional cross-linkers.

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Fanconi Anemia Protein Interactions

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