Human α Spectrin II and the Fanconi Anemia Proteins FANCA and FANCC Interact to Form a Nuclear Complex*

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Fanconi anemia (FA) is a recessively transmitted genetic disorder characterized by bone marrow failure, diverse congenital abnormalities, and an increased incidence of cancer (1–3). One of the distinguishing characteristics of this disease is the marked cellular hypersensitivity to interstrand cross-linking agents, which correlates with a defect in ability to repair this type of damage. We have previously identified an approximately 230-kDa protein present in a nuclear protein complex in normal human lymphoblastoid cells that is involved in repair of DNA interstrand cross-links and shows reduced levels in FA-A cell nuclei. The FA family appears to play a role in the stability or expression of this protein. We now show that p230 is a well-known structural protein, human α spectrin II (αSpII*), and that levels of αSpII* are not only significantly reduced in FA-A cells but also in FA-B, FA-C, and FA-D cells (i.e. in full FA cell lines tested), suggesting a role for these FA proteins in the stability or expression of αSpII*. These studies also show that αSpII* forms a complex in the nucleus with the FA proteins and FANCC proteins. αSpII* may thus act as a scaffold to align or enhance interactions between FA proteins and proteins involved in DNA repair. These results suggest that FA represents a disorder in which there is a deficiency in αSpII*.

EXPERIMENTAL PROCEDURES

Chromatin-associated Protein Extracts—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), FA-B (HSC 230), FA-C (HSC 536), and FA-D (HSC 62) lymphoblastoid cell lines were a gift from Dr. Joel Griswold (Human Genetics, University of California, San Francisco). DNA interstrand cross-links (12-15). A number of the proteins involved in nucleotide excision repair are present in this complex (16). In FA-A and FA-D cells there is a defect in the ability of this complex to incise DNA containing interstrand cross-links (13, 14). We have recently shown that there is a deficiency in FA-A cells in the levels of an approximately 230-kDa protein present in this nuclear complex and that the FANCA gene plays a role in the stability or expression of this protein (16).

We have now determined the identity of the 230-kDa protein and shown that it is the structural protein α spectrin II (αSpII*). We have also shown that levels of αSpII* are significantly reduced in FA-B, FA-C, and FA-D, as well as FA-A cells and that this protein forms a complex in the nucleus with the FANCA and FANCC proteins. Thus αSpII* may act as a scaffold to align or enhance interactions between the FA proteins and other proteins in the nucleus such as those involved in DNA repair. Because nonerythroid α spectrin has been shown to interact with proteins involved in a number of cellular processes, such as DNA synthesis, cell cycle progression, gene expression, signal transduction, and cell growth and differentiation (17–22), a deficiency in this protein in FA cells could have far reaching consequences due to the number of systems affected. This may explain some of the diverse cellular and clinical defects that have been reported in FA (1–3).

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‡‡ The abbreviations used are: FA, Fanconi anemia; mAb, monoclonal antibody; IP, immunoprecipitation; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis.
stained with colloidal gold (Bio-Rad), and the other half was immunoblotted with anti-p230. In this way the 230-kDa band could be identified on the stained membrane. The remainder of the gel was stained with Coomassie, and the 230-kDa band was identified, excised, and sent to the HHMI Biopolymer Facility/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Alternatively, blots were incubated with secondary antibody conjugated to alkaline phosphatase in 1% nonfat dried milk blocker (Bio-Rad) in TTBS, washed in TTBS and incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Bio-Rad). Images were scanned using a Hewlett-Packard Scanjet 4c/T scanner and analyzed using ImageQuant (Molecular Dynamics).

RESULTS
A mAb developed against the 230-kDa protein has been used in its identification. This protein, after separation by gel electrophoresis and identification by Western blot analysis, was digested with trypsin and analyzed by MALDI-MS. The rationale for using this approach is that it is very sensitive and the mass spectrum of the peptide mixture, resulting from the enzymatic digestion of the protein, provides a fingerprint that can be specific enough to identify the protein (27).

Using this method, the 230-kDa protein was identified as human nonerythroid α spectrin (α spectrin II). The calculated peptide masses obtained from the protein digest were searched against two different data bases to determine the number of matched peptides. A ProFound search of the peptides obtained from the 230-kDa protein digest gave a probability score of 1.0e+00 to human α spectrin II with a clean break between this score and the score of the next nonrelated protein. The percentage coverage of the known sequence for this protein was 35% (i.e. the peptide masses obtained for analysis matched to 35% of the known protein sequence). This exceeded the minimum percentage coverage of 25% set as a criterion for a protein match by the Keck Facility. A second ProFound search was performed, after deleting sequences that matched human α spectrin II, and no additional protein was identified. PeptideSearch matched the same protein with 33% coverage. This analysis of the 230-kDa protein was repeated on a second sample obtained from another HeLa cell chromatin-associated protein extract with the same result. Because the isoform of the α spectrin we have identified is unknown, this protein has been designated αSπΠα in accordance with proposed nomenclature (17, 28).

Confirmation of the 230-kDa protein as αSπΠα was made by Western blot analysis. A mAb against an α spectrin, with specificity for mammalian nonerythroid α spectrin (Chemicon), bound to the 230-kDa protein from HeLa chromatin-associated protein extracts (Fig. 1, lane 1); binding of this mAb to human erythroid spectrin (α and β chains) (Fig. 1, lane 2) was used as a control. Similarly, anti-αSπΠβ bound to a spectrin in HeLa cell extracts and to the α and β chains of erythroid spectrin (Fig. 1, lane 4). Both anti-α spectrin and anti-αSπΠβ showed cross-reactivity with α and β spectrin. Using these antibodies, β spectrin was not detected in the HeLa cell chromatin-associated protein extracts. Confirmation that both mAbs were binding to the same protein band was made by analysis of the respective immunoblots banded with gold-stained proteins from a section of the same membrane (data not shown). Further confirmation of the location of β spectrin on these gels was obtained by Western blot analysis using a mAb that principally recognized erythroid β spectrin (data not shown).

Electrophoretic separation of the proteins in the HeLa cell chromatin-associated protein extracts showed that αSπΠβ is not one of the major bands present in these protein extracts (Fig. 2, A, lane 1 and B). The αSπΠβ band ( Fig. 2A, lane 1) lined up exactly with the α spectrin band from erythroid spectrin (Fig. 2A, lane 2), which was run alongside it on the same gel. The identity of these bands was verified by Western blot analysis. Both proteins, according to electrophoretic mobility,
had an apparent molecular mass of approximately 230 kDa, which agrees with previously reported values (230–240 kDa) (18, 29) and is lower than the calculated molecular mass of 284 kDa for nonerythroid and 280 kDa for erythroid (18, 29) and is lower than the calculated molecular mass of 284 kDa for nonerythroid and 280 kDa for erythroid (18, 29) and is lower than the calculated molecular mass of 284 kDa for nonerythroid and 280 kDa for erythroid (18, 29). These results thus show that αSpII*S forms a nuclear complex with these proteins. Faint bands were detected in the pre-immune precipitations (Fig. 5, lane 4) indicating a slight reactivity of the pre-immune sera with spectrin.

The binding of FANCA and FANCC to αSpII*S (top panel) and anti-a spectrin (Chemicon) (second panel) to chromatin-associated proteins from the nuclei of normal human lymphoblastoid cells (lane 1), FA-A cells (HSC 72) (lane 2), and FA-A cells transduced with a retroviral vector expressing the FANCA cDNA (HSC 72–17) (lane 3). This blot was also probed with anti-topoisomerase II, which was used as a loading control (bottom panel, lanes 1–3).

To determine whether αSpII*S interacts with the FANCA and FANCC proteins, immunoprecipitation studies were carried out. Anti-FANCA immunoprecipitation and immunoblotting with either anti-αSpII*S or anti-a spectrin demonstrated that αSpII*S co-immunoprecipitated with FANCA and FANCC from HeLa chromatin-associated protein extracts (Fig. 5, lane 1). Preliminary studies indicate that FANCG also immunoprecipitated with FANCA and FANCC. None of these proteins co-immunoprecipitated from FA-A extracts, which lack FANCA and are deficient in αSpII*S (Fig. 5, lane 2). In extracts from corrected FA-A cells, αSpII*S again co-immunoprecipitated with FANCA and FANCC (Fig. 5, lane 3) and also FANCG (preliminary data). These results thus show that αSpII*S plays a role in the stability or expression of this protein.

The deficiency in αSpII*S in FA-A cells is restored to normal in the corrected cells. Western blot analysis was carried out on binding of anti-αSpII*S (top panel) and anti-a spectrin (Chemicon) (second panel) to chromatin-associated proteins from the nuclei of normal human lymphoblastoid cells (lane 1), FA-A cells (HSC 72) (lane 2), and FA-A cells transduced with a retroviral vector expressing the FANCA cDNA (HSC 72–17) (lane 3). This blot was also probed with anti-topoisomerase II, which was used as a loading control (bottom panel, lanes 1–3).

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The binding of FANCA and FANCC to αSpII*S was confirmed by anti-a spectrin immunoprecipitation (Fig. 6A, lane 1). Anti-a spectrin was used, even though it may be against a different isoform than our αSpII*S, because our anti-αSpII*S is of the IgM class and cannot be used effectively in immunoprecipitations. In FA-A extracts, no FANCA and reduced amounts of αSpII*S and FANCC immunoprecipitated (Fig. 6A, lane 2). This corresponds to no expressed FANCA (Fig. 6B, lane 2) and reduced levels of αSpII*S (Fig. 4, top panel, lane 2) and FANCC (Fig. 6B, lane 2) in FA-A nuclei. Reduced levels of FANCC in FA-A cell nuclei have also been reported by Ya.
The FANCA and FANCC proteins have been shown to form a complex in the nucleus (36, 37). The present results show that \( \alpha \text{Spectrin II}^* \) also forms a complex in the nucleus with these proteins. Because studies suggest that FANCA and FANCC may not bind directly to each other in the nucleus but that their interaction may involve another, as yet unknown, protein (36), it is possible that \( \alpha \text{Spectrin II}^* \) is this protein. These finding suggest that \( \alpha \text{Spectrin II}^* \) may act as a scaffold to align these proteins so as to enhance their interactions. This alignment could be particularly important in repair of interstrand cross-links where recombination may be involved (34, 35). Reduced levels of \( \alpha \text{Spectrin II}^* \) in the nucleus would thus be expected to reduce the efficiency of the repair process rather than inhibit it altogether, consistent with our experimental findings.

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The role of nonerythroid spectrin in the nucleus is not completely understood. Nonerythroid \( \alpha \)-spectrin has been shown to be associated with the nuclear matrix (38). A number of studies suggest that the nuclear matrix is important in DNA repair (39–41). \( \alpha \text{Spectrin II}^* \) may link the repair process to the nuclear matrix. \( \alpha \) spectrin has also been shown to interact with a number of proteins (17, 18) and potentially some of these interactions could occur in the nucleus. For example, \( \alpha \) spectrin contains a calmodulin binding site and has been shown to associate with this protein (19–21) and it also has a SH3 domain, which interacts with tyrosine kinases (17, 20, 22). Collectively these proteins, which potentially could bind to \( \alpha \text{Spectrin II}^* \) in the nucleus, have been shown to interact with proteins involved in DNA synthesis, cell cycle progression, mitosis, gene expression, and signal transduction (17, 19–22, 42). \( \alpha \) spectrin has also been shown to be involved in cell growth and differentiation (17, 18). A deficiency in \( \alpha \text{Spectrin II}^* \) in FA cells could thus have far reaching consequences due to the number of systems affected. This could possibly explain some of the diverse cellular and clinical defects reported in FA, such as cell cycle defects, aberrant induction of apoptosis, and developmental abnormalities (1–3). Identification of other FA genes and their products should help determine how many of the FA proteins form a complex with \( \alpha \text{Spectrin II}^* \) and whether these proteins also play a role in the stability or expression of \( \alpha \text{Spectrin II}^* \).
Elucidation of this relationship may help delineate the function of these FA proteins in the nucleus, the nature of their interaction with aSpII*-a, and their role in the deficiency in aSpII*-a observed in FA cells.

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REFERENCES

1. Glanz, A., and Fraser, F. C. J. (1982) Med. Genet. 19, 412–416
2. Auerbach, A. D., and Allen, R. G. (1991) Cancer Genet. Cytogenet. 51, 1–12
3. Stratledge, C. A., and Buchwald, M. (1993) Am. J. Pediatr. Hematol. Oncol. 14, 177–185
4. Zhen, W., Evans, M. K., Haggerty, C. M., and Bohr, V. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 18715–18723
5. Lambert, M. W., Tsongalis, G. J., Lambert, W. C., Hang, B., and Parrish, D. D. (1994) Biochim. Biophys. Res. Commun. 208, 587–591
6. Lo Ten Foe, J., Rooimans, M. A., Bosnoyan-Collins, L., Alon, N., Wijker, M., Parker, L., Lightfoot, J., Carreau, M., Calleu, D. F., Savea, A., Cheng, N. C., van Berkell, C. G. M., Strunk, H. P., Gille, J. P. J., Pals, G., Kruyt, F. A. E., Pronk, J. C., Arwert, F., Buchwald, M., and Joenje, H. (1996) Nat. Genet. 14, 320–325
7. Fanconi anemia/Brca consortium. (1996) Nat. Genet. 14, 324–328
8. Lambert, M. W., Tsongalis, G. J., Lambert, W. C., Hang, B., and Parrish, D. D. (1997) Biochim. Biophys. Res. Commun. 230, 587–591
9. de Winter, J. P., Waisfisz, Q., Rooimans, M. A., van Berkell, C. G. M., Bosnoyan-Collins, L., Alon, N., Carreau, M., Bender, O., Dewuth, I., Schindler, D., et al. (1998) Nat. Genet. 20, 281–285
10. Lambert, M. W., Tsongalis, G. J., Lambert, W. C., Hang, B., and Parrish, D. D. (1997) Biochim. Biophys. Res. Commun. 230, 587–591
11. Bruks, R. J., Lamberts, J. E., Thelen, M. P., Hwang M., Reardon, J. T., Sancar, A., Zhou, Z.-Q., Walter, C. A., Parris, C. N., and Thompson, L. D. (1996) Mol. Cell. Biol. 16, 6553–6562
12. Yamashita, T., Kupfer, G. M. Vaf, D., Suzuki, K., and Kawashima, S. (1993) J. Biol. Chem. 268, 25239–25243
13. Sancar, A., Zhou, Z.-Q., Walter, C. A., Parris, C. N., and Thompson, L. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13085–13090
14. Kupfer, G. M., Naf, D., Suliman, A., Pulsipher, M., and D’Andrea, A. D. (1997) Nat. Genet. 17, 487–496
15. Bachs, O., Lanini, L., Serratosa, J., Coll, M. J., Bastos, R., Aligue, R., Rius, E., and Carafoli, E. (1999) J. Biol. Chem. 264, 18595–18600
16. Koebler, D. R., and Hanawalt, P. C. (1996) Nucleic Acids Res. 24, 2877–2884
17. Johnston, P. J., MacPhail, S. H., Banath, J. P., and Olive, P. L. (1998) Radiat. Res. 149, 533–542
18. Balaje, A. S., May, A., and Bohr, V. A. (1998) Mutat. Res. 404, 3–11
19. Birge, R. B., Krudsen, B. S., Besser, D., and Hanafusa, H. (1996) Genes Cells 1, 595–613
20. Leto, T. L., Pleasie, S., Forget, B. G., Benz, Jr. E. J., and Marchesi, V. T. (1989) J. Biol. Chem. 264, 5826–5830
21. Wasenius, V.-M., Saraste, M., Salven, P., Ernemaa, M., Holm, L., and Lehto, V.-P. (1989) J. Cell Biol. 108, 79–93
22. Ziemnicka-Kotula, D., Xu, J., Gu, H., Putempska, A., Kim, K. S., Jenkins, E. C., Trenkner, E., and Kotula, L. (1998) J. Biol. Chem. 273, 13681–13692
23. Fa, K.-L., Thuij, P. C., Fujino, T., Digweed, M., Liu, J. M., and Walsh, C. E. (1998) Hum. Genet. 102, 166–169
24. Hang, B., Yeung, A. T., and Lambert, M. W. (1993) Nucleic Acids Res. 21, 4187–4192
25. Williams, K. R., Samandar, S. M., Stone, K. L., Saylor, M., and Rush, J. (1996) in The Protein Protocols Handbook (Walker, J. M., ed), pp. 541–555, Humana Press, Totowa, NJ
26. Laemmli, U. K. (1970) Nature 203, 680–685
27. Cottrell, J. S., and Sutton, C. W. (1996) in Methods in Molecular Biology, Vol. 61: Protein and Peptide Analysis by Mass Spectrometry (Chapman, J. R., ed), pp. 67–82, Humana Press, Totowa, NJ
28. Ma, Y., Zimmer, W. E., Riederer, B. M., Bloom, M. L., Barker, J. E., and Goodman, S. R. (1993) Mol. Brain Res. 18, 87–94
29. Saido, T. C., Yokota, M., Nagao, S., Yamamura, I., Tam, E., Tsuchiya, T., Suzuki, K., and Kawashima, S. (1993) J. Biol. Chem. 268, 25239–25243
30. Moon, R. T., and McMahon, A. P. (1990) J. Biol. Chem. 265, 4427–4433
31. Blech, R. J., and Morrow, J. S. (1989) J. Cell. Biol. 108, 481–493
32. Hu, R.-J., Watanabe, M., and Bennett, V. (1992) J. Biol. Chem. 267, 18715–18723
33. Bennett, V., and Gilligan, D. M. (1993) Annu. Rev. Cell Biol. 9, 27–66
34. Thompson, L. H. (1996) Mutat. Res. 363, 77–88
35. Broekman, K. W., Lusserdin, J. E., Thelen, M. P., Hwang M., Reardon, J. T., Sancar, A., Zhou, Z.-Q., Walter, C. A., Parris, C. N., and Thompson, L. H. (1996) Mol. Cell. Biol. 16, 6553–6562
36. Sancar, A., Zhou, Z.-Q., Walter, C. A., Parris, C. N., and Thompson, L. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13085–13090
37. Kupfer, G. M., Naf, D., Suliman, A., Pulsipher, M., and D’Andrea, A. D. (1997) Nat. Genet. 17, 487–496
38. Bachs, O., Lanini, L., Serratosa, J., Coll, M. J., Bastos, R., Aligue, R., Rius, E., and Carafoli, E. (1999) J. Biol. Chem. 264, 18595–18600
39. Koehler, D. R., and Hanawalt, P. C. (1996) Nucleic Acids Res. 24, 2877–2884
40. Johnston, P. J., MacPhail, S. H., Banath, J. P., and Olive, P. L. (1998) Radiat. Res. 149, 533–542
41. Balaje, A. S., May, A., and Bohr, V. A. (1998) Mutat. Res. 404, 3–11
42. Birge, R. B., Krudsen, B. S., Besser, D., and Hanafusa, H. (1996) Genes Cells 1, 595–613