AKT Induces Transcriptional Activity of PU.1 Through Phosphorylation-Mediated Modifications Within Its Transactivation Domain

Piotr Rieske and Jagan M.R. Pongubala*

Department of Biochemistry, MCP Hahnemann University School of Medicine,
Philadelphia, PA 19102

Running Title: The mechanism of PU.1 activation

Key Words:
Signal transduction, B-cell activation, PU.1, MEKK1, AKT and κE3’-enhancer.

*Address for Correspondence:
Dr. Jagan M.R. Pongubala
Department of Biochemistry
School of Medicine
MCP Hahnemann University
245 N. 15th Street
Philadelphia, PA 19102

Email: jagan.pongubala@drexel.edu
Phone: (215) 762-3245
Fax: (215) 762-4452
SUMMARY

Signal transduction by the antigen receptor (pre-BCR and BCR) complexes is critical for developmental progression of B-lymphocytes, which are defined by assembly and sequential expression of immunoglobulin genes, which in turn are regulated by the enhancer elements. Although proximal antigen-receptor signal transduction pathways are well defined, the precise nuclear factors targeted by these signals remained unknown. Previous studies have demonstrated that tissue restricted transcription factors including PU.1 and PIP function synergistically with c-Fos plus c-Jun to stimulate the κE3’-enhancer in 3T3 cells. In this study, we demonstrate that the functional synergy between these factors is enhanced in response to mitogenic associated protein kinase (MAPK), MEK kinase 1 (MEKK1), in 3T3 cells, where the enhancer is inactive. However in S194 plasmacytoma cells, MEKK1 was able to stimulate the activity of PU.1 but unable to induce the κE3’-enhancer activity. We have found that Ras-PI3K-dependent external regulated kinase, AKT, induces κE3’-enhancer activity in both pre-B and plasmacytoma cells. AKT stimulation of the κE3’-enhancer is primarily due to PU.1 induction and is independent of PU.1 interaction with PIP. Activation of AKT had no effect on the expression levels of PU.1 or its protein-protein interaction with PIP. Using a series of deletion constructs, we have determined that the PU.1 acidic-rich (aa 33-74) transactivation domain is necessary for AKT mediated induction. Substitution analyses within this region indicate that phosphorylation of Ser41, is necessary to respond to AKT. Consistent with these studies, ligation of antigen receptors in A20 B cells mimics AKT activation of PU.1. Taken together, these results provide evidence that PU.1 is a target for phosphatidylinositol 3-kinase dependent signal, AKT leading to inducible or constitutive activation of its target genes.
INTRODUCTION

Signaling components of the antigen receptor complexes on the surface of B cell progenitors (pre-BCR) and B cells (BCR) are necessary for the developmental progression of B-lymphocytes (1-5). BCR engagement rapidly induces an array of signal cascades, particularly the activity of three non-receptor protein tyrosine kinase families (PTKs) including members of Src (Lyn, Fyn and Blk), ZAP-70 (Syk) and Tec (Btk) (6-9). Functional deficiencies in any one of these three family members of PTKs result in defective or aberrant function and impaired development of B cells (10-12). Although various signal molecules have been shown to be necessary for proper development and function of B cells, the effects of these distinct signaling outputs on nuclear target molecules remain to be elucidated. In fact, gene-targeting studies indicate that the developmental progression of B cells is critically dependent on the activity of various transcription factors, which bind to promoter and enhancer elements of immunoglobulin (Ig) genes (Heavy and Light chains) suggesting a link between the signal transduction and transcription factors during B-cell development (13-18). The enhancer elements are implicated not only in regulation of the expression of Ig genes, but also involved in somatic- rearrangement and hypermutation of the same genes (19-21). In fact, disruptions with the κE3’-enhancer results in loss of tissue and developmental stage specific rearrangement of kappa (κ) light chain genes (19).

Previous studies have demonstrated that transcriptional activity of the κE3’-enhancer is critically dependent on binding components of the κE3’-CRE (binds c-Fos, c-Jun, CREM and ATF1), PU.1 plus PIP (binds PU.1 and PIP) and E2A (E12/47) sites. Mutation of any one of these
binding sites greatly reduces enhancer activity (22-25). Among the core binding proteins, the expression of PU.1 and PIP is restricted to cells of hematopoietic lineages. Within the κE3’-enhancer, PU.1 recruits the binding of PIP, through protein-protein interaction to its adjacent DNA binding site (22,23). The recruitment of PIP requires phosphorylation of PU.1 at the amino acid, Ser\textsuperscript{148}. Mutation of this serine residue to alanine (S148A) prevents interaction of PU.1 with PIP as well as the binding of PIP to its adjacent DNA binding sequences. This phosphorylation-mediated interaction of PU.1 appears to induce a conformational change in PIP, thereby allowing it to recognize DNA binding sequences (26). Similar protein-protein interaction between PU.1 and PIP has been detected in the Ig lambda (\(\lambda\)) light chain enhancer and the CD20 promoter (27-29). Interestingly, PIP can bind independently to the IFN-stimulated response element, ISRE (30,31). PIP is expressed exclusively in the lymphoid lineages (27,30,32,33). Gene targeting studies indicate that PU.1 is essential for development of both B cells and macrophages (13,14), while PIP function is necessary for maturation of B and T lymphocytes (34). PU.1\textsuperscript{-} mutants completely lack both lymphoid and myeloid progenitors, whereas PIP\textsuperscript{+} animals exhibit a block in peripheral maturation of B cells and fail to produce antibodies in response to antigenic stimulation. Similarly, T cells of the mutant animals (PIP\textsuperscript{-}) lack proliferative and cytotoxic responses (34).

Our recent studies indicate that both PU.1 and PIP interact with additional factors and function synergistically within the κE3’-enhancer. Such factors include c-Fos plus c-Jun, which binds to the κE3’-CRE site, and E12/E47, which binds to the E2A site (Fig. 1). Through these interactions, PU.1 participates in the assembly of an enhanceosome, a higher order nucleoprotein complex (25). Despite the ability to interact with various transcription factors, PU.1 was found
to be a weak transactivator. Studies of gene regulation have suggested that transcriptional activity of some factors requires phosphorylation-mediated modifications (35,36). For instance, phosphorylation of serine residues at positions S\textsuperscript{63} and S\textsuperscript{73} of c-Jun is important for its activity. These residues are rapidly phosphorylated in response to oncoproteins (v-Sis and Raf) or exposure to UV radiation (37), growth factors (38), or cytokines (TNFα) (39). Both S\textsuperscript{63} and S\textsuperscript{73} of c-Jun are preferentially phosphorylated by JNK1, which is sequentially regulated as result of activation of the Ras-PI3K-responsive protein kinase, MEKK1. In fact, target disruption studies suggest that MEKK1 is essential for JNK activation (40). Therefore, the possibility arose that externally regulated signals could play an important role in stimulation of Ig κE3’-enhancer activity. Therefore, we have focused on the role of the Ras-PI3K-dependent signal molecules, MEKK1 and AKT, on κE3’-enhancer activity and target sites within the enhancer in the current studies.

Here we demonstrate that MEKK1 stimulates the synergy between PU.1 plus PIP and c-Fos plus c-Jun in 3T3 cells. In S194 plasmacytoma cells, where the κE3’-enhancer is active MEKK1 weakly stimulated activity of the PU.1 plus PIP site, but failed to induce enhancer activity. Interestingly, the phosphoinositol 3-kinase (PI3K) dependent signal molecule, AKT, stimulated activity of the PU.1 plus PIP site and induced κE3’-enhancer activity in both pre-B and plasmacytoma cells. Activation of the PU.1 plus PIP site in response to the AKT signal is higher when compared to the stimulation observed in the presence of MEKK1. Mutational analyses of the PU.1 plus PIP site indicated that PU.1 responds to the AKT signals and this response is independent of its interaction with PIP suggesting that AKT induction of the κE3’-enhancer is primarily due to PU.1 stimulation. By mutational analyses, we determined that AKT
stimulation of PU.1 is mediated through the acidic-rich (aa 33-74) region. Activation of PU.1 in response to AKT appears to be due to phosphorylation-mediated modifications within this region. Mutation of PU.1 serine 41 (which is phosphorylated in vivo) to alanine impaired PU.1 induction by AKT signal. Consistent with these studies, we found that cross-linking the BCR mimicked AKT activation of PU.1. These studies indicate that PU.1 serves as a target molecule for Ras-PI3K-mediated signals in B cells providing a role for PU.1 in B-cell proliferation and humoral immunity.

**MATERIALS AND METHODS**

**Construction of Plasmids:** Plasmid constructs containing the TK promoter driving the expression of the enhancer core, κE3’-CRE, PU.1/PIP and E2A were previously described (41). Mutations of the PU.1 plus PIP binding sites were reported earlier (24). Wild type or deletion mutants of c-Fos and c-Jun expression plasmids were kindly supplied by Dr. Frank Rauscher (Wistar Institute, Philadelphia, PA). Plasmids expressing MEKKΔ, MEKKΔ (K432M) were kindly provided by Dr. Michael Karin (University of California, San Diego, CA). The expression plasmids of AKT including HA-Wt AKT, HA-Myr AKT and HA-Myr AKT (K) and retroviral vectors, pBabe GFP and pBabe GFP Myr AKT, were generously provided by Dr. Philip Tsichlis (Thomas Jefferson University, Philadelphia, PA). The reporter plasmid, pT81-Luc containing the minimal TK promoter driving expression of the luciferase gene was kindly provided by Dr. Daniel Tanen (Harvard University, Boston, MA). The pT81 Luc-M5.6 reporter plasmid containing the multimerized PU.1 binding site, was constructed by transferring M5.6 from the TK-CAT plasmid by BamHI and Hind III digestion followed by cloning as a blunt-end
fragment into a blunt-end Hind III site of pT81-Luc. The PU.1 deletion mutants, Δ2-30, Δ33-74 and Δ75-100 were constructed by isolating them from PURI plasmids (42) by EcoRI and ligating them into the EcoRI site of an expression plasmid pCB6 containing the CMV promoter (kindly supplied by Frank Rausher, Wistar Institute, PA). The serine to alanine mutant, S41A was isolated from Bluescript KS by EcoRI digest and cloned into the EcoRI site of the pCB6 expression vector, whereas serine to alanine substitution mutants including S37A, S45A, S41/45A and S37/41/45A were prepared by the overlap-extension PCR method (43). Two substitute mutant primers were generated for each desired amino acid, one on the top strand and one on the bottom strand. The sequences of the mutant primers were as follows: F37, 5'-GACTACTACGCTCTCGTGGGC-3'; R37, 5'-GCCACGAAGGCCTAGTAGTC; F45, 5'-GATGGAGAAGCCCATAGCGAT; 45R ATCGCTATGGGCTTCTCCATC. Two external primers containing EcoRI sites were also used, corresponding to the 5' and 3' ends of PU.1. The sequence of 5' and 3' end primers of PU.1 were, 5'-GCGGAATTCAGCTGGATGTTACAGGCG-3' and 5'-GCGGAATTCTCTCGTGAGGCGGAGGC-3' respectively. Typically, the first PCR amplification was carried out with each mutant primer and corresponding external primer to generate two DNA fragments, each with a newly substituted amino acid. The amplified fragments were gel purified and subjected to the second PCR reaction in the presence of 5' and 3' external primers of PU.1 and full-length cDNAs were generated. The amplified products were digested with EcoRI and ligated into the EcoRI site of the pCB6 expression plasmid. Each PCR reaction was performed by using 2.5 U of Taq Polymerase (Roche Molecular Biochemicals), 2.5 U Taq Extender (Stratagene), a deoxynucleoside triphosphate mix in a final concentration of 0.25 mM, 10 ng template DNA and 250 ng of each primer. The wild type PU.1
cDNA was used as a template to generate the S37A and S45A mutants. The S41/45A mutant was generated using S45A mutant primers and a plasmid containing S41A mutant cDNA as a template. Subsequently, the PU.1 mutant containing serine to alanine mutations S37/S41/45A was generated using S37A mutant primers and S41/45A PU.1 cDNA as a template during PCR amplification. DNA sequences of all inserts were determined to confirm the mutations and to verify no new mutations were introduced by PCR.

**Transient Transfections and Reporter Gene Analysis:** S194 plasmacytoma cells were grown in Dulbecco’s Modified Eagles Medium (DMEM, Life Technologies Inc.) supplemented with 10% horse serum and antibiotics (PS;100 U/ml Penicillin and 0.1 mg/ml Streptomycin), whereas 70Z/3 (pre-B cells) and A20 B cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 5 µM β-mercaptoethanol and antibiotics (PS). 3T3 cells were maintained in DMEM containing 10% fetal calf serum and antibiotics (PS). Transfections in S194 cells were performed by the DEAE-dextran (Pharmacia) procedure (44). Transfections contained 4-5 µg of reporter plasmid, 1 µg β-galactosidase expression plasmid pCH110 (45) and varying concentrations of MEKK1, AKT or PU.1 expression plasmids. The maximum amount of DNA was kept between 6-7 µg during all transfections. 3T3 cells were transfected by the calcium phosphate co-precipitation method of Graham and Van der EB (46). The total amount of DNA per transfection in 3T3 cells varied between 16 to 21 µg. Both S194 and 3T3 cells were harvested 48 hrs post-transfection, cells were lysed by freezing and thawing and the β-galactosidase activity was determined from each cellular extract. CAT assays were carried out according to Gorman et al. using normalized cell extracts (47). Transfections in A20 B cells were performed using FuGene-6 reagent (Roche Molecular Biochemicals) with 3 µg of pT81
Luc reporter alone or containing multimerized PU.1 binding site (pT81 Luc-M5.6) along with 1µg of β-gal expression plasmid. Following transfection, cells were either unstimulated or stimulated with 5 µg/ml of F(ab’)2 anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA). To block AKT activation, cells were incubated for 30 min in the presence of 100 nM wortmannin (PI3K-inhibitor) prior to addition of antibodies for stimulation. After thirty-six hours, cells were harvested and luciferase activity was determined by reading in a luminometer. The luciferase activities were corrected for transfection efficiency by using the β-galactosidase activities.

**Metabolic Labeling and Immunoprecipitation:** Metabolic labeling and immunoprecipitation of PU.1 proteins was performed essentially as described previously (25). Briefly, 3T3 cells were transfected by the calcium phosphate method with 5 µg of plasmid expressing either wild type or various PU.1 mutants. Twenty-four hours post-transfection, cells were pulsed with 0.2 mCi/ml 35S-protein labeling mix (EXPRE 35S Protein Labeling Mix L-35S-Met; NEN, Boston, MA) for 2 hrs., then chased with cold methionine (0.5 mM). Cells were washed twice with PBS and harvested in a lysis buffer (containing 20 mM Tris pH7.6, 50 mM NaCl, 0.5% SDS, 0.5% deoxycholate, 1 mM dithiothreitol (DTT), 10 µg/ml leupeptin, 1 µg/ml pepstatin), sonicated, and clear cellular lysates were collected following centrifugation. Cell lysates were normalized by TCA precipitation of the labeled proteins. Approximately 10X10⁶ cpm counts of each cell lysate were incubated with anti-PU.1 antibodies (Santa Cruz Biotechnology Inc.,) for 2 hrs. at room temperature and the immune complexes were separated by protein-A sepharose CL-4B (Pharmacia Biotech). The beads were washed 3 times with a RIPA buffer (10 mM Tris pH7.4, 0.15 NaCl, 1.0% IGEPAL CA-630, 1% deoxycholate, 0.1% SDS and 0.5% aprotinin) and once
with a high-salt buffer (2 M NaCl, 10 mM Tris pH7.4, 1.0% IGEPAL CA-630, and 0.5% deoxycholate). The immune-complexes were eluted by boiling with SDS-PAGE sample buffer, resolved on 10% SDS-polyacrylamide gels and subjected to autoradiography.

**Retroviral Production**: 293T retroviral packaging cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate and antibiotics (PS). The 293T cells were transiently transfected at >50% confluence on 100 mm dishes with 5 µg of retroviral vector, pBabe GFP or pBabe GFP-Myr AKT along with 5 µg of Ecotropic packaging vector by the calcium phosphate co-precipitation method as described above. Thirty-six hours post-transfection, viral supernatants were collected, filtered, and the viral titers were determined by infecting 3T3 cells.

**Retroviral Infection**: 70Z/3 pre-B cells were grown in RPMI 1640 (Life Technologies Inc.) supplemented with 10% heat-inactivated fetal calf serum, 5 µM β-mercaptoethanol and antibiotics (PS). Prior to retroviral infection, cells were washed and treated with DEAE-Dextran (1 mg/ml) for 30 min and exposed to retroviral supernatants expressing GFP alone, or GFP-Myr AKT, for 4 hrs. at 37°C. Viral supernatants were removed and cells were plated in regular growth medium. Thirty-six hours post-infection, cells were washed with PBS and mini-nuclear extracts were prepared according to the method described by Schreiber et. al., (48).

**Electrophoretic Mobility Shift Assays (EMSA)**: Binding reactions were performed as previously described (41). Briefly, various concentrations of nuclear extracts were pre-incubated with 2 µg of poly (dI-dC) in a binding buffer (10 mM Tris pH 7.6, 50 mM NaCl, 20%
glycerol, 1 mM dithiothreitol, 0.5 mM EDTA) for 5 mins. at room temperature. Approximately 10,000 cpm of \textsuperscript{32}P-labeled DNA containing the PU.1 plus PIP DNA binding site (from the \textgreek{k}E3’-enhancer) was then added to the binding reaction and incubation was continued for an additional 25 mins. The bound complexes were separated on 4% non-denaturing polyacrylamide gels and exposed for autoradiography.

RESULTS

MEKK1 Potentiates the functional synergy between PU.1 plus PIP and c-Fos plus c-Jun:

Previous studies have demonstrated that the \textgreek{k}E3’-enhancer requires multiple core binding proteins for its activity (25). Such factors include c-Fos plus c-Jun, which binds to the \textgreek{k}E3’-CRE site, PU.1 plus PIP which bind to PU.1 and PIP sites and E12/E47, which binds to the E2A site (Fig. 1). Co-transfection of the enhancer with either c-Fos plus c-Jun, or PU.1 plus PIP or E2A alone resulted no enhancer activity in 3T3 cells. However, mixture of c-Fos, c-Jun, PU.1 and PIP caused a dramatic induction in enhance activity in 3T3 cells, where it is normally inactive. Removal of any one of these factors resulted in a loss of enhancer activity (25). Transcriptional activation by c-Jun requires phosphorylation at serines 63 and 73. These sites are rapidly phosphorylated by a protein kinase, JNK1 (49,50), whose activity is regulated through the Ras-responsive protein kinase, MEKK1 (51). Since c-Jun is important for the activity of the \textgreek{k}E3’-enhancer, we sought to determine the role of MEKK1 in functional synergy and enhancer activity. If MEKK1 is important to activity of the \textgreek{k}E3’-enhancer, it should stimulate the enhancer when expressed along with PU.1 plus PIP and c-Fos plus c-Jun. To test this, transfections were carried out in 3T3 cells with a reporter plasmid containing the enhancer
core fragment along with PU.1, PIP, c-Fos and c-Jun either in the absence, or presence, of various concentrations of MEKK1. Parallelly, transfections were carried out with the kinase inactive mutant, MEKKΔ (KM). Expression of MEKK1 caused a significant increase in the functional synergy between PU.1, PIP, c-Fos, and c-Jun and led to stimulation of activity of the enhancer in a concentration-dependent manner. On the other hand, expression of MEKKΔ (KM) resulted in a loss of enhancer activity (Fig. 2). Since MEKK1 stimulates activity of c-Jun, activation of the κE3’-enhancer may be due to stimulation of the c-Jun alone or a combination of c-Jun and PU.1 plus PIP.

**AKT Signals Stimulate Transcriptional Activity of the κE3’-enhancer:** Since, the κE3’-enhancer is B cell specific and the expression of binding factors including PU.1 and PIP is restricted to hematopoietic lineages, we sought to determine the role of MEKK1 on the activity of the κE3’-enhancer in S194 plasmacytoma cells. MEKK1 is an intermediate signal molecule in the MAP kinase pathway and becomes active in response to Ras signals (51). Recent studies suggest that in B cells, AKT is a major signal molecule, which becomes active in response to B cell-receptor (BCR) activation in a Ras-PI3K dependent manner (52). Therefore, we examined the role of AKT in parallel with MEKK1 on the κE3’-enhancer activity. Transfections were carried out with reporter plasmids containing the enhancer core fragment or multimerized (4 copies) binding sites of κE3’-CRE, PU.1 plus PIP or E2A, in the absence, or presence, of constitutively active forms of MEKK1 (MEKKΔ) or AKT (Myr AKT) in S194 plasmacytoma cells. As shown in Fig. 3, expression of MEKK1 had no significant effect on activity of the core fragment. However, activity of the PU.1 and PIP site was stimulated (6 fold) in the presence of MEKK1. No significant MEKK1 induction of κE3’-CRE or E2A was observed. These results
suggest that MEKK1 can stimulate activity of the PU.1 plus PIP binding site, but fails to induce activity of the enhancer. Interestingly, expression of Myr AKT stimulated (5-7 fold) the activity of the enhancer core. This induction appears to be due to stimulation of the PU.1 plus PIP function (17 fold) because very little increase in activity of κE3'-CRE (3 fold) or E2A (4 fold) was observed in response to Myr AKT. Activation of the reporter plasmid containing PU.1 and PIP binding sites in the presence of Myr AKT is significantly higher (Student’s t test; P > 0.001) than activity observed in the presence of MEKK1 (Fig. 3). These results indicate that the κE3'-enhancer can be stimulated by an AKT-mediated signal in plasmacytoma cells. Similar results were obtained in 1-8 pre-B cells (data not shown). AKT stimulation of the κE3'-enhancer is primarily due to activation of the PU.1/PIP site. The signal-mediated activation of the κE3'-enhancer suggests a regulatory mechanism which may control activity of the enhancer during development of B cells.

**AKT Stimulation of PU.1 is Independent of its Interaction with PIP:** The above studies indicate that AKT signal greatly stimulates the activity of PU.1 plus PIP site when compared to MEKK1 and induces κE3'-enhancer activity in B lymphoid cells. Therefore, we focused to study the mechanism of AKT induction of PU.1 plus PIP site. Previous studies have demonstrated that phosphorylation of PU.1 at position S148 is necessary for recruitment of PIP to the κE3'-enhancer. Transcriptional activity of the enhancer is greatly reduced in the presence of a PU.1 mutant containing a serine to alanine mutation at position S148A apparently due to the inability of PU.1 S148A to recruit PIP to its adjacent DNA binding site (22,23). Since AKT can activate the PU.1 plus PIP binding site resulting in activation of the κE3'-enhancer, we wished to determine whether AKT activation is due to induction of PU.1, or PIP, due to augmentation
of protein-protein interaction between PU.1 and PIP. To test this, reporter plasmids containing multimerized (4 copies) 3 bp mutants (Fig. 4B; M5.1-M5.8) across the PU.1 and PIP binding sites were transfected in S194 plasma cells, either in the absence, or presence, of Myr AKT. Previous studies demonstrated that the mutations, which disrupt the binding of PU.1 also abolish the binding of PIP, whereas the mutations disrupting the PIP binding site have no effect on binding PU.1 (22). Therefore, if PU.1 is important for AKT mediated stimulation, mutants which bind PU.1 alone should be stimulated to a level comparable to reporters containing PU.1 plus PIP sites. As shown in Fig. 4B, mutants M5.3 to M5.5, which essentially fail to form PU.1 or PU.1 plus PIP complexes (22), were inactive and unable to respond to the AKT function. However, mutant M5.6, carrying mutations in the PIP site (binds only PU.1 not PIP), was greatly induced by Myr AKT. Similarly, mutants M5.1, M5.2, M5.7 and M5.8, which form PU.1 and PU.1 plus PIP complexes, were able to respond to Myr AKT at a level comparable to the PU.1 binding only mutant (M5.6). These studies suggest that AKT targets PU.1 and stimulates its activity.

We next investigated whether Myr AKT activation of PU.1 is due to AKT function. To test this, transient transfections were conducted in S194 plasma cells using the reporter plasmid containing the PU.1 binding site (M 5.6) in the presence of various concentrations of Wt-AKT, Myr AKT (the Myr signal assists in membrane localization, thereby constitutively stimulating downstream molecules) or a kinase inactive mutant Myr AKT (K), which contains a lysine to methionine mutation (K179M). Expression of wild type AKT caused a nearly 6 fold increase in activity of the reporter plasmid. Consistent with previous studies, expression of Myr AKT caused a significant increase (~17 fold) in activity of the reporter plasmid containing the PU.1
binding site (Fig. 5). On the other hand, the kinase inactive mutant Myr AKT (K) slightly lowered PU.1 activity. These results indicate that AKT kinase activity is needed for PU.1 activation and that membrane localization increases the level of activity.

AKT Activation of PU.1 is not due to Altered PU.1 Expression, DNA Binding, or Protein-Protein Interaction with PIP: The above studies strongly suggest that AKT activation of PU.1 is independent of its interaction with PIP. If true, Myr AKT should have no effect on PU.1 protein-protein interaction with PIP. To test this, we examined the binding pattern of PU.1 and PIP following the expression of Myr AKT by retroviral transduction in 70Z/3 pre-B cells, where the enhancer is inactive to determine the effect of Myr AKT on PU.1. GFP tagged Myr AKT (GFP-Myr AKT) or GFP alone were transduced into 70Z/3 cells (pre-B) and allowed to grow for 36 hrs in regular growth medium. FASC analysis indicated that nearly 90 percent of the cells were infected by retrovirus expressing GFP-Myr AKT or GFP alone. Cells were harvested, mini-nuclear extracts were prepared and subjected to gel electrophoretic mobility shift assays (EMSAs) using an oligonucleotide containing PU.1 and PIP binding sites as a probe. Expression of GFP Myr AKT or GFP alone had no effect on the binding ability of PU.1 and yielded a shift complex of identical size when compared to the bound complex observed with the in vitro translated protein (Fig. 6). Similarly, no significant difference in the binding intensity of PU.1 and PU.1 plus PIP complexes was observed when compared with nuclear extracts obtained from the 70Z/3 pre-B cells transduced with retroviruses expressing GFP alone or GFP-Myr AKT. These results indicate that AKT stimulation of PU.1 was not due to changes in expression levels, DNA binding or protein-protein interaction with PIP.
An Acidic-rich Transactivation Domain is necessary for AKT Stimulation of PU.1: The above studies suggest that AKT-mediated activation of PU.1 is independent of its interaction with PIP. If this is true, deletion of the PEST domain may have little effect in the stimulation of PU.1 activity, whereas removal of other amino acid regions should result in a loss of AKT-mediated stimulation of PU.1. To test this, transfections were carried out in S194 cells with a reporter plasmid containing a PU.1 binding site, but carrying a mutation in the PIP site (Fig. 4B; M5.6), along with plasmids expressing various PU.1 mutants either alone, or in the presence of wild type AKT. AKT stimulation of the wild type PU.1 protein was considered 100 percent. Expression of wild type PU.1 caused a slight increase in activity of the reporter plasmid (Fig. 7). On the other hand, expression of the PU.1 mutant lacking the transactivation domain (Δ33-100) resulted in a loss of activity. This protein functioned in a dominant negative manner and blocked activity of the endogenous protein (65% loss). A weak activity observed by Δ33-100 may be due to its ability to interact with other factors (25). Interestingly, the PU.1 mutant lacking the PEST region (ΔPEST) did not interfere with the endogenous protein but rather caused a slight increase in the activity of the reporter plasmid in the presence of AKT (Fig. 7B). Previous studies suggest that PEST regions are involved in protein degradation. Therefore, it is possible that removal of PEST domain may result in stabilization of PU.1 and thus serves as an efficient target for external regulated signals. The above results suggest that the transactivation domain is necessary for signal mediated activation and this is independent of its interaction with PIP.

Further, to define the minimal amino acids of the transactivation domain necessary for signal-mediated activation, we constructed PU.1 mutants lacking acidic- (Δ2-30 and Δ33-74) or
glutamine- (Δ75-100) rich regions. Interestingly, expression of the deletion mutant Δ2-30 caused a slight decrease in AKT activation, whereas Δ33-74 mutant lowered (65% loss) AKT-mediated stimulation of PU.1. On the other hand, Δ74-100 had no effect on AKT-mediated induction of the reporter plasmid. These experiments suggest that amino acids from 33-74 of the transactivation domain are important for transcriptional activity of PU.1 and are responsible for AKT mediated stimulation.

**AKT Stimulation of PU.1 is due to Phosphorylation Mediated-Modifications within the Transactivation Domain:** The acidic-rich transactivation domain of PU.1 contains two serine phosphorylation sites (S41 and S45). If AKT stimulation of PU.1 requires either of these sites, PU.1 mutants containing serine to alanine mutations at positions S41A, S45A or S41/45A should fail to respond to AKT. To examine this, serine to alanine PU.1 mutants S41A, S45A and S41/45 were expressed with wild type AKT in S194 plasmacytoma cells in the presence of reporter plasmid M5.6. In addition, we prepared an S37A mutant. These residues (S37, S41 and S45) lie with a homology to the consensus sequence (SXXXS) for phosphorylation by IKKs (53). Expression of PU.1 mutant S45A had no effect on AKT-mediated induction. However, expression of PU.1 mutant S41A greatly lowered the AKT induction while S37A showed a slight effect on induction of the reporter. Consistent with these studies, PU.1 mutants S41/45A and S37/41/45 blocked AKT-mediated activation (Fig. 7B). The differences in AKT induction by the PU.1 mutants is not due to differences in expression levels, because they were all expressed at comparable levels when transfected in 3T3 cells (Fig. 7C). In addition, the loss of stimulation of PU.1 mutant S41A or S41/45 is not due to loss of PU.1 DNA binding, because these mutations are able to bind DNA and recruit PIP to the κE3’-enhancer (23). These results
suggest that AKT-mediated modification of PU.1 at amino acid residue S41 is important for its induction and mutation of this single amino acid residue (S41A) fails to respond to the AKT.

**Ligation of BCR stimulates PU.1 activity:** Gene disruption studies have demonstrated that PU.1 is necessary for development of B cells. The block in B cell development occurs at very early stages as the mutants (PU.1−/−) completely lack lymphoid progenitor cells (13,14). Since PU.1 is necessary for expression of Ig genes, it suggests a critical role throughout B cell development. Though PU.1 is important for B cell development, the precise expression levels and phosphorylation-mediated modifications at various stages are not yet established. Binding studies suggest that the expression levels of PU.1 at pre-B and B cell stages are comparable (41). This raises the possibility that the activity of PU.1 may be regulated through post-translational modifications. The above studies establish that AKT signals can induce the activity of PU.1. Recent studies indicate that BCR cross-linking stimulates AKT in a PI3K-dependent manner (52). If PU.1 activity is regulated through AKT signals, stimulation of B cells should induce PU.1 activity. To test this, and to establish a direct physiological link between PI3-Kinase-AKT activation and PU.1 induction, the transcriptional activity of PU.1 was measured following BCR cross-linking of A20 B cells. A20 B cells were transfected with a luciferase reporter alone (pT81 Luc) or containing PU.1 binding site (pT81 Luc-M5.6) and left either unstimulated or stimulated with anti-mouse F(ab’)2 antibodies. Thirty-six hrs post-transfection, cells were harvested and PU.1 activity was determined. Interestingly, BCR cross-linking caused a nearly 7 fold increase in PU.1 activity. No increase in activity of the reporter alone was observed following BCR stimulation (Fig. 8). These studies suggest that BCR signals target PU.1. We next examined whether PU.1 induction in response to BCR cross-linking is due to AKT signal.
If PU.1 induction is due to stimulation of PI3K dependent stimulation of AKT, addition of wortmannin (a PI3K inhibitor) should block PU.1 activity. To test this, A20 cells were transfected with reporter plasmid containing the PU.1 binding site and cells were treated with wortmannin (100 nM) prior to stimulation with anti-F(ab’)2. When cells were treated with wortmannin, BCR-mediated PU.1 induction was blocked suggesting that PU.1 is a target for the PI3K dependent AKT signal (Fig. 8).

**DISCUSSION**

The results presented here indicate that the transcriptional activity of PU.1 can be stimulated by externally regulated signals including MEKK1 and AKT. However, AKT stimulation of PU.1 was markedly higher when compared to activation observed in the presence of MEKK1. Consistent with these results, AKT but not MEKK1 signals were able to induce the transcriptional activity of the κE3’-enhancer. Mutational analyses indicated that the increase in PU.1 activity in response to AKT was due to modifications within the transactivation domain (aa 33-74) and was independent of PU.1 protein-protein interaction with PIP. In addition, we demonstrated that BCR cross-linking stimulates PU.1 activity in a AKT dependent manner.

PU.1 functions as a transcriptional regulator by binding to a purine rich consensus sequence ‘GGAA’, known as the PU-box, in various promoter/enhancer elements (54). Previous studies demonstrated that PU.1 recruits the binding of PIP through phosphorylation-dependent interaction with PU.1 at position Ser 148. This interaction is important for the transcriptional activity of the κE3’-enhancer (22,23). However, stimulation of PU.1 observed in response to the AKT signal was not due to an increase in PU.1 protein-protein interaction with PIP. This is
clearly evidenced by the fact that the relative fold of activation observed with the reporter plasmid containing a PU.1 binding site alone, was comparable with the activity of the reporter containing binding sites for PU.1 and PIP. In addition, PU.1 mutants lacking sequences needed for recruitment of PIP were still stimulated by AKT. Instead, PU.1 activation domain sequences were required for maximal AKT activation. Together, these data suggest that AKT activation of PU.1 is mediated through stimulation of the PU.1 activation domain. Deletional analyses of the transactivation domain indicated that the acidic-rich (aa 33-74) region is important for AKT-mediated activation. Ser to Ala mutational analyses indicated that S41A but not S45A is important for PU.1 induction. However, the significance of phosphorylation of Ser$^{41}$ is yet to be determined in B cells. In macrophages, phosphorylation of PU.1 at position Ser$^{41}$ has been shown to be important for proliferation (55). Possibly AKT induction of PU.1 may result in phosphorylation-mediated modification of Ser$^{41}$, yielding a high affinity interaction with the basal transcriptional machinery. Alternatively, the AKT signal could stimulate the transcriptional activity of PU.1 indirectly by recruiting a co-factor through the phosphorylation-dependent protein-protein interaction. Finally, AKT stimulation of PU.1 may be due to phosphorylation-mediated modification of other transcriptional factors interacting with PU.1. This possibility is unlikely because no change in the binding pattern of PU.1 was observed in the presence of AKT stimulation.

Functional studies indicate that PU.1 regulates the activity of various genes in both B cells and macrophages. In recent years, a number of PU.1 target genes have been identified in both B cells and myeloid lineages. In B cells, PU.1 binding is important for transcriptional activity of the immunoglobulin heavy and light chain (κ and λ) enhancers (22,23,56) and the J chain (57),
AKT signal induces PU.1 activity

mb-1 (58), CD20 (29), and CD72 (59) promoter elements. Similarly, PU.1 is necessary for promoter functions of a number of myeloid specific genes including CD11b (60,61), CD18 (62), c-fes (63,64), granulocyte colony-stimulating (G-CSF) receptor (65), macrophage CSF (M-CSF) receptor, c-fms (66-68), macrophage scavenger receptor (69,70), FCγRIb (71), FCγRIIA (72), IL1β (73), and IL-18 (74). Targeted disruption of PU.1 in mice severely impairs the development of lymphoid (B cells) and myeloid (macrophages and neutrophils) lineages. The block in the development of these lineages occurs at very early stages, as the mutant animals completely lack lymphoid and myeloid progenitor cells, rendering it difficult to determine the precise role of PU.1 during stage- and lineage-specific gene expression. Recently, Harinder Singh’s laboratory demonstrated that reconstitution of PU.1 expression by retroviral transduction in PU.1 deficient hematopoietic progenitor cells allowed them to differentiate into macrophages and B cells in vitro (75). Interestingly, the progenitor cells expressing high levels of PU.1 were found to preferentially differentiate into macrophages, whereas the cells with low levels of PU.1 were committed to differentiate into pre-B cells. These studies suggest that the variation in expression and/or function of PU.1 regulates the lineage commitment of hematopoietic progenitors between lymphoid and myeloid development. Expression of the PU.1 mutant lacking the acidic-rich transactivation (Δ74-100) domain rescued the development of B cells, but substantially reduced the ability of PU.1+/− hematopoietic progenitors to differentiate into macrophages. Interestingly, PU.1 mutants lacking acidic-rich regions (Δ2-100 and Δ2-74) failed to rescue the development of both B cells and macrophages, indicating that an acidic-rich transactivation domain is important for development of both B cells and macrophages (75). The fact that the acidic-rich domain is necessary for transactivation and for stimulation of PU.1 in
response to AKT warrants a possible role of externally regulated signals at the level of cellular development.

An important finding of this study is that PU.1 is stimulated in response to AKT and MEKK1 signals. MEKK1 is an intermediate signal molecule of the MAPK pathway and is involved in cross-talk with various signal pathways, including JNK and p38. In addition, MEKK1 has been implicated in nuclear localization of NF-κB through stimulation of protein serine kinases (IKKs). On the other hand, AKT is a downstream effector of PI3K, which becomes active in a Ras-dependent manner (52). AKT has been shown to play an important role in the regulation of cell survival signals in response to deprivation of growth factors, cytokines and oncogenic Ras (76). This is mediated through phosphorylation-dependent inhibition of proteins associated with apoptosis, including Bad, caspase-9, and the transcription factor Forkhead (77-79). Recent studies indicate that AKT also promotes survival by activating NF-κB (77-80). The AKT activation of NF-κB is due to stimulation of IKKs and is independent of MEKK1 and NIK providing an alternate pathway allowing growth factors such as PDGF to activate NF-κB (81-83). In fact, TNF and IL-1 have been shown to stimulate the PI3K-AKT pathway (84,85). Together, these studies indicate that both MEKK1 and AKT signals activate NF-κB through common signal molecules (IKKs). Consistent with these studies, we demonstrate that the transcriptional activity of PU.1 is stimulated in an AKT or MEKK1-dependent manner, suggesting that PU.1 induction may be mediated through stimulation of IKKs. However, PU.1 stimulation detected in the presence of Mry AKT is higher when compared to activity observed in the presence of MEKK1. This may be due to a prolonged activation signal by AKT or delay in the suppression of AKT signals. Alternatively, activation of PU.1 in response to AKT may
involve multiple mechanisms including stimulation and interaction of PU.1 with other cofactors. Overexpressed MEKK1 or AKT may associate with other cellular proteins leading to the activation of PU.1. The fact that kinase inactive mutants of MEKKΔ (KM) or Myr AKT (K) failed to stimulate PU.1 suggests that activation of PU.1 in response to AKT and MEKK1 may be due to direct or their downstream signal molecules.

Ligation of BCR stimulates AKT signals in a PI3K dependent manner (52,86). In the present study, we provided evidence that PU.1 is a target of the PI3K-AKT signaling pathway and becomes active in response to BCR activation. The loss of PU.1 induction in the presence of wortmannin indicates that kinase signals are important for PU.1 induction in vivo. Since activation of BCR signal pathways and PU.1 are both essential for proper development of B cells, the transcriptional activation of PU.1 by the PI3K-AKT pathway may be an important mechanism by which antigen receptor complexes control the developmental progression of B cells. In fact, activated Ras signals are sufficient to promote the differentiation of Rag-1Δ mutant pro-B cells to B cells. The Rag-1Δ mutant B cells lack Ig H chain genes, but display extensive light chain rearrangement (87). Since, AKT becomes active in a Ras-PI3K dependent manner and stimulates the activity of the κE3’-enhancer, induction of kappa light chain genes in response to Ras-mediated signals may be due to activation of various transcription factors including PU.1.
ACKNOWLEDGMENTS

The authors wish to thank Dr. Philip Tsichlis for generously supplying various AKT expression plasmids and Dr. Michael Karin for providing expression plasmids of MEKK1. We would like to thank Drs. Harinder Singh, Jane Azizkhan-Clifford and Michael Atchison for helpful suggestions. This work was supported by the National Institutes of Health Grant AI 46308 to JP.

LEGENDS:

**Fig. 1.** A) Schematic of the κE3'-enhancer with relative positions of the κE3'-CRE, PU.1 plus PIP and E2A DNA binding sites.

**Fig. 2.** MEKK1 promotes the functional synergy between PU.1 plus PIP and c-Fos plus c-Jun within the κE3'-enhancer. NIH 3T3 cells were transfected by the calcium phosphate method with 5 μg of TKCAT reporter plasmid containing the core fragment and 3 μg of expression plasmids of PU.1, PIP, c-Fos and c-Jun either in the absence or presence of increasing amounts (0.1, 1.0 and 3.0 μg) of MEKKΔ, or kinase-inactive mutant MEKKΔ (KM) as described in materials and methods. Cells were harvested after 48 hrs and CAT activities were determined as described in materials and methods. The level of the activity of the enhancer core in the presence of PU.1, PIP, c-Fos and c-Jun was considered as one fold. The transcriptional activity
of the core fragment in the presence of MEKK1 or MEKKΔ (KM) was calculated relative to the activity observed when the reporter was co-transfected with PU.1, PIP, c-Fos and c-Jun alone.

**Fig. 3.** AKT stimulation of the κE3’-enhancer is mediated through PU.1/PIP site: A) A representative set of CAT assay showing the effect of MEKKΔ and AKT on the enhancer core and its binding sites including the κE3’-CRE, PU.1 plus PIP or E2A. Transient transfections were carried out in S194 plasma cells with reporter plasmids containing the enhancer core, κE3’-CRE, PU.1 plus PIP or E2A either in the absence or presence of MEKKΔ or Myr AKT. B) The activities observed with the enhancer core, κE3’-CRE, PU.1 plus PIP or E2A in the absence of AKT or MEKKΔ is considered one. The relative effect of expression of MEKKΔ or AKT on the activities of κE3’-CRE, PU.1 plus PIP or E2A were calculated by comparing with the activity obtained by respective reporter alone. Relative CAT activities were determined by averaging three or more independent experiments. Error bars indicate standard deviation.

**Fig. 4.** AKT signal stimulates the transcriptional activity of PU.1 in S194 plasmacytoma cells. Transient transfections were performed in S194 plasma cells with reporter plasmids containing multimer binding sites of PU.1 plus PIP or serial 3 bp linker scan mutants across PU.1 and PIP sequences, either in the absence, or presence, of Myr AKT. A) Representative CAT assay of various linker scan mutants in the absence or presence of Myr AKT. B) The nucleotide sequence of wild type or mutant constructs (M5.1 to M5.8) and their relative abilities to bind PU.1 and PU.1 plus PIP complexes is indicated by (−) or (+) (22). The three bp substitution mutations in each mutant construct are underlined. The fold of Myr AKT stimulation of each
construct was determine by comparison with the activity observed by the respective reporter alone. The values represent the mean of two independent experiments.

**Fig. 5.** PU.1 activity is stimulated by AKT signal. Transfections were performed in S194 plasma cells with the TKCAT reporter containing PU.1 binding site but carry mutation in PIP site (M5.6; Fig. 4B) along with Wt AKT, Myr AKT or Myr AKT (K) at various concentrations. The values represent mean of two independent assays.

**Fig. 6.** DNA binding or protein-protein interaction of PU.1 with PIP is not altered by AKT signal. Nuclear extracts were prepared from 70Z/3 cells that were transduced by retrovirus that express either GFP or GFP-Myr AKT as described in materials and methods. Binding assays were carried out using an oligonucleotide containing PU.1 and PIP sites of the κE3'-enhancer. Both PU.1 and PU.1 plus PIP complexes and free probe are indicated.

**Fig. 7.** Phosphorylation mediated modifications within the acidic-rich transactivation domain is necessary for AKT stimulation of PU.1 in S194 plasmacytoma cells: A). Schematic of PU.1 wild type (Wt) and various PU.1 mutant expression constructs used in the transient transfection assays. The sequences deleted or mutated are indicated on the left. B). Identification of amino acid residues necessary for AKT induction of PU.1. Transient transfections were carried out with 4 µg of TKCAT reporter plasmid containing the PU.1 binding site (M5.6, Fig.4B) along with 1 µg of indicated mutant construct in the absence or presence of 1µg of Wt AKT. Forty-eight hours following transfection, the cells were harvested and CAT activity was determined as described in materials and methods. Data are representative of three to five independent
experiments. Error bars indicate standard deviation. C). Expression levels of various PU.1 mutants in 3T3 cells. Three µg of wild type or each PU.1 mutant construct was individually transfected in NIH 3T3 cells by calcium phosphate method. Prior to harvesting (24 hr) the cells, proteins were metabolically labeled, immunoprecipitated and resolved on 15% SDS-PAGE gel.

**Fig. 8.** BCR cross-linking stimulates PU.1 activity. A20 B cells were transfected with a leuciferase reporter alone or containing PU.1 binding site (M5.6). Following transfection, cells were either left un-stimulated or stimulated by cross-linking BCR with anti-F(ab’)_2. After 36 hrs, cells were harvested and luciferase activities were measured. In experiments involving treatment of cells with wortmannin, cells were treated with 100 nM wortamannin prior to ligation of BCR. Shown is the fold of induction of either the in the presence or absence of BCR stimulation. The average and standard error shown are derived from three to five independent experiments.

**Fig. 9.** Schematic of the proposed signal mediated activation of the κE3’-enhancer. Activation of Ras drives AKT signal pathway leading to activation of PU.1. As a result, the functional synergy between PU.1 and other enhancer core binding proteins leads to greater transcriptional activation.
REFERENCES


AKT signal induces PU.1 activity


Fig. 1

![Diagram of Enhancer Core with variations in PU.1/PIP E2A κE3'-CRE]

Fig. 2

![Graph showing fold activity of MEKKΔ and MEKKΔ (KM) with concentrations of 0, 0.1, 1.0, 3.0 µg]

Fig. 3A

![Image with core promoters and variations in MEKKΔ and Myr AKT]
**Fig. 3B**

![Bar graph showing the fold CAT activity for MEKKΔ and Myr AKT.

**Fig. 4**

![DNA gel electrophoresis images for different constructs.

**Fig. 4B**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Binding ability</th>
<th>Fold of AKT Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>++</td>
<td>21.4</td>
</tr>
<tr>
<td>M5.1</td>
<td>++</td>
<td>12.3</td>
</tr>
<tr>
<td>M5.2</td>
<td>++</td>
<td>11.1</td>
</tr>
<tr>
<td>M5.3</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td>M5.4</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>M5.5</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>M5.6</td>
<td>+</td>
<td>19.7</td>
</tr>
<tr>
<td>M5.7</td>
<td>+++</td>
<td>26.2</td>
</tr>
<tr>
<td>M5.8</td>
<td>+++</td>
<td>25.5</td>
</tr>
</tbody>
</table>
Fig. 7C

Fig. 8
Fig. 9

BCR Stimulation

Ras
PI3K
AKT

Enhancer Activation

Synergy
Synergy

Fos/Jun PU.1 PIP E2A
AKT induces transcriptional activity of PU.1 through phosphorylation-mediated modifications within its transactivation domain
Piotr Rieske and Jagan M.R. Pongubala

J. Biol. Chem. published online December 22, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007482200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2000/12/22/jbc.M007482200.citation.full.html#ref-list-1