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A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA

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ABSTRACT **Fanconi anemia (FA) is a recessively inherited disease characterized at the cellular level by spontaneous chromosomal instability and specific hypersensitivity to cross-linking agents. FA is genetically heterogeneous, comprising at least eight complementation groups (A-H). We report that the protein encoded by the gene mutated in complementation group G (FANCG) localizes to the cytoplasm and nucleus of the cell and assembles in a molecular complex with the FANCA protein, both *in vivo* and *in vitro*. Endogenous FANCA/FANCG complex was detected in both non-FA cells and in FA cells from groups D and E. By contrast, no complex was detected in specific cell lines belonging to groups A and G, whereas reduced levels were found in cells from groups B, C, F, and H. Wild-type levels of FANCA/FANCG complex were restored upon correction of the cellular phenotype by transfection or cell fusion experiments, suggesting that this complex is of functional significance in the FA pathway. These results indicate that the cellular FA phenotype can be connected to three biochemical subtypes based on the levels of FANCA/FANCG complex. Disruption of the complex may provide an experimental strategy for chemosensitization of neoplastic cells.**

The autosomal recessive disease Fanconi anemia (FA) is characterized by progressive bone marrow failure, hyperpigmentation of the skin, developmental abnormalities, and an increased cancer risk, particularly acute myeloid leukemia and squamous cell carcinoma (1, 2). Cells derived from FA patients have a highly consistent phenotype characterized by an increased level of spontaneous chromosomal damage and a specific hypersensitivity to bifunctional alkylating (cross-linking) agents, such as mitomycin C and diepoxybutane (1, 2). FA thus may be considered a model disease in which embryonic development, bone marrow function, and cancer predisposition are linked to a pathway that safeguards chromosomal integrity and causes resistance to cross-linking agents. FA is genetically heterogeneous, with at least eight complementation groups (A-H) identified by somatic cell fusion studies (3). Each of these groups probably represents a distinct disease gene (4). The genes mutated in patients of group FA-A (*FANCA*), FA-C (*FANCC*), and FA-G (*FANCG*) have been identified (5–8), whereas those for groups FA-D and FA-E have been mapped to the short arms of chromosomes 3 (9) and 6 (10), respectively.

Because FA patients from different complementation groups have similar clinical and cellular phenotypes, it is possible that the FA gene products participate in a common molecular pathway. Diverse hypotheses have been proposed, implicating FA proteins to function in DNA repair, detoxification, cell cycle regulation, and apoptosis (2, 11), but the primary molecular process defective in FA has remained unresolved. Intriguingly, the identified FA genes are novel and do not share significant homology to known genes or proteins. Therefore, clues about their function may come from studies designed to define the subcellular localization and the interrelationship of the various FA proteins and to identify interacting proteins with a known function. FANCC has been shown to localize predominantly to the cytoplasm in early studies (12, 13), but more recently this protein also has been found in the nuclear compartment (14, 15). Similarly, the first studies using green fluorescent protein (GFP)-tagged FANCA had shown this chimaeric protein to be localized predominantly to the cytoplasm (16), but more recent studies have consistently shown FANCA to be both cytoplasmic and nuclear (14, 17, 18). Indeed, nuclear localization appears to be essential for FANCA function because forced nuclear export using a nuclear export signal tag abolished the complementing activity in FA-A cells (17). A physical interaction between the FANCA and FANCC proteins has been described (14, 18, 19), but contradictory results also have been reported (17). FANCC also has been shown to interact with cytochrome P450 reductase, suggesting a role in a detoxification pathway (20).

The recent identification of the *FANCG* gene (8) led us to explore the subcellular localization of its encoded product and to document a direct physical interaction with FANCA.

MATERIALS AND METHODS

Plasmids. The vector pFAG9 (8) was used for construction of FANCG-GFP hybrid plasmids by subcloning PCR fragments into the vectors pEGFPN1 or pEGFPC1 (CLONTECH). Similarly, a PCR fragment from pFAG9 was subcloned downstream of a double hemagglutinin (HA) tag sequence into the episomal eukaryotic expression vectors pCEP4 and pMEP4 (Invitrogen). For bacterial expression, pGST-FANCG$_{480–622}$ was constructed by inserting a *Xho*I restriction fragment from pFAG9 into pGEX-KG (21). A *Hin*dIII fragment from pFAG9 was subcloned via pBluescript SK$^-$ (Stratagene) vector into pGEX-KG, resulting in pGST-FANCG$_{83–622}$. Sequences encoding FANCA with a carboxyl-terminal FLAG tag were generated by replacing GFP from the expression construct pDR/FANCA-GFP (16) by an 8-aa FLAG tag and inserting the chimaeric cDNA into expression

Abbreviations: FA, Fanconi anemia; GFP, green fluorescent protein; HA, hemagglutinin; GST, glutathione *S*-transferase; IP, immunoprecipitation.

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vectors pCEP4 and pMEP4 for transient and stable expression. FLAG-FANCC was made by replacing the region between the start methionine and the *Bgl*II site of FANCC (7) with oligonucleotides encoding a methionine residue, FLAG tag, and the first six aa of FANCC. The cDNA construct was cloned into the expression vector pMEP4 for stable expression. For bacterial expression, pGST-FANCA$_{1–271}$ was constructed by cloning a PCR fragment encoding the first 271 aa from FANCA (5) with an in-frame stop codon in the pGEX-KG vector. All constructs were sequenced by using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia) and an Amersham Pharmacia ALF express automatic DNA sequencer to ensure their fidelity.

Cell Culture and Transfection. FA patients were diagnosed on the basis of clinical symptoms in combination with a standard chromosomal breakage test showing hypersensitivity to cross-linking agents (2) before establishment of lymphoblastoid cell lines. Reference cell lines representing the eight known complementation groups and culture conditions have been described (3, 22). Lymphoblastoid cell lines from the following FA patients also were investigated: FA-A patients EUFA268 (23), BD32 (24), and EUFA689 (25); FA-C patients GM4510 (26) and EUFA166 (27); FA-D patients PD20 (9), EUFA202 (27), and EUFA423 (unpublished result); FA-E patients EUFA410 and EUFA622 (10), and FA-G patients EUFA316 and EUFA349 (8). For stable expression lymphoblastoid cell lines were transfected by electroporation (28). Mitomycin C-induced growth inhibition tests were performed as described (27, 29). 293-EBNA cells, HeLa cells, and COS-7 cells were transfected by using standard calcium phosphate precipitation. 293/HA-FANCG cells were cloned by limiting dilution after selection in hygromycin (100 μg/ml) to obtain stable cell lines expressing HA-FANCG, which was verified by Western blot analysis.

Immunofluorescence Microscopy. For indirect immunofluorescence, 293 cell derivatives were processed as described (15), by using anti-HA (Boehringer Mannheim) as a primary antibody and Oregon Green-conjugated goat anti-mouse (Molecular Probes) as a secondary antibody, and counter stained with 4′,6-diamidino-2-phenylindole. The stained slides were viewed with a Nikon E8000 at ×400 power.

Generation of FANCA and FANCG Antibodies. Polyclonal antibodies specific for FANCA and FANCG were generated by immunizing guinea pigs with glutathione *S*-transferase (GST) fusion proteins or rabbits with synthetic keyhole limpet hemocyanin (KLH)-conjugated peptides. GST-FANCA$_{1–271}$ and GST-FANCG$_{480–622}$ were expressed in *Escherichia coli* strain DH5α. After lysis in lysis buffer [50 mM Tris, pH 8/10% sucrose/1% Triton X-100/0.2% SDS/5 mM DTT/1 mg/ml protease inhibitor (Pefabloc, Boehringer Mannheim)] containing lysozyme (Sigma) and sonification, GST-FANCA$_{1–271}$ ($M_r \approx$ 60,000) and GST-FANCG$_{480–622}$ ($M_r \approx$ 45,000) were purified by binding to Glutathione Sepharose 4B (Amersham Pharmacia) and elution with 50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM reduced glutathione, and 0.1% Triton X-100. The purified proteins were injected into guinea pigs to obtain FANCA antiserum C2 and FANCG antiserum 12 (30). Rabbit polyclonal antiserum 89 directed against FANCA was generated by immunizing with a mixture of two synthetic KLH-conjugated peptides, CHQSSRSYDHSENSDL (amino acids 992-1006) and CSRQQAAPDADLSQEPHLF (amino acids 1436–1455) as described (31). GST-FANCG$_{83–622}$ fusion protein ($M_r \approx$ 90,000) was isolated as outlined above and injected in rabbits to obtain polyclonal antiserum 43. All antibodies were tested for their specificity in immunoprecipitation (IP) and immunoblotting on lysates from 293-EBNA cells overexpressing epitope-tagged proteins.

IP and Western Blotting. Total cell extracts were prepared by lysis in lysis buffer (10 mM Tris, pH 7.4/150 mM NaCl/1% NP40/0.5% sodium deoxycholate/1 mM EDTA/1 mM DTT/0.5 mg/ml Pefabloc). Lysates were sheared through a 19-gauge needle and clarified by centrifugation at 14,000 rpm in a microcentrifuge. The amount of protein per lysate was normalized to 1 mg (approximately 1×10^7 lymphoblastoid cells) for each IP, by using a Bio-Rad protein assay. Cytoplasmic and nuclear cell extracts were prepared by incubating 2×10^7 293-EBNA cells in hypotonic buffer (10 mM Tris, pH 7.4/1.5 mM $MgCl_2$/10 mMKCl/0.5 mM DTT/0.5 mg/ml Pefabloc) on ice. After 10 min NP-40 (0.1% final concentration) was added and nuclei were pelleted by 10-sec centrifugation at 5,500 × *g*. The cytoplasmic fraction was transferred to a new tube, and nuclei were washed three times with hypotonic buffer. Subsequently, the nuclei were lysed in lysis buffer. For IP cytoplasmic and nuclear extracts as well as total cell extracts were equalized to 50% lysis buffer and 50% hypotonic buffer. Fractionation was checked by Western blotting with mouse mAbs against β-tubulin (Boehringer Mannheim) and topoisomerase II-β (8F8, Ricotti, Pavia, Italy) as cytoplasmic and nuclear markers, respectively. Extracts were precleared with 5 μl of normal guinea pig serum and immunoprecipitated with 5 μl of FANCA or FANCG serum at 4°C. IPs using the HA and FLAG epitopes were performed on lysates of transiently transfected 293-EBNA cells with 1 μg of purified antibody, without preclearance. Antibody-bound proteins were collected with protein A agarose beads (GIBCO/BRL). The beads were washed three times with lysis buffer and suspended in SDS sample buffer. Samples were separated on 8% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore). Membranes were either incubated with rabbit FANCA antiserum 89 or rabbit FANCG antiserum 43 [1:500 diluted in 5% dry milk in TBST (10 mM Tris, pH 7.5/150 mM NaCl/0.05% Tween 20)], purified mouse monoclonal HA antibody 12CA5 (1 μg per 5 ml of TBST), or purified mouse monoclonal FLAG antibody M2 (Kodak, 3 μg per 5 ml of TBST). After washing with TBST, membranes were incubated with peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako) or peroxidase-conjugated goat anti-mouse immunoglobulins (Dako) diluted 1:5,000 in TBST. Membranes were washed and developed by using the ECL Western blotting analysis system (Amersham Pharmacia).

IP of *in Vitro*-Translated Proteins. *In vitro*-translated proteins were generated by using the TNT Coupled Reticulocyte Lysate Systems (Promega) according to the manufacturer's instructions. For IP, proteins were allowed to form complexes in 20 mM Tris·HCl, pH 8.0/50 mM NaCl/2 mM EDTA/0.1% NP40, supplemented with protease inhibitors, for 1 h at 4°C. Rabbit antiserum raised against the amino-terminal portion of FANCA (17) or 12CA5 was added and incubated for 1 h, followed by the addition of protein A/G agarose (Santa Cruz Biotechnology) and incubation for another hour at 4°C. Immunocomplexes were washed three times in NET-gel buffer (50 mM Tris·HCl, pH 7.5/50 mM NaCl/0.1% NP-40/0.2% gelatin) for 30 min at 4°C. Samples were subjected to SDS/PAGE, and gels were treated with glacial acetic acid and 20% 2,5-diphenyloxazole (Sigma) followed by drying and autoradiography.

RESULTS

Predominant Nuclear Localization of HA-Tagged FANCG Protein. To determine the subcellular localization of the FANCG protein, a construct expressing HA-tagged FANCG protein was generated and transfected into human embryonal kidney 293 cells. As shown in Fig. 1, stably expressed HA-FANCG predominantly localized to the nucleus, although variable staining also was seen in the cytoplasmic compartment. A very similar pattern was found by using direct fluorescence microscopy of 293-EBNA, COS-7, and HeLa cells transiently transfected with constructs expressing amino-terminal or carboxyl-terminal GFP-tagged FANCG (not

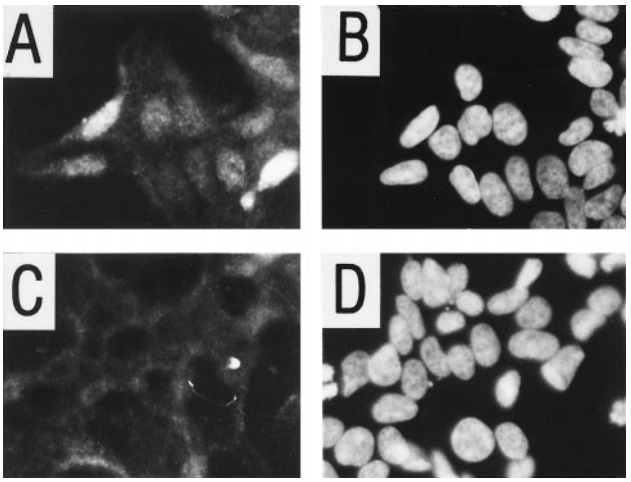

FIG. 1. Predominant nuclear localization of HA-tagged FANCG protein. Indirect immunofluorescence microscopy using HA mAb 12CA5 of human 293 cells stably transfected with HA-FANCG (*A*) or mock transfected (*C*). Nuclei of cells shown in *A* and *C* were stained with 4′,6-diamidino-2-phenylindole (*B* and *D*, respectively). Magnification: ×400.

shown). All tagged FANCG proteins fully complemented the mitomycin C-hypersensitive phenotype of lymphoblastoid FA-G cell lines (not shown).

Co-IP of Transiently Expressed FANCA and FANCG Proteins. Because the localization of FANCG resembles that of FANCA (17, 18), epitope-tagged versions of FANCA and FANCG were used to explore potential interaction between these proteins. Vectors encoding functionally active FANCA-FLAG and HA-FANCG were transiently transfected into 293-EBNA cells. IP of FANCA-FLAG (M_r = 160,000) from 293-EBNA cell lysates showed that HA-FANCG (M_r = 70,000) specifically coprecipitated with FANCA-FLAG (Fig. 2*A*). Reciprocal IP of HA-FANCG from similar lysates confirmed the interaction with FANCA-FLAG (Fig. 2*B*). These results show that transiently expressed FANCA and FANCG interact to form a complex.

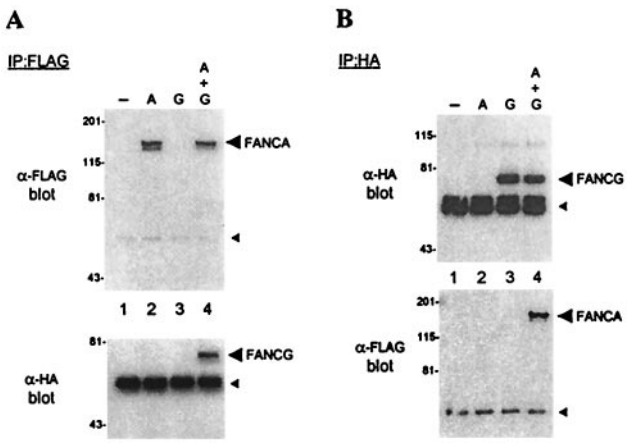

FIG. 2. Co-IP of transiently expressed FANCA and FANCG proteins. Protein from 293-EBNA cell extracts, mock transfected (lanes 1), or expressing FANCA-FLAG (lanes 2), HA-FANCG (lanes 3), or FANCA-FLAG + HA-FANCG (lanes 4) was (*A*) immunoprecipitated with monoclonal FLAG antibody (M2) and immunoblotted with anti-FLAG (*Upper*) or anti-HA (*Lower*) or (*B*) immunoprecipitated with monoclonal HA antibody (12CA5) and immunoblotted with anti-HA (*Upper*) or anti-FLAG (*Lower*). Large arrowheads indicate FANCA-FLAG or HA-FANCG, small arrowheads indicate Ig heavy chains.

Co-IP and Localization of Endogenous FANCA and FANCG Proteins in Wild-Type Human Cells. To determine whether endogenous FANCA binds to FANCG, total cell lysates from 293-EBNA cells and a control lymphoblastoid cell line were immunoprecipitated with anti-FANCG serum and immunoblotted with antiserum specific for FANCA. In the lysates from both cell types FANCA was coprecipitated with FANCG (Fig. 3*A*, lanes 1 and 3), and this coprecipitation was specifically abolished by preincubation with the cognate antigen (GST-$FANCG_{480–622}$) (Fig. 3*A*, lanes 2 and 4). These results demonstrate the presence of endogenous FANCA/FANCG complex in human fetal fibroblasts and lymphoblastoid cells.

The subcellular localization of endogenous FANCG was studied by IP from cytoplasmic and nuclear extracts of 293-EBNA cells. Subsequent immunoblotting revealed that FANCG was present in both compartments of these cells (Fig. 3*B*, *Top*). Similarly, FANCA was precipitated from both cytoplasmic and nuclear extracts as was the FANCA/FANCG complex (Fig. 3*B*, *Bottom* and *Middle*, respectively). FANCA appears as a double band, probably because of proteolytic breakdown. Although IP may increase the ability to detect cross-contamination between the subcellular fractions, it seems unlikely that the FANCA/FANCG complex found in the nuclear fraction is derived from cytoplasmic contamination, because less complex is detected in the cytoplasmic fraction.

FANCA/FANCG Complex Is Not Detected in FA-G and FA-A Cells Lacking Full-Length Protein. We then studied FANCA/FANCG binding in cell lines from FA-A and FA-G patients. In FA-G cell lines, EUFA143, EUFA316, and EUFA349, which lack immunoreactive FANCG (8), no FANCA protein was precipitated with anti-FANCG serum (Fig. 4*A*, lanes 2–4). The FANCA/FANCG interaction was restored in phenotypically corrected EUFA316 cells stably transfected with HA-FANCG (Fig. 4*A*, lane 5). After precipitation with anti-FANCA serum, less FANCA protein was detected in the FA-G cell lines compared with control cells and the corrected EUFA316(HA-FANCG) cells (Fig. 4*B*, *Upper*), suggesting that FANCG somehow contributes to a wild-type level of FANCA. In the latter precipitation HA-tagged FANCG was coprecipitated with FANCA (Fig. 4*B*, *Lower*).

As expected, no FANCA protein was detected upon precipitation of FANCG from the FA-A reference cell line HSC72 (Fig. 5*A*, *Upper*, lane 2), which lacks full-length FANCA protein (Fig. 5*A*, *Lower*, lane 2). Coprecipitation was restored in lysates from HSC72 cells stably transfected with FLAG-tagged FANCA (Fig. 5*A*, lane 3). The absence of wild-type FANCA protein in HSC72 results in a strong reduction of the FANCG protein as shown by FANCG precipitation and subsequent FANCG immunoblotting (Fig. 6*A*, *Lower*, lane 2).

Other FA-A cell lines with homozygous mutations in *FANCA* were studied to determine the effect of these mutations on the FANCA/FANCG interaction. Similar to HSC72, no FANCA was detected after precipitation of FANCG from cell line EUFA268 (Fig. 5*B*, lane 4), which carries a homozygous Q772X mutation in FANCA (23). Interestingly, FANCA proteins with a H1110P mutation (BD32; ref. 24) or with a deletion of phenylalanine 1263 (EUFA689; ref. 25) coprecipitated with FANCG, although less protein was detected in the complex compared with control cells (Fig. 5*B*). This finding indicates that these amino acids are not essential for FANCG binding.

Expression of FANCA/FANCG Complex in Different FA Subgroups. We investigated whether FANCA/FANCG binding was disturbed in cell lines from the remaining FA complementation groups. A reduced amount of FANCA was coprecipitated with FANCG in the reference cell line for complementation group C (HSC536) (Fig. 5*A*, lane 4), which was corrected to control levels after stable transfection of this cell line with FLAG-tagged FANCC (Fig. 5*A*, lane 5). Also, in two

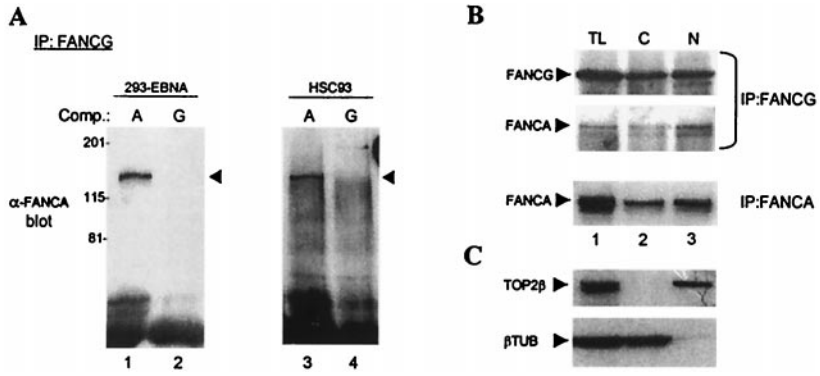

FIG. 3. Co-IP and localization of endogenous FANCA and FANCG proteins in wild-type human cells. (*A*) Cell lysates from control cell lines 293-EBNA and HSC93 were immunoprecipitated with anti-$FANCG_{480-622}$ and immunoblotted with rabbit FANCA antiserum 89. The amount of protein was normalized to 1 mg per IP. Anti-$FANCG_{480-622}$ was preincubated with control antigen GST-$FANCA_{1-271}$ (lanes 1 and 3) or cognate antigen GST-$FANCG_{480-622}$ (lanes 2 and 4). Arrowheads indicate FANCA. (*B*) Total cell extract (lanes 1), cytoplasmic extract (lanes 2), and nuclear extract (lanes 3) from 293-EBNA cells were immunoprecipitated with either anti-$FANCG_{480-622}$ or anti-$FANCA_{1-271}$ as indicated and immunoblotted with rabbit anti-FANCG (*Top*) or rabbit anti-FANCA (*Middle* and *Bottom*). (*C*) Extracts used in *B* were immunoblotted with mouse mAbs directed to topoisomerase II-β as a nuclear marker (*Upper*) or β-tubulin as a cytoplasmic marker (*Lower*).

other FA-C cell lines, less FANCA protein was coprecipitated with FANCG when compared with the control (Fig. 5*C*). Lower levels of FANCA/FANCG complex were observed consistently in FA-C cells.

We also studied the FANCA/FANCG interaction in cells from the FA-B, FA-D, FA-E, FA-F, and FA-H complementation groups. In lysates from the reference cell lines of complementation groups B (HSC230), F (EUFA121), and H (EUFA173), FANCA protein was found to be reproducibly ($n = 3$) reduced when coprecipitated with FANCG (Fig. 6*A*, *Upper*). Coprecipitated FANCA was restored to a level similar to controls in a functionally complemented HSC230x-EUFA173 fusion hybrid cell line (not shown). Immunoblotting with anti-FANCG as well as IP of FANCA suggested that the reduced FANCA/FANCG complex might correlate with a reduced amount of precipitated FANCA or FANCG protein in some of these cell lines (Fig. 6 *A*, *Lower* and *B*). By contrast, FANCA was efficiently coprecipitated with FANCG from the reference cell lines for complementation groups D (HSC62) and E (EUFA130), comparable to the control cell line HSC93 (Fig. 6*A*, *Upper*). To establish whether the cell lines HSC62 and EUFA130 are representative of their complementation groups, cell lines from three additional FA-D and two FA-E patients were studied and found to express wild-type levels of FANCA/FANCG complex (not shown). These results show that the FANCA/FANCG complex is disturbed in some but not all of the FA complementation groups.

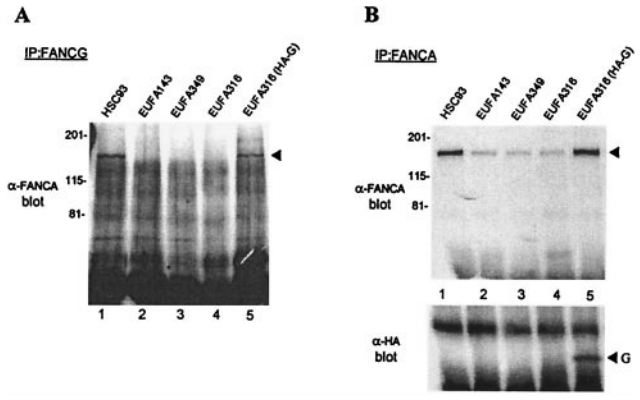

FIG. 4. FANCA/FANCG complex in FA-G cells. (*A*) Cell extracts (1 mg per IP) from control cell line HSC93 (lane 1), FA-G cell lines EUFA143 (lane 2), EUFA349 (lane 3), EUFA316 (lane 4), and FA-G cell line EUFA316 corrected with HA-FANCG in pMEP4 (lane 5) were immunoprecipitated with anti-$FANCG_{480-622}$ and immunoblotted with rabbit FANCA antiserum. (*B*) Similar lysates as in *A* were immunoprecipitated with anti-$FANCA_{1-271}$ and immunoblotted with rabbit anti-FANCA (*Upper*) or with anti-HA (*Lower*). Arrowheads indicate FANCA in *A* and *B* (*Upper*) and HA-FANCG in *B* (*Lower*). Mutations in FA-G lines are: EUFA143, homozygous E105X; EUFA316, frameshift at amino acid 395 and E105X; EUFA349, frameshift at amino acid 479 and E105X (8).

Co-IP of *in Vitro*-Translated FANCA and FANCG. The nature of the observed interaction between FANCA and FANCG was further explored by studying their ability to interact directly as *in vitro*-translated polypeptides. This study also allowed us to determine the region of FANCA that is required for interaction with FANCG, as an approach toward a detailed mapping of their mutual binding domains. HA-FANCG was cotranslated *in vitro* together with either full-length FANCA or a panel of carboxyl-terminal deletion constructs, in the presence of [^{35}S]methionine. HA-FANCG was found to coprecipitate with both full-length FANCA and all of the carboxyl terminal-truncated FANCA proteins tested, the smallest of which comprised the amino-terminal 300 residues of FANCA (Fig. 7*A*). In the reciprocal experiment, FANCA and truncated derivatives coprecipitated with HA-FANCG, further substantiating the interaction (Fig. 7*B*). As shown in Fig. 7*C*, an amino-terminal deletion mutant of FANCA containing residues 342-1455 failed to bind (HA−) FANCG. These findings indicate that the amino-terminal portion of FANCA is necessary and sufficient for direct binding to FANCG. This result is in agreement with the observation that

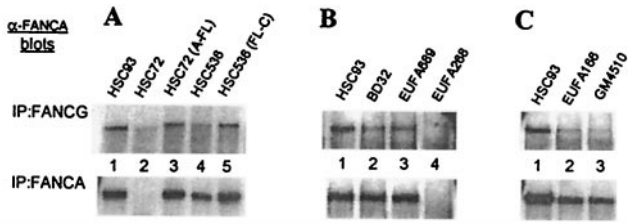

FIG. 5. FANCA/FANCG complex in FA-A and FA-C cells. Immunoblotting of anti-$FANCG_{480-622}$ (*Upper*) or anti-$FANCA_{1-271}$ (*Lower*) precipitated proteins with rabbit anti-FANCA. Cell extracts were prepared from HSC93 control cells (lanes 1), (*A*) HSC72 (lane 2), corrected HSC72(FANCA-FLAG) (lane 3), HSC536 (lane 4), and corrected HSC536(FLAG-FANCC) (lane 5). (*B*) Homozygously mutated FA-A cell lines; BD32 (H1110P) (lane 2), EUFA689 (1263delF) (lane 3), and EUFA268 (Q772X) (lane 4). (*C*) Homozygously mutated FA-C cell lines; EUFA166 (322delG) and GM4510 (IVS4), (lanes 2 and 3, respectively). In all precipitations 1 mg of protein was used.

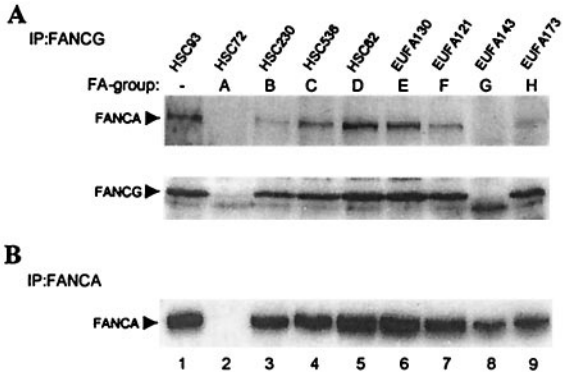

FIG. 6. FANCA/FANCG complex in cell lines representing the eight known complementation groups. (*A*) Total lysate (1 mg per IP) from the indicated cell lines was immunoprecipitated with anti-$FANCG_{480-622}$ and immunoblotted with either rabbit anti-FANCA (*Upper*) or rabbit anti-FANCG (*Lower*). (*B*) Similar aliquots as in *A* were immunoprecipitated with anti-$FANCA_{1-271}$ and immunoblotted with rabbit anti-FANCA.

the mutant FANCA proteins 1263delF and H1110P interact with FANCG (Fig. 5*B*).

DISCUSSION

This study describes subcellular localization of the recently identified FANCG protein as well as a direct interaction between FANCG and FANCA. Indirect immunofluorescence microscopy on human 293 cells stably transfected with functionally active HA-tagged FANCG revealed the protein to be present in both the cytoplasm and the nucleus. As described for FANCA, nuclear FANCG staining was the predominant pattern in the majority of the cells (17, 18). In addition, endogenous FANCG, FANCA, and FANCA/FANCG complex was precipitated from both cytoplasmic and nuclear extracts of 293 cells, although cross-contamination between the subcellular fractions cannot be totally excluded. The interaction of FANCA mutants H1110P and 1263delF with FANCG indicates that the FANCA/FANCG complex formation can take place in the cytoplasm, because these mutants were found defective in nuclear translocation (19, 24). Also, FANCA/FANCG complex was detected in cells from complementation groups (B, C, E, F, and H) in which FANCA previously has been shown to reside exclusively in the cytoplasmic compartment (19). Taken together, these data show that FANCA and FANCG interact in cytoplasm and nucleus and suggest that the location of the FANCA/FANCG complex in the nucleus is essential for a functional FA pathway. This conclusion is supported by the observation that forced localization of FANCA in the cytoplasm abolished its function (17).

Using polyclonal FANCG antibodies, we were able to coimmunoprecipitate FANCA from cell extracts of wild-type cells but not from FA-G cell lines or from FA-A cell lines lacking full-length FANCA protein. The coprecipitation of FANCA was abolished by preincubating anti-FANCG with cognate antigen, confirming that the coprecipitation depends on the precipitation of wild-type FANCG. A survey of cell lines representing the eight known FA complementation groups indicated that FANCA/FANCG complex is reduced in FA-C, FA-F, FA-B, and FA-H cells. An explanation for this observation may be that, although *in vitro*-translated FANCA and FANCG interact directly, the formation or stability of the complex depends on additional factors in living cells. For example, FANCB, FANCC, FANCF, and FANCH might regulate the expression of FANCA or FANCG, or they might be involved in the modification/stabilization of (proteins in) the complex. FANCA protein levels are greatly reduced in FA-G cell lines and are restored to control levels upon stable transfection of HA-FANCG. Similarly, FANCG protein was virtually undetectable in a FA-A cell line. This finding suggests that FANCG binding to FANCA may stabilize these proteins or a putative complex of these proteins and other FA proteins. Similar examples in which a defective or absent protein compromises the stability of an interacting protein have been reported, such as for ERCC1/XPF, Ku70/Ku80, and DNA ligase IV/XRCC4 (32–34). In contrast to the other complementation groups, in all of the FA-D and FA-E cells tested the amounts of FANCA/FANCG complex was very similar to that observed in control cells. Based on the biochemical phenotype of FANCA/FANCG complex levels, three subtypes can be distinguished in FA cells: normal levels (FA-D and FA-E), reduced levels (FA-B, FA-C, FA-F, and FA-H), and absent levels (FA-A and FA-G). However, the amount of complex might depend on the mutations present in specific cells, as observed in different FA-A cell lines. Therefore, identification of the unknown FA genes and more patients for FA-B, FA-F, and FA-H should reveal whether the reduced levels of complex in these cell lines are specific for these complementation groups.

The direct binding of FANCA and FANCG and the observation that in several FA complementation groups this interaction is disturbed demonstrates that at least a subset of FA proteins functions in the same molecular pathway. FA may, in that respect, resemble the autosomal recessive disease xeroderma pigmentosum, where patients carry a defect in one of at least seven genes, *XPA* through *XPG*, all leading to an

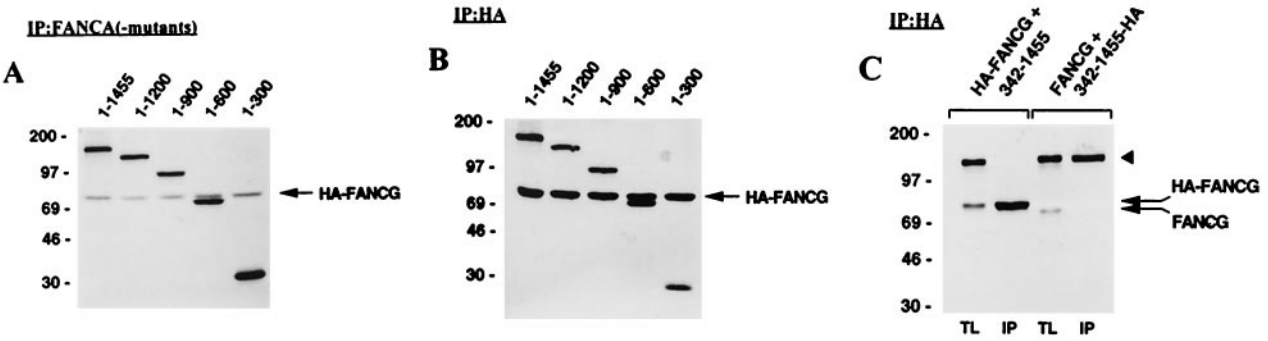

FIG. 7. Co-IP of *in vitro*-translated FANCA and FANCG. Proteins were cotranslated *in vitro* and labeled with [^{35}S]methionine. (*A*) Full-length FANCA (1–1455) and carboxyl terminal-truncated FANCA mutants (1–1200, 1–900, 1–600, and 1–300) were immunoprecipitated with a rabbit polyclonal antibody directed against the amino terminus of FANCA (17). After SDS/PAGE, coprecipitating HA-FANCG was visualized by exposing the dried gel to x-ray film. (*B*) HA-FANCG was immunoprecipitated with anti-HA to identify bound FANCA or carboxyl terminus-deleted forms of FANCA as described in *A*. (*C*) Complex formation was examined between HA-FANCG and FANCA 342-1455 (*Left*) or FANCG and FANCA 342-1455-HA (*Right*) after IP with anti-HA. Total lysate (TL) shows the presence of FANCG (arrows) and amino terminal-deleted FANCA (arrowhead). Immunoprecipitated products are shown in the lanes labeled IP.

extreme sensitivity to sunlight (UV) and skin abnormalities. Proteins defective in the various complementation groups are all components of the nucleotide excision repair machinery (35).

Even though the molecular role of the FA proteins remains to be established, the finding of a presumed functional complex and direct interaction between the FANCA and FANCG proteins may serve as an important foothold to further elucidate the FA pathway. Specifically, mapping of the exact binding domains in FANCA and FANCG may reveal structural clues about the nature of the interaction and its relationship to the function of other proteins participating in the FA pathway. In addition, the existence of a functional FA protein complex may have important implications for experimental systems that call for the introduction of a drug-sensitive cellular phenotype, such as cancer gene therapy. For example, overexpression of peptides that mimic either binding domain may block complex formation and cause a cell to become "FA-like," i.e., apoptosis-prone and hypersensitive to cross-linking chemotherapeutic agents, such as mitomycin C, cyclophosphamide, and cisplatinum.

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