Phosphorylation of Fanconi Anemia (FA) Complementation Group G Protein, FANCG, at Serine 7 Is Important for Function of the FA Pathway*

Received for publication, July 22, 2004, and in revised form, August 2, 2004
Published, JBC Papers in Press, August 6, 2004, DOI 10.1074/jbc.M408323200

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Fanconi anemia (FA) is an autosomal recessive disease of cancer susceptibility. FA cells exhibit a characteristic hypersensitivity to DNA cross-linking agents. The molecular mechanism for the disease is unknown as few of the FA proteins have functional motifs. Several post-translational modifications of the proteins have been described. We and others (Qiao, F., Moss, A., and Kupfer, G. M. (2001) J. Biol. Chem. 276, 23391–23396 and Futaki, M., Watanabe, S., Kajigaya, S., and Liu, J. M. (2001) Biochem. Biophys. Res. Commun. 281, 347–351) have reported that the FANC protein (Fanconi complementation group G) is phosphorylated. We show that in an in vitro kinase reaction FANCG is radioactively labeled. Mass spectrometry analysis detected a peptide containing phosphorylation of serine 7. Using PCR-mediated site-directed mutagenesis we mutated serine 7 to alanine. Only wild-type FANCG cDNA fully corrected FA-G mutant cells. We also tested the effect of human wild-type FANC in Chinese hamster ovary cells in which the FANC homologue is mutant. Human FANC complemented these cells, whereas human FANCG(S7A) did not. Unexpectedly, FANCG(S7A) bound to and stabilized the endogenous forms of the FANCA and FANCC proteins in the FA-G cells. FANCG(S7A) aberrantly localized to globules in chromatin and did not abrogate the internuclear bridges seen in the FA-G mutant cells. Phosphorylation of serine 7 in FANC is functionally important in the FA pathway.

Fanconi anemia (FA) is a genetic disease of cancer susceptibility marked by congenital defects, bone marrow failure, and myeloid leukemia (1–4). To date at least 11 complementation groups have been defined (5–7). Eight genes have been cloned (8–17). However, the gene products resemble no known proteins and have few identifiable functional protein motifs. One exception is the recently cloned FANCL gene, which contains the ubiquitin ligase motif. In addition, the FANCD1 gene has been identified as BRCA2, one of the familial breast cancer genes.

Cells derived from patients with the FA exhibit characteristic hypersensitivity caused by DNA cross-linking agents and generalized decreased survival (18–22). However, no defined biochemical mechanism for this hypersensitivity has been elucidated, although studies have implicated cytokine dysregulation, excessive oxidative damage, defects in DNA repair, and lack of cell cycle control (23–27). Patient and cellular phenotypes across all the complementation groups are similar, suggesting an inter-relatedness or cooperativity between the FA proteins.

This cooperativity has been borne out by work we have done in showing binding of FANCA and FANCC in a protein complex in both nucleus and cytoplasm (28–30). Recent work has found the FANCE, FANCF, FANCG, and FANCL proteins in the complex as well (31–34). A large complex is suggested by our recent work (35), and binding does not occur in any of the complementation groups except the FA-D1, D2, I, and J groups (7).

One clue to FA function lies in the study of the FANCA protein, which contains a classic bipartite nuclear localization signal and is phosphorylated. Generally, FANCA nuclear localization, phosphorylation, and binding to FANCC are abolished in all complementation groups except the FA-D1 and D2 groups (28–30, 33). This suggests that a nuclear event is critical to the normal function of the FA proteins, and the aberrant proteins in the FA-D groups may have a role downstream of the FA complex in the nucleus. However, some have found FANCA point mutants that are expressed, translocated to the nucleus, and are phosphorylated to some extent. Some of these mutants are of intermediate MMC sensitivity (36).

Over the years, little information has been found that addresses the regulation of the FA proteins. mRNA or protein levels change little in response to DNA damage or the cell cycle. Our recent work has revealed that at least a subset of the FA proteins resides in the nucleus bound to chromatin, where increased protein binding occurs in response to DNA damage (37). One of the non-core complex FA proteins, FANCD2, becomes monoubiquitinated in response to DNA damage (38).

In addition, we have shown during the cell cycle that the FA proteins detach from chromatin during mitosis, and FANCG becomes phosphorylated while remaining part of the complex (37). One group has demonstrated that FANCG has a isoform seen in asynchronous cells that is phosphatase sensitive (39).

In this paper we report the identification of a phosphopeptide from endogenous, in vivo FANCG. The phosphorylated amino acid, serine 7, is functionally important in a way that is evolutionarily conserved between hamster and human.
Materials and Methods

Production of FLAG-FANCG and Mutants—pMMP-FLAG-FANCG was made as previously described (37). All PCR were done using high fidelity Taq polymerase (Amersham Biosciences). Primers homologous to FANCG with restriction sites for ligation into pcDNA-CFP were synthesized on pMMP-FLAG-FANCG, and the resulting product was restricted and ligated into pcDNA-CFP. Primers homologous to FANCG nucleotides were used in a polymerase chain reaction using a Stratagene QuickChange kit. Each set of primers contained a one-nucleotide difference resulting in the change of amino acid 7. PCR products were sequenced to confirm the mutation as well as to eliminate the possibility of PCR-induced mutations. The resulting pMMP constructs were transfected into 293TG producer cells, and viral supernatants were collected daily between 3 and 7 days after transfection. Retroviral supernatants were used for subsequent transduction. pcDNA-CFP-FANCG constructs were maxi-prepped using standard techniques (Qiagen). Cells were subsequently transfected using LipofectAMINE reagent (Amersham Biosciences).

Cell Culture—Cells were grown at 37°C in a 5% CO2 incubator. HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, FA-MAXA monkey GM914 cells, corrected GM6914 + FLAG-FANCA cells, FA-D2 mutant PD20 cells, FA-D2 corrected 20–315 cells, and FA-G mutant cells 32SV were grown in F12 + 15% fetal bovine serum. 293sv producer cells were grown as described (37). HeLa cells were also transduced with pMMP-FLAG-FANCA, and the resulting cells were selected with puromycin. FA-G mutant EUPA10 lymphoblasts were grown in RPMI1640 + 15% fetal bovine serum, the Chinese hamster ovary (CHO) cell line AA8 and FANCG/XRC9 mutants NM3 and UV40 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum as described previously (40).

Lysate Preparation and Immunoprecipitation—For whole cell lysate, pelleted cells were extracted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors (1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 20 mM β-glycerophosphate). The extract was tested for protein concentration by the Bradford assay. Two mg of protein in 1 ml of the buffer containing protease and phosphatase inhibitors was incubated with 2 µg of both anti-N and anti-C-terminus FANCA antibodies or anti-FLAG affinity gel for 1 h at 4°C. 30 µl of Protein A-Sepharose (Amersham Biosciences) was added to the anti-FANCA immunoprecipitations, and the resulting mixture was rotated at 4°C for an additional hour. The beads were then washed 3 times in Tris-buffered saline (TBS; 50 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 0.1% Triton X-100, protease, and phosphatase inhibitors and dried. 50 µl of loading buffer was added (25).

SDS-PAGE and Immunoblotting—SDS-PAGE was performed, followed by gel transfer in 25 mM Tris and 200 mM glycine onto nylon-supported nitrocellulose (Amersham Biosciences). Filters were blocked for 1 h in 5% bovine serum albumin in TBS and then incubated in TBS plus Tween 20 (TBS-T) containing primary antibody overnight at room temperature. Filters were then washed in TBS-T, incubated with horseradish peroxidase-linked secondary antibody (Amersham Biosciences), washed again, and visualized by chemiluminescence (28).

Immunoblotting was performed using FANCG and FANCA antisera, as previously described (37). A rabbit polyclonal antisera raised against a peptide containing phosphoserine 7 of FANCG was provided by Upstate USA. This crude serum was purified first over a column containing unphosphorylated peptide covalently linked to a matrix (Pierce). The flow through was then passed over a column containing phosphorylated peptide. A specific anti-phosphoserine 7 of FANCG was then eluted from this column with 0.1 M glycine, pH 2.5, and neutralized with Tris-HCl, pH 9.5. A FANCD2 polyclonal antisera (rabbit) was made by providing a FANCD2 fusion protein for immunization (Protoitech, following the procedure described by Timmers et al. (16). Densitometry was performed on blots using Scion Image software.

In Situ Chromatin Preparation and Intercellular Bridging—Cells grown on chamber slides were first washed in cold PBS. Soluble protein was removed by extraction with 250 µl of a buffer containing 293TG culture supernatant, Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 1 mM EGTA, 20 mM vanadyl ribose complex, 1 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride] containing 0.5% Triton X-100 for 2 min at 4°C. The structure remaining was extensively cross-linked by treatment with 4% formaldehyde in CSK for 40 min at 4°C. The slides were covered with mounting solution containing 2 µg/ml DAPI (Vector Laboratories, Inc.) after washing with cold PBS (37) (41).

For bridging experiments, cells were plated into chamber slides and treated with or without the indicated doses of MMC for 4 days. Cells were fixed in 4% paraformaldehyde, mounted with medium with 10 µg/ml DAPI (Vector Laboratories, Inc.), and mounted under a coverslip. Internuclear bridges were assessed by counting 5 random views, for a total of 500–1000 cells, repeating 3 times per group. For fluorescent microscopy and analysis, an inverted fluorescent microscope (Nikon) was used, with images captured by digital camera (Hamamatsu).

Chromatin Fiber Preparation—Chromatin fibers were prepared according to method of Blower et al. (42). Cells were trypsinized 48 h after transduction, washed twice with cold PBS, and then suspended in hypotonic solution (75 mM KCl in PBS) for 10 min. Cells were adjusted to a density of 1–2 × 106 cells/ml were cytospun onto charged slides (double funnels) at 800 rpm with high acceleration for 4 min, 150 µl of cells per slide. For attached monolayer cells, 1 × 105 cells/ml were used, and for suspension cultures, 2 × 107 cells/ml. After cytospin, slides were immersed in a Coplin jar filled with lysis buffer (25 mM Tris-Cl, pH 7.5, 0.5 µM NaCl, 1% Triton X-100, 0.2 µM mea) for 3–10 min. The slide was slowly lifted vertically from the lysis buffer, and the chromatin was allowed to stream down the slide. After fixation in 4% formaldehyde in PBS-Tween 20 (0.05%) for 20 min, the slides were covered with mounting solution containing 2 µg/ml DAPI (Vector Laboratories, Inc.) after wash in PBS. For fluorescent microscopy and analysis, an inverted fluorescent microscope (Nikon) was used, with images captured using a digital camera (Hamamatsu).

Mass Spectrometry Analysis—FLAG-FANCA affinity immunoprecipitations were conducted on crude lysates from HeLa + FLAG-FANCA and HeLa + vector. Elutions from the FLAG affinity gel were executed in 0.5 ml of Tris-buffered saline in 100 µl FLAG peptide. The eluate was buffered exchanged into 100 mM NaHCO3.

The main digestion and extraction of peptides from the aqueous mixture were performed, as in Shevchenko et al. (43, 44). Peptides were separated on microcapillary columns by reverse phase nanoflow HPLC (Applied Biosystems or Agilent HPLC) with a gradient consisting of 0.1% acetic acid and 70% acetone at 0.1% acetic acid on either an Applied Biosystems or Agilent HPLC as described (45). Online analysis of the chromatographically separated tryptic peptides was performed using a Bruker microESI ionization source coupled to a Bruker LCQ ion trap mass spectrometer. Data-dependent analysis was performed, in which one full MS scan from m/z of 300–2000 was performed followed by five MS/MS scans of the most abundant ions present in the full mass scan (46). All MS/MS scans were performed with an isolation window of 3 Da (precursor m/z = 1.5 Da) and using 35% collision energy.

ProteinFarm in-house software was used as an interface between the raw mass spectrometry data and databases and a data mining program, SEQUEST (46). After deletion of poor quality, spectra and conversion to .dta file format, SEQUEST was used to search against a FANCA data base. Peptides with a cross-correlation score above 2 and each of those spectra were manually confirmed for protein validation.

Immobilized Metal Affinity Chromatography Enrichment of Phosphorylated Proteins—Immunoblotting of total cell lysates was performed using 100 µl of FeCl3 (Aldrich). After the sample containing tryptic peptides from the previous section was applied to the column, non-binding peptides were removed by washing with a solution of 100 mM NaCl (Aldrich) in acetonitrile (Malinckrodt, Paris, KY), water, and glacial acetic acid (Aldrich) (25:74:1, v/v/v). This column was then connected in line to a second fused silica column packed with 4–20 µm C18 (YMC, Wilmington, NC). Phosphorylated peptides were eluted onto the C18 column with 5 µl of 50 mM NaH2PO4 (Aldrich; pH 9.0). The C18 column was disconnected and rinsed with several column volumes of 0.1% acetic acid to remove salts and excess sodium phosphate buffer prior to mass spectrometric analysis. The peptides were then gradient-eluted directly into the ion trap mass spectrometer as described in the previous section. Synthetic peptides were synthesized and analyzed, as previously described (43).

In Vitro Kinase Assays—In vitro kinase reactions were performed by incubation of immunoprecipitated beads and 10 µCi of [γ-32P]ATP in 25 µl of buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM NaCl, 100 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 1 mM EGTA, 20 mM vanadyl ribose complex, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) containing 0.5% Triton X-100 for 2 min at 4°C. The structure remaining was extensively cross-linked by treatment with 4% formaldehyde in CSK for 40 min at 4°C. The slides were covered with mounting solution containing 2 µg/ml DAPI (Vector Laboratories, Inc.) after washing with cold PBS (37) (41).
stained with crystal violet followed by extraction in SDS-methanol. Absorbance was measured by A_{595}. MMC sensitivity in CHO cell lines was determined by clonal survival assays as described previously (40).

For lymphoblasts, 2 ml of cells were seeded into flasks at a density of 2 × 10^6 cells/ml. MMC was added at the indicated doses (0–300 nM) with duplicate or triplicate flasks being prepared for each dose. Following 24 h incubation at 37 °C, a further 2 ml of growth medium was added, along with sufficient MMC to maintain the indicated (starting) dose. This process was repeated for a further 2 days (i.e., the volume of medium in the flask was doubled each 24 h, while maintaining the MMC concentration). After 4 days, 4 ml of the resulting 16-ml cultures were transferred to new flasks. 12 ml of fresh medium containing MMC was added and the cells were incubated for a further 3–4 days. Resulting cell densities in each individual culture were determined by hemocytometer counts (a minimum of four counts for each culture) and normalized to the cell numbers obtained in untreated cultures. Data shown represent the means of a minimum of five independent experiments. p values were calculated using t test.

Cell Cycle Analysis—500,000 cells of EUFA143 cells with various constructs of FANCN were treated with 1 μM MMC. After the wash in PBS, the cells were resuspended in 0.1% citrate, 0.03% Nonidet P-40, 100 μg/ml propidium iodide, and 10 μg/ml RNase and analyzed by FACS (47).

RESULTS

FANCA and FANCG Can Be Phosphorylated in Vitro—Previous work has shown that both FANCA and FANCG are phosphorylated. To demonstrate that FANCG was a phosphoprotein, we immunoprecipitated FANCA using anti-FANCA antibody in whole cell lysate from HeLa + vector cells followed by an in vitro kinase reaction (Fig. 1A). An immune-specific phosphorylated protein was evident at 70 and 150 kDa upon autoradiography. Subsequent immunoblotting of the gel demonstrated that the 70-kDa band represented FANCG. FANCA was detected by immunoblotting as well at the same position as the 150-kDa band seen at autoradiography. This is consistent with previous literature showing that FANCA can be in vitro phosphorylated (48).

We also immunoprecipitated FLAG-FANCA from whole cell lysates made from GM6914 FA-A mutant cells containing either vector alone or FLAG-FANCA constructs, using anti-FLAG affinity beads and incubated the washed immunoprecipitates in kinase buffer in the presence of [γ-32P]ATP. The resulting gel was transferred to membrane and subjected to autoradiography. In Fig. 1B, 150- and 70-kDa proteins were specifically phosphorylated in the FLAG-FANCA containing cell extract (lane 1). Immunoblotting indicated these to be FANCA and FANCG, respectively. Immunoblotting of 100 μg of whole cell lysate from each cell line with Ku80 antiserum demonstrated equal amounts of protein in the cell lysates (Fig. 1C).

FANCG Is Phosphorylated in Vivo—To detect and sequence the peptide containing the phosphorylation site for FANCG and demonstrate in vivo phosphorylation, we performed anti-FLAG immunoprecipitations on lysate made from HeLa cells transduced with FLAG-FANCA. The immunoprecipitates were eluted using FLAG peptide. After trypsin digestion, the resulting peptide mixture was loaded onto an immobilized metal affinity chromatography column. Ion trap mass spectrometry data revealed the presence of a 14-amino acid tryptic peptide containing serine phosphorylation at residue 7 of FANCG (Fig. 2A). The peak indicated by the asterisk represents the loss of 49 mass units from the phosphopeptide parent mass that is characteristic of phosphopeptides fragmented in an ion trap resulting from the liability of the phospho group. Peptides corresponding to FANCG (4–17) were synthesized containing phosphorylated or non-phosphorylated serine 7 and analyzed by an ion trap mass spectrometer. Only the spectra generated from the synthetic peptide containing phosphorylated serine 7 matched the peptide spectra originally acquired and sequenced in the trypsin-digested FANCG preparation (Fig. 2B).

FANCG(S7A) Fails to Fully Correct FA-G Mutant Cells—To assess the functional importance of the phosphorylation of serine 7 of FANCG, we mutated serine 7 to aspartic acid, a phosphomimetic amino acids, in a whole cell lysate from HeLa cells treated with 150 nM MMC. After infection of EUFA143; HeLa, lane 1; FA-A mutant + vector, lane 2; FA-A mutant + FANCA, lane 3. Pre, preimmune; vec, vector.

Fig. 1. FANCA and FANCG can be phosphorylated in vitro. A, whole cell lysate from HeLa cells was immunoprecipitated with anti-FANCA antiserum or preimmune serum. The resulting immunoprecipitates were subjected to in vitro kinase assay in the presence of [γ-32P]ATP. Autoradiography revealed a specific band corresponding to FANCG. Immunoblotting confirmed the presence of FANCG. B, whole cell lysates from GM6914 FA-A mutant + vector or + FLAG-FANCA cells were immunoprecipitated with FLAG affinity gel. The resulting beads were washed and subjected to in vitro kinase reaction in the presence of [γ-32P]ATP. Autoradiography revealed specific bands corresponding to FANCA and FANCG. Subsequent immunoblotting confirmed bands corresponding to FANCA and FANCG. FA-A mutant + FLAG-FANCA, lane 1; FA-A mutant + vector, lane 2. C, whole cell lysates from the above cell lines were run by SDS-PAGE and immunoblotted with Ku80 antiserum. HeLa, lane 1; FA-A mutant + vector, lane 2; FA-A mutant + FANCA, lane 3. Pre, preimmune; vec, vector.

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acid, also failed to fully correct. This finding confirms that the phosphorylation of serine is indeed functionally important to the \textit{in vivo} role of the FA pathway, although the serine cannot be simply substituted for by a phosphomimetic amino acid. To demonstrate that a similar amino acid could correct, we substituted threonine, a site for physiological phosphorylation, for serine 7 and expressed the FANCG construct in 326SV cells. In this case, the FANCG(S7T) was able to fully correct (Fig. 3B). Immunoblotting revealed that wild-type and mutant FANCG species were expressed at equivalent levels (Fig. 3C).

Another phenotype associated with FA cells is increased G2 accumulation after MMC treatment (26, 49, 50). This phenotype is reversed in mutant cells after provision of the specific complementing cDNA. To assess the effect of these mitotic phosphorylation mutations on cell cycle, we subjected the EUFA143 cells to FACS analysis after MMC treatment. Treatment of mutant cells resulted in 40% G2-M cells, whereas cells corrected with wild-type FANCG exhibited only 20%. None of the point mutants was able to fully correct this excessive G2-M accumulation ($p < 0.001$) (Fig. 3D). S7D mutant was not statistically significantly different from S7A ($p = 0.09$). This graph represents at least three independent experiments.

An additional phenotype that has been established in most FA cell lines has been the absence of monoubiquitination of FANCD2 following DNA damage or during S phase, as seen in wild-type cells (38). This modification appears to depend on the integrity of the FA core complex. To determine the ability of the FANCG mutants to confer ability of FANCD2 to become monoubiquitinated, whole cell extracts made from EUFA143 cells containing the indicated forms of FANCG were run by SDS-PAGE. Subsequent immunoblotting with a FANCD2 antiserum demonstrated that only wild-type FANCG-containing cells exhibited a robust FANCD2 monoubiquitination, whereas FANCG(S7A) and FANCG(S7D) showed little (Fig. 3E). As expected, no monoubiquitinated FANCD2 could be detected in the vector only control. These data are consistent with the previous data showing that serine 7 is vital for normal FA core complex function.
Genomic instability is inherent in the FA phenotype. In addition to chromosomal instability noted by many FA investigators, we have noted internuclear bridging peculiar to FA mutant cells. Internuclear bridging is a phenomenon seen in cells displaying genomic instability (51, 52). Cells with abnormal chromosomal segregation and chromosomal structures such as those in FA can form internuclear bridging, presumably because of lagging chromosomes (53). To demonstrate this...
visually in vivo, we used DAPI to stain FA-G mutant 326SV fibroblasts containing vector only, FANCG(S7A), FANCG(S7D), and wild-type FANCG in the presence or absence of MMC. Direct fluorescence demonstrated that both mutant and FANCG(S7A) expressing cells contained increased nuclear bridges, which was enhanced after MMC treatment. An example of an observed internuclear bridge is shown in Fig. 4A in FA-G + FANCG(S7A) cells. These bridges were seen to an equal extent in the vector and FANCG(S7A) containing cells, whereas significantly fewer were seen in the corrected cells (p < 0.001) (Fig. 4B). S7D and S7A mutants were not statistically significantly different from vector only (p = 0.08).

To demonstrate this phenotype in other FA cells, we analyzed mutant and corrected FA-D2 and FA-A cells. Internuclear bridging was characteristic of the FA cells that were studied, as exemplified by the FA-D2 FA-A complementation groups, with mutant FA-D2 and FA-A cells containing more bridges than corrected FA-D2 (20–315) (p < 0.001) and FA-A (GM6914 + FLAG-FANCA) (p < 0.001), respectively (Fig. 4, C and D).

FANCG Serine 7 Phosphorylation

Previous reports have suggested that reduced levels of FANCA and FANCG exist in FA-A and FA-G mutant cell lines, respectively, and that FANCA and FANCG stabilize each other (54). Numerous others have also reported that at least 5 of the known FA proteins bind in a complex (55–57). To test for binding of mutant FANCG proteins we performed anti-FLAG immunoprecipitations from EUFA143 FA-G mutant cells. As previously reported very low or non-detectable levels of FANCA, FANCC, and FANCG were evident by immunoblotting in mutant cells (Fig. 5A). As expected, expression of FANCG stimulated increased expression of FANCA. Unexpectedly, FANCG also resulted in increased expression of FANCC, which was not previously reported. Also, the mutant FANCG(S7A) stabilized both FANCA and FANCC and coprecipitated with both in the same manner as the wild-type FANCG. FANCC was present in lower amounts in the complex, a finding consistently seen in our immunoprecipitation-immunoblotting experiments. Densitometry performed on the
FANCA, FANCG, and FANCC bands revealed that only FANCC was present in lower amounts in the immunoprecipitated complex. FANCA and FANCG were present at 90 and 92% of the amount in the FANCG(S7A) extract as in the wild-type FANCG extract, whereas FANCC dropped to 54%. Ku80 immunoblotting confirmed equal loading and use of similar amounts of crude lysates for the immunoprecipitations. Equal amounts of wild-type FANCG and FANCG(S7A) were observed in crude extracts of both human FA-G mutant EUFA143 and 326 SV cell lines after transduction of the respective FANCG species (Fig. 3). Given that most FA-G mutant cells result in lack of expression of FANCG, the presence of expression of FANCG(S7A) and its stabilization of FANCA may account for intermediate MMC sensitivity versus vector only cells.

We have also shown that FANCG is phosphorylated at mitosis (37). To test if serine 7 is a mitotic phosphorylation site, we arrested HeLa cells containing FLAG-FANCG(wt), FLAG-FANCG(S7A), or vector alone with 1 μM nocodazole and immunoprecipitated with FLAG affinity gel. All mitotic forms were present, indicating that serine 7 does not account for FANCG mitotic phosphorylation (Fig. 5B). In vitro kinase assays performed on FA-G mutant cells containing FANCG(S7A) still showed labeled FANCG (data not shown), further supporting that multiple phosphorylation sites exist on FANCG. Ku80 immunoblot on crude lysates served as a control for the lysates from which the FLAG immunoprecipitations were conducted.

Phosphoserine 7 FANCG Antiserum Detects Phosphorylated FANCG—To confirm in vivo labeling of FANCG, we analyzed FANCG for phosphorylation using a phosphospecific antibody raised against phosphoserine 7 of FANCG. We performed FLAG immunoprecipitations using extracts from HeLa cells transduced with either FLAG-FANCG or FLAG-FANCG(S7A) followed by immunoblotting with anti-FANCG antiserum showed that the previously described FANCG mitotic isoforms (29) were not abolished by mutation at serine 7 of FANCG. Ku80 immunoblotting served as controls for crude lysates from which the immunoprecipitations were performed. Mitotic (lanes 1–3) and asynchronous (lanes 4–6) HeLa cells; +wild-type FANCG, lanes 1 and 4; +FANCG(S7A), lanes 2 and 5; +vector, lanes 3 and 6. Vec, vector; wt, wild-type; IP, immunoprecipitation.
FANCG(S7A) was undetectable by the phosphoserine 7 antobody (lane 4). To detect endogenous phosphorylated FANCG, we immunoprecipitated FANCA from HeLa whole cell extract from cells treated with 1 μM MMC, using an anti-FANCA antiserum. After SDS-PAGE and transfer to membrane, the blots were immunoblotted successively with phosphoserine 7 FANCG antiserum and FANCG antiserum. The phospho-specific antibody detected endogenous phosphorylated FANCG (Fig. 6B, lanes 3 and 4), which was confirmed as FANCG after membrane stripping and subsequent FANCG immunoblotting. An increase in signal was seen in phosphorylated FANCG after MMC treatment (lane 4).

FANCG(S7A) Fails to Correct FA-G Mutant CHO Cells—FANC was first cloned as XRCC9 using the mutant CHO cell
C and UV40 (Fig. 7B) as well as the wild-type CHO line AA8. NM3 and UV40 both contain frameshift mutations that cause premature truncation of FANCG in exons 3 and 1, respectively, and fail to express FANCG protein (59). Wild-type human FANCG corrected the MMC sensitivity phenotype of the NM3 (D$_{37}$ = 120 nM versus D$_{37}$ = 28 nM for vector only control; p < 0.001) and UV40 (D$_{37}$ = 106 nM versus D$_{37}$ = 9.5 nM for vector only control; p < 0.001) cells near that of the wild-type parental AA8, confirming previous reports (40, 58). However, FANCG(S7A) failed to fully correct the MMC sensitivity of NM3 (D$_{37}$ = 40 nM; p < 0.001) and UV40 (D$_{37}$ = 41 nM; p < 0.001), producing an intermediate phenotype between that of wild-type FANCG and vector-only controls (Fig. 7, B and C). FANCG(S7D) behaved similarly to FANCG(S7A) in NM3 cells (D$_{37}$ = 28 nM; p = 0.26). Fig. 7D demonstrates that the level of human FANCG protein expression was similar in NM3, UV40, and AA8 transductants. Given the similar levels of expression of wild-type FANCG and FANCG(S7A), the incomplete correction observed is most likely a direct consequence of the amino acid substitution in FANCG(S7A). Expression of human wild-type FANCG in AA8 at a level higher than endogenous hamster protein (Fig. 7D) did not alter the MMC sensitivity of these cells (D$_{37}$ of AA8 + FANCG = 119 nM; D$_{37}$ of AA8 + FANCG(S7A) = 124 nM; D$_{37}$ of AA8 + FANCG(S7D) = 100 nM), as they exhibited a response similar to AA8 cells transduced with empty vector (D$_{37}$ = 124 nM). These data support the fact that serine 7 is important for FA function in a way that is conserved evolutionarily between hamster and human.

**FANCG(S7A) Abnormally Localizes to Chromatin—**To test the effect of the S7A mutation on FANCG localization, we mutated a CFP-FANCG construct via PCR-mediated mutagenesis. The wild-type and resulting mutants were transfected into mutant FA-G 326SV cells, which were subsequently analyzed by fluorescent microscopy. In recent work, we have shown that CFP-FANCG localizes to chromatin and chromatin fibers (60). Interestingly, the FANCG(S7A) was localized to the nucleus in a similar fashion as wild-type FANCG in whole cells (Fig. 8, panel A). The cells were then subjected to chromatin preparation in situ. This analysis showed that both wild-type FANCG and FANCG(S7A) localized to chromatin (Fig. 8, panel B). However, while wild-type protein localized to fine foci, the FANCG(S7A) localized to qualitatively different, large, globular foci, suggesting that mutation of FANCG results in aberrantly localized FA core complex. Analysis of chromatin fibers also demonstrated the large globular appearance of FANCG(S7A) (Fig. 8, panel C). In these micrographs, fluorescence of FANCG(S7A) appeared restricted to DAPI staining areas consistent with heterochromatin.

![Diagram](http://www.jbc.org/)

**Fig. 7.** FANCG(S7A) fails to fully correct FA-G mutant CHO cells. A, alignment of hamster and human FANCG shows that serine 7 is conserved between hamster and human. Constructs containing the empty vector, wild-type FANCG, and FANCG(S7A) were transduced into mutant CHO cell lines NM3 (B) and UV40 (C). As in the human cell line UV40, which is 20-fold hypersensitive (40, 58). Alignment analysis shows that the human FANCG protein shares 70% residue identity to the hamster protein, and this homology extends to the region of serine 7 in the human FANCG (Fig. 7A). To test the effect of the wild-type FANCG and FANCG(S7A) constructs in CHO cells, the FANCG constructs were transduced into the CHO FANCG mutant cells lines NM3 (Fig. 7B) and UV40 (Fig. 7C) as well as the wild-type CHO line AA8. NM3 and UV40 both contain frameshift mutations that cause premature truncation of FANCG in exons 3 and 1, respectively, and fail to express FANCG protein (59). Wild-type human FANCG corrected the MMC sensitivity phenotype of the NM3 (D$_{37}$ = 120 nM versus D$_{37}$ = 28 nM for vector only control; p < 0.001)) and UV40 (D$_{37}$ = 106 nM versus D$_{37}$ = 9.5 nM for vector only control; p < 0.001) cells near that of the wild-type parental AA8, confirming previous reports (40, 58). However, FANCG(S7A) failed to fully correct the MMC sensitivity of NM3 (D$_{37}$ = 40 nM; p < 0.001) and UV40 (D$_{37}$ = 41 nM; p < 0.001), producing an intermediate phenotype between that of wild-type FANCG and vector-only controls (Fig. 7, B and C). FANCG(S7D) behaved similarly to FANCG(S7A) in NM3 cells (D$_{37}$ = 28 nM; p = 0.26). Fig. 7D demonstrates that the level of human FANCG protein expression was similar in NM3, UV40, and AA8 transductants. Given the similar levels of expression of wild-type FANCG and FANCG(S7A), the incomplete correction observed is most likely a direct consequence of the amino acid substitution in FANCG(S7A). Expression of human wild-type FANCG in AA8 at a level higher than endogenous hamster protein (Fig. 7D) did not alter the MMC sensitivity of these cells (D$_{37}$ of AA8 + FANCG = 119 nM; D$_{37}$ of AA8 + FANCG(S7A) = 124 nM; D$_{37}$ of AA8 + FANCG(S7D) = 100 nM), as they exhibited a response similar to AA8 cells transduced with empty vector (D$_{37}$ = 124 nM). These data support the fact that serine 7 is important for FA function in a way that is conserved evolutionarily between hamster and human.

**FANCG(S7A) Abnormally Localizes to Chromatin—**To test the effect of the S7A mutation on FANCG localization, we mutated a CFP-FANCG construct via PCR-mediated mutagenesis. The wild-type and resulting mutants were transfected into mutant FA-G 326SV cells, which were subsequently analyzed by fluorescent microscopy. In recent work, we have shown that CFP-FANCG localizes to chromatin and chromatin fibers (60). Interestingly, the FANCG(S7A) was localized to the nucleus in a similar fashion as wild-type FANCG in whole cells (Fig. 8, panel A). The cells were then subjected to chromatin preparation in situ. This analysis showed that both wild-type FANCG and FANCG(S7A) localized to chromatin (Fig. 8, panel B). However, while wild-type protein localized to fine foci, the FANCG(S7A) localized to qualitatively different, large, globular foci, suggesting that mutation of FANCG results in aberrantly localized FA core complex. Analysis of chromatin fibers also demonstrated the large globular appearance of FANCG(S7A) (Fig. 8, panel C). In these micrographs, fluorescence of FANCG(S7A) appeared restricted to DAPI staining areas consistent with heterochromatin.
DISCUSSION

Because of the few functional motifs in FA proteins, the biochemical pathway delineated by the FA proteins remains unknown. Recent work detailing the ubiquitination and phosphorylation of FANC D2 and its interaction with BRCA1 reveals some insight (38, 61). In addition, the recent cloning of FANCL has revealed a protein containing a ubiquitin ligase motif thought to account for D2 monoubiquitination (17). However, the other proteins that are part of the FA core complex are intact even in FA-D2 cells, implying that the bulk of the FA proteins participate in an upstream event that remains poorly defined. One of the members of the complex, FANCA, has been shown to be phosphorylated, but the site of phosphorylation or its stimulus is unknown (36). We and others (37, 39) also have demonstrated that FANCG is phosphorylated, including at mitosis. One recent report details that FANCG has TPR motifs that may be important for FA core complex assembly and stability (62). Our data showing FA cells containing internuclear bridging is intriguing, and we are working to assess the uniformity of this phenomenon across all groups and cells in FA.

In this work we report the first described phosphorylation site for the FANCG protein. In previous work we have reported that FANCG is phosphorylated at mitosis (37). However, the specific phosphoamino acid identified in this study, serine 7, is apparently not responsible, because mutation of serine 7 does not knock out the mitotic isoforms. In addition, mutation of serine 7 does not knock out our ability to radiolabel FANCG. Nonetheless, by mutagenizing serine 7 we have shown that it is indeed important for physiologic FA function by demonstrating that mutant FANCG has an impaired ability to correct mutant cells. In addition, we have utilized another system to illustrate the importance of serine 7 in the FA pathway, using hamster cells mutant for FANCG. Human FANCG corrects the MMC hypersensitivity phenotype in mutant FANCG CHO cells, whereas the expression of S7A results in only partial correction. In contrast, the mouse sequence contains a proline at this position. Whereas the mouse fancg gene has been shown to correct human mutant FA-G cells (63), human FANCG cannot correct mouse fancg -/+ knockout cells.2 Thus, mouse and human FANCG are not simply interchangeable, and replacement of serine 7 with proline, as occurs in the mouse, does not result in MMC sensitivity correction.2 Interestingly, the FANCG(S7A) mutant is itself stable and appears to stabilize FANCA through their interaction. This phenomenon might explain at least in part the partial function that is seen in FANCG(S7A). The notion of partial function of FANCA and FANCC proteins has also been proposed (36, 64).

FA has been long classified an autosomal recessive disease, implying that physiologic mutant proteins are incapable of binding to FA proteins. Indeed this is likely given that most FA mutations appear to result in gross deletions or insertions with relatively low levels of protein expression. Curiously, the S7A mutant is expressed and participates in the FA complex. This ability to bind likely accounts for lack of complete MMC hypersensitivity in both hamster and human.

A most basic question remains: what role does the phosphorylation event play in cell metabolism and how is the phosphorylation stimulated? The mitotic isoforms are preserved in cells expressing the S7A mutant of FANCG. Because the mobility of the wild-type FANCG and the FANCG(S7A) is similar, it remains unclear what is the stimulation of the event. The production of a new reagent to detect serine 7 phosphorylation should be helpful to analyze the stimulus for this event.

2 G. M. Kupfer, unpublished data.

FIG. 8. FANCG(S7A) abnormally localizes in chromatin. Wild-type ECFP-FANCG and ECFP-FANCG(S7A) were transfected into FA-G mutant 326SV cells and subjected to analysis by fluorescent microscopy. A, both wild-type FANCG and FANCG(S7A) were seen in the nucleus and cytoplasm in whole cells. B, in situ chromatin and C, chromatin fiber preparations revealed that while wild-type FANCG localized in numerous, fine foci, FANCG(S7A) localized in qualitatively different, large globular structures. CFP, cyano fluorescent protein.
Given the continued lack of ascribed function for the FA pro-signal noted after MMC treatment (Fig. 4C in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased signal noted after MMC treatment (Fig. 4C) in our early use of this antibody are evident in the increased
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J. Biol. Chem. 2004, 279:46035-46045.
doi: 10.1074/jbc.M408323200 originally published online August 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408323200

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