The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG

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Received 29 June 2000; Revised and Accepted 31 August 2000

Fanconi anemia (FA) is a chromosomal instability syndrome associated with a strong predisposition to cancer, particularly acute myeloid leukemia and squamous cell carcinoma. At the cellular level, FA is characterized by spontaneous chromosomal breakage and a unique hypersensitivity to DNA cross-linking agents. Complementation analysis has indicated that at least seven distinct genes are involved in the pathogenesis of FA. Despite the identification of four of these genes (FANCA, FANCC, FANCF and FANCG), the nature of the ‘FA pathway’ has remained enigmatic, as the FA proteins lack sequence homologies or motifs that could point to a molecular function. To further define this pathway, we studied the subcellular localizations and mutual interactions of the FA proteins, including the recently identified FANCF protein, in human lymphoblasts. FANCF was found predominantly in the nucleus, where it complexes with FANCA, FANCC and FANCG. These interactions were detected in wild-type and FA-D lymphoblasts, but not in lymphoblasts of other FA complementation groups. This implies that each of the FA proteins, except FANCD, is required for these complexes to form. Similarly, we show that the interaction between FANCA and FANCC is restricted to wild-type and FA-D cells. Furthermore, we document the subcellular localization of FANCA and the FANCA/FANCG complex in all FA complementation groups. Our results, along with published data, culminate in a model in which a multi-protein FA complex serves a nuclear function to maintain genomic integrity.

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive disorder characterized by diverse clinical symptoms, including progressive bone marrow failure, developmental anomalies, growth retardation, hyperpigmentation of the skin and predisposition to cancer, particularly acute myeloid leukemia and squamous cell carcinoma (1). FA cells exhibit spontaneous chromosome breakage and are uniquely hypersensitive to DNA cross-linking agents such as mitomycin C (MMC), cisplatin and diepoxybutane. In addition, FA cells have an abnormal cell cycle profile, described as an elongation or arrest in the G2 phase and this abnormality is exacerbated by treatment with MMC (2,3). Because of associated cancer predisposition and genomic instability, FA has been classified along with xeroderma pigmentosum, Bloom syndrome, ataxia telangiectasia, Werner syndrome and hereditary nonpolyposis colorectal cancer as one of the ‘caretaker gene diseases’ (4), which are collectively thought to have a defect in the ‘handling’ of DNA damage. Intriguingly, FA is the only caretaker gene disease in which the defect has not been defined in molecular terms.

Complementation analysis using lymphoblastoid cell lines from FA patients has resulted in eight complementation groups (5), each of which was thought to be connected to a distinct disease gene. However, the reference cell line for complementation group H (EUFA173) has recently been reassigned to group A, reducing the number of FA complementation groups and suspected FA genes to seven (6). Four of these genes have been identified: FANCA, FANCC, FANCF and FANCG (7–11), whereas FANCD and FANCE have been mapped to chromosomes 3p25.3 and 6p21–22, respectively (12–14). The startling feature of the FA genes is that they all encode novel proteins whose amino acid sequences have failed to provide immediate clues for a molecular function. However, the similarity of clinical symptoms and cellular features found in FA patients representing the various complementation groups strongly suggests that FA proteins function in an integrated pathway.

The FA proteins FANCA, FANCC and FANCG have been shown to localize in both the cytoplasm and the nucleus of the cell (15–24). The nuclear localization of FANCA and FANCC is critical for function (17,20,21,25–28). Enforced transport of FANCA out of the nucleus with a heterologous nuclear export signal abolished its ability to correct the phenotype of FA-A cells (19). FANCA and FANCC are therefore thought to be involved in a nuclear process, which is strengthened by the recent observations that FANCA, FANCC and FANCG

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Human Molecular Genetics, 2000, Vol. 9, No. 18 2665–2674

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interact in a nuclear complex (17,22–24). On the other hand, forced nuclear localization of FANCC abolished its complementing activity (29), suggesting that FANCC may have temporal and spatial requirements for function, or may have a function in the cytoplasm as well. In this respect, a role for FANCC has been proposed in a cellular detoxification pathway, through binding to the cytosolic domain of NADPH cytochrome-P450 reductase (30).

The recent identification of a fourth FA gene, FANCF (11), has allowed a further characterization of the FA pathway. Here we examined the subcellular localization of the FANCF protein and its ability to interact with other FA proteins, in lymphoblasts representing all currently known FA complementation groups. We report that FANCF is located predominantly in the nucleus and interacts with the FA proteins FANCA, FANCC and FANCG. We also document the localization of FANCA and the FANCA–FANCG complex in all FA complementation groups and demonstrate the interaction between FANCA and FANCC. The results described in this paper culminate in a working model that accommodates all that is currently known on the interrelationship among the different FA proteins, their subcellular localizations and their engagement in a nuclear complex. The absence of this complex is correlated with the cellular FA phenotype, i.e. spontaneous chromosomal breakage and hypersensitivity to DNA crosslinking agents, indicating that an intact FA protein complex plays an essential role in the maintenance of genomic integrity.

RESULTS

FANCF is predominantly localized in the nucleus

To determine the subcellular localization of FANCF, a plasmid encoding a FANCF–green fluorescent protein (GFP) fusion protein was constructed and transiently transfected into HEK293 cells. Fluorescence microscopy of the transfected cells showed a predominantly nuclear FANCF–GFP staining (Fig. 1A). The FANCF–GFP protein complemented the MMC-hypersensitive phenotype of the FA-F lymphoblastoid cell line EUFA698 similar to wild-type FANCF (Fig. 1B) and had the expected molecular weight (Fig. 1C). The predominant nuclear localization of FANCF found by fluorescence microscopy was confirmed by immunoprecipitation of endogenous FANCF from fractionated wild-type lymphoblasts followed by immunoblotting with FANCF-specific antisera (Fig. 2A). To determine whether the presence of FANCF in the nucleus depends on wild-type levels of other FA proteins, we studied the subcellular localization of FANCF in several FA cell lines. FANCF was found predominantly in the nuclear fractions of lymphoblastoid cell lines representing all known FA complementation groups (Fig. 2A), as well as in fractionated BD32 (FA-A), EUFA178 (FA-B), GM4510 (FA-C), PD20 (FA-D) and EUFA410 (FA-E) (data not shown), suggesting that FANCF does not require other FA proteins for its accumulation in the nuclear compartment. The relatively small amount of FANCF protein detected in the cytoplasmic fractions might be authentic, or could result from minor cross-contamination between the subcellular fractions. In this respect, it should be noted that the molecular weight of FANCF allows diffusion of nuclear FANCF through the nuclear pores, during the fractionation procedure (31). Visualization of endogenous FANCF by immunofluorescence is needed to exactly quantify the amount of cytoplasmic FANCF. In any case, our results show that FANCF has a predominant nuclear localization, irrespective of the FA complementation group.

The subcellular distribution of FANCF is different from that of FANCA

To compare the subcellular localization of FANCF with the localization of FANCA, similar experiments were performed on fractionated lysates using FANCA-specific antisera. FANCA was clearly detected in both cytoplasmic and nuclear fractions (Fig. 2A). The proportion of cytoplasmic and nuclear FANCA varied among the different complementation groups. In wild-type and FA-D cells, relatively high proportions of FANCA were detected in nuclear fractions (Fig. 2A) whereas, in FA-B cells and FA-A cell line EUFA173, FANCA was mainly present in cytoplasmic fractions. Similar results were obtained in FA-D cell line PD20 and in FA-B cell line EUFA178 (data not shown). FANCA proteins encoded by genes containing a missense mutation (termed H1110P) or a microdeletion (1263delF) were also found predominantly in the cytoplasmic fractions (Fig. 2B), indicating that the C-terminal region contributes to the nuclear localization of FANCA. In FA-C, FA-E, FA-F and FA-G cells FANCA was distributed essentially evenly between cytoplasmic and nuclear fractions (Fig. 2A). The presence of FANCA in the nuclear fraction of FA-C cells contrasts with previous reports that postulated a role for FANCC in the nuclear accumulation of FANCA (17,20,25). Therefore, we studied the localization of FANCA in two additional FA-C cell lines. Interestingly, in these cell lines FANCA was also present in the nucleus (Fig. 2B). Thus, our data argue against the involvement of FANCC in the nuclear translocation of FANCA.

In summary, our results show that FANCF is always localized predominantly in the nucleus of the cell, whereas FANCA is present in varying amounts in the cytoplasm and nucleus. Moreover, the appearance of FANCF in the nucleus seems to be independent of other FA proteins, whereas the nuclear localization of FANCA requires the putative FANCB protein as well as a functional FANCA C-terminus.

FANCA and FANCF interact in vivo

A possible interaction between FANCA and FANCF was investigated by co-immunoprecipitation experiments. After immunoprecipitation of FANCA, with either a polyclonal antiserum against the FANCA N-terminus or a monoclonal antibody against the FANCA C-terminus, FANCF was detected in wild-type lymphoblasts, but not in FA-A or FA-F lymphoblasts (Fig. 3A). In the reciprocal experiment, FANCA was specifically co-precipitated with FANCF in wild-type lymphoblasts, but not in FA-F lymphoblasts, by using polyclonal antiserum directed against either the N- or C-terminus of FANCA (Fig. 3B). The interaction between FANCA and FANCF was also observed in FA-D lymphoblasts, but was absent, or strongly reduced, in lymphoblasts from FA complementation groups B, C, E and G (Fig. 3C), indicating that each of the corresponding FA proteins is required for the formation or stabilization of the FANCA–FANCF complex.

In FA-F lymphoblasts EUFA121 and EUFA698, which lack the FANCA–FANCF complex (Fig. 3), FANCA is equally
distributed over the cytoplasmic and nuclear compartments (Fig. 4A). Phenotypic correction of EUFA698 lymphoblasts specifically elevated nuclear FANCA to an amount comparable to wild-type, without affecting the cytoplasmic FANCA level (Fig. 4A). Expression of FANCF in EUFA698 also stabilized the interaction between FANCA and FANCG as shown by the co-precipitation of FANCA with FANCG from total cell extracts (Fig. 4B). These results suggest that the interaction between FANCA and FANCF is required for stabilization of FANCA in the nucleus, possibly by stabilizing the FANCA–FANCG complex.

**FANCF forms a complex with FANCG and FANCC**

The observed molecular complexes between FANCA and FANCF prompted us to investigate the interaction between FANCF and the other identified FA proteins, i.e. FANCG and FANCC, which have been found in a complex with FANCA (17,20,22–25). Co-immunoprecipitation experiments with FANCG- and FANCC-specific polyclonal antisera were performed on total cell lysates representing all known FA complementation groups. After FANCG immunoprecipitation, FANCF was detected in wild-type and FA-D lymphoblasts (Fig. 5A). Similarly, FANCF and FANCA were co-immunoprecipitated with FANCC in wild-type and FA-D lymphoblasts (Fig. 5B). The interactions among FANCA, FANCC and FANCF were consistently found in all the currently known FA-D cell lines (Fig. 5C). In EUFA698 (Fig. 5B) and other FA-F lymphoblastoid cell lines BD497, EUFA121, EUFA927 and EUFA1228 (data not shown), a weak FANCA–FANCC interaction was detected, suggesting that FANCF stabilizes the FANCA–FANCC complex. EUFA1228 carries a homozygous

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**Figure 1.** Functionally active FANCF–GFP is predominantly localized in the nucleus. (A) HEK293 cells were transiently transfected with expression vector pMEP4 encoding FANCF–GFP (upper panels) or with expression vector pEGFP-N1 encoding GFP (lower panels). Fluorescence microscopy of the transfected cells at 400× magnification shows GFP staining in left panels. The same cells were stained with DNA-specific dye DAPI to show the nuclei (right panels). (B) Mitomycin C (MMC) hypersensitivity of FA-F lymphoblastoid cell line EUFA698 is corrected after stable transfection with FANCF cDNA clone 3 (11) or expression vector pMEP4 encoding FANCF–GFP. Lymphoblastoid cell line HSC93 is shown as a wild-type control [mean IC₅₀ ± SD for wild-type lymphoblasts is 40 ± 18 nM (n = 13)]. (C) FANCF protein expression in the stably transfected lymphoblastoid cell lines used in the MMC test. FANCF is visualized by immunoprecipitation of cell lysates (prepared in lysis buffer 1) with guinea pig antiserum against GST–FANCF244–374 followed by immunoblotting with rabbit antiserum against GST–FANCF1–374. Molecular weight markers are indicated. Ig, immunoglobulin heavy chain.
8 bp deletion in the FANCF gene, resulting in a frameshift at codon 296 (G296V + 10 amino acids). This cell line expresses a 35 kDa truncated FANCF protein, which does not interact with FANCA (data not shown), indicating that the C-terminus of FANCF is involved in this interaction. Taken together, these results show that FANCG and FANCC form a complex with FANCF in wild-type and FA-D lymphoblasts, but not in other FA complementation groups.

Subcellular localization of FA protein complexes

Immunoprecipitations on cytoplasmic and nuclear fractions of wild-type and FA lymphoblasts were performed to determine the subcellular localization of the FA protein complexes. We found that, although FANCA was precipitated from both cytoplasmic and nuclear fractions of wild-type lymphoblastoid cells, FANCF and FANCG were co-precipitated with FANCA predominantly from the nuclear fraction (Fig. 6A). Similarly, the majority of the FANCA–FANCF–FANCG complex was detected in the nuclear fractions after FANCG immunoprecipitation, whereas FANCG was present in both nuclear and cytoplasmic fractions (Fig. 6A). FANCA–FANCG complexes were also precipitated from nuclear fractions of FA-C, FA-D, FA-E and FA-F lymphoblasts, but were barely detectable in nuclear fractions of FA-B and EUFA173 cells (Fig. 6B). Addi-
tionally, FANCA–FANCC–FANCF complex was found in the nucleus of wild-type lymphoblasts (HSC93) and FA-F lymphoblasts (EUFA698 and EUFA121) were immunoprecipitated with anti-FANCA 1–271 and FANCA was visualized by immunoblotting with anti-FANCA serum 89. EUFA698 was complemented by stable transfection of functionally active FANCF–flag (EUFA698 + FANCF). Topoisomerase II β (TOP2β) and β-tubulin (βTUB) were used as nuclear and cytoplasmic markers, respectively. Equal amounts of protein from total cell extracts (prepared in lysis buffer 1) of wild-type lymphoblasts (HSC93) and FA-F lymphoblasts (EUFA698 and EUFA121) were immunoprecipitated with anti-FANCG 40–622. FANCA and FANCG were visualized by immunoblotting with anti-FANCA serum 89 and anti-FANCG 40–622, respectively. FANCG is represented by the upper band of the doublet. EUFA698 was complemented by stable transfection of FANCF–flag (EUFA698 + FANCF).

**In vitro translated FANCF does not interact with FANCA, FANCC or FANCG**

The in vivo interactions between FANCF and the other FA proteins were further explored by studying their ability to interact directly as in vitro translated proteins. FANCF was serially co-translated with epitope-tagged FANCA, FANCC or FANCG, all shown previously to be functionally active in complementation assays. Complexes were immunoprecipitated with specific antisera described for the in vivo interaction studies. When FANCF and each of the other FA proteins were present in the lysate, the FANCF antibody precipitated only FANCF (Fig. 7). Similarly, antibodies specific for FANCA, FANCC or FANCG precipitated only their cognate proteins, without co-precipitating FANCF (Fig. 7). The experimental conditions used were identical to those used to demonstrate a direct interaction between FANCA and FANCG (23,24).
These data imply that the interactions between FANCF and FANCA, FANCC and FANCG are indirect and/or depend on modification(s) or interaction(s) with other proteins. In vitro translation experiments with different combinations of FA proteins are necessary to further explore these possibilities.

**DISCUSSION**

The present study provides new information about the interactions and subcellular compartmentalization of all currently known FA proteins. We show that the recently identified FANCF protein has a predominant nuclear localization and that it forms nuclear complexes with the FA proteins FANCA, FANCC and FANCG. The absence of these complexes in all currently known FA complementation groups except group D strongly suggests that assembly of the complex is a sequential process that involves most of the other FA proteins. The notion that FA proteins collaborate in a novel pathway that maintains genomic integrity is supported by our findings that FA protein complexes are found mainly in the nucleus and that these complexes are absent or reduced in most FA complementation groups.

The predominantly nuclear localization of FANCF was demonstrated by fluorescence microscopy of HEK293 cells expressing functionally active FANCF–GFP and confirmed by immunoprecipitation of endogenous FANCF from fractionated lymphoblasts. The localization of FANCF was found to be predominantly nuclear in all FA complementation groups, indicating that the nuclear localization of FANCF occurs independently of other known FA proteins. Since FANCF lacks a recognizable nuclear localization signal, the nuclear translocation mechanism for FANCF is presently unclear and might depend on other proteins that are still to be discovered.

In comparison with FANCF, FANCA was clearly detected in both the cytoplasmic and nuclear fractions of asynchronous cells, whereas its distribution varied among different FA complementation group cell lines. In wild-type and FA-D cells, more FANCA was reproducibly detected in nuclear fractions than in cytoplasmic fractions, whereas both in FA-B cells and in FA-A cells expressing inactivated FANCA proteins with alterations in the C-terminal region [missense mutations (R951Q and H1110P) or microdeletion (1263delF)], FANCA was found predominantly in the cytoplasm. These results confirm previous reports (25,27) and are consistent with the notion that the FA protein defective in FA-B cells is involved in the nuclear accumulation of FANCA. An intact FANCA C-terminus is apparently also required for its proper subcellular compartmentalization. One possible interpretation is that FANCB regulates the phosphorylation of FANCA, consistent with the observations that (i) phosphorylated FANCA has been
detected in both wild-type and FA-D lymphoblasts, but not in FA-B cells (25); and (ii) the FANCA mutant H1110P is not phosphorylated and fails to accumulate in the nucleus (27,32). Whether a putative kinase is inactive in FA-B cells remains to be established. Modifications of FANCA such as phosphorylation might change its conformation, which could result in the exposure of the FANCA N-terminus, which has been shown to be important for the nuclear localization of FANCA (20,21) and binding of FANCG (22–24).

In contrast to previous reports (17,20,25), we detected FANCA in the nucleus of FA-C, FA-E, FA-F and FA-G cells. In the previous studies, it was suggested that FANCC is essential for the nuclear accumulation of FANCA and that retention of FANCA in the cytoplasm of FA-C, FA-E, FA-F and FA-G cells was secondary to a failure in FANCA–FANCC complex formation. However, in our hands FANCA appeared in the nuclear compartment of FA-C cell lines containing different inactivating mutations in the FANCC gene, which argues against a role for FANCC in the nuclear accumulation or transport of FANCA. Visualization of FANCA by immunoblotting might be more difficult in these cell lines, whereas differences in fractionation protocols could account for the apparent discrepancy between our results and those previously reported. To make our results interpretable, fractionation experiments were carefully controlled by using widely accepted cytoplasmic (β-tubulin) and nuclear (topoisomerase II-β) markers and by using equal amounts of cytoplasmic and nuclear protein in our immunoprecipitations. We recognize that immunoprecipitation increases the sensitivity of the assay and might detect minor cross-contamination between the subcellular fractions. However, we were also able to detect FANCA in the nuclear fractions of FA-C, FA-E, FA-F and FA-G cells by direct immunoblotting with the FANCA-specific monoclonal antibody 5G9 (J.P. de Winter, unpublished data).

Several studies have shown evidence for the formation of molecular complexes among FANCA, FANCC and FANCG (17,20,22–25), but the interaction between FANCA and FANCC has been controversial (19). Here, we confirm the existence of a FANCA–FANCC complex in wild-type and FA-D lymphoblasts and extend previous observations by demonstrating interactions of FANCA, FANCC and FANCG with FANCF. These interactions were predominantly, if not exclusively, found in the nuclear compartment of wild-type lymphoblastoid cells as well as in total cell lysates of lymphoblasts from FA-D patients. It is tempting to speculate from these results that the FA proteins function as a single nuclear multi-protein complex, but such a complex still needs to be formally demonstrated, for example by co-migration in sucrose gradients or gel filtration. The absence of this FA protein complex in cells of most FA complementation groups predicts that the complex assembles by a sequential process in which several of the FA proteins are involved. This hypothesis would explain why FANCF does not interact with FANCA, FANCC and FANCG when co-translated in vitro. Similarly, the interaction of FANCC with FANCA and FANCG was not found with in vitro translated products (22). These interactions clearly differ from the interaction between FA and FANCG, because FANCA–FANCG complexes are found, at variable levels, in most FA complementation groups and can be observed with in vitro translated proteins (22–24). This implies that the FANCA–FANCC interaction is an early event in the FA pathway that precedes other essential interactions. In FA-F cells, reduced levels of FANCA–FANCG and FANCA–FANCG complexes were found, suggesting that binding of FANCF is required for stabilization of these complexes. In the absence of FANCF, nuclear FANCA levels were also reduced, which indicates that the stabilization of FANCA-containing complexes is essential for stabilization or retention of FANCA in the nuclear compartment. Since FANCF is localized predominantly in the nucleus of the cell, FANCF might have a role in the assembly of a nuclear multiprotein complex.

Based on the results presented in this and previous studies, we propose a working model that accommodates all currently known features of the FA proteins (Fig. 8). FANCA and FANCG are localized in the cytoplasm of the cell and can interact directly, which requires the N-terminus of FANCA and the C-terminus of FANCG (22–24). This interaction stabilizes FANCA and FANCG protein levels (22,24) and is indispensable for complex formation among the other FA proteins, as all known FA protein complexes are lacking in FA-A and FA-G cells. The interaction between FANCA and FANCG is apparently not essential for nuclear localization of FANCA, since in FA-G cells FANCA is detected in the nucleus. In the absence of functionally active FANCB (in FA-B cells) or when the C-terminus of FANCA carries an inactivating mutation, the FANCA–FANCG complex is unstable (22,24) and is retained in the cytoplasm. We infer that modification of the FANCA C-terminus by the putative FANCB protein initiates the nuclear translocation of the FANCA–FANCG complex and allows interaction with cytoplasmic FANCC, which stabilizes this complex. In the absence of full-length FANCC (in the FANCC IVS4+4A→T and 322delG mutants) the FANCA–FANCG complex is unstable (24), but still capable of entering the nucleus. FANCC mutant L554P might have a low affinity for the FANCA–FANCG complex, enough to partially stabilize

**Figure 7.** Immunoprecipitation of _in vitro_ translated FA proteins. FANCF was co-translated and [35S]methionine-labeled _in vitro_ with functionally active FANCA–FLAG, FLAG–FANCC or HA–FANCG, using reticulocyte lysates. Radiolabeled proteins were immunoprecipitated with guinea pig anti-FANCA271 (IP A), anti-FANCC105 (IP C), anti-FANCG480–622 (IP G) or anti-FANCA–FLAG, FLAG–FANCC or HA–FANCG, using reticulocyte lysates. After SDS–PAGE, precipitated proteins were visualized by exposing the dried gel to X-ray film. Total lysate (TL) shows the presence of the co-translated FA proteins. Immunoprecipitated products are shown in the lanes labeled with IP.
The only direct binding, i.e. confirmed with interaction mediated by the putative B protein (step 2), which allows interaction with C (step 3) and subsequent accumulation of the complex in the nucleus (step 4). Binding of C is not essential for nuclear entry of the A–G complex (dotted arrow), although its presence appears to have a stabilizing effect on the complex as a whole, possibly by allowing interaction with other FA proteins. In the nucleus, E may have a role in the stabilization of the A–C–G complex and the recruitment of F (step 5). The putative D protein is inferred to act downstream of the other FA gene products, predicting a nuclear localization. D presumably interacts with the depicted complex (step 6) to result in a complex that acts to maintain genomic stability. The presence of the FA proteins in cytoplasm and/or nucleus is mainly based on information obtained from EBV-immortalized B lymphoblasts. E and F are found almost exclusively in the nucleus, whereas A, C and G are also found in the cytoplasm. Even though F has no nuclear localization signal (NLS), this protein is nuclear in all complementation groups, so the only direct binding, i.e. confirmed with interaction with other FA proteins does not occur in FA-B cells, the putative B protein is presumed to be important for this modification. The only direct binding, i.e. confirmed with interaction with other FA proteins is between A and G; all other tested interactions (A–C, G–C, A–F, C–F and G–F) are indirect.

Figure 8. Model for the sequential interactions and subcellular localizations of FA proteins. Letters indicate the seven different FA proteins, five of which (A, C, E–G) have been identified. The model involves six steps culminating in a single nuclear complex that incorporates A, C, E, F and G, although the existence of several smaller complexes is not excluded. The first step is the formation of an A–G complex from minor fractions of cytoplasmic A and G (step 1). This complex is stabilized by a modification of A, presumably a phosphorylation event mediated by the putative B protein (step 2), which allows interaction with C (step 3) and subsequent accumulation of the complex in the nucleus (step 4). The presence of the FA proteins in cytoplasm and/or nucleus is mainly based on information obtained from EBV-immortalized B lymphoblasts. E and F are found almost exclusively in the nucleus, whereas A, C and G are also found in the cytoplasm. Even though F has no nuclear localization signal (NLS), this protein is nuclear in all complementation groups, so the only direct binding, i.e. confirmed with interaction with other FA proteins does not occur in FA-B cells, the putative B protein is presumed to be important for this modification. The only direct binding, i.e. confirmed with interaction with other FA proteins is between A and G; all other tested interactions (A–C, G–C, A–F, C–F and G–F) are indirect.

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In summary, our results suggest that the FA proteins function as a multiprotein nuclear complex that assembles as a result of sequential interactions. Although the exact nature of the process driven by this complex is still unknown, it is inferred to be essential for chromosomal stability and the prevention of carcinogenic DNA alterations. Resolving its function awaits identification of other cellular components that participate in or interact with this complex.

MATERIALS AND METHODS

Patients and cell lines

Patients were diagnosed on the basis of clinical symptoms in combination with a standard chromosomal breakage test showing hypersensitivity to cross-linking agents (1). With informed consent from the patients and/or their families, blood samples were collected for establishment of lymphoblastoid cell lines. The following FA patients were investigated: FA-A patients BD32 (homozygous for H1110P) and EUFA689 (homozygous for 1263delF), FA-B patient EUFA178, FA-C patients GM4510 (homozygous for IVS4+4A→T), EUFA158 and EUFA166 (both homozygous for 322delG), FA-D patients PD20, EUFA008 and EUFA423, FA-E patient EUFA410 and FA-F patients BD497 (homozygous for 484–485del), EUFA698 (homozygous for 230–252del), EUFA927 (homozygous for Y109X) and EUFA1228 (homozygous for 887–894del). Reference cell lines representing the currently known complementation groups are: HSC72 (FA-A; homozygous for an in-frame deletion of exons 18–28), HSC230 (FA-B), HSC536 (FA-C; heterozygous for L554P and a deletion of exons 1 and 2), HSC62 (FA-D), EUFA130 (FA-E), EUFA121 (FA-F; heterozygous for Q6X and 349–393del) and EUFA143 (FA-G; homozygous for E105X). The reference cell line for complementation group H (EUFA173) has recently been reassigned to group A with a deletion of exons 17–31 and a missense mutation R951Q in FANCA (6). HSC93 lymphoblasts were from a healthy control individual. MMC-induced growth inhibition tests were carried out as described (5,34).

Plasmid constructs

For the generation of FANCF antibodies several GST fusion proteins were constructed by subcloning fragments from FANCF cDNA clone 10 (11) in the prokaryotic expression vector pGEX-KG (35). Constructs expressing GST–FANCF1–374 and GST–FANCF1–387 were made by subcloning BamHI–EcoRI and BamHI–XhoI restriction fragments from clone 10 directly into pGEX-KG. To obtain a construct expressing GST–FANCF1–374 and EcoRI–BamHI fragment from clone 10 was
first subcloned in pBluescript SK-1, digested with EcoRV and XbaI and then subcloned in the Smal and XbaI sites of pGEX-KG. Constructs encoding FANCF–GFP and FANCF–FLAG chimeric proteins were generated by replacing the stop codon in clone 10 by a HindIII site using synthetic oligonucleotides. The modified cDNA was then subcloned into the eukaryotic expression vector pEGFP-N1 (Clontech, Palo Alto, CA) to obtain a construct encoding FANCF–GFP or into pCEP4 (Invitrogen, Carlsbad, CA), which contains the FLAG epitope and a stop. For functional analysis after stable transfection, FANCF–GFP was subcloned in expression vector pMEP4 (Invitrogen). Constructs expressing functionally active FA NCA–FLAG, FLAG–FANCC and HA–FANCG have been described (24).

Generation of specific antibodies

Polyclonal antisera against either FANCF N- or C-terminus were obtained by immunizing guinea pigs with GST–FANCF1–245 or GST–FANCF244–374, as described (24). The fusion proteins were expressed in Escherichia coli strain BL21 at 30°C and purified from bacterial lysates (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1.5% sarkosyl, 4% Triton X-100, 50 mM dithiothreitol (DTT), protease inhibitors [Pefabloc (1 mg/ml; Boehringer, Mannheim, Germany), leupeptin (2.5 µg/ml), pepstatin (2.5 µg/ml) and apro tinin (2.5 µg/ml)] and lysozyme (10 mg/ml), by binding to glutathione–Sepharose 4B (Pharmacia, Uppsala, Sweden). Fusion proteins were eluted with 75 mM HEPES pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% Triton X-100 and 20 mM reduced glutathione. Rabbit polyclonal antisera against FANCF was generated by immunizing rabbits with purified GST–FANCF1–374, as described (24). All antibodies were tested for their specificity by immunoprecipitation and immunoblotting using lysates from 293-EBNA cells overexpressing FANCF–FLAG. Guinea pig polyclonal antisera against FANCC6–105 were generated by immunizing guinea pigs with GST–FANCC, which was purified as described (18). Monoclonal FANCA antibody 5G9 was raised against a KLH-coupled synthetic peptide (KLH–C–srqaapdadlsqephlf) representing amino acids 1436–1455 of human FANCA by standard methods (36). Guinea pig polyclonal antisera against GST–FANCC6–105 and rabbit polyclonal antisera against FANCA C-terminus (antisera 89) and GST–FANCC6–105 have been described (24).

Cellular fractionation, immunoprecipitation and immunoblotting

Cytoplasmic and nuclear extracts were prepared from 2 × 10^7 exponentially growing lymphoblasts, as described (24). Total cell extracts were made by lysis of 2 × 10^7 exponentially growing lymphoblasts in 1 ml of lysis buffer 1 (100 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM DTT and 0.5 mg/ml Pefabloc) or in 1 ml of lysis buffer 2 (50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet NP40, 0.5 mg/ml Pefabloc, 1 mg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin) as described (24). For immunoprecipitation experiments aliquots of extract were used containing equal amounts of protein, as determined with a Bio–Rad (Hercules, CA) protein assay. Immunoblotting was performed as described (24).

Immunoprecipitation of in vitro translated proteins

In vitro translated proteins were generated from cDNA constructs encoding FANCA–flag, flag–FANCC, HA–FANCG or FANCF in expression vector pcDNA3 (Invitrogen) using the TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI) according to the manufacturer’s instructions. For immunoprecipitation, proteins were allowed to form complexes in IP buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.1% Nonidet NP40, supplemented with protease inhibitors) for 1 h at 4°C. Guinea pig antiserum raised against FANCA1–271, FANCC6–105, FANCF244–374 or FANCG480–622 was added and incubated for 1 h. Subsequently, antibody-bound proteins were collected by the addition of Protein A agarose (Life Technologies, Rockville, MD) and incubation for 1 h at 4°C. Immunocomplexes were washed three times in IP buffer for 30 min at 4°C. Samples were subjected to SDS–PAGE and gels were treated with 30% methanol/7% acetic acid (v/v) and Amplify fluorographic reagent (Amersham, Little Chalfont, UK) followed by drying and autoradiography.

ACKNOWLEDGEMENTS

We thank N. Cool, F.M. di Summa, I. Grujis, M. Levitus, A.B. Oostra, M.A. Rooimans, J. Steltenpool and C.G.M. van Berkel for contributing to the complementation studies and C.G. Mathew, M. Gro mpe and M. Buchwald for cell lines BD32, BD497, PD20 and HSC cell lines. We also thank Tanja Reuter and Kirsten Silvey for stimulating discussions. Financial support was from the Dutch Cancer Society (VU-97-1565), the Fanconi Anemia Research Fund (Eugene, OR), the FA patient support organizations in Germany, France, Italy and The Netherlands, the Commission of the European Union (contracts PL931562 and BMH4-98-3784) and the National Institutes of Health (HL56045).

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