Fanconi anemia (FA) is an autosomal recessive disease marked by congenital defects, bone marrow failure, and cancer susceptibility. FA cells exhibit a characteristic hypersensitivity to DNA crosslinking agents such as mitomycin C. The molecular mechanism for the disease remains elusive, but at least 6 FA proteins are known to be part of what is termed the FA core complex. We used affinity pulldown of FLAG-FANCA to pull down the FA complex from whole-cell extracts. Mass spectrometry detected previously reported FA-binding proteins, including FANCA, FANCC, FANCG, cdc2, and GRP94, thus validating the approach. We further describe a method of purification of the FA core complex in an effort to find novel complex components and biochemical activity to define the function of the complex. By using conventional chromatographic fractionation of subcellular preparations, we report: (i) the FA core complex exists in a cytoplasmic form at 500–600 kDa; (ii) a larger, 750-kDa cytoplasmic form is seen only at mitosis; (iii) a nuclear form achieves a size of 2 megaDaltons; and (iv) a distinct 1-megaDalton FA core complex exists bound to chromatin that contains phosphorylated FANCA after undergoing DNA damage. We are continuing our analysis using mass spectrometry in an effort to characterize novel binding proteins. These data will help define the biochemical role of the FA core complex in normal cell physiology as well as in the development of the FA disease state.

Received for publication, January 6, 2004, and in revised form, March 18, 2004
Published, JBC Papers in Press, April 13, 2004, DOI 10.1074/jbc.M400091200

Andrei Thomashevskis, Anthony A. Highs, Mary Drozdís, Jeffrey Shabanowitzís, Donald F. Hunt§§, Patrick A. Grant¶¶, and Gary M. Kupfer†††††† From the Departments of §Microbiology, ¶¶Pediatrics, ‡Pathology, and |Biochemistry and Molecular Genetics, University of Virginia Health System, and ‡Department of Chemistry, University of Virginia, Charlottesville, Virginia 22908

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 25, Issue of June 18, pp. 26201–26209, 2004

This paper is available on line at http://www.jbc.org

Fanconi anemia (FA) is a genetic disease of cancer susceptibility marked by congenital defects, bone marrow failure, and myeloid leukemia (1–5). To date, at least 11 complementation groups have been defined (6–8), and eight genes have been cloned (9–18). However, none of the gene products resemble any known proteins, and all have few identifiable functional protein motifs.

Cells derived from patients with the disease exhibit characteristic hypersensitivity caused by DNA crosslinking agents and generalized decreased survival (19–23). In addition, a well described G2-phase cell-cycle delay has also been described that is thought to be secondary to a defective S or G2 checkpoint (24–26). Others have implicated cytokine signaling, apoptotic and oxidative damage defects (19, 27–29). However, no defined biochemical mechanism for crosslinker hypersensitivity has been elucidated. Patient and cellular phenotypes across all of the complementation groups are similar, suggesting an interrelatedness 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 (30–32). Subsequent work has found the FANCE, FANCF, FANCG, and FANCL proteins in the complex as well (33–38). Although reports describing binding partners to the FA core complex, including GRP94, cdc2, STAT1, and FAZF, have been published, little progress has been made in providing a unifying theory of FA protein function (27, 39, 40).

The core complex of FA described above fails to form in all of the complementation groups except FA-D2. The FANCD2 protein is ubiquitinated in response to DNA damage and in S-phase, which is dependent upon the existence of the FA core complex. In addition, FANCD2 binds to BRCA1, and FANCD1 is BRCA2, providing links to better described pathways of DNA repair and genome surveillance, including homologous recombination (17, 41).

FANCD2 ubiquitination is not the only modification that has been shown to be functionally important. Several reports have shown that FANCA phosphorylation is vital to FA pathway function and is missing in all but FA-D1 and FA-D2 cells (32, 42, 43).

A recent study details the purification of a BLM-containing FA core complex, using FANCA antibody, in which several sizes of complex were noted in a whole-cell extract (44). Heli-case activity was also coprecipitated. This approach yielded the latest cloned FA gene, FANCL (18). The coprecipitation of FA proteins with another genomic instability syndrome protein, BLM, puts FA at the nexus of DNA repair syndromes.

In this study, we attempt to validate a more conventional approach in concert with modern methods to purify the FA core complex. We use a series of chromatographic methods coupled with an immunoaffinity step to characterize the FA core complex with respect to size and composition. In doing so, we show a means to identify complex components that not only can shed light upon normal FA function but also has the potential to demonstrate biochemical activity. We also demonstrate that the FA core complex is not simply one complex; it is actually composed of at least four distinct complexes, depending upon its subcellular localization. Purification also allows us to isolate a phosphorylated form of FANCA, which is DNA-damage inducible.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Box 441 Jordan Hall, University of Virginia, Charlottesville, VA 22908. Tel.: 434-243-6289; Fax: 434-982-1071; E-mail: gbba@virginia.edu.
‡ The abbreviations used are: FA, Fanconi anemia; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MMC, mitomycin C; TBS, Tris-buffered saline; MDa, megaDaltons.
MATERIALS AND METHODS

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. Whereas FA-A mutant GM6914 cells were grown in 15% fetal bovine serum. Each cell line was transduced with pMMP-FLAG-FANCA or pMMP vector alone, and the resulting cells were selected with puromycin. Cells were treated for 24 h in 0.1 μM mitomycin C (MMC), a dose producing a 90% kill in mutant cells and a 10% kill in wild-type cells.

 Supernatant from GM6914 cells was clarified by spinning at high speed. The nuclear pellet was collected at 14,000 rpm in a microfuge for 10 min was termed the chromatin fraction. Supernatant was dialyzed, reduced, and subjected to protease inhibitors (1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate).

Chromatin Preparation—Procedures for permeabilization and sequential subnuclear extraction were adapted from the methods of Bertaina et al. (45) and were detailed in our previous work (48). In brief, cells pelleted (100 g) from one large plate were resuspended and permeabilized in 5 ml of low salt buffer (10 mM Heps, pH 7.4, 10 mM KCl, 50 mM NaCl, 1% Triton X-100), protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate).

Cell Fractionation—Cells were collected similarly and resuspended in 10 mM Heps, pH 7.4, 40 mM KCl, or 2 mM MgCl. After 20–30 strokes on ice with a Dounce homogenizer, the cytoplasmic extract was separated from the nuclei by spinning at 1500 rpm. The cytoplasmic extract was clarified by spinning at high speed. The nuclear pellet was further extracted in the lysis buffer using 500 mM NaCl as above.

Chromatin Preparation—Procedures for permeabilization and sequential subnuclear extraction were adapted from the methods of Bertaina et al. (45) and were detailed in our previous work (48). In brief, cells pelleted (100 g) from one large plate were resuspended and permeabilized in 5 ml of low salt buffer (10 mM Heps, pH 7.4, 10 mM KCl, 50 mM NaCl, 1% Triton X-100), protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4) for 15 min at 4 °C. After washing, the nuclei were resuspended in 200 μl of permeabilization buffer containing 30 units of DNase I (RNase Free, Roche Applied Science) for 15 min at room temperature and an additional 15 min at 37 °C. Chromatin proteins were extracted by adding extraction buffer (1% Triton X-100, 50 mM Heps, pH 7.4, 150 mM NaCl, 30 mM sodium phosphate, 10 mM NiCl, and 1 mM EDTA) containing protease and phosphatase inhibitors for 10 min at 4 °C. Supernatant collected at 14,000 rpm in a microfuge for 10 min was termed the chromatin fraction.

Affinity Purification of the FA Complex—To demonstrate the validity of purification of epitope-tagged FA complex, we performed batch FLAG-affinity precipitation of FLAG-FANCA from GM6914 + FLAG-FANCA and GM6914 + vector control cells in whole-cell extract (500 mM NaCl). Immunoprecipitation was performed with covalently bound anti-FLAG-affinity resin. After extensive washing, the precipitates were eluted with FLAG peptide, and the resulting eluates were concentrated and buffer-replaced with 100 mM NH4HCO3. Thereafter, these eluates were analyzed by tandem mass time-of-flight ion-spray MS. Peptide sequence was detected by correlating to the FA proteins FANCA, FANCC, and FANCNG (Fig. 1A). Additionally, cdc2 and GRP94, which are both known and previously reported binding proteins of FANCA (Fig. 1B; Refs. 40, 47), were detected.

Whole-cell Extract Contains Two Different FA Complexes—We and others have demonstrated that the FA complexes exist in both the cytoplasm and nucleus. However, it is unclear which function the FA complexes play and what the complex composition is in each compartment. Thus, isolation and purification of the FA complexes would serve to define them to determine potential functions. To ascertain characteristics of FA protein purification, we prepared whole-cell lysate in 150 mM NaCl from GM6914 + FANCA cells and applied it to a gravity anion-exchange column. Immunoblotting for FANCA revealed that most protein was bound and eluted in the 0.3 and 0.6 M fractions (data not shown). These fractions were pooled, rediluted to 150 mM NaCl, and run over an anion-exchange (MonoQ, Amersham Biosciences) column by HPLC. Proteins were eluted from the column using a salt gradient. Subsequent immuno-
blotting of fractions revealed two distinct peaks containing FANCA: one eluted with low salt and the other eluted with high salt (Fig. 2A).

Fractions from these two separate peaks were pooled separately and passed over a gel-filtration column (Superose 6, Amersham Biosciences). Immunoblotting for FANCA revealed that the low salt fractions corresponded to a FANCA-containing complex of 600 kDa, whereas the high salt fractions corresponded to a FANCA-containing complex of 2 MDa (Fig. 2B), as compared with a set of molecular mass standards.

**Stepwise Purification of FANCA-containing Complexes**

Published data from this lab and others (30, 48, 51) have revealed that FA proteins are contained in both nucleus and cytoplasm. Reasoning that these cellular compartments might contain differently sized and differently composed FA complexes, we performed a stepwise scheme of purification, as detailed in the scheme in Fig. 3. First, we fractionated FA-A mutant GM6914/H11001 FLAG-FANCA or GM6914/H11001 vector cells (negative control), either untreated or treated with 0.1 mM MMC into cytoplasmic and nuclear extracts. In all subsequent figures, the vector control was conducted in parallel but is not shown except for the final affinity step. Because of the significant precipitation of proteins in nuclear extracts made with high salt and subsequently lowered to low salt (data not shown), we decided to make nuclear extracts at 500 mM NaCl while maintaining the cytoplasmic extract at 50 mM salt, as called for during the simple fractionation procedure. Second, we passed the extract (20 mg) from the cytoplasmic fraction over a gravity anion-exchange column and eluted with a step gradient of NaCl. FANCA was detected predominantly in the 0.3 and 0.6 M NaCl step elutions (Fig. 4A). These fractions were then diluted back to 100 mM, passed over an anion-exchange column, and eluted using a 100–500 mM NaCl gradient. Immunoblotting showed FANCA in lower salt fractions, which is consistent with the data in Fig. 2 (data not shown). These FANCA fractions were then concentrated and loaded on a gel filtration column. Immunoblotting revealed FANCA in fractions corresponding to a predicted size of ~600 kDa (Fig. 4B), which is

**Fig. 1.** *Affinity purification of the FA complex.* A, whole-cell extract was prepared from FA-A mutant cells corrected with FLAG-FANCA. After immunoprecipitation with anti-FLAG-affinity gel, the FA complexes were eluted with FLAG peptide, and the resulting solution was digested with trypsin and analyzed by MS. Peptides from FANCA, FANCG, and FANCC were detected. B, peptides from previously described binding proteins cdc2, GRP94, and HSP70 were also detected.
consistent with the finding of a complex at that size in the whole-cell extract in Fig. 2. Overall, we noted that less FANCA was present in the cytoplasmic extract of MMC-treated cells. Total protein was 200 μg, a 100-fold decrease of the starting material.

Similarly, we assayed the nuclear extract, starting with 20 mg of protein. First, the nuclei made in the fractionation were extracted in 500 mM NaCl and passed through a P11 column, where FANCA was predominantly detected in the flow-through (Fig. 5A). The flow-through was loaded directly on a gel filtration column after concentration. Immunoblotting revealed FANCA in a 2-MDa complex (Fig. 5B), indicating that the second peak in the analysis of whole-cell extracts in Fig. 2, B and C likely corresponds to a nuclear form of the FA complex.

Previous work in our lab with different tagged FANCA and FANCG constructs co-expressed in FANCA- and FANCG-null mutant cells, respectively, revealed that homodimerization of FANCA or FANCG is unlikely (data not shown). These data indicate that the FA nuclear complex consists of additional proteins added after presumed transport from the cytoplasm to the nucleus. To further purify the complex, we pooled the FANCA fractions and passed them over a heparin column. The FANCA fractions in extract from MMC-treated cells were detected in flow-through and early in the elution (data not shown) but did not bind at all in the untreated group. Total protein at this step was ~200 μg as well, denoting a 100-fold purification.

Fig. 2. Whole-cell extract contains two different FA complexes. A, whole-cell extract was prepared from HeLa cells in 500 mM NaCl and successively passed over gravity anion-exchange and gradient anion-exchange columns. B, two complexes were noted; each was then subjected to gel filtration. Each step was followed by FANCA immunoblotting. Immunoblotting revealed the presence of 500- to 600-kDa and 2-MDa complexes containing FANCA. wce, whole-cell extract.

Fig. 3. A scheme of FA core complex purification. Details of sequential purification of cytoplasmic, nuclear, and chromatin extracts are given. Each step was monitored by FANCA immunoblotting. M, molar.
Fanconi Anemia Core Complex

FIG. 4. Step-wise purification of cytoplasmic FA core complex. FA-A mutant cells infected with vector control or FLAG-FANCA were collected after treatment with no or 0.1 μM MMC. Cells were fractionated into cytoplasmic and nuclear extracts. Cytoplasmic extract was passed over gravity anion-exchange and gradient anion-exchange columns (A), and a gel filtration column (B). Immunoblotting with FANCA antiserum revealed the presence of FA complex at 500–600 kDa, with diminished amounts of complex after MMC treatment. M, molar; i, input; FT, flow-through; nc, crude nuclear extract; pc, positive control (whole-cell extract from FA-A mutant + FLAG-FANCA); pc, negative control (FA-A mutant + vector).

Fig. 5. Stepwise purification of nuclear FA core complex. Nuclear extracts were passed over P11 column (A), gel filtration and heparin-Sepharose column (B). Immunoblotting with FANCA antiserum revealed a 2-MDa FA complex associated with increased amounts of complex after MMC treatment. M, molar; i, input; FT, flow-through; w, wash; nc, negative control; pc, positive control.

Interestingly, none of the FANCA from non-drug-treated cells appeared to bind to the heparin column at all. Overall, we detected increased FANCA-containing complex in nuclear extract from MMC-treated cells.

Chromatin Extract Contains a 1-MDa Form of the FA Complex—We have previously reported that a subset of the FA proteins resides in a protein fraction liberated by DNase treatment and is thus termed chromatin extract (48). We also have reported that increased FA proteins localize to chromatin after MMC treatment (48). To characterize the FA complex residing on chromatin, we first extracted chromatin by DNase treatment of FA-A mutant GM6914 + FLAG-FANCA or + vector cells with or without MMC treatment after permeabilization and removal of cytoplasmic and soluble nuclear proteins. We used approximately the same number of cells as in the production of the nuclear and cytoplasmic extracts above. The resulting extract consisted of 5 mg of protein in 200 mM salt. As in the previous sections, we passed the chromatin extract successively over a gravity anion-exchange column (Fig. 6A), an anion-exchange chromatography column (data not shown), and gel filtration (Fig. 6B), detecting the FA complex by FANCA immunoblotting. The chromatin FA complex was detected as a 1-MDa complex, a form smaller than that seen in nuclear extract but larger than the cytoplasmic complex. A much more pronounced amount of FA proteins was seen throughout chromatin fraction chromatography on extract from cells treated with 0.1 μM MMC, which is consistent with our published data demonstrating greater chromatin localization upon DNA damage (48). Interestingly, a higher mobility form of FANCA was noted only in the fractions from chromatin made from MMC-treated cells. The higher molecular mass forms of the complex in fractions 12–14 did not contain this isoform, suggesting they may be part of the higher molecular mass nuclear complex.

Cytoplasmic Extract from Mitotic Cells Contains both Small and Large FA Complex—We have previously shown that the FA complex cannot be detected in a nuclear extract at mitosis and that the FANCG protein, although still part of the complex, becomes phosphorylated (48). To define the complex in which phosphorylated FANCG resides, we purified the FA complex as above from cytoplasmic extract made from HeLa + FLAG-FANCG or + vector cells arrested at mitosis after a 16-h incubation in 1 μM nocodazole versus that made from an asynchronous cell population. All purification steps were followed by FANCA immunoblotting. The mitotic extract displayed a similar behavior to asynchronous extract over gravity (Fig. 7A) and column anion-exchange chromatography (data not shown). These FANCA fractions were then passed over a gel filtration column. The cytoplasmic FA core complex in HeLa cells was the same as in the FA-A-corrected cells in Fig. 4 (GM6914 + FLAG-FANCA). Compared with cytoplasmic extract from asynchronous cells displaying the 600-kDa form of the FA complex, we observed a form existing in higher molecular mass fractions in the mitotic extract corresponding to 750 kDa (Fig. 7B). The higher mobility forms of FANCG seen in mitosis were also apparent (see Fig. 8D), confirming that the complex is intact at the time of egress from chromatin and suggesting that the
larger complex in the mitotic cytoplasmic extract consists of additional proteins.

Affinity Purification and Silver-staining of FA Complexes—
Based on our experience with this scheme of chromatography, three column purification steps still result in complex mixtures. To further purify the different preparations, we incubated pooled fractions from chromatographic preparations in the earlier sections containing the FA proteins with anti-FLAG-affinity gel. The result of this immunoprecipitation was run by SDS-PAGE for immunoblotting. In Fig. 8, A–D are shown cytoplasmic, nuclear, chromatin, and mitotic preparations, respectively. All four compartments contained FA core complex consisting of at least FANCC, FANC, and FANCG coprecipitating with FLAG-FANCA expressed from pMMP-FLAG-FANCA. In the cytoplasmic preparation, a clear decrease in FA core complex was seen after MMC treatment (Fig. 8A). However, no FANCE coprecipitation was detected in the cytoplasm. In contrast, a clear increase in magnitude was evident in the nuclear fraction of MMC-treated cells (Fig. 8B), and FANCE coprecipitated with FLAG-FANCA. Consistent with these data, the chromatin pulldown also displayed increased FA core complex (Fig. 8C), which is in agreement with our published data (48). FANCC and FANCE coprecipitation with FLAG-FANCA in chromatin is shown in Fig. 9. A comparison of mitotic versus asynchronous extracts shows that the complex was intact at the time of egress from the nucleus at mitosis (Fig. 8D). Again, FANCE is not detectable in either the mitotic or asynchronous FA cytoplasmic core complex. FANCE is presented separately for clarity, as it tends to be difficult to resolve from FANCG on SDS-PAGE. Overall, these data are consistent with the idea that an inducible shift occurs from cytoplasm to nucleus after DNA damage and back again after mitosis. Although we have not shown if the increase in nuclear/chromatin FA core complex levels represents active nuclear import or increased nuclear/diminished cytoplasmic FA protein stability, the net result is increased nuclear/chromatin FA proteins.

To see whether additional proteins become part of the FA core complex after MMC treatment, we ran chromat-in-immunoprecipitated proteins from the affinity step above on SDS-PAGE and silver-stained the resulting gel. All proteins in the complex were markedly higher in magnitude after MMC treatment (marked by an *), and several additional binding proteins can be seen (Fig. 8E, ➔). FA-A mutant GM6914 cells both untreated and treated with MMC were included as negative controls and run in parallel. Any band from both the negative controls and in the FLAG-FANCA-containing cells was considered nonspecific. Immunoblotting on a parallel gel revealed the relative positions of the coprecipitating FA proteins, as indicated.

Protein extracts were made from ~20 plates of cells. Nuclear and cytoplasmic extracts each totaled 20 mg, whereas chromatin extracts totaled ~7.5 mg. Purification by chromatography resulted in a 100-fold diminution in protein amount for all three types of extracts. Affinity-purification resulted in another 2-fold purification, resulting in an ~200-fold overall.

**FANCA Is a Chromatin-bound Phosphoprotein in Response to MMC Treatment**—FANCA immunoprecipitation of chromatin extract from MMC-treated cells detected a higher mobility FANCA form (Fig. 6B). This result was confirmed as an isoform of FANCA, after immunoprecipitation with anti-FLAG-affinity gel and FANCA immunoblotting (Fig. 8C). The two FANCA isoforms are not due to dimerization of FANCA, because the mutant FA-A cell line GM6914 used in this experiment contained no endogenous FANCA; thus, the only FANCA species was FLAG-FANCA. Past reports have shown that FANCA is phosphorylated and that mutant cells display a lack of FANCA phosphorylation (32, 49). To demonstrate that FANCA is a phosphoprotein, we performed phosphatase reactions with immunoprecipitated FANCA from chromatin extract made from MMC-treated cells (as in the previous section). Both λ and PP2A phosphatase reactions caused the high mobility form of FANCA, which was inhibited by phosphatase inhibitors (Fig. 9), to disappear. Incubation in reaction buffer at 30 °C had no effect upon the two FANCA isoforms. The phosphatase reaction did not affect the coprecipitation of either FANCC or FANCE.

**DISCUSSION**

In this paper we describe the purification of the FA core complex in its four forms: asynchronous cytoplasmic, mitotic, chromatin, and nuclear. In addition, we show evidence to confirm our earlier findings that increased FA core complex is present in chromatin after MMC treatment and that it displays egress from the nucleus at mitosis. These data suggest that the complex is translocated into the nucleus where it becomes much larger and presumably becomes activated for a particular biochemical function. MMC treatment results in a number of changes in the FA complex, including a higher mobility FANCA form on chromatin, an increase in the amount of nuclear complex, and a decrease in the amount of cytoplasmic complex. These data are summarized in Table I.

FANCA has been reported to be phosphorylated in a functionally important manner (49). However, an additional iso-

**Fig. 7.** Stepwise purification mitotic FA complexes. HeLa cells with or without FLAG-FANCA were subjected to no or 1 μM nocodazole treatment to arrest cells in mitosis. Cytoplasmic extracts were prepared and subjected to stepwise purification over gravity anion-exchange and gradient anion-exchange columns (A), and a gel filtration column (B). Immunoblotting with FANCA and FANCG antisera revealed a larger FA complex in the cytoplasm of mitotic cells, along with additional FANCG isoforms. M, molar; i, input; FT, flow-through.
FIG. 8. FLAG-affinity pulldown of chromatography-purified FA complexes. Fractions containing FANCA from the stepwise purification of cytoplasm, nuclear, chromatin, and mitotic extracts were pooled and subjected to affinity purification using FLAG-affinity gel to immunoprecipitate FLAG-FANCA. The pulldowns were all immunoblotted subsequently with antisera against FANCA, FANCC, FANCE, FANCF, and FANCG. Cytoplasm (A), nuclear (B), chromatin (C), and mitotic preparations (D) all showed the presence of FA proteins. Anti-FANCE and anti-FANCC blots are shown in Fig. 9 for chromatin. Cytoplasm preparations displayed diminished FA core complex after MMC and no FANCE treatment, whereas nuclear and chromatin preparations showed an increased FANCE coprecipitation. FANCE blots are shown separately in B and D because FANCE tends to run close to FANCG. E, purified chromatin extracts from FA-A mutant cells + vector or + FLAG-FANCA with or without MMC treatment were subjected to anti-FLAG-affinity gel pulldown. Silver-staining of the resulting SDS-PAGE showed multiple specific coprecipitating proteins (*) in increased amounts; →, proteins seen only in MMC-treated cells. Immunoblotting on a similar preparation revealed the likely FANCA, FANCC, FANCE, FANCF, and FANCG positions on the gel. WCE, whole-cell extract; pc, positive control (FA-A mutant GM6914 + FLAG-FANCA); nc, negative control (FA-A mutant GM6914 + vector); mit, mitotic; asy, asynchronous.
Form has not been noted, and a cytoplasmic kinase has been implicated (42, 43). We did not see the higher mobility isoform of FANCA unless we performed chromatography. Even then, the phosphorylated isoform was noted only in chromatin preparations after DNA damage. This finding does not rule out that FANCA is also phosphorylated in the cytoplasm, producing an isoform with identical mobility to unphosphorylated FANCA.

Importantly, we have described a rational approach for purification of the native complex and its binding partners that will allow identification of proteins and functional motifs and which can subsequently be tested biochemically using these extracts. This approach has already shown promise, as we have confirmed the ability to detect the pulldown protein and the known FA-binding proteins (FANCA, FANCC, FANCG, cdc2, and GRP94) by tandem mass time-of-flight ion-spray MS (27, 39, 40). We have also found by MS at least two novel binding proteins with obvious functional motifs, the binding of which we are currently confirming by immunoprecipitation, immunoblotting experiments, and biochemical testing. In addition, we show that cytoplasmic extract displays less FA protein after MMC treatment, an observation we believe is consistent with our data showing localization of FA core complex in the cytoplasm only in early G1 phase, followed by exclusive nuclear localization from the border between G1-S until mitosis. This would explain the increase in chromatin binding seen after MMC treatment, whereby more cells are arrested during S or G2/M phase of the cell cycle. In fact, because MMC treatment will induce an increase in S and G2 percentages even in wild-type cells, the possibility exists that the primary effect on the FA core complex is upon the cell cycle. We are currently investigating this possibility.

To date, eight FA genes have been cloned, but their gene products have yielded relatively little information. Advances have been made in the recent elucidation that FANCD2 binds to BRCA1 and that BRCA2 equals FANCD1 (17, 41, 50). Also, the FANCD2 protein has been shown to be ubiquitinated in a manner that is in part dependent upon the wild-type presence of the FA core complex. It is the FA core complex about which little is known. In fact, none of the members of the core complex (FANCA, FANCC, FANCF, FANCG, FANCE, FANCL) has lower eukaryotic homologs, and few functional motifs exist, although FANCL has apparent ubiquitin ligase activity (18). Therefore, it is critical that all complex components be identified, as these may have functional motifs whose biochemical activities can be tested in the setting of the FA core complex. Thus, methods to purify the FA complex are crucial for identifying these proteins as well as providing material for testing function. A recent report by Wang and co-workers (44) details binding of the BLM helicase to the core complex in a 2-MDa complex, as well as the existence of two smaller FANCA-containing complexes. This work suggests the presence of both large and small complexes in a 200 m NaCl-extracted nuclear extract. Our data both confirms the report of Wang and co-workers and separates the complexes: the small form is cytoplasmic, the large form is nuclear, and the intermediate form binds to chromatin. On the other hand, using our conditions, we were unable to detect BLM in our preparations.

The cytoplasmic form of the FA core complex is likely to be critical, as past work shows that forced, exclusive expression of FANCC in the nucleus of mutant FA-C cells fails to correct the cells to wild-type MMC sensitivity (51). In addition, the 1-MDa chromatin complex, which is localized to chromatin in a DNA damage inducible fashion, is only found when extracted using DNase (48). Finally, a fourth unique complex is found at mitotic egress from condensed chromosomes. These findings are suggestive of a very complicated FA pathway, with roles and signaling in cytoplasm, soluble nuclear, and chromatin compartments. The FA core complex seems to be tied closely to cell-cycle regulation.

As presented in this paper, we have performed simple affinity purification and have indeed identified several known specific binding proteins by MS. It is important to establish con-

![Fig. 9. FANCA is a chromatin-bound phosphoprotein in response to MMC treatment. Beads from FLAG-affinity pulldown performed on purified chromatin were incubated in λ or PP2A phosphatase reactions with or without phosphatase inhibitors (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate). Phosphatase removed the higher FANCA isoform and diminished the overall FANCA protein level. No higher isoform was detectable on crude chromatin extract that was not purified by chromatography. FANCC and FANCE coprecipitation was unaffected by phosphatase reaction. PC, positive control (FA-A mutant GM6914 cells + FLAG-FANCA); NC, negative control (FA-A mutant GM6914 cells + vector); WCE, whole-cell extract.](image)

| Compartment                  | Size          | FA protein components |
|------------------------------|---------------|-----------------------|
| Cytoplasm, asynchronous      | 600 kDa       | A, C, F, G            |
| + MMC                        | Decreased protein | A, C, F, phospho-G    |
| Cytoplasm, mitotic           | 750 kDa       | A, C, E, F, G         |
| Nucleus                      | 2 MDa         | A, C, E, F, G         |
| + MMC                        | Increased protein | A, C, E, F, G         |
| Chromatin                    | 1 MDa         | A, C, E, F, G         |
| + MMC                        | Increased protein | phospho-A             |

2 G. M. Kupfer, unpublished data.

3 J. Mi, submitted for publication.
