Leukemia virus long terminal repeat activates NFκB pathway by a TLR3-dependent mechanism

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Abstract

The long terminal repeat (LTR) region of leukemia viruses plays a critical role in tissue tropism and pathogenic potential of the viruses. We have previously reported that U3-LTR from Moloney murine and feline leukemia viruses (Mo-MuLV and FeLV) upregulates specific cellular genes in trans in an integration-independent way. The U3-LTR region necessary for this action does not encode a protein but instead makes a specific RNA transcript. Because several cellular genes transactivated by the U3-LTR can also be activated by NFκB, and because the antiapoptotic and growth promoting activities of NFκB have been implicated in leukemogenesis, we investigated whether FeLV U3-LTR can activate NFκB signaling. Here, we demonstrate that FeLV U3-LTR indeed upregulates the NFκB signaling pathway via activation of Ras-Raf-IκB kinase (IKK) and degradation of IκB. LTR-mediated transcriptional activation of genes did not require new protein synthesis suggesting an active role of the LTR transcript in the process. Using Toll-like receptor (TLR) deficient HEK293 cells and PKR−/− mouse embryo fibroblasts, we further demonstrate that although dsRNA-activated protein kinase R (PKR) is not necessary, TLR3 is required for the activation of NFκB by the LTR. Our study thus demonstrates involvement of a TLR3-dependent but PKR-independent dsRNA-mediated signaling pathway for NFκB activation and thus provides a new mechanistic explanation of LTR-mediated cellular gene transactivation.

Keywords: Leukemia virus; LTR; Transactivation; NFκB; TLR3

Introduction

The long terminal repeat (LTR) region of nontransforming retroviruses such as leukemia viruses plays a critical role in their replication and pathogenesis (DesGroseillers and Jolicoeur, 1984; Fan, 1990; Lenz et al., 1984). These repeat regions (U3-R-U5) are generated during reverse transcription of the viral genome and are present at both ends of the proviral sequence integrated in the host genome. The U3-LTR region of leukemia viruses contains multiple transcription factor binding sites that provide tissue-specific expression capacity to the virus (Golemis et al., 1989; Short et al., 1987). Further, these transcription factor-binding sites can also enhance the transcriptional activity of cellular genes adjacent to the site of integration of these viruses (Hayward et al., 1981). Activation of a proto-oncogene adjacent to the site of integration can result in abnormal expression of the gene and may ultimately lead to tumorigenesis. Insertional activation of proto-oncogenes c-myc, pim-1, pvt-1, fvi-2, int-1/vent-1 and others have been reported in various leukemia virus pathogenesis (Fan, 1997; Morrison et al., 1995; Selten et al., 1985).

Long before tumor development, a state of preleukemic hyperplasia is seen in the leukemia virus infected animals. It has been suggested that this preleukemic hyperplasia provides more cells susceptible to virus infection and therefore increases the chance of insertional activation of proto-oncogenes (Brightman et al., 1990; Davis et al., 1987). Interestingly, insertion of polyoma virus transcriptional enhancer element PyF101 into the Moloney murine leukemia virus (Mo-MuLV) U3-LTR greatly reduces the preleukemic hyperplasia and the leukemogenic potential of the virus, but does not interfere with normal...
replication of the virus (Brightman et al., 1991, 1993; Davis et al., 1985). This finding suggested that the LTR may have some role in the induction of preleukemic hyperplasia in addition to its activity as an enhancer element. Other studies demonstrated that transient or stable expression of the U3-LTR region of Mo-MuLV or feline leukemia virus (FeLV) in fibroblasts of murine or feline origin, as well as in human lymphoid cell lines, induces expression of specific cellular genes, such as collagenase IV, monocyte chemotactic protein 1 (MCP-1), c-jun and MHC class I (Faller et al., 1997a, 1997b; Ghosh and Faller, 1999; Koka et al., 1991; Wilson et al., 1987). Upregulation of these genes occurs at the level of transcription and is independent of the physical location of the LTR or the responding cellular genes. The LTR from endogenous FeLV, however, which is nonpathogenic, does not possess this transactivation activity (Ghosh et al., 2000). Whether the cellular gene transactivational activity of the LTR is necessary for preleukemic hyperplasia has not been determined.

The mechanism of LTR-mediated transactivation of cellular genes has not been elucidated. We have previously reported that only the U3 region of the LTR is necessary for this transactivational activity and, in case of FeLV, it can be as small as 210 bp (Choi and Faller, 1995; Ghosh and Faller, 1999; Ghosh et al., 2000). The U3-LTR region sufficient for gene transactivational activity does not contain a readable protein-coding frame. A specific RNA transcript for this region has been consistently detected in cells expressing only the U3-LTR as well as cells infected with the full-length virus (Ghosh and Faller, 1999). We have also demonstrated that LTR from nonpathogenic endogenous FeLV, which cannot transactivate cellular genes, does not make LTR-specific transcript (Ghosh et al., 2000). Since the U3-LTR is unable to make a protein product, it is likely that the RNA transcript generated acts as a mediator of cellular gene transactivation.

Double-stranded RNA (dsRNA) is a strong inducer of several signal transduction pathways including activation of the transcription factor NFκB (Geiss et al., 2001; Ghosh et al., 1998; Siebenlist et al., 1994). NFκB is intimately associated with cellular defense mechanisms as it is activated by a variety of inflammatory cytokines, growth factors and stress inducing agents (Karin and Ben-Neriah, 2000; Kopp and Ghosh, 1995). Recent evidences also suggest that NFκB plays an important role in cell survival and antipapoptotic responses (Baldwin, 2001; Yamamoto and Gaynor, 2001). In nonstimulated cells, NFκB exists as a cytoplasmic heterodimeric complex composed mainly of p50 and RelA proteins bound to inhibitory proteins of IκB family. Upon stimulation, the IκB protein is phosphorylated and degraded in the proteasome allowing free NFκB heterodimer to migrate to the nucleus where it binds to its cognate binding sites on the promoters of various target genes and enhances their expression. Because of the diverse range of genes that NFκB can modulate, it also has been an important target molecule for many viruses including tumor-igenic viruses, such as Epstein–Barr virus (EBV), hepatitis C virus (HCV), human T-lymphotropic virus (HTLV) and also human immunodeficiency virus (HIV) (Hiscott et al., 2001; Santoro et al., 2003). Although different viruses use different strategies to control NFκB, many of them converge to the activation of IκB kinase (IKK). Double stranded RNA-dependent protein kinase R (PKR) and some members of the Toll-like receptor (TLR) family (such as TLR3) have been shown to act as an intermediate in the activation of the NFκB pathway by RNA (Alexopoulou et al., 2001; Williams, 1997, 2001).

The ability of the LTR to mediate cellular gene transactivation through the production of an RNA transcript and the observation that many of the cellular genes transactivated by the LTR are known to be NFκB responsive indicated that LTR might activate NFκB signaling. In this study, we investigated such a possibility and report that leukemia virus LTR activates NFκB signaling pathway. We also investigated the role of PKR and RNA-activated TLRs in this process. Using cell lines deficient in either PKR or TLRs, we found that although PKR is dispensable, TLR3 is necessary for LTR-mediated activation of NFκB. Our results thus provide a new insight into the mechanism of leukemia virus LTR-mediated cellular gene activation.

**Results**

**U3-LTR activates NFκB-dependent gene expression**

Numerous reports have demonstrated that the leukemia virus LTR directly influences propagation of the virus through the transcription factor binding sites they contain. There are recent indications that the LTR may also play a role in target lymphocyte proliferation during preleukemic hyperplasia. Our previous studies demonstrated that the LTR can upregulate expression of specific cellular genes, some of which can also be activated by NFκB, through the generation of a noncoding RNA transcript (Ghosh and Faller, 1999; Ghosh et al., 2000). RNA secondary structure analysis predicted that the putative LTR transcript possessed strong secondary structure (data not shown). Since dsRNA is a strong activator of NFκB, we examined whether NFκB signaling is activated in cells that are expressing LTR. We cotransfected NFκB-dependent reporter plasmid NFBCO-CAT with FeLV U3-LTR construct 61E-LTR into Balb-3T3 cells. The NFBCO-CAT construct contains two NFκB binding sites from β-interferon gene adjacent to a minimal β-globin promoter (Richardson and Gilmore, 1991). We compared upregulation of this NFBCO-CAT reporter by the 61E-LTR along with other promoter-reporters we have previously shown to be transactivated by the LTR. In Fig. 1A, we show that the 61E-LTR can activate expression of NFBCO-CAT reporter up to 22-fold. In parallel, we show that the LTR construct can induce expression of reporters containing promoters for genes such as collagenase IV, MCP-1, MHC-I, but not IL-6, thus corroborating our previously published report (Ghosh and Faller, 1999). We next tested whether activation of NFκB-dependent gene expression by the LTR was directly related to increased DNA-binding activity of NFκB protein. To address this issue, in transient transfection assays, we used another reporter construct OBCO-CAT, which is the same as the NFκB-dependent reporter NFBCO-CAT except...
that the two NFkB binding sites are absent (Richardson and Gilmore, 1991). In Fig. 1B, we demonstrate that 61E-LTR can activate NFkB-CAT but not the NFkB binding site-deleted reporter OBCO-CAT suggesting that these sites are necessary for NFkB activation by the LTR. This activation is dose-dependent as the fold induction of NFBCO-CAT increases with increasing amount of 61E-LTR.

We next tested whether LTR can act on other NFkB-dependent reporter constructs. We used two luciferase reporter constructs, 3XKB-Luc (containing three copies of MHC class I \( \kappa \)B element upstream of a minimal \( c-fos \) promoter) and PBIIX-luc (containing two copies of immunoglobulin light chain \( \kappa \)B element upstream of a minimal \( c-fos \) promoter) (Kopp and Ghosh, 1994; Mitchell and Sugden, 1995). As shown in Fig. 1C, both these reporters were also strongly induced by 61E-LTR (7–10-fold). It may be noted, however, that the basal activity with PBIIX-luc reporter was significantly lower and higher amounts of reporter plasmid than that of the 3XKB-luc reporter were necessary to achieve comparable basal activity.

**LTR enhances nuclear translocation of p65**

We determined whether upregulation of NFkB-dependent gene expression by the LTR was due to the increase of NFkB protein in the nucleus. We measured the RelA protein (p65, most common member of NFkB family of proteins) level in the nucleus following expression of LTR. Nuclear extracts were prepared from Balb-3T3 cells at multiple time points following transfection with 61E-LTR or vector plasmid pTZ and were analyzed for the p65/RelA protein by Western immunoblotting. The level of common nuclear protein PCNA in these samples was tested for sample loading control. Fig. 2A demonstrates that p65/RelA protein level in LTR transfected cells increased up to 2.2 times compared to pTZ vector transfected cells. Transfection efficiency in these experiments was approximately 25%. The increase in p65 level could be seen within 2 h post-transfection, reached a peak by 4 h and decreased gradually over time. Transient increase of NFkB level of this nature following virus infection has been previously reported with vesicular stomatitis virus (VSV) and measles virus (MeV) (Boulares et al., 1996; Donze et al., 2004; Helin et al., 2001). The p65 level in pTZ vector transfected cells increased insignificantly during the course of the study. These results show that expression of LTR increased the level of p65/RelA protein in the nucleus.

To test whether NFkB activation by the LTR indeed affects expression of endogenous NFkB-dependent genes, we tested the level of two genes, cyclin D1 and Bcl-2, in 61E-LTR
transfected cells. Cyclin D1 helps progression of cell cycle in G1 phase and Bcl-2 acts as an antiapoptotic protein, both of which are regulated by NFκB (Guttridge et al., 1999; Wang et al., 1998). As shown in Fig. 2B, both cyclin D1 and Bcl-2 levels were increased significantly in LTR expressing cells over backbone vector expressing cells. This result therefore, clearly demonstrates that NFκB activation by the LTR leads to upregulation of endogenous NFκB-dependent genes.

**Full-length FeLV also activates NFκB**

We tested whether LTR would activate NFκB in the context of full-length virus. To address this issue, we first tested whether full-length FeLV orMuLV could activate NFκB. We cotransfected Balb-3T3 cells withNFBCO-CAT and either 61E-LTR or pTZ19 by lipofectamine method at indicated time post-transfection. The time denotes period after the initial 3 h incubation with DNA and lipofectamine. Twenty micrograms of protein for each sample was separated in 10% SDS-PAGE and analyzed for p65 or PCNA by Western immunoblotting. Band intensities were determined directly on the film using LabWorks Image Analysis Program (UVP Inc, Upland, CA). Fold inductions were calculated after the band intensities were normalized for equal PCNA loading.

**Activation of endogenous cyclin D1 and Bcl-2 protein.** Twenty micrograms of whole cell lysates from Balb-3T3 cells transfected with either 61E-LTR or pTZ19 vector was analyzed for cyclin D1 or Bcl-2 level by Western immunoblotting. Lysates were prepared 40 h after transfection. Same blots were further analyzed for β-actin protein following stripping of the antibodies used in first immunoblotting.

![Fig. 2. Translocation of p65 and expression of endogenous NFκB-dependent genes by the LTR.](image)

**Fig. 2. Translocation of p65 and expression of endogenous NFκB-dependent genes by the LTR.** (A) Analysis of nuclear p65 following expression of LTR. Nuclear extracts were prepared from Balb-3T3 cells transfected with 61E-LTR or pTZ19 by lipofectamine method at indicated time post-transfection. The time denotes period after the initial 3 h incubation with DNA and lipofectamine. Twenty micrograms of protein for each sample was separated in 10% SDS-PAGE and analyzed for p65 or PCNA by Western immunoblotting. Band intensities were determined directly on the film using LabWorks Image Analysis Program (UVP Inc, Upland, CA). Fold inductions were calculated after the band intensities were normalized for equal PCNA loading. (B) Activation of endogenous cyclin D1 and Bcl-2 protein. Twenty micrograms of whole cell lysates from Balb-3T3 cells transfected with either 61E-LTR or pTZ19 vector was analyzed for cyclin D1 or Bcl-2 level by Western immunoblotting. Lysates were prepared 40 h after transfection. Same blots were further analyzed for β-actin protein following stripping of the antibodies used in first immunoblotting.

![Fig. 3. Full length FeLV activates NFκB in an LTR-dependent manner.](image)

**Fig. 3. Full length FeLV activates NFκB in an LTR-dependent manner.** (A) Analysis of NFκB activation by full-length and U3-LTR constructs from FeLV and Mo-MuLV. Balb-3T3 cells were cotransfected with 7.5 μg of NFBCO-CAT reporter and 7.5 μg of 61E-LTR (FeLV U3-LTR) or GMNX (Mo-MuLV U3-LTR) or 61E (full-length FeLV) or Mov9 (full-length Mo-MuLV) or pTZ19 vector plasmid by DEAE-dextran method. Cells were harvested 48 h post-transfection for CAT assays as described in Fig. 1. Similar results were obtained in three independent experiments and the fold-induction values for the presented chromatogram are indicated. (B) Analysis of NFκB activation by the full-length FeLV containing mutation in the U3-LTR. Balb-3T3 cells were cotransfected with 100 ng of 3XKB-luc reporter and 300 ng of either wild-type or mutant FeLV U3-LTR constructs (61E-LTR or EDD2, respectively), or wild-type or mutant full-length FeLV constructs (61E or 61E-Mut, respectively). Cells were harvested 48 h post-transfection for luciferase assays as in Fig. 1.
collagenase or MCP-1 genes like the wild-type virus (Abujamra et al., 2003). As shown in Fig. 3B, both the EDD2 and the 61E-Mut were unable to activate NFκB-dependent luciferase reporter gene expression in transient transfection experiment. This result demonstrated that U3-LTR portion of the full-length FeLV is responsible for activation of NFκB-dependent gene expression by the virus.

Proteasomal degradation of the inhibitory protein IκB is necessary for NFκB activation by the LTR

Activation of NFκB-dependent gene expression primarily depends on phosphorylation of inhibitory protein IκB followed by its proteasomal degradation in the cytoplasm and subsequent translocation of free NFκB heterodimer to the nucleus. To determine whether proteasomal activity is necessary for the activation of NFκB-dependent gene expression by the LTR, we used the drug lactacystin that inhibits chymotrypsin- and trypsin-like activities of proteasome (Dick et al., 1997). In transient transfection experiments with 61E-LTR and NFκB-dependent luciferase reporter 3XKB-luc, we tested the effect of 5 μM Lactacystin on reporter activity. As shown in Fig. 4A, lactacystin, but not the DMSO solvent, inhibited induction of luciferase reporter by the LTR significantly by 24 h post-transfection. As a control, we tested upregulation of the same reporter by p65/RelA expression plasmid and the effect of lactacystin on it. Because proteasomal activity is not necessary for p65/RelA-mediated upregulation of 3XKB-Luc, as expected, similar concentrations of lactacystin did not affect reporter activation. This result demonstrated that active proteasome function is necessary for NFκB activation by the LTR.

To determine whether NFκB activation by the LTR actually leads to degradation of the inhibitory protein IκB, we analyzed cytoplasmic IκB-α level over time in 61E-LTR transfected cells. As shown in Fig. 4B, IκB-α level decreased within 1 h after transfection, reached a minimum level within 2–3 h and went back to steady state level thereafter. This trend was very

Fig. 4. Activation of NFκB by the LTR require proteasomal activity, phosphorylation and degradation of IκB. (A) Effect of proteasomal inhibitor lactacystin on LTR-mediated activation of NFκB. Balb-3T3 cells were cotransfected with 100 ng 3XKB-luc reporter and 300 ng 61E-LTR or p65/relA expression plasmid by lipofectamine plus method. Lactacystin (5μM) or DMSO solvent control was added on to the cells along with DNA-lipofectamine mixture. Cells were harvested 24 h post-transfection for luciferase assay. (B) Induction of IκB-α degradation by the LTR. Balb-3T3 cells transfected with 61E-LTR or pTZ19 similarly as in Fig. 2A but instead cytoplasmic extracts were prepared at indicated time period and analyzed for IκB-α by Western immunoblotting. Same blots were later analyzed for β-actin. (C) Effect of expression of super repressor dn-IκB. Balb-3T3 cells were cotransfected with 100 ng 3XKB-luc reporter and 300 ng 61E-LTR and various amounts of dn-IκB plasmid (50 ng, 100 ng or 200 ng) by lipofectamine plus method as indicated in Fig. 1. Cells were harvested 24 h post-transfection for luciferase assay. (D) Effect of inhibition of IκB-kinase (IKK) activity. Balb-3T3 cells were cotransfected with 3XKB-luc reporter and 61E-LTR along with expression plasmids for dominant negative form of IKK subunits (dn-IKK1 and dn-IKK2, 300 ng) as appropriate and processed for luciferase assay.
much reciprocal to the increase of nuclear p65 level following LTR expression as reported in Fig. 2A. This result thus demonstrated that LTR expression leads to IkBα degradation, which facilitates subsequent translocation of p65 to the nucleus.

To determine whether NFκB activation by the LTR requires phosphorylation of the inhibitory protein IkB, we conducted two different experiments. First, we used a dominant negative form of IkB (dn-IkB) that has serine to alanine mutation at positions 32 and 36, which prevent its phosphorylation, polyubiquitylation and subsequent proteasomal degradation. Overexpression of this dn-IkB thus sequesters endogenous NFκB in the cytoplasm, blocking its dissociation and translocation into the nucleus. As shown in Fig. 4C, dn-IkB expression strongly inhibits LTR-mediated activation of NFκB-dependent reporter 3xKB-Luc in transient transfection experiments. In the second approach, we inhibited the upstream kinase that phosphorylates IkB and determined how it affects LTR-mediated transactivation. In response to stimulation, IkB is phosphorylated by IkB-kinase (IKK) complex. IKK complex is composed of two kinases, IKKa, IKKβ and another modulatory component IKKy. Different external stimuli for activation of NFκB pathway preferentially activate either IKKa or IKKβ although they both can phosphorylate IkB. We used dominant negative forms of IKKa (serine to alanine mutation at positions 176 and 180, dn-IKK1) and IKKβ (serine to alanine mutation at positions 177 and 181, dn-IKK2) which can interact with upstream regulators but are unable to phosphorylate IkB (Mercurio et al., 1997). As shown in Fig. 4D, dn-IKK2 but not dn-IKK1, inhibits the ability of the LTR to activate NFκB-dependent reporter. This result collectively showed that phosphorylation of IkB is necessary for NFκB activation by the LTR.

**Gene transactivation function of the LTR does not require new protein synthesis**

To determine if synthesis of any new protein is necessary for NFκB activation by the LTR, we cotransfected 61E-LTR and 3XKB-luc reporter in Balb-3T3 cells as before but in the presence or absence of the protein synthesis inhibitor cycloheximide, and measured transactivation by luciferase assay. In parallel, we analyzed transcription of the luciferase gene by measuring luciferase mRNA production by RT-PCR analysis. We chose two different concentrations of cycloheximide, 5 μM for 18 h or 1 μM for 24 h. Both treatments of cycloheximide inhibited >95% new protein synthesis in Balb-3T3 cells as determined by 35S-methionine incorporation (data not shown). As shown in Fig. 5A, cycloheximide at both 5 μM and 1 μM concentrations almost completely inhibited LTR-mediated activation of NFκB-dependent luciferase activity as expected, demonstrating effective suppression of protein synthesis. In untreated cells, however, 7-fold activation of luciferase reporter by the LTR was observed, as in previous experiments. RT-PCR analysis performed on the total RNA extracted from cycloheximide untreated cells showed that there was a strong induction of luciferase RNA expression in cells transfected with 61E-LTR whereas no such induction was seen in cells transfected with vector pTZ19 (Fig. 5B). Interestingly, presence of 1 μM cycloheximide did not inhibit induction of luciferase RNA expression by the LTR. Similar result was also obtained with cells treated with 5 μM cycloheximide (data not shown). PCR performed on the same RNA samples without any reverse transcription confirmed the absence of any contaminating luciferase reporter DNA. The production of β-actin RNA was similar in all samples irrespective of cycloheximide treatment. This result demonstrated that new protein synthesis was not required for LTR-mediated activation of NFκB.

We designed another experiment to determine the role of new protein synthesis in transactivation by using dn-IκB, which strongly inhibits LTR-mediated activation of NFκB. We reasoned that since protein synthesis is necessary for dn-IκB-mediated effect, dn-IκB should not be able to inhibit LTR-mediated luciferase RNA production in the presence of cycloheximide. Result presented in Fig. 5C shows that activation of the luciferase reporter activity by 61E-LTR, in the presence of dn-IκB, was abolished both in the presence and in the absence of cycloheximide, as expected. RT-PCR experiment showed that expression of dn-IκB completely inhibited LTR-mediated luciferase RNA production in the absence of cycloheximide. However, luciferase RNA production was detected in cycloheximide-treated cells cotransfected with LTR and dn-IκB, as predicted (Fig. 5D). These data demonstrated further that new protein synthesis is not necessary for transactivation. Further, complete inhibition of luciferase RNA production by the LTR in presence of dn-IκB also suggested against any mechanism by which LTR may act directly as a coactivator of gene transcription.

**PKR−/− MEFs support activation of NFκB by the LTR**

RNA folding analysis indicated that LTR transcript can assume stable stem–loop structure, making dsRNA-dependent kinase PKR, a known activator of NFκB, a potential target in the LTR-mediated transactivation process. To investigate potential involvement of the PKR in this signaling cascade, we analyzed the activation of NFκB-dependent reporter by the LTR in PKR−/− MEFs and compared to normal PKR+/+ MEFs and Balb-3T3 cells. We verified the status of PKR protein in these cells by Western blot analysis of whole cell lysates. As expected, PKR−/− MEFs did not express PKR, whereas the other two cell lines did express the protein (Fig. 6A). Next, we cotransfected these three cell lines with wild-type or mutant U3-LTR (61E-LTR or EDD2), full-length virus (61E or 61E-Mut) or pTZ19 vector and 3XKB-Luc and measured luciferase activity in the cell lysates 48 h after transfection. Cells were also transfected with 100 ng of green florescence protein in separate wells to compare transfection efficiency. As shown in Fig. 6B, even in the absence of PKR expression, 61E-LTR was able to activate the NFκB reporter, and the level of induction (6–8-fold) was comparable to that seen in either PKR−/− MEFs and Balb-3T3 cells (as shown in Fig. 3). Comparison of GFP expression in these three cells showed that transfection efficiency in PKR−/− cells was 40–50%, compared to 25–30% in Balb-3T3 cells and 15–20% in PKR+/+ MEFs. Lower transfection efficiency in PKR+/+ MEFs compared to PKR−/−
lines has also been reported in other studies (Elia et al., 2004). These results demonstrated that PKR is not necessary for NFκB activation by the LTR.

**TLR3 acts as an intermediate in LTR-mediated signaling**

As an alternate mechanism of activation of RNA-mediated signaling event, we looked into the possibility that LTR may activate TLR signaling. TLRs are an integral part of cellular innate immune response, which are activated by various extracellular ligands of microbial origin including dsRNA, single stranded RNA (ssRNA), lipopolysaccharides and CpG DNA (Kawai and Akira, 2005; Takeda et al., 2003). Activation of these receptors initiates a number of complex signaling cascades leading to activation of both NFκB and MAPK pathways. Of the 11 members of the TLR family identified to date, TLR3, TLR7 and TLR8 are activated by RNA molecule (Heil et al., 2004). However, dsRNA is the primary activator for TLR3 (Alexopoulou et al., 2001). Although, TLRs are primarily cell surface molecules, TLR3, -7, -8 and -9 are also retained in endocytic compartment (Heil et al., 2003). To test whether TLR3 signaling is activated by the LTR, we tested HEK293 cells (which are negative for all TLR expression) and HEK293 cells stably expressing mouse-TLR3 for their ability to transactivate gene expression. As shown in Fig. 6C, parental HEK293 cells did not support activation of NFκB-dependent reporter expression by 61E-LTR but HEK293 that expresses m-TLR3 (HEK293/m-TLR3) strongly activated expression of the reporter. Transfection of p65 expression plasmid in both cells resulted in huge activation of the reporter in both cell types suggesting that the failure of reporter activation in HEK293 cells by the LTR is due to the absence of TLR3 and not because of transfection efficiency. That HEK293/m-TLR3 cells were really expressing m-TLR3 was verified by the fact that synthetic dsRNA polyI:C

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**Fig. 5. New protein synthesis is not necessary for LTR-mediated gene transactivation. (A) Effect of cycloheximide on the activation of NFκB-dependent luciferase reporters by the LTR. Cotransfection experiments were carried out with 3XKB-luc reporter and 61E-LTR or vector pTZ19 plasmid in Balb-3T3 cells by lipofectamine plus method. Cycloheximide (5 μM or 1μM CHX) was added to cells in appropriate wells 30 min after the DNA-lipofectamine complex was added onto the cells. Cells were harvested for luciferase assay at 18 h (for 5 μM CHX treatment) or 24 h (for 1 μM CHX treatment) post-transfection. (B) Effect of cycloheximide on transcription of luciferase reporter gene by the LTR. Total cellular RNA (2 μg) from a second set of Balb-3T3 cells cotransfected and cycloheximide-treated essentially similarly as above, were subjected to RT-PCR analysis for luciferase mRNA. RT-PCR figure shows data for 24 h treatment group only. Essentially similar results were obtained with 18 h treatment group. In order to demonstrate absence of DNA contamination in RNA preparations, one set of PCR amplification for each sample was carried out without any RT and loaded onto the lanes marked as ‘-’. All RNA samples were also tested for β-actin mRNA by RT-PCR performed in similar manner as for luciferase mRNA. Lane C represents PCR control with no RNA sample. Products were separated on 2% agarose gel. The 100 bp DNA ladder was included in the left lane. The amplified product for luciferase mRNA (250 bp) and β-actin mRNA (353 bp) are indicated by an arrow. (C) Effect of cycloheximide on the inhibitory effect of dn-IκB. Balb-3T3 cells were cotransfected with 3XKB-luc reporter and 61E-LTR or vector pTZ19 plasmid as above. In addition, some wells were also cotransfected with 200 ng of dn-IκB as indicated. One set of LTR and LTR + dn-IκB transfected cells were treated with 1 μM cycloheximide. Cells from all transfected wells were harvested at 24 h post-transfection for luciferase assay. (D) Effect of cycloheximide and dn-IκB on transcriptional activation of luciferase reporter gene by the LTR. Total cellular RNA from a similar set of transfected cells as in section C were subjected to RT-PCR analysis for luciferase and β-actin mRNA essentially as described in section B.**
strongly activated NFκB reporter in these cells but not in HEK293 cells. Synthetic agonist for ssRNA-activated TLR7 and TLR8, loxoribine, however, failed to activate reporter similarly in either cell lines. These results strongly suggested that TLR3-mediated signaling is involved in LTR-mediated activation of NFκB-dependent genes.

Ras plays a role in NFκB activation by the LTR

We have previously demonstrated that inhibitor of MEK1/2 abrogates collagenase IV gene transactivation by the LTR which suggested a necessary role of MAPK pathway in the process. To determine whether NFκB activation is required for upregulation of MAPK pathway by the LTR, we tested the effect of expression of dn-IκB (which inhibits NFκB activation) on LTR-mediated activation of collagenase IV and MCP-1 genes. In transient transfection assays, dn-IκB was cotransfected with −517 coll-CAT or MCP-1-CAT and 61E-LTR or p65 expression plasmid (a). Cells were harvested for luciferase assay 48 h after transfection. As a control, same set of transfection was also carried out on Balb-3T3 cells as in Fig. 3B. (C) Role of TLR3 in the activation of NFκB pathway by the LTR. TLR deficient regular HEK293 cells and HEK293 cells stably expressing mouse TLR3 (from Invivogen) were cotransfected by lipofectamine plus method with 100 ng of 3XKB-Luc reporter and 300 ng of pTZ19 vector, 61E-LTR or p65 expression plasmid as indicated. Synthetic dsRNA polyI:C (100 μg/ml) or loxoribine (1 μM) was added to the media 8 h before harvesting the cells for luciferase assay at 24 h post-transfection.

Fig. 6. Role of PKR and TLR3 in the activation of NFκB by the LTR. (A) Analysis of PKR expression in the cell lines used in this study. Whole cell lysates (15 μg total protein) from actively growing Balb-3T3, PKR+/+ or PKR−/− MEFs were separated on 10% SDS-PAGE, and Western immunoblotting analysis was performed using polyclonal anti-PKR serum. Lysates were also analyzed in separate SDS-PAGE for β-Actin using specific monoclonal antibody. (B) Analysis of NFκB-dependent reporter gene activation in PKR−/− cells. PKR−/− and PKR+/+ cells were cotransfected by lipofectamine plus method with 100 ng 3XKB-luc and 300 ng of either wild-type (b) or mutant (c) FeLV U3-LTR constructs (61E-LTR or EDD2, respectively), wild-type (d) or mutant (e) full-length FeLV constructs (61E or 61E-Mut, respectively) or pTZ19 vector plasmid (a). Cells were harvested for luciferase assay 48 h after transfection. As a control, same set of transfection was also carried out on Balb-3T3 cells as in Fig. 3B. (C) Role of TLR3 in the activation of NFκB pathway by the LTR. TLR deficient regular HEK293 cells and HEK293 cells stably expressing mouse TLR3 (from Invivogen) were cotransfected by lipofectamine plus method with 100 ng of 3XKB-Luc reporter and 300 ng of pTZ19 vector, 61E-LTR or p65 expression plasmid as indicated. Synthetic dsRNA polyI:C (100 μg/ml) or loxoribine (1 μM) was added to the media 8 h before harvesting the cells for luciferase assay at 24 h post-transfection.
Ras and Raf-1 are necessary for NF-κB-Luc reporter. These data demonstrated that functional inhibitors of various upstream regulators of Ras-Raf-MAPK pathway for their ability to abrogate LTR-mediated NF-κB activation. We cotransfected 61E-LTR and 3XKb-Luc reporter along with dominant negative Ras (N17-Ras) or dominant negative Raf-1 (Raf-BXB-K375W) constructs were used at 300 ng per well. The MEK1/2 inhibitor PD98059 (50 μM) was added to the cells in appropriate wells along with DNA-lipofectamine mixture.

Fig. 7. Analysis of upstream signaling pathways leading to the activation of NF-κB by the LTR. (A) Inhibition of NF-κB activation does not affect collagenase IV and MCP-1 activation by the LTR. Balb-3T3 cells were cotransfected with −517coll-CAT, NFBCO-CAT or MCP-1-CAT together with pTZ19 vector or 61E-LTR and dn-IκB as described in Fig. 1A. (B) Analysis of role of Ras-Raf-MAPK pathway in NF-κB activation. Balb-3T3 cells were cotransfected with 3XKB-luc and 61E-LTR or pTZ19 vector along with inhibitors as indicated. The dominant negative Ras (RasN17) and dnRaf-1 (Raf-BXB-K375W) constructs were used at 300 ng per well. The MEK1/2 inhibitor PD98059 (50 μM) was added to the cells in appropriate wells along with DNA-lipofectamine mixture.

Discussion

In this study, we demonstrated that the U3-LTR region of FeLV activates NF-κB-dependent gene expression. Previous studies have shown that insertion of unrelated sequences into the leukemia virus U3-LTR region leads to a defect in preleukemic events in pathogenesis such as hematopoietic hyperplasia and production of recombinant mink cell focus-inducing viruses but without affecting virus replication and spread (Brightman et al., 1993). This suggested that U3-LTR might influence target cell growth by some mechanism intrinsic to LTR. We have previously demonstrated that the U3-LTR region of Mo-MuLV and FeLV can transactivate expression of specific cellular genes and this activity correlated with the ability of the LTR to make specific RNA transcript (Ghosh and Faller, 1999; Ghosh et al., 2000). In this study, we investigated whether this transactivating property of the LTR might function through activation of NF-κB. Further, we focused on NF-κB signaling pathway because it can be activated by dsRNA and could potentially mediate the induction of many of the host genes regulated by the LTR. Although NF-κB is an important regulator of host immune response against a variety of external stimuli, its role in various cancers has also been described. Reticuloendotheliosis virus encoded Rel protein, whose cellular counterpart c-Rel is a member of NF-κB complex, causes lymphoid tumors in chickens (Chen et al., 1983; Wilhelmsen et al., 1984). Further, the genes for c-Rel, NF-κB2 (p100/52) and Bcl-3 proteins, all members of NF-κB complex, are located within regions of chromosomes that are often involved in rearrangement or deletion (Fraichiolla et al., 1993; McKeithan et al., 1997; Rayet and Gelinas, 1999). NF-κB is also known to activate genes that are involved in the control of cell growth and apoptosis. Antiapoptotic genes such as Bcl-2 family members (Bcl-XL and A1/Bfl-1), cellular inhibitors of apoptosis (c-IAP and IXAP) and TRAFs or genes such as cyclin D1 which promote cell cycle progression, can be directly activated by NF-κB and thus facilitate tumorigenesis (Guttridge et al., 1999; Wang et al., 1998, 1999; Wu et al., 1998).

In fact, many tumor cell lines express high level of NF-κB in the nucleus and conversely, inhibition of NF-κB expression in many transformed cells induces apoptosis (Sovak et al., 1997; Wang et al., 1996). Thus, our finding that the LTR alone can activate the NF-κB pathway is significant and suggests that this activation could be a mechanism by which LTR may exert cell proliferative activity.

NF-κB is activated by various external stimuli, including growth factor and mitogens, stress-inducing agents, cytokines, bacterial lipopolysaccharides and viral infection. The signaling pathways activated by these stimuli are diverse and include a variety of secondary signal transducer molecules such as TNF-α receptor-associated factors (TRAFs), NF-κB-inducing kinase (NIK), TLRs, TGF-β-activated kinase (TAK1), MAPK kinase kinase 1 (MEKK1), protein kinase C and PKR (Karim and Lin, 2002; Yamamoto and Gaynor, 2004). Most of these signaling molecules ultimately activate IKK. Although phosphorylation of IκB by IKK and its subsequent proteasomal degradation followed by nuclear translocation of dimeric NF-κB are the
mechanisms of NFκB activation by most stimuli, two other atypical pathways have also been described. NFκB activation by hypoxia treatment or UV radiation does not involve IkBα phosphorylation at serine 32 and 36 (Imbert et al., 1996; Li and Karin, 1998). In our experiments, the super-repressor dn-IκBα completely abrogated LTR-mediated NFκB activation, indicating that IkBα phosphorylation at position 32 and 36 was required and no atypical method of activation was involved. Similarly, the ability of lactacystin to inhibit LTR-mediated transactivation, as shown in Fig. 4, indicated that proteasomal activity is also an integral part of this transactivation process. Transactivation experiments with dominant-negative IKKs demonstrated that IKKβ is specifically required for NFκB activation by the LTR, linking it to the same pathway as that activated by proinflammatory cytokines (Li et al., 1999; Sizemore et al., 2002; Tanaka et al., 1999).

The central importance of NFκB activation in cellular physiology has made it an attractive target for viruses and many viruses have developed various strategies to alter the NFκB signaling pathway. In most of these strategies, virus-encoded proteins interact with different signaling molecules that ultimately activate IKK. For example, EBV latent membrane protein (LMP1), HCV core protein, rotavirus VP4 protein, HBV HBx protein, HTLV-1 tax protein, HIV gp120 membrane protein (LMP1), HCV core protein, rotavirus VP4 protein, HBV HBx protein, HTLV-1 tax protein, HIV gp120 and tat protein and influenza virus proteins can all activate IKK (Cahir-McFarland et al., 2000; Demarchi et al., 1999; Flory et al., 1998, 2000; Harhay and Sun, 1999; Kim et al., 2001; LaMonica et al., 2001; Sylla et al., 1998; You et al., 1999). On the other hand, measles virus and vesicular stomatitis virus-mediated activation of NFκB has been attributed to cellular PKR activation possibly by viral RNA molecules (Donze et al., 2004). A recent comprehensive review of various pathways to modulate NFκB activity employed by viruses is available (Santoro et al., 2003). We demonstrated in this report that the full-length wild-type FeLV, but not the virus with a mutation in U3-LTR, can activate NFκB. We also demonstrated that activation of NFκB-dependent gene expression by the LTR takes place in the absence of any new protein synthesis. We have previously demonstrated a causal relationship between transcript production and transactivational ability of the LTR (Ghosh et al., 2000). Taken together, our data show that NFκB activation by FeLV is an RNA-mediated event.

Several studies have implicated an essential role of PKR in the activation of NFκB by dsRNA. For example, dsRNA has been shown to activate NFκB binding to the β-interferon promoter in many different cell lines and the nucleoside analog 2-amino purine (2-AP), a PKR inhibitor, blocked such activity (Visvanathan and Goodbourn, 1989). Further, selective degrada- tion of PKR RNA in HeLa cells by antisense oligonucleotide also inhibited dsRNA-mediated activation of NFκB (Maran et al., 1994). Synthetic dsRNA (poly-C)-mediated activation of NFκB was also perturbed in PKR−/− cells, suggesting further the role of PKR in the process (Yang et al., 1995). In other experiments, IKKβ has been shown to be essential for PKR-mediated signaling in response to dsRNA or vesicular stomatitis virus (Chu et al., 1999). Possible involvement of LTR transcript and requirement of IKKβ for NFκB activation in our study thus suggested that PKR could be involved in the process. However, by using PKR−/− MEFs, we demonstrate here that LTR-mediated activation of NFκB is independent of PKR activity. Two previous studies demonstrated that such PKR-independent alternate dsRNA-dependent NFκB activation pathways indeed exist. One such study demonstrated that dsRNA-mediated activation of β-IFN and inflammatory cytokine does not require PKR or RNase-L (Iordanov et al., 2001). In another study, both PKR-dependent and -independent pathways have been demonstrated for dsRNA-mediated activation of macrophages (Maggi et al., 2000). Furthermore, it has been shown that PKR is not required for dsRNA-induced NFκB activation in rat islets (Blair et al., 2001).

Several recent studies demonstrated that association of TLRs with their specific ligands activates both NFκB and MAPK pathways (Kawai and Akira, 2005; Wang et al., 2001). Various virus-associated molecular patterns such as, dsRNA, ssRNA, CpG DNA and envelope glycoproteins have already been reported to be the targets of various TLRs (Boehme and Compton, 2004). Our demonstration that TLR3 is required for LTR-mediated activation suggests that LTR transcript is another member of these targets. Most importantly, the necessity of TLR3 in NFκB activation by the LTR suggests that this could be the missing link between LTR transcript and cellular gene transactivation.

Since LTR also activates other cellular genes such as collagenase IV and MCP-1, and there are evidences that NFκB can modulate their expression (Takeshita et al., 1999; Ueda et al., 1997), it was possible that NFκB acts a mediator for their upregulation in LTR expressing cells. But we could not demonstrate any effect of dn-IκBα on the LTR-mediated expression of collagenase IV or MCP-1. However, our studies with dominant negative forms of MAPK pathway members showed that, in fact, Ras and Raf-1 act as upstream regulators of NFκB signaling by the LTR. Ras and Raf-1 have been previously shown to activate NFκB (Arsura et al., 2000; Baumann et al., 2000; Finco and Baldwin, 1993). Rat liver epithelial cells transformed with oncogenic Ras or Raf show upregulation of NFκB and this activity could be inhibited by the expression of dn-IKK1 or dn-IKK2. Specifically, dn-IKK2 was more potent in inhibiting Raf-induced NFκB activation in Raf-transformed cells, whereas both Ras- and PI3-kinase-mediated pathways were involved in Ras-transformed cells and both IKK1 and IKK2 were necessary for the latter. In our system, RasN17, Raf-BXB-K375W and dn-IKK2 (but not dn-IKK1) inhibited LTR-mediated activation of NFκB. This suggested a Ras-Raf-IKK-IκB pathway of NFκB activation. Our finding of Ras as an upstream regulator of LTR-mediated cellular gene transactivation is consistent with our previous finding which demonstrated that MEK1/2 inhibitor PD98059 inhibits activation of collagenase IV gene expression by the LTR (Ghosh and Faller, 1999). It is therefore conceivable that both NFκB and MAPK activation by the LTR acts in synergy in disease pathogenesis.

Data presented in this report also suggest a mechanism by which both NFκB as well as MAPK pathway can be activated following interaction of LTR transcript with TLR3. Such association could lead to activation of Ras as has been
previously demonstrated for CpG DNA and TLR9 interaction (Yeo et al., 2003). Activated Ras subsequently initiates Ras-Raf-IKK-IkB signaling cascade that liberates NF-κB, facilitating its translocation to the nucleus and ultimately enhances NF-κB-dependent gene expression. Activated Ras and Raf can also independently upregulate MAPK signaling pathway.

**Materials and methods**

**Cells**

Balb-3T3 and HEK293 cells were obtained from American Type Culture Collection and maintained in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml) with 10% donor calf serum or fetal calf serum, respectively, at 37 °C in a humidified incubator under 5% CO2. Wild-type and PKR-negative mouse embryo fibroblasts (MEF) (PKR+/+ and PKR−/−, respectively) were provided by B. Williams from the Cleveland Clinic Foundation and were maintained in DMEM containing 10% fetal calf serum and antibiotics. HEK293 cells stably expressing mouse TLR3 were purchased from Invivogen (San Diego, CA) and maintained in DMEM containing 10% fetal calf serum, penicillin, streptomycin and blasticidin (10 μg/ml).

**Plasmids**

Replication competent FeLV full-length clone 61E and U3-LTR construct 61E-LTR (which contain sequences from −307 to +34) have been described previously (Ghosh and Faller, 1999). Replication competent Mo-MuLV full-length clone Mov9 and U3-LTR construct GMNX (which contain sequences from −419 to −147) also have been described (Choi and Faller, 1995). EDD2 is a derivative of 61E-LTR with 8 base substitutions that abrogates its transactivational activity toward collagenase IV promoter. 61E-Mut is a full-length FeLV expression plasmid for green fluorescence protein (EGFP) from Stratagene. In some transfection experiments, Lactacystin (Clasto-Lactacytin β-lactone, Calbiochem), cycloheximide (Sigma Chemicals), PD98059 (Calbiochem), poly(I:C) (Sigma Chemicals) or Loxiribine (Invivogen) was used as described in the Results section and figure legends. All transfection experiments were repeated at least three times and standard deviations were determined from these replicates.

**RT-PCR**

Analysis of requirement for new protein synthesis during gene transactivation was performed by RT-PCR. RNA was extracted from various transfected cells, untreated or treated with cycloheximide. Cells were washed once with PBS, harvested and total cellular RNA was extracted by Trizol extraction method according to manufacturer’s protocol (Invitrogen). All RNA preparations were digested with RNase free DNase (Promega) at a concentration of 0.1 unit/μl, followed by phenol-chloroform extraction, ethanol precipitation and suspension in DEPC-treated water. DNase digestions were repeated to ensure complete removal of any contaminating DNA. First strand cDNA synthesis for luciferase and β-actin mRNA was performed on 2 μg of total RNA at 50 °C using Thermoscript cDNA synthesis kit (Invitrogen). Antisense primers used for reverse transcription had the following sequences: 5′-ATAATGTGCGTGGCCCC3′ (luciferase) and 5′-CAGGACATGATCTGGGTCATCTTCTC-3′ (β-actin). One-twentieth of the RT product was used in PCR reactions (30 s denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C) using antisense primers as above and following sense primers: 5′-GCTCTGGGATGCTTGTTCACA-3′ (luciferase) and 5′-GCTGCTGCTGCAACGCGCTTCTC-3′ (β-actin). RNA samples without any reverse transcription were used as a negative control in all PCR reactions. In order to maintain a linear range during amplification, PCR was carried out for only 25 cycles.

**Nuclear extracts**

Cells were harvested by scraping, washed once with PBS and twice with hypotonic buffer (10 mM HEPES, pH 7.9; 1.5
nM MgCl₂; 10 mM KCl) at 4 °C. Cells were then allowed to swell in the presence of 1 ml hypotonic buffer (per 10^7 cells) for 20 min on ice. Cells were harvested and resuspended in 0.4 ml hypotonic buffer containing 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin and 0.2% NP-40 and left on ice for another 5 min. Cytoplasmic membrane was disrupted by vortexing vigorously for 30 s and the nuclei were pelleted by high speed centrifugation at 4 °C for 20 s. Nuclei were suspended in high salt extraction buffer (20 mM HEPES, pH 7.9; 1.5 mM MgCl₂; 420 mM NaCl; 0.5 mM DTT; 0.5 mM PMSF; 1 μg/ml aprotinin; 25% glycerol) and incubated for 30 min on ice for nuclear proteins to leach out. The nuclear extract was clarified by centrifugation in microfuge for 10 min at 4 °C.

**Immunoblotting**

Whole cell extracts (WCE) were prepared in RIPA buffer (25 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.1% SDS; 1.0% sodium deoxycholate; 1.0% Triton X-100) containing proteinase inhibitor cocktail from Roche. Twenty micrograms of nuclear extract or WCE was separated on a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20 and were incubated overnight with the following antibodies as appropriate: rabbit polyclonal anti-p65/RelA (Santa Cruz sc-372), anti-IkBα (Santa Cruz sc-371), anti-PKR (Santa Cruz sc-708), mouse monoclonal anti-PCNA (Santa Cruz sc-56) and anti-β-actin (Oncogene CP01). Goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Oncogene) was used as secondary antibody. Bound antibodies were detected by Western Lightning Chemiluminescence kit (PerkinElmer Life Sciences). Prestained molecular weight protein markers (Benchmark marker, Invitrogen) were used to determine molecular weight of the detected bands.

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**References**


