Nucleophosmin Interacts with and Inhibits the Catalytic Function of Eukaryotic Initiation Factor 2 Kinase PKR*

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In normal cells the protein kinase PKR effects apoptosis in response to various extra and intracellular cues and can also function to suppress the neoplastic phenotype. Because most neoplastic cells are resistant to certain apoptotic cues, we reasoned that an early molecular event in carcinogenesis or leukemogenesis might be the inactivation of PKR by expression or activation of intracellular PKR inhibitors. Seeking novel PKR-modulating proteins we report here that nucleophosmin (NPM), a protein frequently overexpressed in a variety of human malignancies, binds to PKR, and inhibits its activation. Co-immunoprecipitation and in vitro binding experiments showed that NPM associated with PKR. Kinase assays demonstrated that recombinant NPM inhibited PKR activation in a dose-dependent manner. In addition, purified recombinant NPM was phosphorylated by activated PKR. Most importantly, overexpression of NPM suppressed PKR activity, enhanced protein synthesis, and inhibited apoptosis. Lymphoblasts from patients with Fanconi anemia (FA) expressed low levels of NPM, which correlated with high ground-state activation of PKR and cellular hypersensitivity to apoptotic cues, but enforced expression of NPM in these mutant cells reduced aberrant apoptotic responses. Inhibition of PKR by NPM may be one mechanism by which neoplastic clones evolve in sporadic malignancies and in neoplastic cells arising in the context of the cancer predisposition syndrome, Fanconi anemia.

Sustained cellular proliferation and resistance to apoptotic cues are characteristic features of neoplastic cells. Ongoing protein synthesis is required for both of these processes, so factors that directly control mRNA translation also can directly or indirectly influence apoptotic activity. One such factor is the interferon (IFN)1-inducible, double-stranded RNA (dsRNA)-dependent protein kinase PKR (1, 2), a pivotal antiviral response protein in eukaryotic cells (3, 4) that functions to inhibit mRNA translation. In the ground state PKR is unphosphorylated but by binding dsRNA the molecule dimerizes and autoprophosphorylates on multiple serine and threonine residues (5, 6). Phosphorylated PKR inactivates the eukaryotic initiation factor 2a subunit (eIF-2a) by phosphorylating it on Ser-51 (7).

PKR has tumor suppressor activity (2). Enforced expression of dominant negative mutants of either PKR or eIF-2a induced malignant transformation of mouse cells (8–12). Transforming Ras genes and the nuclear proto-oncogene c-Myc inhibit PKR activity (13, 14). The functional tumor suppressor activity of PKR derives, at least in part, from its capacity to induce apoptosis. Because of its pro-apoptotic effects, overexpression of PKR in mouse, insect, and yeast cells causes growth inhibition and cell death (2, 8, 15). Indeed, inappropriate activation of PKR has been associated with certain disease states characterized by high level apoptotic activity. For example, activation, in the brain, of PKR and the PKR-like kinase PERK has been implicated in the pathogenesis of Alzheimer’s, Parkinson’s, and Huntington’s diseases (16–19) and activation of PKR in bone marrow may play a role in the pathogenesis of bone marrow failure in children and adults with Fanconi anemia (20). Taking into account the high prevalence of myeloid leukemia in Fanconi anemia, excessive activation of PKR in hematopoietic stem cells may also serve as a selective force for the emergence of adapted stem cells that have acquired the capacity to suppress PKR activation or expression, a somatic change that might account for clonal evolution and leukemia (21).

For these reasons, we sought to identify factors that bind to and inhibit PKR reasoning that enhanced expression of such factors could represent an early adaptive response of a stem cell in a highly apoptotic environment. We report here that the nucleolar protein nucleophosmin (NPM), known to be overexpressed in tumor cells, actively proliferating cells (22–26), and stem cells (stemcell.princeton.edu) is such a factor. We find that NPM expression is suppressed in Fanconi anemia cells, increases when these cells are complemented with the proper FA gene, modulates PKR activity in normal cells, and, in gain-of-function analyses, complements the apoptotic phenotype of FA cells. We suggest that the capacity of NPM to suppress PKR activation is one mechanism by which NPM promotes cell proliferation, suppresses apoptosis, and maintains states of transformation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Mouse embryo fibroblasts (MEF) from wild-type (PKR−/−) and PKR knockout (PKR+/+) mice (27), were maintained in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS). Normal JY and mutant HSC536N lymphoblasts derived from a Fanconi anemia patient (FA-C) were maintained in RPMI media 1640 (Invitrogen) supplemented with 15%
heat-inactivated FCS. HeLa cells and human normal fibroblasts were grown in Dulbecco’s modified Eagle’s medium with 10% FCS and αMEM with 20% FCS, respectively. The factor-dependent myeloid cell line MO7e and chronic myelogenous leukemia line K562 were maintained in various media in accordance to the requirements. MEF were transfected with 100 μg/ml of poly(I)poly(C) (Amersham Biosciences) and lysed 2 h post-transfection. For treatments of lymphoblasts, cells were stimulated with recombinant human IFN-γ (10 ng/ml) and TNFα (10 ng/ml) (R & D Systems, Minneapolis, MN) for the indicated time periods followed by immunoprecipitation or flow cytometric analysis.

In Vitro dsRNA Binding Assays, NPM Isolation and Microsequencing, and Mass Spectrometry Analysis—Whole cell extracts (WCE) were prepared in Nonidet P-40 (Nonidet P-40) lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 mM MgCl2, 2 mM CaCl2, 0.1 mM ATP, 5% glycerol, and a mixture of protease inhibitors including 1% aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and 2 mM sodioethanolamine. 500 μl of WCE containing 2 mg of total proteins were mixed with 50 μl of poly(I)poly(C)-agarose (Amersham Biosciences) beads and rocked at 4 °C for 60 min. The beads were then washed three times with 500 μl of Nonidet P-40 lysis buffer. Proteins bound to beads were eluted from the beads by heating the samples at 94 °C for 5 min in 2× Laemmli SDS sample buffer, separated by SDS-PAGE, stained with Ponceau S, and subjected to immunoblot analysis. To isolate the 40-kDa protein for sequence analysis, WCE from ~106 cells were passed through poly(I)poly(C)-agarose columns. After three washes, the bound proteins were eluted in 6× Laemmli SDS sample buffer, resolved by SDS-PAGE and transferred to a polyvinylidene difluoride protein transfer and sequencing membrane (Schleicher & Schuell). The 40-kDa band was visualized by Ponceau S staining, excised, and subjected to protein microsequencing analysis (Shriners Hospital Peptide Sequencing Core Facility, Portland, OR).

For mass spectrometry analysis, whole cell extracts from normal lymphoblasts were immunoprecipitated with polyclonal PKR antibody (Santa Cruz Biotechnology) as described below. The immunocomplexes were then separated by SDS-PAGE and visualized by Coomassie Blue stain. The excised protein bands were subjected to in-gel digestion. Protein identity following in-gel digestion was determined by atmospheric pressure matrix assisted laser desorption ionization mass spectrometry (AP-MALDI). Gel digests were purified using a reverse phase C18 membrane (20–60 μg) and subjected to in-gel digestion. The indicated amounts of GST-PKR and eIF-2α were used in binding assays. For binding, 30 μl of [35S]methionine-labeled or unlabeled (cold) full-length PKR, NPM, and their truncated derivatives were synthesized in vitro using the Tnt wheat-germ coupled transcription/translation system (Promega, Madison, WI). All in vitro translated products were treated with RNAse A (300 μg/ml) at 30 °C for 1 h before use in binding assays. For binding, 30 μl of [35S]methionine-labeled proteins was incubated with equal amounts of cold proteins, as indicated, at 30 °C for 15 min. The mixtures were immunoprecipitated with anti-PKR or anti-NPM antibodies as described above, and analyzed by SDS-PAGE and autoradiography.

In Vitro Protein Interactions—[35S]Sulfur-labeled or unlabeled (cold) full-length PKR, NPM, and their truncated derivatives were synthesized in vitro using the Tnt wheat-germ coupled transcription/translation system (Promega, Madison, WI). All in vitro translated products were treated with RNAse A (300 μg/ml) at 30 °C for 1 h before use in binding assays. For binding, 30 μl of [35S]methionine-labeled proteins was incubated with equal amounts of cold proteins, as indicated, at 30 °C for 15 min. The mixtures were immunoprecipitated with anti-PKR or anti-NPM antibodies as described above, and analyzed by SDS-PAGE and autoradiography.

Expression and Purification of the GST-PKR fusion proteins were performed according to the protocol described previously (29). For binding, GST-PKR fusion proteins bound to glutathione-Sepharose beads were incubated with WCE (1 mg of total protein) prepared in Nonidet P-40 lysis buffer. After incubation at 30 °C for 20 min, the beads were recovered, washed, and analyzed by SDS-PAGE and immunoblotting analysis using antibodies against human PKR and NPM (Santa Cruz Biotechnology).

In Vivo Protein Interactions—[32P]Orthophosphate-labeled or unlabeled (cold) full-length PKR, NPM, and their truncated derivatives were synthesized in vivo using the Tnt wheat-germ coupled transcription/translation system (Promega, Madison, WI). All in vitro translated products were treated with RNAse A (300 μg/ml) at 30 °C for 1 h before use in binding assays. For binding, 30 μl of [32P]Orthophosphate-labeled proteins was incubated with equal amounts of cold proteins, as indicated, at 30 °C for 15 min. The mixtures were immunoprecipitated with anti-PKR or anti-NPM antibodies as described above, and analyzed by SDS-PAGE and autoradiography.

Transfection and Transduction—dsRNA transfections were carried out as previously described (29). PKR nullizygous (PKR−/−) MEFs were transiently transfected with the empty vector p5XFLAG-CMV (2 μg), pFLAG-PKR (1 μg), or indicated amounts of pFLAG-NPM, along with a pEGFP plasmid (BD Biosciences Clontech, Palo Alto, CA) that served as a transfection control for transfection efficiency. Mock and transduced cells were treated with dsRNA followed by detection for the exogenously expressed proteins, examination of apoptotic morphology, and analyses of the phosphorylation of PKR and eIF2α and the rates of protein synthesis. The Moloney (pLXSN) retroviral vectors encoding FANCC or NPM were transduced into HESC56N lymphoblasts by a previously described protocol (28). As sets of isogenic lines were selected for G418 (300 μg/ml) resistance, and subjected to analyses for PKR activity and apoptosis.

Analysis of Cell Viability and Apoptosis—Cell viability was measured by trypan blue exclusion analysis. To quantify apoptotic cells, we used a polyclonal antibody to the active form of caspase 3 in a flow cytometric assay to detect cells in the early stages of apoptosis. The flow cytometric assays were performed by the procedure described elsewhere (29).

In Vivo 32P and 35S Labeling of Proteins—Cells were starved for 60 min in phosphate-free medium containing 10% dialyzed fetal bovine serum, and were treated with 100 μg/ml of poly(I)poly(C) or combination of poly(I)poly(C) and TNFα (10 ng/ml each) for the indicated time periods followed by labeling for 3 h with [32P]Orthophosphate (150 μCi/ml, PerkinElmer Life Sciences). After labeling for 3 h, whole cell lysates were prepared as described above and were subjected to immunoprecipitation with antibodies specific for mPKR or eIF2α. [32P]Phosphate-labeled immunocomplexes were analyzed by SDS-PAGE and autoradiography. In vivo phosphorylation analysis was performed on these samples to determine the quantity of PKR or eIF2α precipitated by the respective antibody. For 35S labeling, 0.5 × 106 MEF were seeded in a 60-mm dish and cultured overnight. Cells were rinsed with methionine-cysteine-free Dulbecco’s modified...
Eagle's medium and treated with dsRNA and IFN/H9253 where indicated. Labeling was again performed in the same medium containing 50Ci/ml of [35S]methionine-cysteine labeling mix (PerkinElmer Life Sciences) and incubated at 37°C for 60 min. Whole cell lysates were prepared in Nonidet P-40 lysis buffer and protein concentration was determined. Equal amounts of protein were analyzed on SDS-PAGE followed by autoradiography. Protein synthesis was measured by the incorporation of [35S]methionine and [35S]cysteine into trichloroacetic acid-precipitable proteins.

RESULTS

Induced NPM Binding to dsRNA Columns in Normal Lymphoblasts—We recently showed that mutant FA-C cells were hypersensitive to IFN/H9253 and dsRNA. Equal amounts of whole cell extracts (WCE; 2 μg of total proteins each sample) were applied to a poly(I)/poly(C)-agarose column. The bound proteins were eluted and analyzed by SDS-PAGE, followed by Ponceau S staining. B, amino acid sequences of four peptides derived from microsequencing of the 40-kDa protein, which is identical to the human nucleophosmin (NPM; Ref. 30). Parentheses indicate probable but not definite residues. C, proteins obtained from anti-PKR immunoprecipitates identified by mass spectrometry analysis. Top, proteins obtained from anti-PKR immunoprecipitates identified by mass spectrometry analysis. Middle, MS/MS spectrum of peptide 278–281 of NPM. Observed fragment ions matching those predicted from the sequence below are indicated along with their measured m/z values. Bottom, the calculated m/z values of the expected b and y series fragment ions from singly charged peptide 278–281 of NPM. D, association of NPM with GST-PKR. GST alone, or GST-PKR was incubated with WCE from normal JY lymphoblasts treated with (+) or without (−) IFNγ and TNFα (10 ng/ml each), and the bound proteins were analyzed by immunoblotting with antibodies against NPM (upper) or PKR (lower). The same WCE (100 μg) was electrophoresed directly as input controls.

Fig. 1. Identification of NPM as a PKR-interacting protein. A, dsRNA-agarose affinity chromatography showing enhanced binding of NPM to dsRNA in normal lymphoblasts treated with IFNγ and TNFα. Equal amounts of whole cell extracts (WCE; 2 μg of total proteins each sample) were applied to a poly(I)/poly(C)-agarose column. The bound proteins were eluted and analyzed by SDS-PAGE, followed by Ponceau S staining. B, amino acid sequences of four peptides derived from microsequencing of the 40-kDa protein, which is identical to the human nucleophosmin (NPM; Ref. 30). Parentheses indicate probable but not definite residues. C, proteins obtained from anti-PKR immunoprecipitates identified by mass spectrometry analysis. Top, proteins obtained from anti-PKR immunoprecipitates identified by mass spectrometry analysis. Middle, MS/MS spectrum of peptide 278–281 of NPM. Observed fragment ions matching those predicted from the sequence below are indicated along with their measured m/z values. Bottom, the calculated m/z values of the expected b and y series fragment ions from singly charged peptide 278–281 of NPM. D, association of NPM with GST-PKR. GST alone, or GST-PKR was incubated with WCE from normal JY lymphoblasts treated with (+) or without (−) IFNγ and TNFα (10 ng/ml each), and the bound proteins were analyzed by immunoblotting with antibodies against NPM (upper) or PKR (lower). The same WCE (100 μg) was electrophoresed directly as input controls.
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Fig. 2. NPM co-immunoprecipitates with PKR. A, whole cell extracts (WCE; 1 mg of total proteins in 500 μl of Nonidet P-40 lysis buffer as described under “Experimental Procedures”) of MEF from wild-type (PKR+/+) and PKR knockout (PKR−/−) mice treated with (+) or without (−) 100 μg/ml of dsRNA were immunoprecipitated with antibody against NPM (lanes 5–8), and the immunocomplexes were subjected to immunoblotting analysis with anti-PKR (upper panel) or anti-NPM (lower panel). 100 μg of whole cell extracts (WCE) were run as input controls (lanes 1–4). IP, immunoprecipitation. B, whole cell extracts (WCE; 1 mg of total proteins in 500 μl of Nonidet P-40 lysis buffer) of wild-type PKR+/+, PKR−/−, MEFs treated with (+) or without (−) 100 μg/ml of dsRNA, and incubated with RNase A (300 μg/ml; lanes 5 and 6) or DNase I (100 μg/ml; lanes 7 and 8) at 30 °C for 1 h before being immunoprecipitated with antibody against PKR (lanes 3–8), and the immunocomplexes were subjected to immunoblotting analysis with anti-PKR (lanes 1–4) or anti-NPM (lanes 5–8). 100 μg of whole cell extracts (WCE) were run as input controls (lanes 1–4). D, immunoprecipitation confirms that the anti-PKR antibody used in the co-immunoprecipitation experiments does not interact with purified recombinant NPM. GST-PKR and GST-NPM fusion proteins bound to glutathione-Sepharose beads were eluted by adding an appropriate volume of the elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). Eluted GST-PKR (0.2 μg in 200 μl) or GST-NPM (0.5 μg in 200 μl) were immunoprecipitated with IgG (lanes 3 and 4) or anti-PKR (lanes 5 and 6) antibodies, and analyzed by SDS-PAGE followed by immunoblotting. 20 μl of the GST-PKR (lane 1) and 10 μl of the GST-NPM (lane 2) elutes without antibody were run as input controls. Lanes 7 and 8 show the amounts of GST-PKR and GST-NPM retained in the supernatants after anti-PKR immunoprecipitation, respectively.

NPM Binds to GST-PKR—To confirm that NPM associated with PKR, we performed an in vitro binding assay with GST-PKR fusion proteins and tested the bound products by immunoblotting using an anti-NPM antibody. Because stimulation of the lymphoblasts with IFNγ and TNFα increased substantially the NPM protein recovered from the dsRNA column, we also asked whether treatment of IFNγ and TNFα might enhance interaction between PKR and NPM. We thus used whole cell lysates from the normal lymphoblasts stimulated with both IFNγ and TNFα in the binding assays. As shown in Fig. 1D, NPM did bind to GST-PKR (top, lanes 5 and 6) but not to GST alone (lanes 3 and 4). Treatments with IFNγ and TNFα only marginally increased the association of NPM with GST-PKR (compare lane 5 with lane 6). Reprobing the blot with antibody to PKR revealed similar amounts of GST-PKR in the binding reactions (Fig. 1D, bottom). Note that there is an additional lower band revealed by anti-PKR antibody in GST-PKR pull-down samples, which runs at approximately the position of endogenous PKR (Fig. 1D, bottom, lanes 5 and 6). We suspected the bacterially expressed GST-PKR could have dimerized with the endogenous PKR in the whole cell extracts of the normal lymphoblasts. We confirmed this by incubating GST-PKR (twice the amount we used in the pull-down) with phosphate-buffered saline buffer and demonstrating the absence of the lower band (lane 7).

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Subsequently, we performed immunoprecipitation experiments with these whole lymphoblasts using anti-PKR antibody to isolate potential PKR-interacting proteins in hematopoietic cells. As assessed by Coomassie Blue staining of the SDS-PAGE of anti-PKR immunoprecipitates there were six unique bands (as compared with IgG control), which were then removed from the gel and subjected to mass spectrometric analysis. NPM was one of the proteins that co-immunoprecipitated with PKR (Fig. 1C). NPM was identified by tandem mass spectrometric analysis of peptides produced from in-gel digestion (Fig. 1C, middle and bottom panels). The 3 major ions in the MS spectra of the peptide digest were selected for MS/MS analysis. The top ranking matches for the MS/MS spectra of the two most intense ions with m/z values of 1819.8 and 1835.7 were for peptide 278–291 of NPM (Xcorr values of 2.14 and 1.5, respectively). Interestingly, among these six potential PKR-interacting proteins, five are involved in protein synthesis.2

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Association of Endogenous PKR and NPM in MEFs and Human Cell Lines—Given that binding of NPM to dsRNA increased after treatment of normal lymphoblasts with IFNγ and TNFα and that ground state and cytokine induced PKR activation is higher than normal in FA-C lymphoblasts, (Ref. 20; see below), we reasoned that interaction of NPM with PKR might modulate PKR activation or activity. We first confirmed that NPM and PKR co-immunoprecipitated in lysates of MEF from wild-type (PKR+/+) but not from PKR−/− cells (27) (Fig. 2A). Treatment of the cells with dsRNA slightly increased the association of NPM with PKR (compare lane 5 with lane 6). Since NPM binds both RNA and DNA (31), we sought to assure that the NPM-PKR interaction was direct and not dependent upon the presence of nucleic acids by comparing the interaction between NPM and PKR in untreated and RNase- or DNase-treated lysates. The association of these two proteins was influenced by treatment with neither enzyme (Fig. 2B, lanes 3–8).

To determine whether the interaction of NPM with PKR occurred in other cell types, we performed coimmunoprecipitation using lysates from HeLa, a normal human foreskin fibroblast line NFF-6, and the myeloid leukemic cell lines MØ7e and K562. Association of NPM with PKR could be detected in all these cell types with particularly strong interactions observed in the leukemic cell lines MØ7e and K562 cells (Fig. 2C). To demonstrate the specificity of the anti-PKR antibody used in co-immunoprecipitation experiments, we used purified recombinant GST-PKR and NPM proteins in an anti-PKR immunoprecipitation experiment in which the potential capacity of the anti-PKR antibody to bind to NPM directly has been ruled out. Specifically, the anti-PKR antibody did not precipitate NPM when purified recombinant NPM was incubated with the PKR antibody (Fig. 2D, lane 6), thus excluding the possibility that the PKR antibody interacts with NPM.

NPM Interacts with PKR in Vitro—We performed in vitro binding assays using 35S-labeled proteins synthesized in a wheat germ-coupled transcription/translation system. Labeled PKR was coimmunoprecipitated by anti-NPM antibody when unlabeled NPM was present (Fig. 3A, lane 4). Conversely, NPM co-precipitated with PKR using an anti-PKR antibody in the presence of unlabeled PKR (lane 6). Increased amounts of dsRNA did not influence the interaction between the two proteins (lanes 7–9).

To test whether dsRNA binding was required for the interaction, RNA binding-defective mutants of both proteins were used. It is known that PKR encodes two dsRNA-binding motifs (DRBM) in its N terminus while the kinase domain (KD) is located in the C terminus (6, 32, 33). We constructed two RNA binding-defective mutants of PKR, PKRΔDRBM (deletion of both DRBMs) and PKRΔKD (three point mutations in the first DRBM; Ref. 34), and constructed a kinase-defective mutant, PKRΔKD. These mutant proteins were synthesized in vitro and labeled with 35S, mixed with unlabeled full-length protein and co-immunoprecipitated with antibody to the unlabeled protein. Fig. 3B shows that PKRΔDRBM (lane 7) and PKRΔKD (lane 9) retained the ability to interact with NPM as well as the kinase-defective mutant PKRΔKD (lane 8). In contrast, two truncated NPM constructs NPMΔRBD and NPMΔN that are defective in RNA binding and oligomerization (35), respectively, lost their ability to bind PKR (lanes 15 and 16).

NPM Is a Substrate of PKR Phosphorylation—We next determined whether NPM is a substrate for PKR. We performed in vitro kinase assays using bacterially expressed recombinant PKR and NPM proteins fused to glutathione S-transferase (GST). In Fig. 4, the presence of dsRNA induced phosphorylation of PKR but not NPM, and NPM was phosphorylated by PKR in a dsRNA-dependent manner (upper panel). The lower panel shows the relative amounts of the recombinant proteins that were included in the kinase reactions.

Inhibition of PKR by NPM in Vitro and in Vivo—To assess the effect of NPM on PKR activity, we performed two series of in vitro kinase assays to determine whether: 1) NPM inhibits phosphorylation of PKR; 2) NPM reduces the kinase activity of PKR. In the first assay, we incubated recombinant GST-PKR with increasing amounts of NPM in the presence of dsRNA and 32P-labeled proteins. As shown in Fig. 5A, even in the absence of activator dsRNA, recombinant GST-PKR was slightly phosphorylated (lane 1), suggesting that a small part of the bacterially expressed PKR had been activated. As expected, phosphorylation of PKR was greatly enhanced in the presence of dsRNA (lane 2), and, most importantly, was partially inhibited by increasing amounts of recombinant NPM (lanes 5–9).
Additional text: Nucleophosmin Inhibits PKR

The relative amounts of the recombinant GST-PKR and GST-NPM proteins included in the kinase reactions are shown in the middle and bottom panels, respectively. This inhibition of PKR phosphorylation was coincident with phosphorylation of NPM.

A second series of kinase assays was carried out in which recombinant eIF-2α was added to the reaction. The results (Fig. 5B) show that eIF-2α was phosphorylated by activated PKR (lane 5), but that no eIF-2α phosphorylation occurred in the reaction mixtures containing eIF-2α only (lane 3) or eIF-2α plus NPM (lane 6). Like its effect on PKR phosphorylation, NPM inhibited eIF-2α phosphorylation in a dose-dependent manner (lanes 7–9).

To confirm the biological effects of NPM-PKR interaction in vivo, we transiently co-transfected PKR nullizygous (PKR<sup>−/−</sup>) MEF with a human PKR plasmid and increasing amounts of a plasmid encoding FLAG-tagged NPM, along with a pEGFP plasmid that served as an internal control for transfection efficiency. The transfected cells were subsequently treated with dsRNA followed by detection for the exogenously expressed proteins, and quantification of PKR and eIF2α phosphorylation, the rates of protein synthesis, and cell survival. As shown in the top panel of Fig. 6A, the correct FLAG-tagged PKR (lanes 3 and 4) and NPM (lanes 5 and 6) were expressed in the corresponding transfecants but not in those transfected with the empty vectors (lanes 1 and 2). Co-transfection of PKR (1 µg) and increasing amounts of NPM (0.5–10 µg) cDNA resulted in expression of both proteins in a dose-dependent manner (lanes 7–10). The addition of dsRNA had no effect on the expression of these proteins (lanes 4 and 6–10).

To examine the effect of NPM overexpression on activation of PKR in response to dsRNA, we labeled transfectants with <sup>32</sup>Porthophosphate and measured levels of <sup>32</sup>P-labeled PKR immunoprecipitated with a monoclonal PKR antibody. (PKR<sup>−/−</sup>) cells transfected with vector alone contained neither phosphorylated (P-PKR) nor total PKR (Fig. 6B, lanes 1 and 2). This is expected because these cells are devoid of PKR (27). In contrast, PKR transfecants were highly responsive to dsRNA treatments, as evidenced by the dense phosphorylated (P−)
PKR band (lane 4). Co-expression of NPM led to a dose-dependent decrease of phosphorylated PKR (lanes 7–10).

To further substantiate the idea that overexpression of NPM inhibits PKR activation, we examined the phosphorylation of eIF2α in cell extracts from the transfectants with a phosphospecific antibody to phosphoserine 51 of eIF2α. Consistent with the levels of PKR phosphorylation (Fig. 6C), increasing expression of NPM resulted in decreasing phosphorylation of eIF2α (lanes 7–10). Phosphorylation of eIF2α was PKR-dependent, as no eIF2α phosphorylation was detected in cells transfected with the empty vector or plasmids expressing only NPM (lanes 1, 2, 5, and 6). Re-probing the same immunoblot with anti-eIF2α revealed that all samples contained similar amounts of total eIF2α proteins. The data reveal that overexpression of NPM suppresses the activation of PKR.

Measurement of global protein synthesis rate and cell survival confirmed the indirect effect of NPM on eIF2α activity. MEF transfectants were treated with dsRNA and metabolically labeled with [35S]methionine-[35S]cysteine. General translation was measured as a function of [35S]methionine-[35S]cysteine incorporation into trichloroacetic acid-precipitated proteins. Protein synthesis was significantly reduced by dsRNA treatment in PKR transfectants compared with cells without the treatment (Fig. 6D). That [35S] incorporation in NPM-expressing cells was similar to that of control cells (carrying only the empty vector) even in the face of dsRNA treatment indicates that reduction in the rates of protein synthesis is the direct result of PKR activation. Increasing expression of NPM progressively restored protein synthesis, confirming that NPM expression relieves translational inhibition induced by PKR. We also found that overexpression of NPM increased survival of PKR transfectants treated with dsRNA (data not shown).

**Enforced Expression of NPM Inhibits PKR-mediated Apoptosis of Fanconi Anemia Lymphoblasts**—The observation that NPM overexpression led to inhibition of PKR activity and cell survival in MEF prompted us to examine its capacity to suppress aberrant PKR activation in human cells. To this end, the availability of the Fanconi anemia C (FA-C) patient-derived lymphoblast line (HSC536N) provided us a unique opportunity, as the HSC536N lymphoblasts are hypersensitive to IFN-γ and TNFα (36) and express extremely low levels of NPM (see below). We enforced NPM expression by retroviral gene transfer in the HSC536N lymphoblasts. As shown in Fig. 7A, NPM level was significantly lower in FA-C mutant HSC536N cells than in the corresponding normal (JY) and corrected (HSC536N/FANCC) cells (compare lane 1 with lanes 2 and 4). Retroviral expression increased NPM levels moderately in HSC536N cells (HSC536N/NPM; lane 3). Flow cytometric analysis of IFN-γ- and TNFα-mediated apoptosis demonstrated that enforced expression of NPM reduced apoptotic NSC536N cells by ~60% (Table I). Since PKR in FANCC mutant cells is constitutively activated and hyperactivatable by cytokines (20) we asked whether inhibition of apoptosis by NPM expression was due to suppression of PKR activation in cytokine-exposed (2, 37) cells. Indeed, expression of NPM in HSC536N cells led to a decrease in PKR phosphorylation, both in the ground state and after treatment with IFN-γ and TNFα (Fig. 7B, compare lanes 1–3 with lanes 7–9). These data are consistent with the notion that NPM modulates stress-induced apoptosis and promotes cell survival through suppression of the indicated plasmids, treated with [●] or without [□] dsRNA, and labeled in a methionine-cysteine-free medium containing 50 μCi/ml of [35S]methionine-cysteine labeling mix for 80 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. Values represent means ± S.D. of two independent experiments.
Fig. 7. NPM expression reduces PKR-mediated apoptosis in Fanconi anemia lymphoblasts. A. levels of NPM and PKR expression in Fanconi anemia lymphoblast line HSC536N (lane 1), HSC536N cells transduced with normal FANCC cDNA (lane 2), HSC536N cells transduced with the NPM cDNA (lane 3), and a normal lymphoblast line (lane 4). Whole cell extracts containing 100 μg of total proteins each lane were analyzed by direct immunoblotting using antibodies specific for NPM (upper) or PKR proteins (lower). B. in vivo phosphorylation of PKR in lymphoblast lines HSC536N, HSC536N/FANCC, and HSC536N/NPM. Cells were labeled, postincubation with IFN-γ (10 ng/ml) for an additional 3 h. Cells were lysed and equal amounts of whole cell extracts (500 μg of total proteins each sample) were immunoprecipitated with antibody specific for PKR. [32P] phosphate-incorporated PKR immunocomplexes were analyzed by SDS-PAGE followed by autoradiography (upper), or immunoblotted with anti-PKR for total PKR protein (lower). P-PKR refers to phosphorylated PKR.

PKR activation and reinforce the essential apoptotic role played by PKR in the FA-C phenotype.

DISCUSSION

NPM is a multifunctional nucleolar protein highly expressed in tumor cells, stem cells (stemcell.princeton.edu) and proliferating cells (22, 23), and has been proposed as a tumor marker for human ovarian (24), prostate (25), and gastric (26) cancers. Conversely, NPM expression is suppressed in cells undergoing differentiation or apoptosis and in malignant cells induced to undergo growth arrest or programmed cell death (38–40). In addition, repression of NPM expression by antisense strategies potentiated drug-induced apoptosis in HL-60 leukemic cells (41, 42).

PKR serves as a negative regulator of translational control and thus plays roles in regulation of cell proliferation and apoptosis (1, 2). The present study demonstrates that NPM is a cellular PKR inhibitor and suggests that its capacity to influence survival and proliferation of malignant cells (22–26, 38–40) reflects, at least in part, its function as a suppressor of PKR-induced apoptosis. The results reported herein derived from both in vitro and in vivo studies suggest that NPM suppresses apoptosis through inhibition of PKR autophosphorylation. Specifically, using in vitro kinase assays NPM inhibited PKR autophosphorylation in a dose-dependent manner (Fig. 5), an effect confirmed in vivo by co-expressing the two proteins in PKR nullzygous MEF and observing that overexpression of NPM not only suppressed PKR activation, but it enhanced protein synthesis and increased fractional cell survival (Fig. 6, data not shown) as well. The mechanism by which NPM inhibits PKR autophosphorylation is not yet clear. NPM has been shown to contain molecular chaperone activities (43); therefore, by interacting with PKR, NPM could alter the conformation of the kinase and prevent it from dimerizing, a prerequisite for PKR autophosphorylation. Alternatively, high levels of NPM may provide a nucleation site for Hsp70 on the PKR molecule. Under normal circumstances, Hsp70 requires FANCC to bind to PKR (44).

Reduced expression of NPM (Fig. 7A) may be a feature of hematopoietic cells from patients with Fanconi anemia (FA), at least of the C complementation group. This hematopoietic disorder is characterized by bone marrow failure and high relative risk of myelogenous leukemia (45). Suppression of NPM correlates with the typical phenotype of FA hematopoietic cells, that is, cellular hypersensitivity to apoptosis induced by mitogenic inhibitors IFNγ and TNFα (Ref. 36; Table I) through PKR activation (20). It is important to emphasize that the apoptotic phenotype in FANCC mutant cells depends entirely on high level PKR activation and can be completely suppressed in FANCC mutant cells by enforcing expression of a dominant negative mutant of PKR (20). Moderate elevation of NPM expression (by retroviral gene transfer) rescued the mutant cells from apoptosis induced by IFNγ and TNFα (Table I) at least in part, by suppression of PKR activation (Fig. 7B).

It is interesting to note that among the six proteins we obtained with anti-PKR immunoprecipitation and mass spectrometric analysis, five, including NPM, are known to be involved in protein synthesis (Fig. 1C). PKR and the PKR-like kinase PERK have been implicated as important pathogenic factors in the pathogenesis of Alzheimer’s, Parkinson’s, and Huntington’s diseases (16–19). Two other proteins functioning in protein synthesis, the ribosomal protein S19 (RPS19) and dyskenin encoded by DKC1, are associated with Diamond-
Blackfan anemia and Dyskeratosis Congenita, both diseases with increased incidence of hypoplastic anemia (46–48). This indicates that proteins involved in translational control play important roles in maintaining survival of hematopoietic progenitor cells.

We also found that the level of NPM is elevated in the myeloid leukemia (UoC-M1) cells, which have retained a FA phenotype but have lost the capacity to undergo apoptosis. (Ref. 49 and data not shown). At this time, it is not clear whether these extreme elevations of NPM expression represent a cause of transformation or an effect of other molecular leukemogenic events. Taking into account the critical role of PKR in effecting excessive apoptosis in FA cells, we argue that one mechanism by which preleukemic stem cells evolve, particularly in FA-C cells, may be to increase expression or activation state of NPM. That UoC-M1 cells exhibit FA-like defects but have high level NPM expression supports this notion. Whether enhanced NPM expression plays a primary role in leukemogenic events. Taking into account the critical role of PKR (Ref. 49 and data not shown). At this time, it is not clear whether these extreme elevations of NPM expression represent a cause of transformation or an effect of other molecular leukemogenic events. Taking into account the critical role of PKR in effecting excessive apoptosis in FA cells, we argue that one mechanism by which preleukemic stem cells evolve, particularly in FA-C cells, may be to increase expression or activation state of NPM. That UoC-M1 cells exhibit FA-like defects but have high level NPM expression supports this notion. Whether enhanced NPM expression plays a primary role in leukemogenic events.
Nucleophosmin Interacts with and Inhibits the Catalytic Function of Eukaryotic Initiation Factor 2 Kinase PKR
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