The Fanconi Anemia Proteins Functionally Interact with the Protein Kinase Regulated by RNA (PKR)*

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Protein kinase regulated by RNA (PKR) plays critical roles in cell growth and apoptosis and is implicated as a potential pathogenic factor of Alzheimer’s, Parkinson’s, and Huntington’s diseases. Here we report that this pro-apoptotic kinase is also involved in Fanconi anemia (FA), a disease characterized by bone marrow (BM) failure and leukemia. We have used a BM extract to show that three FA proteins, FANCA, FANCC, and FANCG, functionally interact with the PKR kinase, which in turn regulates translational control. By using a combined immunoprecipitation and reconstituted kinase assay, in which an active PKR kinase complex was captured from a normal cell extract, we demonstrated functional interactions between the FA proteins and the PKR kinase. In primary human BM cells, mutations in the FANCA, FANCC, and FANCG genes markedly increase the amount of PKR bound to FANCC, and this PKR accumulation is correlated with elevated PKR activation and hypersensitivity of BM progenitor cells to growth repression mediated by the inhibitory cytokines interferon-γ and tumor necrosis factor-α. Specific inhibition of PKR by 2-aminopurine in these FA BM cells attenuates PKR activation and apoptosis induction. In lymphoblasts derived from an FA-C patient, overexpression of a dominant negative mutant PKR (PKRDN) suppressed PKR activation and apoptosis induced by interferon-γ and tumor necrosis factor-α. Furthermore, by using genetically matched wild-type and PKR-null cells, we demonstrated that forced expression of a patient-derived FA-C mutant (FANCC1554Δ) augmented double-stranded RNA-induced PKR activation and cell death. Thus, inappropriate activation of PKR as a consequence of certain FA mutations might play a role in bone marrow failure that frequently occurred in FA.

The Protein kinase regulated by RNA (PKR)¶ plays a critical role in translational control (1). PKR has been subjected to intensive investigations (2) because of its antiviral function. The underlying mechanism by which PKR mediates antiviral defense in mammalian cells is the inhibition of translation through phosphorylation of eukaryotic initiation factor 2-α (eIF-2α) (2). It is known that PKR becomes autophosphorylated on multiple serine/threonine residues following viral infection by dsRNA produced by viral gene expression or viral replication. Upon activation by autophosphorylation, PKR phosphorylates Ser-51 on eIF-2α, which in turn blocks the initiation of protein synthesis (3). Another important part of the cellular functions of PKR is its role in regulation of cell proliferation, survival, and apoptosis. Because of its pro-apoptotic effects, forced expression of PKR in mouse, insect, and yeast cells causes translational inhibition and cell death (4, 5).

Indeed, inappropriate activation of PKR has been associated with certain disease states characterized by high levels of apoptosis. For example, PKR and the PKR-like kinase PERK have been implicated as important pathogenic factors in the Alzheimer’s, Parkinson’s, and Huntington’s diseases (6–9).

Research on Fanconi anemia (FA) has recently generated great interest because the disease serves as an excellent model for hematopoietic failure and leukemic evolution, and because FA proteins function in cellular responses to a variety of stresses including signals of DNA damage and apoptosis. FA is an autosomal recessive disease characterized by progressive bone marrow failure, variable congenital anomalies, and a predisposition to cancer (10–12). Cells from FA patients are hypersensitive to DNA cross-linking agents such as mitomycin C and diepoxybutane. FA is genetically heterogeneous, with at least 11 complementation groups identified thus far (13). The genes encoding the groups A (FANCA), C (FANCC), G (FANCG), D1 (FANCD1/BRC2A), D2 (FANCD2), E (FANCE), F (FANCFF), and L (FANCL) have been cloned (14–21). The biological functions of these FA gene products remain mostly unknown.

We demonstrated previously that one of the FA proteins, FANCC, functions to protect cells from cytotoxicity mediated by PKR (22, 23). Because PKR was found to be constitutively activated in FA cells with mutations in the FANCC gene (22), we reasoned that activation of PKR in bone marrow may play a role in the pathogenesis of bone marrow failure in children with FA. We thus sought to investigate the mechanism by which FANCC modulates PKR activity. We found that suppression of the PKR kinase in FA cells required a coordinated action

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† The abbreviations used are: PKR, protein kinase regulated by RNA; 2-AP, 2-aminopurine; dsRNA, double-stranded RNA; eIF-2α, eukaryotic initiation factor 2-α; FA, Fanconi anemia; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; BM, bone marrow; MEF, mouse embryo fibroblasts; BM-MNCs, bone marrow mononuclear cells; WCE, whole cell extracts; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; CFU, colony-forming units; BFU, burst-forming units; WT, wild type; Hsp, heat shock protein.

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of FANCC and the molecular chaperone Hsp70, a cytoprotective factor we have previously shown to act in concert with FANCC to protect hematopoietic cells from cytotoxicity induced by mitogenic inhibitors interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) (24). In this report, we demonstrate that three FA proteins, FANCA, FANCC, and FANCG, functionally inter-act with the PKR kinase. Mutations in these FA genes cause abnormal accumulation of PKR kinase bound to the FANCC protein, which is correlated with elevated PKR activation and hypersensitivity of BM progenitor cells to growth repression mediated by inhibitory cytokines IFN-γ and TNF-α. Our results suggest that inappropriate activation of PKR as a consequence of certain FA mutations might play a role in bone marrow failure frequently occurring in FA.

EXPERIMENTAL PROCEDURES

Cell Culture, Patient Material, and Treatments—Normal and mutant lymphoblast lines derived from patients diagnosed with Fanconia anemia (FA) were maintained in RPMI media 1640 (Invitrogen) supplemented with 15% fetal bovine serum. Cells were stimulated with recombinant human IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) (R & D Systems) for 16 h. Mouse embryo fibroblasts (MEF) from wild-type (PKR+/+) and PKR knockout (PKR−/−) mice (25), were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. MEFs were transfected with 50 μg/ml of poly(rI: rC) (dsRNA) (Amersham Biosciences) and lysed 24 h post-transfection. Bone marrow mononuclear cells (BM-MNCs) of healthy donors and patients with a diagnosis of FA in complementation groups A or G were obtained in accordance to guidelines from the Institutional Review Board of the Cincinnati Children’s Hospital Medical Center. BM-MNCs were cultured in RPMI 1640 medium containing 20% dialyzed fetal bovine serum and supplemented with stem cell factor (100 ng/ml), thrombopoietin (100 ng/ml), and G-CSF (100 ng/ml). All growth factors were purchased from R&D Systems. On day 3 of culture, cells were stimulated with IFN-γ and TNF-α (1 ng/ml each) for 16 h before lysis.

Construction of Retroviral Expression Vectors and Transduction—The full-length human PKR (kindly provided by Dr. G. N. Barber, Emory University, Atlanta, GA; GenBank accession number NM002759; see Ref. 26) was amplified by PCR, using Pfu DNA polymerase (Stratagene). The resulting PCR fragment was subcloned into the NotI site of retroviral vector SF91 (a generous gift from Dr. Christopher Baum, Cincinnati Children’s Hospital Medical Center) to create SF91-PKR. The dominant negative mutant PKR (PKR GG129E; see Ref. 27) was constructed by site-directed mutagenesis using SF91-PKR as the template and primers (sense, 5′-GGGAAGACGTAGACCTTGTATTAGACGTGTTAAAAATATAATAC-3′; antisense, 5′-GTTATTATA-TTTAACCCTGCTAAACCTGATTCTTTCC-3′). The FA-C patient-derived mutant FANCC L554P cDNA (28) was removed from pLXSN-L554P (24) and subcloned into the NotI site of SF91 as described above. The SF91 plasmids (10 μg each) were used to produce retroviral supernatant. Lymphoblasts or MEFs were exposed to the retroviral supernatant with BM-MNC extracts, the FANCC antibodies were precipitated with BM-MNC extracts, the FANCC antibodies were washed, and resuspended in 100 μl of staining buffer, and analyzed by flow cytometry by using a FACSCalibur (BD Biosciences). Camptothecin-treated cells were harvested, washed with phosphate-buffered saline, and resuspended at a concentration of 1 × 10⁷/ml in staining buffer containing phosphate-buffered saline, 2% fetal calf serum, 0.1% sodium azide. This cell suspension (400 μl) was then added to 400 μl of Cytofix/Cytoperm (BD Biosciences) and incubated on ice to permeabilize the cells. The cells were then washed with 2 ml of Perm/Wash buffer (Pharmingen) and resuspended in 100 μl of Perm/Wash buffer. Purified rabbit immunoglobulin G (IgG; 10 μl; Pharmingen) was then added to each sample as a blocking antibody to prevent the nonspecific uptake of fluorochrome-conjugated antibody. After 15 min, 20 μl of phycoerythrin- or fluorescein-conjugated anti-α-skeletal actin (3×) was added, and samples were incubated for 30 min in the dark at room temperature. Cells were washed with 2 ml of Perm/Wash buffer, resuspended in 500 μl of staining buffer, and analyzed by flow cytometry by using a FACSCalibur (BD Biosciences). Camptothecin-treated cells were used as a positive control.

RESULTS

Mutations in FANCA, FANCC, and FANCG Genes Lead to Abnormal Activation of PKR and Translational Repression—
The increased association of the PKR kinase with the FANCC protein in lymphoblasts derived from an FA patient carrying an FANCC mutation (23) prompted us to examine the association of FANCC, PKR, and Hsp70 in cells from FA patients bearing mutations in other FA genes. Fig. IA shows the input controls of whole cell extracts of normal and FA lymphoblasts used in anti-FANCC immunoprecipitation. In addition to the previously demonstrated binary PKR-FANCC complex in FANCC−/− lymphoblasts (Fig. 1B, lane 2), we observed accumulation of PKR on FANCC in FANCA−/− and FANCG−/− cells (Fig. 1B, lanes 4 and 6). Functional complementation with normal FA cDNAs completely eliminated the accumulation of PKR on the FANCC protein in the corresponding FA mutant lymphoblasts (Fig. 1B, lanes 3, 5, and 7). The anti-FANCC immunocomplexes from all mutant FA cells except FANCC−/− lymphoblasts contain amounts of Hsp70 comparable with those obtained with lymphoblasts derived from a healthy donor (Fig. 1B). This would be expected because the FANCC protein in these FA cells is presumably normal and capable of binding to Hsp70 (24).

Hematopoietic cells from FA patients and Fancc knockout mice are hypersensitive to the treatment of the mitogenic inhibitors IFN-γ and TNF-α (11, 22, 29–31). We and others (22, 32) have demonstrated that these inhibitory cytokines can induce apoptosis in FA cells and other cells through the activation of the pro-apoptotic protein kinase PKR and subsequent inhibition of protein synthesis. We sought to determine whether stimulation with IFN-γ and TNF-α could overactivate the PKR kinase in lymphoblasts derived from FA patients carrying FANCA, FANCC, or FANCG mutations. Fig. 2A shows the input controls of whole cell extracts of normal and FA lymphoblasts used in these experiments. Significantly, treatment of the FANCA−/−, FANCC−/−, and FANCG−/− lymphoblasts with IFN-γ and TNF-α further increased the accumulation of PKR on FANCC in FANCA−/−, FANCC−/−, and FANCG−/− lymphoblasts (Fig. 2B, lanes 4, 6, and 8). To determine whether the increased amounts of PKR bound to FANCC might be associated with PKR activation, we labeled the lymphoblasts with [32P]orthophosphate and measured levels of 32P-labeled PKR immunoprecipitated with a monoclonal PKR antibody. As expected, treatment of normal and FA lymphoblasts with IFN-γ and TNF-α induced PKR phosphorylation (Fig. 2C). However, IFN-γ and TNF-α stimulation led to a much higher PKR activation in FANCA−/−, FANCC−/−, and FANCG−/− lymphoblasts than in normal cells (compare Fig. 2C, lanes 4, 6, and 8 with lane 2), findings that are consistent with the observation that treatment of these FANCA lymphoblasts with the inhibitory cytokines increased the accumulation of the PKR kinase on FANCC in FANCA−/−, FANCC−/−, and FANCG−/− lymphoblasts (Fig. 2B). Therefore, these results provide direct evidence linking the nonproductive interaction between PKR and the FA proteins to deregulation of the pro-apoptotic protein kinase PKR.

Because PKR activation leads to translational repression (1, 2), we reasoned that global translational activity in FANCA−/−, FANCC−/−, and FANCG−/− cells should be reduced to a greater extent than in normal cells after exposure to IFN-γ and TNF-α. Protein synthesis was significantly reduced by IFN-γ and TNF-α treatment in FANCA−/−, FANCC−/−, and FANCG−/− lymphoblasts, demonstrating a 60–80% decrease after 16 h (Fig. 2D). Taken together, these results thus support the notion of links between abnormal PKR activation and deregulation of translational control in FA hematopoietic cells well documented to undergo excessive apoptosis in response to IFN-γ and TNF-α (11, 22, 29–31).

PKR Activity Is Elevated in FA Bone Marrow Cells—To verify the role of PKR signaling in primary human hematopoietic cells, we sought to characterize the interaction between FA proteins and the PKR kinase in BM cells from healthy donors and FA patients. Since the bone marrow uniformly fails in FA patients and abnormalities of other organ systems are sporadic (10, 11), we first chose a normal human BM cell extract that can be assayed for PKR kinase activity but has no overexpressed proteins or interfering elements. To improve the immunoprecipitation, we coated the FANCC antibody with Dynabeads before incubation with the BM cell extracts. As shown in Fig. 3A, essentially all the FANCC protein present in the normal BM mononuclear cells could be pulled down by the antibody beads, as indicated by the absence of FANCC in the supernatant or the wash fraction (Fig. 3A, lanes 4–6). With rabbit whole IgG as the precipitation control, FANCC was detected in the supernatant but not in the immunoprecipitated fraction (Fig. 3A, lanes 7 and 8). Further analysis of the anti-FANCC immunoprecipitate revealed that detectable amounts of FANCA, FANCG, PKR, and Hsp70 could be found in the fraction bound to the anti-FANCC beads (Fig. 3A, lane 4). To examine PKR-FA protein interaction in primary FA BM cells, two FA patients, genotyped in the complementation groups A
Fig. 2. FA mutations lead to abnormal activation of PKR and translational repression. A, 100 μg of the indicated WCEs of normal and FA lymphoblasts stimulated with or without IFN-γ and TNF-α (10 ng/ml each) for 2 h were run as input controls for B. B, WCEs in A (500 μg of total proteins) were subjected to immunoprecipitation with antibody against FANCC and analyzed with anti-PKR (top) or anti-Hsp70 (bottom). C, in vivo phosphorylation of PKR in cytokine-stimulated normal and FA lymphoblasts. [32P]Phosphate-incorporated PKR immunocomplexes were analyzed by SDS-PAGE followed by autoradiography (top) and immunoblotting (bottom). D, rate of protein synthesis in cytokine-stimulated normal and FA lymphoblasts. Cells were treated with (■) or without (□) IFN-γ and TNF-α, and labeled with [35S]methionine-cysteine labeling mix for 60 min. The rate of protein synthesis was measured as a function of the incorporation of [35S]methionine and [35S]cysteine into trichloroacetic acid-precipitable proteins. Data represent means ± S.D. of two independent experiments.

(FA-A) and G (FA-G), respectively, were recruited for the study. The results with the immunoprecipitates from the FA-A and FA-G patients were similar to those obtained with the FA mutant cell lines (Fig. 2). Specifically, these immunocomplexes contain significantly more FANCC-bound PKR than that in the immunoprecipitate of the normal control under the same conditions (Fig. 3B). In these FA BM cells, about 25–30% of total PKR was accumulated on the FANCC protein as compared with less than 5% in the normal BM cells (Fig. 3B, compare lane 4 with lanes 6 and 8). FANCC from both FA-A and FA-G BM-MNCs coimmunoprecipitated with small amounts of Hsp70, suggesting that the FANCC protein in both the FA-A and FA-G BM cells retains the capacity to bind this chaperone protein. However, no coimmunoprecipitated FANCA or FANCG was detected (Fig. 3B, lanes 6 and 8), consistent with reports by others that mutations in the FANCA or FANCG genes abolish the interactions between the FA proteins (12, 18).

To examine PKR activation in primary FA BM cells, we determined the levels of PKR and eIF-2α phosphorylation in the cell extracts from normal and FA BM cells stimulated with IFN-γ and TNF-α, using the respective phospho-specific PKR and eIF-2α antibodies. Normal BM cells showed a low level of activated PKR after treatment with IFN-γ and TNF-α (Fig. 3C, lane 1). However, there was 3–4-fold more phosphorylated PKR detected in the BM cells from the FA patients than in normal BM cells (Fig. 3C, compare lane 1 with lanes 2 and 3). Significantly, the degree to which increased PKR was phosphorylated correlated positively with the accumulation of PKR on the FANCC protein in these BM cells (compare Fig. 3, B with C).

Treatment of FA cells with combinations of either IFN-γ and double-stranded RNA or IFN-γ and TNF-α dramatically increases phosphorylation of the eukaryotic translation initiation factor eIF-2α as a result of PKR activation (22, 23). In MCF-7 breast cancer cells, TNF-α stimulation causes a PKR-dependent elevation of phosphorylated eIF-2α and subsequent translational repression (20). We thus determined the levels of eIF-2α phosphorylation in BM cell extracts with a phosphospecific antibody to phosphoserine 51 of eIF-2α. We observed 2–3-fold higher phosphorylated eIF-2α in FA BM cell extracts than in normal BM cells (Fig. 3D, top panel, compare lane 1 with lanes 2 and 3). The amounts of total eIF-2α proteins were comparable among these different BM cell extracts (Fig. 3D, bottom panel).

Apoptosis Induction by IFN-γ and TNF-α or dsRNA in FA Cells Is Dependent on PKR Activation—We next examined the biological consequences of the abnormal PKR activation observed in the FA BM cells. It is known that BM cells from FA patients overproduce IFN-γ and TNF-α, which repress the growth of BM progenitor cells through mechanisms involving apoptosis (30, 31). To investigate whether de-regulated PKR activation could sensitize the FA BM cells to the inhibitory cytokines, we grew BM-committed progenitor cells in a semi-solid assay for the growth of erythroid colony-forming and burst-forming units (CFU-E and BFU-E, respectively) in the presence of IFN-γ and TNF-α. In the absence of IFN-γ and
TNF-α, the normal control had a mean number of CFU-E and BFU-E (1×10⁵ seeded BM-MNCs) of 108.4 and 86.6, respectively (Fig. 4, A and B). BM cells from the FA-A patient showed significant inhibition of colony growth with average numbers of 33.2 for CFU-E and 28.8 for BFU-E. When cells were cultured in the presence of IFN-γ and TNF-α at 1 ng/ml each, a dose that does not have an effect on the growth of the normal progenitors, the mean numbers of CFU-E and BFU-E formed by the FA BM cells were significantly decreased to 8.6 and 4.9, respectively (Fig. 4, A and B). Collectively, these results suggest that FA mutations could cause abnormal PKR activation, leading to growth inhibition of hematopoietic progenitors.

To determine specifically the role of PKR activation in eIF-2α phosphorylation and apoptotic death in FA BM cells, we treated normal and FA BM cells with IFN-γ and TNF-α in the presence of 2-aminopurine (2-AP), the nucleoside analog known to inhibit the kinase activity and PKR-dependent phosphorylation of eIF-2α (33). As shown in Fig. 4C, 2-AP treatments suppressed PKR activation as manifested by the inhibition of both PKR and eIF-2α phosphorylation (Fig. 4C, lanes 4–6). Thus, the ability of 2-AP to block eIF-2α phosphorylation indicates that enhanced eIF-2α phosphorylation observed in FA BM cells indeed is a downstream effect of PKR activation. We next evaluated the effect of 2-AP on apoptotic induction by IFN-γ and TNF-α in FA-A BM cells. A high concentration of 2-AP (10 mM) alone did not induce significant apoptosis in both normal and FA BM cells (Fig. 4D). However, in cells treated with IFN-γ and TNF-α, 2-AP blocked apoptosis induction in FA-A BM cells (Fig. 4D). Thus, the inhibition of apoptosis by 2-AP appeared to be the consequence of its suppression of PKR activation.

To confirm the contribution of PKR activation to the FA
apolipoprotein pathway, we overexpressed a dominant negative mutant, PKR{sup}*{sub}296R (catalytically inactive; see Ref. 27), in lymphoblasts derived from an FA-C patient and subsequently quantified statistical significance between normal and FA-A samples at expression of PKR K296R markedly reduced phosphorylation of eIF-2α in untreated WT cells (Fig. 5C, lane 3). This likely resulted from the constitutive PKR activation in the FA-C lymphoblasts, as reported previously (23). PKR{sup}*{sub}296R expression completely abolished this constitutive eIF-2α phosphorylation in FA-C mutant lymphoblasts (Fig. 5A, lane 3). Flow cytometric analysis of apoptotic death (Fig. 5B) showed that ~40% of empty vector-transduced FA-C cells underwent apoptosis after exposure to IFN-γ and TNF-α (compared with less than 10% of normal cells). PKR{sup}*{sub}296R expression reduced apoptosis of FA-C cells treated with IFN-γ and TNF-α by more than 70% (Fig. 5B).

We evaluated further the effect of FA mutation on dsRNA-induced PKR activation by expressing the FA-C patient-derived mutant FANCC{sup}*{sub}554P cDNA in WT and PKR-null MEFs. WT cells were highly responsive to dsRNA treatments, as indicated by the dense phosphorylated (P−−) PKR band (Fig. 5C, lane 4). Remarkably, forced expression of FANCC{sup}*{sub}554P led to detectable PKR phosphorylation in untreated WT cells (Fig. 5C, lane 5) and a significant increase of phosphorylated PKR in dsRNA-treated WT cells (lane 6). In contrast, PKR{sup}o{sup}o cells transduced with vector alone or FANCC{sup}*{sub}554P contained neither phosphorylated (P-PKR) nor total PKR (Fig. 5C, lanes 3, 4, 7, and 8). This is expected because these cells are devoid of PKR (25). To substantiate further the idea that overexpression of FA mutant augments PKR activation, we examined the survival of cells expressing the FANCC{sup}*{sub}554P protein. Although there was adequate expression of the FANCC{sup}*{sub}554P protein in both WT and PKR-null MEFs (Fig. 5C), the effect of the mutant FA protein on cell death was observed only in WT MEFs (Fig. 5D), suggesting a PKR-dependent effect of the mutant FA protein. Significantly, expression of the FANCC{sup}*{sub}554P protein resulted in substantial cell death in untreated WT cells (Fig. 5D). Collectively, these results support our hypothesis that FA mutations could cause abnormal PKR activation, leading to cellular apoptosis.

**Functional Interaction between PKR and the FA Proteins Leads to Control of PKR Activity**—To gain insight into the mechanism of molecular interaction between PKR and the FA proteins, we wished to determine whether functionally significant amounts of other components of the complex had coprecipitated with the FANCC immunocomplex. We performed PKR kinase assays using both the anti-FANCC immunopre-
in the absence of either FANCA or FANCG (Fig. 6 B–C) reaction mixture containing the BM immunoprecipitate (Fig. 6 D) each purified component was individually omitted from the activation of individual components to the control of PKR kinase activity, (Fig. 6 B)–activation. This was confirmed by phosphorylation of the kinase (Fig. 6 C)–which was found when FANCC or Hsp70 was omitted (Fig. 6 C)–significant amount of eIF2α phosphorylation (P-eIF2α; top), total eIF2α (middle), or β-tubulin (bottom). B, cells indicated in A were analyzed for IFN-γ and TNF-α-induced apoptosis by quantification of the fraction of exposed cells containing activated caspase 3. C, WT and PKR-null MEFs were transduced with retroviruses carrying empty vector or expressing the FA-C patient-derived mutant FANCC L544P cDNA and treated with dsRNA. WCEs (100 μg of total proteins) were analyzed for levels of PKR phosphorylation (P-PKR), total PKR, FLAG-FANCC, or β-tubulin. D, percentage of cell death in vector or FANCC L544P-transduced PKR+/+ and PKR−/− MEFs after treatment with 50 μg of poly(rC) (dsRNA). Cell survival was analyzed 48 h after transduction and 24 h after dsRNA treatment.

To verify independently the functional interaction between these components, we performed a similar assay using the anti-FANCC immunoprecipitate isolated from the extract of FANCA−/− lymphoblasts exposed to IFN-γ and TNF-α, as described in Fig. 2. The immunoprecipitate from the IFN-γ- and TNF-α-stimulated FANCA−/− extract had an activity to phosphorylate eIF2α significantly higher than that of the reconstituted reaction mixture supplemented with the complete purified components (Fig. 6 C, left, compare lane 2 with lane 5). Omission of either the purified FANCA or FANCG resulted in an increased eIF2α phosphorylation to the level comparable with that in the reaction mixture containing the immunoprecipitate only (Fig. 6 C, left, compare lanes 6 and 8 with lane 2), indicating that these two FA proteins are important for the control of PKR kinase activity. As in the previous assay with normal immunoprecipitate (Fig. 6 B), removal of FANCC or Hsp70 from the complete reaction mixture had no further increase in eIF2α phosphorylation compared with the full reaction (Fig. 6 C, left, compare lanes 7 and 9 with lane 5). Taken together, these results reveal different strength of interactions between the components of the FA-PKR signaling complex and suggest special functional significance of these interactions.

**DISCUSSION**

FA constitutes the most frequent genetic cause of BM failure (reviewed in Refs. 11 and 12). Mutations in three of the 11 FA genes, FANCA, FANCC, and FANCG, account for nearly 90% of
FA patients diagnosed worldwide (13). In this report, we have used a BM extract to show that these three FA proteins functionally interact with the pro-apoptotic kinase PKR. We have also provided evidence that this interaction plays a role in the regulation of the stress kinase PKR and the downstream translational control. In primary human BM cells, disruption of the interaction between PKR and the FA proteins correlates with elevated PKR activation and hypersensitivity of BM progenitor cells to growth repression mediated by the inhibitory cytokines IFN-γ and TNF-α. Specific inhibition of PKR by 2-AP in these FA BM cells attenuates PKR activation and apoptosis induction by IFN-γ and TNF-α. In lymphoblasts derived from an FA-C patient, overexpression of a dominant negative mutant PKR (PKR\textsubscript{K206R}) suppressed PKR activation and apoptosis induced by IFN-γ and TNF-α. Furthermore, by using genetically matched WT and PKR-null cells, we demonstrated that forced expression of a patient-derived FA-C mutant (FANCC\textsubscript{T554P}) augmented dsRNA-induced PKR activation and cell death. We also used reconstituted kinase assay to demonstrate functional interactions between the FA proteins and PKR. Collectively, these results support our hypothesis that inappropriate activation of PKR as a consequence of certain FA mutations might play a role in bone marrow failure as frequently occurred in FA.

Our present results suggest that FANCC plays an essential role in PKR signaling in hematopoietic cells. The accumulation of PKR on the FANCC protein in FA lymphoblast cell lines (Figs. 1 and 2) and primary BM cells from FA patients (Fig. 3) suggests that FANCC binds to PKR and then recruits
other components to the complex, resulting in the displacement of FANCC and control of PKR activity. The release of FANCC from the complex may depend upon a functional interaction between FANCC and the other two FA proteins (12, 18) or the association of FANCC with Hsp70 (23, 24). Disruption of these interactions would be expected to lead to the kind of abnormal PKR activation in FA cells (Fig. 2B and Fig. 3, C and D). In addition, overexpression of the patient-derived FA-C mutant (FANCC[L534P]) enhanced dsRNA-induced PKR activation and cell death (Fig. 5). A special or perhaps the central role of FANCC in PKR signaling is anticipated from the work of many laboratories on the function of FANCC that has established a consistent signaling abnormality in hematopoietic cells bearing FANCC mutations. It has been shown that suppression of FANCC expression represses clonal growth of normal erythroid and granulocyte-macrophage progenitor cells, and disruption of the FANCC gene, in mice, renders hematopoietic progenitor cells hypersensitive to the pro-apoptotic effect of IFN-γ and TNF-α (11, 29–31). The multifunctional property of the FANCC protein appears to explain the clinical observations that patients in complementation group C had a significantly more severe phenotype than other complementation groups (11).

In addition to FANCC, the FANCA and FANC G proteins are necessary for the control of the PKR kinase activity. This strengthens the idea of a primary function of the two FA proteins in the FA-PKR interaction. Such a function would also explain why FANCA−/− and FANCG−/− cells accumulate abnormal FANCC-PKR-Hsp70 ternary complexes (Figs. 1–3). What might be the function of FANCA and FANC G in the PKR signaling pathway is unclear at this stage. We only recovered relatively small amounts of FANCA and FANC G proteins in human BM cells even after washing the anti-FANCC immuno-complex with a mild ionic strength buffer (Fig. 3), suggesting a weak or transient interaction of the two FA proteins with the rest of the associated proteins. However, the importance of this weak or transient interaction was demonstrated by a reconstituted kinase assay, in which omission of either FA protein resulted in a marked increase in PKR activation (Fig. 6). The existence of interactions of various strengths between the components of the FA-PKR complex is not surprising because there is evidence that tight association has been observed between FANCA and FANC G or FANCC and Hsp70 by yeast two-hybrid analysis or immunoprecipitation (24, 34, 35), whereas weaker interactions were detected between FANCA or FANC G and FANCC by antibody methods (36, 37). Thus, there are specific contacts between components of the complex, some of which may reflect contacts made between components during stress response and different steps of the complex assembly.

One objective of our study was to look for evidence that FA interacts with PKR and controls the kinase activity in hematopoietic cells. To this end, we used a combined immunoprecipitation and reconstituted kinase assay in which an active PKR kinase complex was captured from a normal extract by FANCC antibody-coated paramagnetic beads. It is noteworthy that the anti-FANCC immunocomplex can only partially suppress PKR activation, even after washing the beads with a buffer of low ionic strength (50 mM KCl). However, we were unable to reconstitute the complex to nearly full strength in controlling PKR activity by adding the purified components of the complex (Fig. 6B). It appears that reconstitution of the anti-FANCC immunoprecipitate of normal cells with purified FANCA and FANC G is sufficient for full control of the PKR kinase, as addition of FANCC or Hsp70 to the reaction mixtures did not further reduce PKR activity. The physiological relevance of the FA-PKR interaction was demonstrated by the results obtained from experiments with BM cells of the FA patients. The anti-FANCC immunoprecipitates from the FA patients are similar to those obtained with the FA mutant cell lines (Figs. 1–3). Specifically, these immunocomplexes contain significantly more FANCC-bound PKR and exhibit higher levels of the kinase activity of PKR as compared with those in the immunoprecipitate of the normal control under the same conditions. Furthermore, these biochemical alterations correlate with IFN-γ- and TNF-α-mediated growth inhibition of the BM progenitor cells of the FA-A patient (Fig. 4). We observed a 3–4-fold increase of FANCC-bound PKR in BM cell extracts of the FA-A and FA-G patients (Fig. 3) and a lymphoblast cell line that expresses a missense mutated FANCC protein (Figs. 1 and 2). This finding suggests two possible scenarios for the function of the FA proteins in PKR signaling. First, the three FA proteins bind to PKR as a pre-assembled complex in response to stresses and then recruit the PKR inhibitor to the complex. In this way, the FA proteins may function as a scaffold network. So far, no biochemical activities have been found for any of the FA proteins. However, FA proteins have been detected in at least three biologically functional complexes (18, 23, 38). The multifunctionality of the FA proteins may well play a putative scaffold role in multiple biological processes. In the PKR signaling pathway, for example, the FA proteins can assemble a functional signaling module and facilitate the control of the pro-apoptotic kinase mediated by a cellular PKR inhibitor. The second scenario concerns the accumulation of PKR on FANCC in extracts of BM cells from FA patients and of FA lymphoblasts. This suggests that the kinase, and perhaps together with other associated factors, remains trapped after its assembly on the FANCC protein, most likely due to a block in subsequent steps of maturation and dissociation of the complex. This strongly argues for a role of the FANCA and FANC G proteins in either the replacement of the FANCC or the transfer of PKR to a cellular PKR inhibitor.

In summary, our results suggest that through interaction between FANCC and PKR, the three FA proteins can form an initial complex with the kinase, followed by the recruitment of the molecular chaperone Hsp70 to the complex. Hsp70 may exert its chaperone function by displacing the FA proteins from the initial complex and exposing the kinase to a cellular PKR inhibitor, which prevents phosphorylation of PKR and eIF-2α and ablates protein synthesis to continue and cells to survive. In FA cells, mutations in the three FA genes cause the abnormal accumulation of PKR on the FANCC protein. As a result, when hematopoietic cells from these FA patients are exposed to environmental cues such as viral infection, growth-inhibitory cytokines (IFN-γ and TNF-α), or oxidative stress (e.g. reactive oxygen species), the pro-apoptotic kinase PKR becomes activated by autophosphorylation, leading to the phosphorylation of eIF-2α and cell death. Uncontrolled activation of PKR may contribute in part to excessive cell death, leading to the universal hallmark of FA, bone marrow failure.

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