A deficiency in a 230 kDa DNA repair protein in Fanconi anemia complementation group A cells is corrected by the FANCA cDNA

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Introduction

Fanconi anemia (FA) is a recessively transmitted genetic disorder characterized by bone marrow failure, diverse congenital abnormalities and a markedly increased incidence of acute myelogenous leukemia, numerous types of carcinomas and other malignancies (1–3). Cells from FA patients are hypersensitive to DNA interstrand cross-linking agents and this hypersensitivity correlates with a defect in ability to repair this type of damage to their DNA. We have isolated a DNA endonuclease complex from the nuclei of normal human cells which is involved in repair of DNA interstrand cross-links and have shown that in FA complementation group A (FA-A) cells there is a defect in ability of this complex to incise DNA containing interstrand cross-links. In order to identify the specific protein(s) in this complex which is defective in FA-A cells, monoclonal antibodies (mAbs) were developed against proteins in the normal complex. One of these mAbs, which is against a protein with a molecular weight of ~230 kDa, completely inhibited the ability of the normal complex to incise cross-linked DNA. Western blot analysis has shown that there is a deficiency in this protein in FA-A cells. Electrophoretic analysis has also indicated that there are reduced levels of this protein in FA-A compared with normal cells. Studies carried out utilizing FA-A cells which have been stably transduced with a retroviral vector expressing the FANCA cDNA have shown that the DNA repair defect in these cells has been corrected; levels of unscheduled DNA synthesis are at least as great as those of normal human cells. In addition, in the transduced cells the deficiency in the 230 kDa protein has been corrected, as determined by both western blot and electrophoretic analysis. These results indicate that the FANCA gene plays a role in the expression or stability of the 230 kDa protein.

Abbreviations: BCIP-NBT, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium; CS, Cockayne syndrome; FA, Fanconi anemia; FA-A, Fanconi anemia complementation group A; mAb, monoclonal antibody; MMC, mitomycin C; 8-MOP, 8-methoxypsoralen; NER, nucleotide excision repair; PBS, phosphate-buffered saline; TMP, 4,5’,8-trimethylpsoralen; UDS, unscheduled DNA synthesis.

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by western blot analysis. The results show that a monoclonal antibody (mAb) has been produced against an ~230 kDa protein in normal cells which is deficient in FA-A cells. In addition, this deficiency is corrected in FA-A cells stably transduced with a retroviral vector expressing FANCA cDNA, indicating a role for the FANCA gene in the expression or stability of the 230 kDa protein.

**Materials and methods**

**Extraction of DNA endonuclease complexes**

Normal human (GM 1989 and GM 3299) lymphoblastoid cell lines were obtained from the Coriell Institute for Medical Research (Camden, NJ). FA-A (HSC 72 and HSC 99) lymphoblastoid cell lines were a gift from Dr. Manuel Buchwald. FA-A lymphoblastoid cells (HSC 72) were stably transduced with the pG1 retroviral vector expressing FANCA cDNA (HSC72-17) (41). The transduced FA-A cells were obtained following selection in MMC (41). All cell lines were grown in suspension culture in RPMI 1640 medium as previously described and routinely checked for mycoplasma using an American Type Culture Collection PCR-based mycoplasm detection kit (22). HeLa cells were obtained from Cellex Biosciences (Minneapolis, MN). Cell nuclei were isolated and the chromatin-associated proteins were extracted from them in a series of steps as previously described (22,39). DNA endonuclease complexes with specificity for different types of DNA damage were separated from the chromatin-associated proteins on an isoelectric focusing column and dialyzed against 50 mM potassium phosphate, pH 7.1, 1 mM β-mercaptoethanol, 1 mM EDTA, 0.25 mM phenylmethylsulfonylfluoride and 40% ethylene glycol as previously described (22,39,40).

The activity of one of these endonuclease complexes, pI 4.6, which we have previously shown to have specificity for DNA interstrand cross-links, was examined using as substrate closed, circular PM2 DNA (Boehringer Mannheim, Indianapolis, IN) and a gel electrophoretic assay which measures the number of endonuclease-mediated incisions per DNA molecule (22). Interstrand cross-links were introduced into the DNA by reacting the DNA with 8-MOP (Sigma Chemical Co., St Louis, MO) and two doses of UVA light so as to produce mainly interstrand cross-links (22,39).

**Production and isolation of monoclonal antibodies**

For development of mAbs against proteins in the endonuclease complex, pI 4.6, Balb/c mice were given a primary immunization by injecting them i.p. with 165 μg of normal human chromatin-associated protein extract in phosphate-buffered saline (PBS) and complete Freund’s adjuvant (Sigma Chemical Co.) (42). For the secondary and tertiary boosts, the endonuclease complex, pI 4.6, was emulsified with liposomes and used without the addition of Freund’s adjuvant. Encapsulation by liposomes was carried out by Drs R. Mannino and S.Gould-Fogerite of our department (43,44). The mice were boosted twice i.p. with this sample for the secondary and tertiary injections (25 and 30 μg, respectively). This method has been shown to generate strong antibody responses to peptides without the use of carrier proteins or Freund’s adjuvant (43).

Three days after the final boost the mouse spleens were removed and the splenocytes harvested. The splenocytes were added together with Sp2/O-Ag14, myeloma cells (American Type Culture Collection, Rockville, MD) and fusion was carried out with the addition of 50% polyethylene glycol 1500 (Boehringer Mannheim). The fused cells were diluted in a selection medium [RPMI 1640 medium, 20% fetal calf serum, 10% Hybridoma Cloning Supplement (Boehringer Mannheim), 1 mg/l asparagine, 13.6 mg/l hypoxanthine, 150 mg/l o xoacacetate, 50 mg/l pyruvate, 8.2 mg/l bovine insulin, 100 000 U/l penicillin-G, 10 mg/l streptomycin sulfate, 2.6 mg/l amphotericin B and 2 mM l-glutamine], plated in microculture plates and incubated at 37°C in 5% CO2 (42). After 10 days, the supernatants of the hybridomas were screened in an immunoblot assay in which proteins in the normal endonuclease complex were bound to nitrocellulose membranes (42). Goat anti-mouse IgG conjugated to alkaline phosphatase (BioRad, Richmond, CA) was used as secondary antibody. The blots were developed using a peptide derived from the C-terminus of the FANCA protein (CRQQAAP-3). The C-terminal antiserum was produced by immunizing rabbits with a synthetic peptide corresponding to the C-terminal region of the FANCA protein (42). Western blot analysis was carried out on the normal endonuclease complexes using antibodies against a number of the proteins involved in nucleotide excision repair (NER). The polyclonal and monoclonal antibodies tested were generous gifts from the following individuals: XPA, Dr. C.James Ingles (University of Toronto); RPA, Dr Mark Wold (University of Iowa College of Medicine); TFIH (XPB, XPD, p62, p44, p34), Dr Danny Reinberg (Wood Johnson Medical School); XPG, Dr Mark MacInnes (Los Alamos National Laboratory); RER, Dr Koos Jaspers (Erasmus University). A polyclonal antibody against PCNA (Sigma Chemical Co.) was also tested. For analysis of these proteins, the secondary antibody used was goat anti-rabbit IgG conjugated to alkaline phosphatase and immunoblots were developed using BCIP-NBT, as above. Western blot analysis of HHR23B was carried out using one of the mAbs we developed (108) (Fig. 2) and a recombinant HHR23B (a generous gift from Dr Richard Wood, Imperial Cancer Research Fund).

**Electrophoretic separation of the normal and FA-A proteins**

The normal and FA-A chromatin-associated proteins were separated by SDS–PAGE on a 7–9.5% gradient gel (16×16 cm) to allow better separation of bands in the 200 kDa range and above. After separation, the proteins were electrophoretically transferred onto nitrocellulose membrane and stained with colloidal gold (BioRad). The positions of the 230 kDa protein band and topoisomerase II were confirmed by western blot analysis of a section of the unstained membrane containing the separated normal proteins. Molecular weight markers were NOVEX SeeBlue and BioRad Kaleidoscope pre-stained standards.
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Fig. 1. Western blot analysis of the proteins involved in NER that are present in the DNA endonuclease complex from normal human lymphoblastoid cells which recognizes DNA interstrand cross-links. Samples (8.5 µg) were subjected to 5–15% gradient SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes. Membranes were probed with antibodies to the proteins shown in the figure. For HHR23B, binding of mAb 108, isolated in the present study; to recombinant HHR23B is shown.

Analysis of the 40 kDa protein
HeLa chromatin-associated protein extracts were separated by SDS–PAGE on an 18% gel (16×16 cm). Half of the gel was stained with Coomassie blue and half was electrophoretically transferred onto a nitrocellulose membrane. Western blot analysis of the proteins on the membrane was performed using the mAb to the 40 kDa protein. This blot was used to determine the position of the 40 kDa protein in the stained gel. The 40 kDa protein was excised from the gel and sent to the HHMI Biomembrane Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University for amino acid sequencing.

Measurement of UDS
The ability of FA-A cells which had been stably transduced with a retroviral vector expressing the FANCA cDNA to repair DNA damaged was examined by measuring levels of UDS. Normal and FA-A cells in culture were treated with 10 nM MMC (Sigma Chemical Co.) for 1 h at 37°C. Cells were then pulsed for 2 h with 10 µCi/ml [3H]thymidine (sp. act. 61 Ci/mol) (ICN Radiochemicals, Costa Mesa, CA) at 37°C, washed with PBS, attached to slides pretreated with poly-L-lysine (0.1 mg/ml) and prepared for autoradiography as previously described (47). Slides were stained with Feulgen reagent and cells with 40–150 grains/nucleus were classified as undergoing UDS. Cells with higher numbers of grains were not counted and were considered to be in S phase (24,47). This assay is a direct measure of DNA repair synthesis in which the single-stranded gap formed in NER is filled in with new nucleotides.

Results
Western blot analysis of NER proteins present in the endonuclease complex, pI 4.6
The endonuclease complex, pI 4.6, from normal human lymphoblastoid cells, which recognizes DNA interstrand cross-links, was examined by western blot analysis for the presence of proteins involved in NER. Antibodies against known NER proteins were examined for ability to bind to proteins in this complex. It can be seen in Figure 1 that XPA, RPA, TFIH (XPB, XPD, p62, p44, p34), XPG, XPF, ERCC1 and PCNA are associated with this complex. HHR23B is also present, as described below.

Analysis of mAbs against proteins in the DNA endonuclease complex, pI 4.6
In order to determine what additional proteins are associated with this complex, mAbs were developed against these proteins. Immunoblot analysis of the supernatants of the hyridoma clones, which were developed against proteins in the endonuclease complex, pI 4.6, showed that there were a number of clones which produced antibodies to proteins in the complex. Twenty-one of these supernatants were initially purified by passage over a protein A column, via FPLC. These purified mAbs were then examined in functional assays to determine their ability to influence the activity of the normal complex, pI 4.6, on DNA treated with 8-MOP plus UVA light. Functional analysis showed that four of the mAbs (numbers 79, 98, 108 and 142) completely inhibited endonuclease activity on 8-MOP plus UVA damaged DNA and four mAbs (numbers 65, 66, 75 and 99) stimulated endonuclease activity (Figure 2). The solid horizontal line in Figure 2 represents 100% of the normal endonuclease activity observed on damaged DNA.

Three of the mAbs that completely inhibited endonuclease activity, 108, 98 and 79, were examined by western blot analysis against normal human chromatin-associated proteins. These mAbs bound to proteins with molecular weights of ~58 kDa (data not shown), 230 (Figure 3A, upper, lane 1) and 40 kDa (Figure 3B, bottom, lane 1), respectively. These three mAbs were isotypically using two different methods, mAbs 79 and 98 were found to be of the IgM class with light chains of the k type. mAb 108 was of the IgG3 class/subclass. Since mAb 108 bound to a protein with a molecular weight of 58 kDa on SDS gels, studies were carried out to determine whether it was the NER protein HHR23B, which is of the same molecular weight and is known to form a complex with the XPC protein and stimulate its activity (48,49). Western blot analysis showed that mAb 108 bound to a recombinant HHR23B protein (Figure 1); it also bound to this protein from the normal complex which migrated alongside recombinant HHR23B on the western blot (data not shown). These results indicate that HHR23B is also a component of our endonuclease complex.

Further studies were carried out on mAbs 79 and 98. Since these mAbs were of the IgM class, they were subsequently purified using size exclusion chromatography and their purity confirmed by SDS–PAGE. In order to ensure that mAb binding to these proteins was not just specific for proteins from human
western blot analysis of the HeLa proteins (Figure 4, upper, lane 2) were the same as those obtained using the normal lymphoblastoid cell proteins (Figure 4, upper, lane 1), indicating that mAb binding to the 230 and 40 kDa (data not shown) proteins was not specific for cell type. This blot was stripped and reprobed with a mAb against topoisomerase II, which was used as an internal control for protein loading (Figure 4, bottom). Topoisomerase II activities have previously been shown to be similar in FA-A cells, in particular the FA-A cell lines used in the present study, as compared with normal human cells (50,51).

Since the mAbs against the 40 and 230 kDa proteins were of the IgM class, it was important to ascertain that their ability to inhibit incision activity of the endonuclease complex was not due to their specific class of antibody. Further examination of several of the other purified mAbs showed that mAbs 37 and 94, which had no effect on endonuclease activity (Figure 2), were also of the IgM class. In addition, mAb 108, which also completely inhibited endonuclease activity (Figure 2), was of the IgG class. These results thus indicate that inhibition of endonuclease activity by mAbs against the 40 and 230 kDa proteins was not due to the fact that they were of the IgM class.

Western blot analysis was also performed using native gel electrophoresis for separation of the chromatin-associated proteins. The results showed that purified mAb 98 did not exhibit binding to any of the proteins that had been electroblotted onto the nitrocellulose membrane. A faint band was observed at the very top of the membrane, indicating that the 230 kDa protein had not migrated into the gel during electrophoresis (data not shown). This could be due to the fact that the 230 kDa protein is part of a protein complex that is too large to enter the gel during native PAGE and, as a result, the majority of the 230 kDa protein does not transfer to the nitrocellulose membrane for detection by western blot analysis.

Binding of mAbs to the 40 and 230 kDa proteins in FA-A proteins
mAbs 79 and 98 were examined for their ability to bind to chromatin-associated proteins from FA-A cells. Western blot analysis showed that there was decreased binding of mAb 98 to the 230 kDa protein from FA-A cells compared with binding of this same protein from normal cells (Figure 3A, upper). This decrease in mAb binding was seen with the 230 kDa protein from two different FA-A cells lines, HSC 72 and HSC 99 (Figure 3A, upper, lanes 2 and 3). This same blot was reprobed with a mAb against topoisomerase II (Fig. 3, bottom), which was used as control for amount of protein loaded per lane. Densitometric analysis of the western blots showed that mAb binding to the 230 kDa protein from HSC 72 and HSC 99 cells was only 35 and 37%, respectively, of that to this protein from normal cells. In contrast, mAb 79 showed the same degree of binding (100%) to the 40 kDa protein from FA-A cells as it did to this protein from normal cells (Figure 3B, bottom, lanes 1–3). Thus this protein also served as an internal control to indicate that the proteins in the FA-A extracts were not degraded.

Binding of mAb 98 to the 230 kDa protein in both normal and FA-A cells was dependent upon the concentration of the protein extracts used. Binding increased as the protein concentration was increased (Figure 5A). At all concentrations tested, reduced binding of the mAb to the 230 kDa protein from FA-A cells was observed compared with mAb binding to this protein from normal or HeLa cells (Figure 5B).
Identification of the 40 kDa protein

The 40 kDa HeLa protein band in the Coomassie stained SDS–PAGE gel, which was sent for analysis to the Keck facility at Yale University, was digested with trypsin, separated via reverse phase HPLC and individual peak fractions screened for purity via matrix-assisted laser desorption ionization mass spectrometry. One fraction was selected for Edman sequencing. The resulting 17 residue sequence exactly matched the p40 ribosomal protein. The observed and predicted average masses differed by <0.2%. The high sequencing yield of this peptide indicated that the peptide sequence obtained was derived from a major protein component in the SDS gel band. To reconfirm that the mAb we had isolated and which was used in identification of the p40 ribosomal protein was against this protein, the mAb was sent to Dr Mark Sobel (NCI, Bethesda, MD) for analysis. The results of western blot analysis showed that our mAb bound to the p40 protein in extracts from a variety of cell lines and to the recombinant p40 ribosomal protein (Dr M. Sobel, personal communication).

Correction of the DNA repair defect in FA-A cells expressing the FA-A cDNA

Studies were carried out to determine whether in FA-A cells which had been stably transduced with a retroviral vector expressing the FANCA cDNA, the DNA repair defect had been corrected. Examination of these cells, after treatment with MMC, showed that levels of UDS were slightly greater than 100% of those observed in normal cells, indicating that the repair defect had been corrected (Figure 6). In contrast, levels of UDS in similarly treated FA-A cells which were not expressing the FANCA cDNA were only 28% of normal (Figure 6).

Correction of the deficiency in the 230 kDa protein in FA-A cells expressing the FA-A cDNA

In order to determine whether there was any relationship between the FANCA gene and the deficiency in the 230 kDa protein from FA-A cells, chromatin-associated protein extracts were prepared from FA-A cells which had been transduced with a retroviral vector expressing the FANCA cDNA. Western blot analysis of these extracts from the transduced FA-A cells showed that the mAb against the 230 kDa protein bound to this protein from the transduced cells (Figure 7A, upper, lane 3) to the same degree as it did to this protein from normal cells (Figure 7A, upper, lane 1). Binding to the transduced FA-A cell protein was ~120% of that to the normal cell protein. Expression of the FANCA protein was also examined in the transduced cells. Reprobing of this same blot with a polyclonal antiserum against the C-terminus of FANCA showed that levels of FANCA in extracts from the transduced cells were similar to those in normal cells, whereas no expression of this protein was detected in these extracts from mutant FA-A cells (Figure 7A, bottom, lanes 3, 1 and 2, respectively). This same blot was reprobed with a mAb against topoisomerase II, which was used as an internal loading control (Figure 7A, middle).

Several other proteins associated with the endonuclease complex of which p230 is a component were examined by western blot analysis to determine if their levels changed in
Fig. 7. Western blot analysis of the 230 kDa protein, p40 and XPF in FA-A cells transduced with a retroviral vector expressing the FANCA cDNA. Chromatin-associated nuclear proteins (8.5 µg in each lane) from normal, FA-A (HSC 72) and FA-A (HSC 72–17) transduced cells were separated by SDS–PAGE and transferred to a nitrocellulose membrane. (A) (Upper) The membrane was probed with the mAb against the 230 kDa protein. The arrow indicates mAb binding to the 230 kDa protein. The data are representative of five experiments. (Middle) The membrane was reprobed with a mAb against topoisomerase II which was used as a loading control. (Bottom) The membrane was reprobed with affinity-purified polyclonal antiserum against the FANCA protein. The position of molecular weight markers is indicated. The arrow indicates binding of the FANCA antiserum to the FANCA protein. (B) (Bottom) A second membrane was probed with a mAb against p40; (middle) this membrane was reprobed with a polyclonal antibody against XPF; (upper) this membrane was reprobed with a mAb against topoisomerase II which was used as a loading control.

Discussion

Though FA-A cells have been shown to be defective in ability to repair DNA interstrand cross-links (19–25), the exact nature of the repair defect has not been elucidated. In addition, it is not known whether there is any relationship between this repair defect and the FANCA gene. We have isolated a DNA endonuclease complex with specificity for DNA interstrand cross-links from the nuclei of normal human cells (22,39) and have demonstrated that this complex from FA-A cells is defective in ability to recognize and incise DNA containing interstrand cross-links (22,24). We have shown that the normal complex contains a number of the proteins involved in NER (XPA, RPA, TFIIH, HHR23B, XPG, ERCC1, XPF and PCNA). Since the complex we have described exists in the absence of DNA lesions, it may correspond to the repairsome, a complex of proteins postulated to contain all of the essential NER proteins which assembles in the absence of DNA damage (52–54). As an aid in determining what additional proteins are associated with this complex, mAbs against proteins in the normal human complex have been developed. A number of these mAbs were found to completely inhibit ability of the endonuclease complex to incise DNA containing interstrand cross-links produced by 8-MOP plus UVA light. Of particular
interest is a mAb directed against a protein of ~230 kDa which, upon western blot analysis and gold staining of the electrophoretically separated protein, was found to be deficient in FA-A cells. Further examination has shown that the deficiency in this protein is corrected in FA-A cells stably transduced with a retroviral vector expressing the FANCA cDNA.

Our studies have previously demonstrated, at several different levels, that in FA-A cells there is a defect in the initial damage recognition and incision steps in the repair of DNA interstrand cross-links (22,24,40). First, FA-A cells show reduced UDS in response to 8-MOP plus UVA light (24); second, there is reduced ability of the FA-A endonuclease complex with specificity for DNA interstrand cross-links to incise DNA treated with 8-MOP or 4,5',8-trimethylpsoralen (TMP) plus UVA light (22); third, there is a reduction in the ability of FA-A endonuclease to produce dual incisions at the site of a single TMP interstrand cross-link (K.Kumaresan and M.W.Lambert, in preparation); fourth, there is a deficiency in a damage recognition protein with specificity for TMP interstrand cross-links, which could possibly play a role in the recruitment of the endonucleases and other repair proteins to the site of damage (40). A repair defect in FA-A cells has also been demonstrated in studies from other laboratories (19–25), though there has been some variability in the reported degree of the defect (reviewed in refs 8,22,55). Some of this variability could be due to the fact that a number of these investigations were carried out before classification of cell lines by complementation analysis was available and that a number of different types of assay systems were utilized. In addition, it has recently been shown that ~25% of patients with FA have evidence of spontaneously occurring mosaicism in which some circulating cells have reverted to the wild-type and have a normal MMC sensitivity (56). It is possible that this type of reversion of the FA phenotype could account for some of the variability reported regarding a DNA repair defect in FA cells.

The present results suggest that the 230 kDa protein, which is deficient in nuclear chromatin-associated protein extracts from FA-A cells compared with these same extracts from normal cells, is also involved in the repair of DNA interstrand cross-links. The mAb directed against this protein is able to completely inhibit the ability of the normal complex to incise a plasmid DNA containing TMP interstrand cross-links and we have also shown that it is able to inhibit the ability of the complex to produce dual incisions in a substrate containing a site-directed TMP interstrand cross-link (unpublished observations). These results point to a role for the 230 kDa protein in the initial steps of the repair process. The reduced levels of binding of the mAb to this protein in FA-A cells, which is 35–37% of the level of binding to the normal protein, corresponds to a reduced level of this protein in FA-A cells (38% of normal), as determined by electrophoretic analysis of the protein in normal and FA-A cells. This in turn correlates with the reduced levels of DNA repair (UDS) in FA-A cells (28% of normal UDS) in response to MMC.

A mAb has also been developed against a 40 kDa protein in our protein complex with specificity for DNA interstrand cross-links. This protein has been isolated and shown by sequence analysis to be ribosomal protein p40. The mAb against p40 completely inhibits the activity of the normal complex on cross-linked DNA. Unlike the 230 kDa protein, this protein is not deficient in FA-A cells. Protein p40 is a multifunctional protein involved in translation and plays a key role in DNA replication (57–60). The present findings, which indicate that p40 also plays a role in repair of DNA interstrand cross-links, also suggests that this protein may have both ribosomal and non-ribosomal functions. Other ribosomal proteins have been found to be multifunctional. Drosophila ribosomal protein S3 and Drosophila and human ribosomal protein PO, for example, have been shown to be involved in repair of apurinic/apyrimidinic sites in DNA and to be tightly associated with the nuclear matrix and chromatin, as well as associated with ribosomes and involved in protein translation (60–65). The human PO gene is also inducible in human cells by bifunctional alkylation agents that cause DNA intra- and interstrand cross-links (61). In addition, mammalian ribosomal protein S3 has been shown to have UV endonuclease activity (64) and this protein in Escherichia coli plays a role in inter- and/or intramolecular recombination (66). Thus p40 may be another multifunctional ribosomal protein which plays a role in DNA repair. The finding that the 230 kDa protein and not p40 is deficient in FA-A cells, that both of these proteins are components of the endonuclease complex which recognizes DNA interstrand cross-links and that in FA-A cells this complex is deficient in ability to incise damaged DNA (22) may indicate that the defect in this complex in FA-A cells is related to the deficiency of the 230 kDa protein.

The FANCA gene has recently been cloned (12,13). The protein encoded by this gene has been shown to be located in the nucleus as well as in the cytoplasm (67–70). Our data also demonstrate that the FANCA protein is present in the nucleus of normal human cells and of the corrected FA-A cells, as evidenced by western blot analysis, which shows that FANCA is present in our chromatin-associated protein extracts isolated from these cell nuclei. Its function is currently not known. The FANCA protein has been shown to form a complex with the FANCC protein (68,69). This complex is found both in the cytoplasm and the nucleus, where it has been suggested that it may play a role in DNA repair, transcriptional activation, DNA repair, splicing or cell cycle regulation (68). Whether there is an association between the 230 kDa protein described in the present study, the other proteins present in the complex we have isolated and the FANCA and FANCC proteins is currently under investigation. Preliminary evidence indicates that the 230 kDa protein interacts with the FANCA and FANCC proteins in the nucleus to form a complex.

The sensitivity of FA-A cells to DNA interstrand crosslinking agents has been corrected in FA-A cells transduced with a retroviral vector containing the FANCA cDNA (41,68). This includes normalization of levels of cell survival, cell cycle kinetics and chromosome breakage in the presence of MMC. The present study shows that levels of UDS in transduced FA-A cells, following exposure to MMC, are similar to those found in normal cells, indicating that the repair defect in these cells has also been corrected by the FANCA cDNA. In addition, the present study demonstrates, by both western blot analysis and gold staining of the electrophoretically separated protein, that the deficiency in the 230 kDa protein in FA-A cells is corrected in the transduced cells, just as are the levels of expression of the FANCA protein. Since it has been shown that transduction of the FA-A (HSC 72) cell line examined in the present study with a vector carrying a non-FANCA cDNA (i.e. FANCC cDNA) does not correct the MMC sensitivity of these cells (41) and since the uncorrected FA-A cells have a homozygous deletion for the FANCA gene (71), the correction of deficiencies in p230 and the FANCA protein

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observed in the transduced FA-A cells are due to the \textit{FANCA} cDNA rather than to stimulation of endogenous factors. These results thus indicate that the \textit{FANCA} gene plays a role in the expression or stability of the 230 kDa protein, which our studies suggest has a role in repair of DNA interstrand cross-links. This in turn indicates that there is a relationship between the \textit{FANCA} gene and the repair of this type of damage.

The defect in DNA repair (i.e. reduced levels of UDS) that we observe in FA-A cells in response to MMC or 8-MOP plus UVA (22,24), the reduced ability of the FA-A endonuclease complex with specificity for DNA interstrand cross-links to incise this type of damaged substrate (22) and the reduced levels of the 230 kDa protein in FA-A cells all involve an ~65–75% reduction from normal levels. It could be argued that this defect in a DNA repair function alone is not sufficient to account for the clinical features of FA. However, it is possible that the protein involved in the FA-A repair defect has another function in the cell. Many DNA repair proteins have been shown to have dual functions (reviewed in ref. 72).

For example, in Cockayne syndrome (CS), the degree of the DNA repair defect cannot account for the severity of the clinical symptoms associated with this disorder (73). However, it has recently been shown that the CS complementation group B protein resides in a large protein complex associated with RNA polymerase II (73) and that there is reduced RNA polymerase II transcription in CS group B cells (74). Based on these studies it has been hypothesized that CS is due to a combined DNA repair and transcription deficiency (73,75). Similarly, it is possible that the 230 kDa repair protein, which is deficient in FA-A, may have a second, as yet unknown, role that may be important in the pathophysiology of the disorder.

There may be a multistep process that leads to the clinical manifestations of FA-A, which could either be organized as a linear cascade or form a network (8), and this may account for the wide variety of cellular deficiencies reported to be associated with this disorder. Thus, ascertaining the relationship between the 230 kDa repair protein and the \textit{FANCA} gene will be an important step in elucidating the relationship between the DNA repair defect and the gene defect in FA-A.

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