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New insights into the Fanconi anemia pathway from an isogenic *FancG* hamster CHO mutant

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Abstract

The Fanconi anemia (FA) proteins overlap with those of homologous recombination through FANCD1/BRCA2, but the biochemical functions of other FA proteins are largely unknown. By constructing and characterizing a null *fancg* mutant (KO40) of hamster CHO cells, we show that FancG protects cells against a broad spectrum of genotoxic agents. KO40 is consistently hypersensitive to both alkylating agents that produce monoadducts and those that produce interstrand crosslinks. KO40 cells were no more sensitive to mitomycin C (3×) and diepoxybutane (2×) than to 6-thioguanine (5×), ethylnitrosourea (3×), or methyl methanesulfonate (MMS) (3×). These results contrast with the pattern of selective sensitivity to DNA crosslinking agents seen historically with cell lines from FA patients. The hypersensitivity of KO40 to MMS was not associated with a higher level of initial DNA single-strand breaks; nor was there a defect in removing MNU-induced methyl groups from DNA. Both control and MMS-treated synchronized G1-phase KO40 cells progressed through S phase at a normal rate but showed a lengthening of G2 phase compared with wild type. MMS-treated and untreated early S-phase KO40 cells had increased levels of Rad51 foci compared with wild type. Asynchronous KO40 treated with ionizing radiation (IR) exhibited a normal Rad51 focus response, consistent with KO40 having only slight sensitivity to killing by IR. The plating efficiency and doubling time of KO40 cells were nearly normal, and they showed no increase in spontaneous chromosomal aberrations or sister chromatid exchanges. Collectively, our results do not support a role for FancG during DNA replication that deals specifically with processing DNA crosslinks. Nor do they suggest that the main function of the FA protein "pathway" is to promote efficient homologous recombination. We propose that the primary function of FA proteins is to maintain chromosomal continuity by stabilizing replication forks that encounter nicks, gaps, or replication-blocking lesions.

Keywords: FANCG; Fanconi anemia; Replication fork; Rad51 foci; Homologous recombination

1. Introduction

Fanconi anemia (FA) is a rare autosomal recessive disorder characterized by progressive bone marrow failure and cancer susceptibility, especially for acute myelogenous leukemia [1]. FA is genetically heterogeneous, consisting of at least eight complementation groups for which genes have been identified: *FANCA*, *C*, *D1*, *D2*, *E*, *F*, *G*, and *FANCL/PHF9* [2,3]. The genes for groups B, I, and J are unidentified [4]. The role of the FA protein "pathway" in recovery of cells from DNA damaging agents is under intense investigation. FA A/C/E/F/G/L proteins interact to form a nuclear complex [3,5–10]. The integrity of this complex is essential for the monoubiquitination of FANCD2 following mitomycin C (MMC) or ionizing radiation (IR) treatment; this modifica-

Abbreviations: MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; MNU, methylnitrosourea; ENU, ethylnitrosourea; CNU, chloroethylnitrosourea; IR, ionizing radiation; MMC, mitomycin C; HR, homologous recombination; HRR, homologous recombinational repair; ROS, reactive oxygen species; SCE, sister chromatid exchange; DSB, double-strand break; S^6G, 6-thioguanine

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tion of FANCD2 appears essential for normal resitance to genotoxic agents. Interestingly, FANCD1, D2, and L are the only FA proteins evolutionarily conserved in non-vertebrates [3,11,12].

Recently, the *FANCD1* gene was found to be identical with *BRCA2* [13], a participant in the formation of Rad51 nucleoprotein filaments during homologous recombinational repair (HRR) [14]. FA-D1 and FA-J mutant cells are able to monoubiquitinate FANCD2 at Lys561 [15], whereas cells in the other groups cannot ubiquitinate FANCD2 during S phase of the cell cycle [4,16]. The finding that FANCD1 is a bonafide homologous recombination protein suggests that the main function of the FA proteins may be to promote HRR [2]. Indeed, monoubiquitinated FANCD2 colocalizes with BRCA1 and Rad51 in nuclear foci [15,16].

The phenotype of FA cells includes increased chromosomal breakage/exchange, apoptosis, and reactive oxygen species (ROS), as well as prolongation of the G2 phase (for reviews see [2,17–19]). One possible mechanism is that elevated ROS leads to genotoxic stress. Many studies have suggested defects in oxygen metabolism in FA cells (reviewed in [20]); at least FANCC [21,22] and FANCG [23] appear to have important roles in redox metabolism.

Historically, FA has often been viewed as a DNA repair deficiency disorder, largely because FA cells are consistently hypersensitive to DNA cross-linking agents and have increased chromosome fragility [24,25]. The evidence for defects in DNA repair has been inconsistent and contradictory [26,27]. The lack of homology between many FA proteins and DNA repair proteins in lower organisms argues against the involvement of FA proteins in the enzymology of DNA repair.

Previously we cloned the human *XRCC9* gene by phenotypic correction of the MMC sensitivity of a mutagen-derived CHO mutant (UV40) [28]. Despite the UV sensitivity of UV40, *XRCC9* proved to be identical to *FANCG* cloned from FA-G lymphoblasts [29]. This convergence was unexpected because UV sensitivity is generally not associated with FA cells. In order to have a better-defined, isogenic CHO *fancg* system, we produced a knockout event by gene targeting. Our studies of this new mutant show a broad spectrum of genotoxin sensitivity that is inconsistent with a specific deficiency in processing DNA interstrand crosslinks. The increased sensitivity to methylating damage in *fancg* mutant cells is not due to increased initial damage. The phenotype of *fancg* cells suggests a more general role for FA proteins than participating in HRR when DNA replication encounters lesions.

2. Materials and methods

2.1. Cell culture and centrifugal elutriation

CHO cells were grown in monolayer or suspension culture in αMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were counted and analyzed on a Coulter® Multisizer II during subculturing. Cell doubling time was determined for suspension cultures diluted twice weekly by averaging 20 consecutive measurements. In synchronization experiments, 2×10^8 cells were resuspended in elutriation buffer (80% PBS and 20% culture medium). The buffer and cells were kept on ice, and the centrifuge was set at 8 °C. Centrifugal elutriation was conducted in elutriation buffer on a J6-M1 centrifuge (Beckman Coulter) at 2800 rpm with an initial flow rate of 19–20 ml/min and ∼20 fractions were collected as the flow rate was incremented by 1 ml/min.

2.2. FancG targeting vector construction

Gene-targeting plasmid pUC-FancG.TV was derived from pUC19. The multiple cloning region of pUC19 was replaced with a new cloning region containing a short section of the neomycin gene (*neo*) beginning at the ATG start codon and ending at the *Eag*I restriction site by inserting an 82-mer duplex oligonucleotide between *Eco*RI and *Hin*dIII. The remaining *neo* sequences from *Eag*I to *Sal*I were cloned from IRES-Neo-pA (a gift from John Sedivy, Brown University) to create plasmid pUC-neo. Two *FancG* recombinant PCR fragments were amplified from a CHO *FancG* genomic BAC clone (BAC 174) and inserted into the two cloning sites of pUC-neo. The upstream *FancG* PCR fragment from intron 1 to exon 3 was 1419 bp and cloned in frame with *neo*, and the downstream *FancG* PCR fragment from exon 7 to exon 14 was 3987 bp. The thymidine kinase gene (*tk*) was isolated from pSSC-9 [30] and blunt-end ligated into the final vector to create pUC-FancG.TV. Plasmid pUC-FancG.con was created as a positive control for gene targeting by defining PCR reactions that would identify *FancG* knockout clones. pUC-FancG.con contains an additional 655 nt 5′ of the upstream *FancG* fragment inserted into pUC-FancG.TV. PCR reactions were performed on 0.5 μg genomic DNA using High-Fidelity Platinum® Taq DNA Polymerase (Invitrogen). PCR primer sequences are available upon request.

2.3. Gene targeting and DNA transfection

For gene targeting, 3×10^7 cells were rinsed with and resuspended in 1 ml cold electroporation buffer (20 mM HEPES (pH 7), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose), mixed with 10 μg linearized pUC-FancG.TV DNA, electroporated at 250 V/1600 μF, incubated for 5 min on ice, and plated in T150 flasks for 24 h to allow for *neo* expression. Cells were plated into 10 cm dishes at $\sim 2 \times 10^6$ cells per dish in 20 ml medium containing 1.7 mg/ml G418 (Gibco Invitrogen) and incubated for 5 days, after which the medium was replaced with fresh medium (supplemented with 10% dialyzed serum) containing 0.1 μM 2′-fluoro-2′-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil (FIAU); cells were incubated for an additional 5 days. Each dish contained a pool of ∼150 drug-resistant colonies, which were harvested for freezing and DNA isolation (QIAamp® DNA Blood Mini Kit, Qiagen Inc). The