Discriminating self from nonself is crucial to innate immunity that employs immune receptors to sense molecular patterns associated with pathogens [1]. Human PKR is a serine/threonine kinase induced by interferons (IFNs) and characterized by a conserved kinase domain in the C-terminal region of the protein and two double stranded RNA (dsRNA) binding domains (dsRBDs) localized to the N-terminal regulatory region [2-4]. Binding of viral dsRNA to the dsRBDs causes PKR to undergo autophosphorylation at multiple kinase residues and subsequent activation [5-7]. The alpha subunit of eukaryotic initiation factor 2 (eIF2α) is the best characterized cellular substrate that is phosphorylated by PKR. This post-translational modification results in inhibition of cellular protein synthesis [8, 9]. In addition to its function as a critical regulator of translation, we have previously implicated PKR in transcriptional signaling of NFκB and IRF1 and identified the IkB as a novel substrate for PKR [10, 11].

Recently, it has been shown that natural bacterial RNA can induce secretion of proinflammatory cytokines and type I IFN in TLR7-dependent manner. However the endosomal TLR7 can also detect viral single stranded RNA (ssRNA) [12-17]. Furthermore, recent investigations have also identified the NALP3 inflammasome as an additional cytosolic mechanism involved in the recognition of different patterns of RNA species including RNA from bacterial and viral origins and poly I:C [18, 19]. Similarly, the RNA helicase RIG-I was first proposed as a specific sensor for dsRNA, however, subsequent studies identified the 5’-triphosphate RNA as a ligand for RIG-I [20, 21]. It is widely accepted that PKR recognizes viral dsRNA as nonself because it is normally absent in mammalian cells. However, it has been recently reported that in vitro transcribed RNA with 5’-triphosphate end, which is characteristic of bacterial transcripts, is a ligand for PKR [22]. Although long dsRNA was first pro-
posed as a ligand for PKR, a diverse range of biological RNA patterns with various structural elements including bulges, stem-loops, and internal loops can also regulate the kinase activity of PKR [23-29]. These observations emphasized a unifying theme in innate immunity whereby the host could use the same immunoreceptor to detect various subspecies of foreign RNA. Although bacterial RNA shares certain structural and signaling features with viral RNA, regulation of PKR signaling by natural RNA derived from bacterial origin has not been previously reported.

JAK/STAT signaling plays central roles in regulating diverse biological responses including cellular immunity, proliferation, apoptosis, and development. To exert their biological effects on targeted cells, IFNs bind to their receptors which results in the activation of intrinsic receptor tyrosine-kinase activity or receptor-associated kinases such as the JAK family of kinases [30]. The activated JAKs in turn phosphorylate specific tyrosine motifs in receptor endodomains to recruit specific monomeric STATs [31]. Type I IFNs phosphorylates STAT1 and STAT2, which form IFN-stimulated gene factor-3 (ISGF-3), a ternary complex that also includes IFN response factor-9 (IRF9). This multimeric transcription factor translocates into the nucleus and regulates gene expression by binding to interferon stimulated response element (ISRE) in the promoters of regulated genes [32, 33]. IFN-γ induces the formation of STAT1 homodimers which in turn translocates into the nucleus, where they bind to distinct γ-activated sequence (GAS) in the promoters of target genes.

Many genes that are activated by viral dsRNA can also be induced by IFNs including the PKR. Although much progress has been made in identifying the signaling pathways that mediate the initial responses to IFNs and viral dsRNA, the role that bacterial RNA plays in regulating these responses has remained largely unknown. Herein, we identified the bacterial RNA as a novel activator of PKR and provided a link between IFN signaling and RNA-dependent PKR activation.

Materials and methods

Cell lines and treatments

The human fibrosarcoma cell line 2fTGH was derived from HT1080 cells. The parental 2fTGH cells and its deficient variants: U1A (TYK2-/-), U4A (JAK1-/-), and γ2A (JAK2 -/-) were previously described [34-36]. U1AR, U4AR, and γ2AR cells are U1A, U4A and γ2A cells restored with TYK2, JAK1, and JAK2 respectively by stable transfection with expression vectors. Cells were grown to about 70% confluency in DMEM supplemented with 10% fetal bovine serum and 1% of penicillin/streptomycin and then the growth medium was replaced with unsupplemented base DMEM for 18 h before treatment. Synthetic dsRNA (poly I:C) was purchased from Sigma Chemical Co. and resuspended in sterile distilled H₂O. Approximately 2 x 10⁶ cells were stimulated with 100 µg/ml natural or synthetic RNAs to test their potential to regulate activation of PKR, and JAK/STAT signaling.

Isolation of bacterial RNA

Pathogenic isolates of E. coli (O18:K1:H7; ATCC #700973) were used for this study. Hot phenol combined with enzymatic lysis was carried out to isolate bacterial total RNA. Exponentially growing bacteria were harvested and resuspended in a lysis buffer of TE and 2 mg/ml lysozyme (Sigma Aldrich, St.Louis, USA). Samples were brought to 2% SDS and water-saturated phenol was added and incubated for 5 minutes at 64ºC. Following centrifugation, the aqueous phase was transferred and equal amounts of chloroform were added. The RNA was precipitated by 0.1 vol 3M sodium acetate and 2.5 vol ethanol. The RNA pellets were washed and resuspended in DEPC-treated water. The integrity and purity of RNA species were determined by electrophoresis on agarose and polyacrylamide gels and A260/A280 ratios. Extracted crude RNA was treated with RNase-free DNase I (Roche, Indianapolis, USA) to remove contaminant DNA and further purified by the Qiagen method (Qiagen, Valencia, USA) as per the manufacturer’s instructions. Aliquots of RNA samples were incubated in the presence of a heterogeneous mixture of ribonucleases [(1 U per 2 µl of RNA at 1 µg/µl for 1 h) (Roche, Indianapolis, IN)] as described [37] and analyzed by denaturing agarose or polyacrylamide gel electrophoresis for quality assurance.

Calf intestinal alkaline phosphatase (CIP) treatment of RNA

Dephosphorylation was performed as described
JAK kinases phosphorylate PKR [20] with few modifications: 1 μg bacterial RNA was treated with 30 U of CIP (Roche) in a dephosphorylation buffer (50 mM Tris·HCl, pH8.5/0.1 mM EDTA) for 2 h at 37°C in the presence of 10 U of RNase inhibitor (RNaseOut, Invitrogen). Following CIP treatment, bacterial RNA was purified by the Qiagen method as described above. The dephosphorylated RNA was used to treat the cells.

**Immunoprecipitation and immunoblotting**

Cells were challenged with RNAs to test their ability to induce activation of certain endogenous proteins. The treated and non-treated cells were washed three times with cold PBS, and then pellets were resuspended in RIPA lysis buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) plus a protease inhibitor mixture and incubated on ice. For Immunoprecipitation, 500 μg of the protein extracts were first precleared with the addition of 50 μl of 50% protein G-Sepharose beads (Santa Cruz) for 1 h at 4°C under rotation and recovered. The supernatants were incubated with 5 μl of antibodies specific for the targeted proteins for 30 min on ice and then 50 μl of 50% suspension of protein G-beads in RIPA were added, and incubation was continued overnight with rotation. The beads were washed twice with RIPA, collected, and immunoprecipitates were subjected to electrophoresis. For detection of tyrosine phosphorylated proteins, membranes were probed with the anti-phosphotyrosine monoclonal antibodies PY20 (Signal Transduction Laboratories) and 4G10 (Upstate Biotechnology Incorporated). For Western blot analysis, cellular lysates were resolved by SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked and subjected to immunoblotting analysis using antibodies specific for, JAK1, JAK2, TYK2, PKR, eIF2α, phosphoSer eIF2α (Cell Signaling), while phosphoThr 446 PKR was from Invitrogen. When required, membranes were stripped of previous antibody and reprobed for total protein forms or co-immunoprecipitation experiments.

**Results**

Bacterial RNA shares certain structural and signaling features with viral RNA and can serve as a PAMP. Because tyrosine phosphorylation is the major determinant of activation of JAK family kinases, we first examined the ability of bacterial RNA to activate endogenous JAKs. The 2TGH cells were left untreated or treated with bacterial RNA and then TYK2, JAK1, or JAK2 proteins were immunoprecipitated from these cells. Immunoblotting analyses with phosphotyrosine specific antibody shows that purified TYK2, JAK1, and JAK2 were efficiently tyrosine phosphorylated by bacterial RNA in the parental 2TGH cells (Figure 1A-C, lanes 3 and 4). The amount of phosphotyrosine JAK proteins were reduced when the cells were treated with di-
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Binding of viral dsRNA causes PKR to undergo autophosphorylation at multiple kinase residues which is essential for optimal PKR activation and eIF2α docking. To investigate tyrosine phosphorylation, we treated the parental HT1080 and 2fTGH cells with bacterial RNA and then immunoprecipitated PKR from these cells. Immunoblotting analyses of PKR with phosphotyrosine specific antibodies indicated that bacterial RNA was able to induce tyrosine phosphorylation of endogenous PKR (Figure 2A and B, lane 3). The efficiency of bacterial RNA to induce tyrosine phosphorylation of PKR was comparable to that induced by poly I:C (Figure 2A and B, lane 5). The amount of tyrosine phosphorylated PKR protein was greatly reduced in the cells treated with digested bacterial RNA (Figure 2A and B, lane 2). To investigate whether the tyrosine phosphorylation of PKR induced by bacterial RNA requires the JAK kinases, we repeated similar experiments on JAK mutants. Unlike the parental cell lines, all JAK mutant cells U1A, U4A, and γ2A display defects in tyrosine phosphorylation of PKR indicating the essential role of these kinases in mediating the bacterial RNA-induced tyrosine phosphorylation of PKR (Figure 2C-E).

We next evaluated the potency of bacterial RNA to modulate PKR activity in comparison to poly I:C. Western blot analysis shows that stimulation of 2fTGH cells with 100 µg/ml RNA derived from E. coli activated PKR on threonine 446 to an extent comparable to that generated by poly I:C (Figure 3A, top panel, lanes 3 and 4). While the 5'-triphosphate RNA is also characteristic of bacterial mRNA, cellular transcripts acquires a 7'-methylguanosine (7mG) cap structure at its 5'-end [38]. To investigate the dependence of PKR activation on the presence of 5'-triphosphate, we treated bacterial RNA with CIP to eliminate this feature and challenged the cells to examine the endogenous PKR activation. Interestingly, we found no difference in the activation of endogenous PKR and induction of eIF2α by the CIP-treated RNA in comparison to the bacterial RNA that was not treated with CIP or poly I:C (Figure 3B, top and third panels, lanes 2-4). To understand how bacterial RNA activates the PKR kinase, we first subjected the bacterial RNA to digestion with a cocktail of RNase that attacks the double strands secondary structures. We found that the potential of bacterial RNA to activate PKR was sensitive to RNase that removes the base-paired secondary structures indicating the dependence of PKR on this feature as a key for the molecular recognition.
JAK kinases phosphorylate PKR and signaling (Figure 3A, top panel, lane 2). This result also excludes the possibility of the presence of any potential contaminants in the RNA preparations that may activate PKR. To investigate the biological significance of PKR activation by bacterial RNA, we tested whether bacterial RNA could induce the phosphorylation of eIF2α. Immunoblotting experiments revealed enhanced phosphorylation of eIF2α on Ser 51 corresponding to the PKR activation levels (Figure 3A, third panel, lanes 3 and 4). Again, RNase treatment of bacterial RNA resulted in a reduction of the eIF2α phosphorylation (Figure 3A, third panel, lane 2). The 2fTGH cell line was originally derived from HT1080 cells to obtain mutant cell lines defective in interferon signaling [35, 36]. To rule out the possibility that a mutation in another gene was responsible for the effects on PKR, we repeated similar experiments on HT1080 cells. Consistent with our previous observations, we determined that bacterial RNA activates PKR on threonine 446 and induces the eIF2α phosphorylation, while RNase-digested RNA fails to exert these effects (Figure 3C). Although much progress has been made in identifying the PKR signaling pathways that are induced by either dsRNA or IFNs, the role of bacterial RNA in regulating these responses has not been reported. To determine the participation of different JAK/STAT signaling components in bacterial RNA-induced PKR activation, we treated mutant cell lines lacking a single JAK protein with natural RNA derived from E. coli.

Unlike the parental 2fTGH and HT1080 cells, TYK2 mutants (U1A) cells were defective in PKR activation induced by bacterial RNA (Figure 4A, top panel). To test the impact of TYK2 on PKR function, we examined the status of bacterial RNA induced eIF2α phosphorylation in U1A cells. Western blot analysis reveals that eIF2α phosphorylation was also impaired in U1A cells corresponding to deficiency in PKR activation (Figure 4A, third panel). The defects in PKR activation and eIF2α phosphorylation in U1A cells were reversed by transfecting these cells with full-length cDNA for TYK2 (U1A-R) excluding the possibility that a mutation in another gene was responsible for these effects (Figure 4B, top and third panels, lanes 3-4). Again, RNase-digestion of bacterial RNA failed to induce PKR activation and eIF2α phosphorylation confirming that bacterial RNA is the actual inducer of these effects (Figure 4B, top and third panels, lane 2). We next repeated similar experiments on cell lines lacking JAK1 (U4A), or JAK2 (γ2A). Interestingly, we found that PKR activation and eIF2α phos-
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Figure 4. JAK mutants are defective in PKR activation and eIF2α phosphorylation induced by bacterial RNA. Mutant cell lines lacking TYK2 (A), JAK1 (C), or JAK2 (E) cells were not treated (NT) or treated with 100 µg/ml RNase-digested E. coli RNA (DERNA), intact E. coli RNA (ERNA), or synthetic analogue to viral dsRNA (poly I:C, as a positive control). Cell lysates were separated by SDS-PAGE and probed with antibodies to phospho-Thr446 on PKR (top panel) or phospho-Ser51 on eIF2α (third panel). Membranes were stripped and reprobed with antibodies to total PKR (second panel) or total eIF2α (bottom panel). Similar experiments were repeated with the rescue cells: (B) U1A cells stably transfected with TYK2 cDNA (U1A-R), (D) U4A cells stably transfected with JAK1 cDNA (U4A-R) and (F) γ2A cells stably transfected with JAK2 cDNA (γ2A-R).

Phosphorylation induced by bacterial RNA were also impaired in both JAK1 and JAK2 mutants (Figure 4C and E). Reconstitution of these mutants with JAK1 and JAK2 cDNAs restores the PKR activation and induction of eIF2α phosphorylation suggesting the non-redundant role of these kinases in PKR activation (Figure 4D and F). These data demonstrated the requirement of JAKs for PKR activation and implicated JAK kinases in translational control of PKR. Since the JAK mutants are also defective in IFN signaling, the above observations suggested that bacterial RNA may use or depend on components common to IFN signaling to activate PKR. Thus, the patterns of bacterial RNA-induced tyrosine phosphorylation of JAKs (Figure 1A-C) were similar to those of PKR activation on threonine 446 (Figure 3A, top panel). We have noticed that bacterial RNA-induced endogenous PKR phosphorylation on threonine 446 occurred under the same conditions that induced phosphorylation of tyrosine (Figure 2A and B compared to Figure 3A and D). Furthermore, bacterial RNA-dependent phosphorylation of PKR on either threonine 446 or tyrosine requires the JAK kinases suggesting the positive impact of tyrosine phosphorylation on PKR activation.

These observations implicate JAK activation with tyrosine phosphorylation of PKR. However, whether PKR is a direct substrate for activated JAK kinases remains undetermined. To test this, we first investigated why activation of PKR by bacterial RNA depends on the JAK kinases. We examined the possibility of physical association between endogenous PKR and JAKs. Co-immunoprecipitation experiments performed on 2TGH cells stimulated with or without bacterial RNA show that PKR interacts with JAKs in non-
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Discussion

Although type I IFNs that act locally and systemically to induce cellular responses are generally considered as antiviral cytokines, recent investigations have indicated that detection of bacterial RNA by TLRs and the cytosolic RNA sensors can also trigger the production of IFNs [12, 16, 39]. IFNs exert their biological effects on target cells through the utilization of specific JAKs and tyrosine phosphorylation of STAT proteins. However, the role of JAK/STAT in bacterial RNA signaling and whether this pathway regulates PKR activity has not been described. In this study, we show that there is a positive impact of tyrosine phosphorylation on PKR activation. We also show that bacterial RNA is a novel activator of JAKs and demonstrated the requirement of JAKs for PKR activation and eIF2α phosphorylation and thus implicated JAK kinases in translational control of PKR. Although STAT proteins are the best characterized substrates for JAKs, we identified the eIF2α kinase, PKR as an additional substrate for JAKs. These data provide new insights into the substrate recognition properties of the JAKs and suggest the potential of alternative substrates for JAKs in cellular signaling and bacterial RNA immunity. Binding of dsRNA to PKR is thought to cause conformational changes that allow PKR to autophosphorylate, therefore switching its substrate specificity. It has been previously reported that the bacterial and yeast eIF2α kinases PKR and HRI can phosphorylate eIF2α even when serine 51 is mutated to Tyr at residue 51 in vivo [40]. Tyrosine phosphorylation is essential for efficient dsRNA-binding, dimerization, in addition it is needed for optimal threonine phosphorylation and activation of PKR. Biologically, tyrosine phosphorylation of PKR was shown to mediate the antiviral and antiproliferative properties of the kinase [6]. Therefore, bacterial RNA-induced PKR tyrosine phosphorylation presents an important link between IFN signaling and translational regulation through the eIF2α phosphorylation. In regard to alternative substrate recognition and biological roles of PKR, the identification of tyrosine phosphorylation of PKR is important. Although it is thought that the primary function of PKR is the regulation of translation via phosphorylation of eIF2α, we have previously identified the IκB as an additional substrate for PKR and implicated PKR in transcriptional control [10]. We also show that mice devoid of functional PKR are impaired in cell signaling pathways including IFN- and dsRNA-induced antiviral responses and thus PKR is a signal transducer [11, 41].

Herein, we show that natural RNA derived from bacterial origin is a novel activator of PKR. Sev-
eral lines of investigation have supported the idea that immunoreceptors sense bacterial RNA while ignoring the endogenous RNA. For example, it has been shown that bacterial RNA activates human dendritic cells (DCs) to secrete IL-12, TNF-α and other cytokines in a TLR-dependent manner, while mammalian RNA failed to exert such immunostimulatory potential [16]. It was also observed that bacterial but not murine RNA, induced IL-1β and IL-18 secretion from macrophages in a Nalp3-dependent fashion [19]. We next determined that bacterial RNA has structural features involved in the activation of PKR and thus PKR is a molecular sensor activated in the presence bacterial RNA. We studied the bacterial RNA modification requirements for endogenous PKR signaling. We concluded that among the other structural elements contained in bacterial RNA, double strandedness is a major element that is required for activation of PKR.

In addition to TLRs as key sensors of pathogens at the cell surface or phagocytic vacuole, recent studies have identified cytosolic surveillance pathways that specifically distinguish intracellular from extracellular bacterial pathogens. Cytosolic recognition of pathogenic bacteria can activate signaling pathways and induces specific gene expression program [42, 43]. Bacterial ligands including RNA activate cytosolic innate immune receptors by ligands generated in the phagosome and transported to the cytosol or secreted in the cytosol of immunostimulatory bacterial ligands [39, 44, 45]. In parallel to our findings, recent studies have shown that TLR7 and RIG-1 pathways which are considered exclusively as viral RNA sensors, detect bacterial RNA ligands released from ingested bacteria [12, 39, 46]. We propose that bacterial RNA generated in the phagosome or derived from lysed bacteria could be transported into the host cytosol and leads to PKR activation. In addition to bacterial cell wall components and toxins which proposed as the principal PAMPs, recent studies have indicated that both purified bacterial nucleic acids and synthetic oligonucleotides can also induce the production of inflammatory cytokines, reactive oxygen/nitrogen species, and thus implicated in a range of human diseases [47-49]. We have previously demonstrated that bacterial RNA derived from S. aureus and E. coli depress myocardial functions and suggested the contribution of bacterial RNA to cardiac depression during bacterial sepsis [37]. We also reported that human sera derived from septic shock patients exposed to bacterial infections induces apoptosis, activates transcriptional factors STAT, NfκB, IRF1, and differentially regulates cardiac gene expression [50-52].

In conclusion, we identified an unexpected role for the viral RNA-sensing PKR in recognition of bacterial RNA. Tyrosine phosphorylation of PKR provides new insights into the substrate recognition for JAKs and links JAK/STAT signaling to translational control of PKR. These observations provide novel insights on bacterial RNA signaling and the molecular mechanisms by which the immune system detects bacterial infection.

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