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Synergism of cytoplasmic kinases in IL6-induced ligand-independent activation of androgen receptor in prostate cancer cells

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IL6 is a pleiotropic cytokine which has been implicated in ligand-independent activation of androgen receptor in prostate cancer cells. Here, we present the evidence that two cytoplasmic kinases Pim1 and Etk are involved in this process. We showed that Pim1 is expressed in all prostate cancer cell lines examined. Both the expression level and the kinase activity of Pim1 are regulated by IL6 in these cells. Furthermore, we showed that IL6 downstream tyrosine kinase Etk can induce tyrosine phosphorylation of Pim1 which is correlated with its kinase activity. Mutation of the conserved Tyrosine 218 in the activation loop results in reduced kinase activity of Pim1. Interestingly, Etk can also be activated by Pim1 when they are coexpressed in prostate cancer cells, suggesting a possible positive feedback loop between Etk and Pim1. It appears that both Pim1 and Etk are required for IL6-induced activation of androgen receptor-mediated transcription in prostate cancer cells because overexpression of the kinase-deficient form of either Pim1 or Etk dramatically blocks the IL6 effect. Coexpression of the two kinases together but neither one alone is sufficient to activate ARE-containing promoter. Taken together, our data suggest a synergism of Ser/Thr kinase Pim1 and tyrosine kinase Etk in IL6 signaling and provide new insights into ligand-independent activation of androgen receptor in prostate cancer cells.
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Introduction

IL6 is a pleiotropic cytokine originally identified as a regulator of immune and inflammatory responses (Hirano, 1992). IL6 induces cellular differentiation and exerts growth inhibition, growth stimulation, and antiapoptosis in different cellular contexts. Deregulation of IL6 has been reported in several human carcinomas including myeloma, ovarian cancer and breast cancer (Abe *et al*., 1991; Plante *et al*., 1994; Ueda *et al*., 1994). Mounting evidence suggests that IL6 may also play a role in regulation of prostate cancer (PCA) cell growth and metastasis. Prostatic tumor characteristically metastasizes to bone, lymph node and liver, where IL6 is present (Siegall *et al*., 1990; Siegsmund *et al*., 1994). Patients with advanced PCA have elevated systemic serum IL6, which is correlated with the tumor burden (Akimoto *et al*., 1998; Adler *et al*., 1999). Interestingly, the expression of IL6 is regulated by androgen. With aging, serum androgen levels decline while serum IL6 levels increase (Daynes *et al*., 1993). The orchiectomy in a murine model results in the elevated serum IL6, which is reversible by testosterone administration (Bellido *et al*., 1995). A recent study showed that androgen suppresses the expression of IL6 through androgen receptor and *I*kB in a PCA cell line LNCaP (Keller *et al*., 1996). A report from Pastan's lab showed that IL6 receptors are expressed at significant level in 100% of PCA samples and cell lines surveyed (Siegall *et al*., 1990; Siegsmund *et al*., 1994). The IL6 autocrine loop is present in all androgen-independent PCA cells tested so far, suggesting an intimate link between IL6 autocrine loop and androgen-independent phenotype (Okamoto *et al*., 1998). This is further supported by a recent report that a neutralizing antibody against IL6 inhibits the growth of PC3 cells in nude mice (Smith and Keller, 2001). IL6 has also been shown to increase nuclear androgen receptor (AR) level in PCA cells and activate AR-mediated transcription activity in LNCaP cells in the absence of androgen (Chen *et al*., 2000; Lin *et al*., 2001; Ueda *et al*., 2002). However, the detailed mechanisms by which IL6 activates ligand-independent activation of AR remain elusive although p300 has been shown required for this process (Debes *et al*., 2002). Several major signaling pathways are activated by IL6 in PCA cells, including PI3-kinase, JAK-Stats and Ras-MAPK pathways (Smith *et al*., 2001). In our previous studies, we showed that IL6 induces the activation of tyrosine kinase Etk through the PI3-kinase, and Etk is required for IL6-induced neuroendocrine differentiation. Etk has also been implicated in regulation of cell migration, cytoskeletal reorganization and vesicle

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trafficking (Chen *et al*., 2001; Kim *et al*., 2002; Yang *et al*., 2002). A recent report also suggested a role of Etk in androgen-independent growth of PCA cells induced by neuropeptides (Lee *et al*., 2001).

The serine/threonine kinase Pim1 was originally identified as a frequently activated cellular gene by retrovirus insertion (Cuypers *et al*., 1984). Our current knowledge on Pim1 kinase is largely derived from studies in immune systems. The transgenic mice over-expressing Pim1 under the control of the immunoglobin enhancer Eu are highly susceptible to developing T lymphoma after MuLV infection and chemical carcinogen exposure (Breuer *et al*., 1989). Under physiological conditions, the expression of Pim1 is rapidly induced by various cytokines, including IL2, IL3 and IL6. The proliferative response to these cytokines is impaired in cells from Pim1-deficient mice (Lilly *et al*., 1992; 1999). Pim1 has been shown to play a critical role in IL6-initiated cell cycle progression and antiapoptosis in B cells (Shirogane *et al*., 1999). Recently, Pim1 has emerged as a potential important kinase in prostate cancer. In a recent gene and tissue array study of 738 prostate tumor specimens, Pim1 was found upregulated in more than 50% of the samples examined (Dhanasekaran *et al*., 2001), strongly suggesting a potential role of Pim1 in prostate cancer progression. However, very little is known about the functions of Pim1 in prostate cancer. Here, we present the evidence that Pim1 kinase is regulated by IL6 in prostate cancer cells and involved in IL6-induced ligand-independent activation of androgen receptor-mediated transcriptional activity.

Materials and methods

Cell culture and transfection

All cell lines used in this study were purchased from ATCC. COS-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum. LNCaP and PC3 cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum. 22Rv1 cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum. Transfections were performed by using Fugene 6 (Roche) according to the manufacturer's instruction.

DNA constructs and antibodies

T7-tagged Etk and its mutants were cloned in pcDNA3 vector as described previously (Qiu *et al*., 1998). Flag-tagged Pim1 and Pim1KM constructs were kindly provided by Dr Hirano (Shirogane *et al*., 1999). Site mutagenesis was carried out by using PCR-based QuickChange kit (Stratagene). Mutations were verified by sequencing. Anti-T7 antibody was purchased from Novagen. Anti-Flag antibody was from Sigma. Anti-Pim1 and HRP conjugated anti-phosphotyrosine antibody were from Santa Cruz. Polyclonal Etk antibody was developed as described previously (Qiu *et al*., 1998). The Probasin luciferase reporter construct ARR2-Luc was generated by insertion of the minimal probasin promoter ARR2, kindly provided by Dr R Matusik (Zhang *et al*., 2000) into the polyclonal linker region of pGL2. A promoter-less RL (Promega) was used as the internal control.

Immunoprecipitation and Western blot

The transfected cells were lysed in the buffer (20 mM Tris/HCl pH 7.4,. 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mg/ml aprotinin, 1 mg/ml leupterin and 1mM PMSF). Insoluble material was removed by centrifugation, and antibodies were added to lysates for 1 h at 4°C. Antibodies were collected with protein A or protein G-sepharose beads, and protein complexes were washed three times at 4°C with the lysis buffer. Immunoblotting was performed as previously described (Kim *et al*., 2002). Briefly, blots were incubated with primary antibodies, 1 : 5000 dilution of anti-T7 tag, 1 : 2000 dilution of anti-phosphotyrosine, 1 : 1000 dilution of anti-Flag tag, 1 : 100 dilution of anti-Pim1 at room temperature for 1 h, and followed by detection with horseradish peroxidase-conjugated secondary antibody.

In vitro *kinase (IVK) assay*

Pim1 IVK assays were performed as reported previously (Zhu *et al*., 2002). Briefly, anti-Flag immunoprecipitates were washed twice with Pim1 kinase buffer (25 mM HEPES, 10 mM $MgCl_2$, 1 mM DTT). The immune complex was then incubated at room temperature for 15 min in 30 μl of the kinase buffer with 0.1 μg/ml histone1 and 10 μCi of [γ-^{32}P]ATP. The reaction was separated by 10% SDS–polyacrylamide gel electrophoresis. After the gels were dried, the phosphorylation of histone1 was visualized by autoradiography. Etk IVK assays were carried out as described previously (Qiu *et al*., 1998).

Luciferase assay

LNCaP cells grown in a 24-well plate coated with polylysine were transfected with ARR2-Luc and the internal control (a promoter-less RL) in the medium containing 10% charcoal-stripped FBS (Hyclone). The conventional interanal control TK-RL was replaced by a promoter-less RL because of the strong response of TK promoter to IL6. 24 h post-transfection, the cells were incubated with fresh phenol-red free serum-free medium with or without IL-6 (10 ng/ml) for 12 h. Luciferase activities were measured by using dual luciferase assay system according to the manufacturer's instruction (Promega). The results are presented as the fold induction that is the relative luciferase activity of the treated cells divided by that of the control. Lucifease assays were carried out in triplicates.

Cell proliferation assay

22Rv1 cells were transfected with vector, T7-EtkKQ and FLAG-Pim1KM, respectively. After 24 h, the transfected cells were seeded on 96-well plates and incubated in RPMI1640 supplemented with 10% fetal bovine serum for 24 h in order to allow the cells to attach. The cells were then cultured in serum-free medium with or without 25 ng/ml IL6 for another 24 h. The proliferation rate was measured using WST-1 reagent (Roche Molecular Biochemicals). An aliquot of cells were lysed and followed by Western blotting with anti-T7 or anti-Flag to monitor the transfection efficiency.

Results

It has been reported that Pim1 is upregulated in 50% of prostate tumor samples by using high-density tissue microarrays (Dhanasekaran *et al*., 2001). Therefore, we examined the expression of Piml in several well-established prostate cancer cell lines including LNCaP,

22Rv1 and PC3 cells by Western blot using a monoclonal antibody specific for Pim1. Consistent with previous studies (Amson *et al*., 1989; Saris *et al*., 1991), Pim1 is detected as doublets around 33 kDa in these human prostate cancer cell lines. There is very minimal expression of Pim1 in LNCaP while much higher in 22Rv1 and PC3 (Figure 1a). Based on previous studies in immune systems, Pim1 is rapidly induced by various cytokines (Lilly *et al*., 1992; Sato *et al*., 1993; Jaster *et al*., 1999; Matikainen *et al*., 1999). Therefore, we examined the effect of IL6 on Pim1 expression in PCA cells. As shown in Figure 1b, Pim1 protein level is dramatically increased in LNCaP cells after 12 h treatment with 10 ng/ml of IL6. Similar induction of Pim1 was also observed in PC3 cells when they were treated with 50 ng/ml IL6, although the basal level of Pim1 is significantly higher in these cells. We further performed semiquantitative RT–PCR analysis to confirm that the increase of Pim1 expression is resulted from IL6-induced transcription of Pim1 mRNA (Figure 1c). To test whether the higher basal level of Pim1 expression is resulted from the IL6 autocrine loop present in PC3 (Okamoto *et al*., 1998), the cells were treated with IL6 neutralizing antibody for 24 h. Our results show that IL6 neutralizing antibody significantly suppressed the Pim1 expression in PC3 (Figure 1d). Taken together, these data suggest that IL6 can induce Pim1 expression in prostate cancer cells.

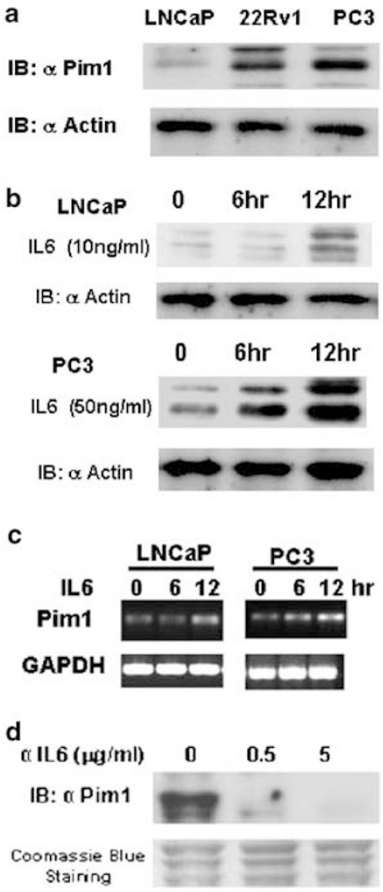

Figure 1 Regulation of Pim1 expression by IL6. (**a**) Expression of Pim1 in PCA cells. The total cell lysates from the indicated cell lines were subjected to immunoblotting with a monoclonal anti-Pim1 antibody (top). The same blot was then probed with anti-actin to monitor the sample loading (bottom). (**b**) Induction of Pim1 by IL-6. LNCaP and PC3 cells were treated with IL6 10 and 50 ng/ml, respectively for the times indicated. Immunoblotting with anti-Pim1 and anti-actin were performed as above. (**c**) Effects of IL6 on the mRNA level of Pim1 in PCA cells. The cells were treated with IL6 as above. Total RNAs were isolated, followed by semiquantative RT–PCR by using a pair of primers specific for Pim1. GAPDH was used as a control. (**d**) Effects of neutralizing antibody for IL6 on Pim1 protein level in PC3 cells. PC3 cells were treated with neutralizing antibody against IL6 at the indicated concentrations for 24 h. The total cell lysates from these cells were subjected to immunoblotting with an anti-Pim1 antibody

To test whether Pim1 kinase activity can also be promoted by IL6 treatment, the flag-tagged wild-type Pim1 and kinase-inactive Pim1KM were transfected into LNCaP cells, respectively. The cells were then serum-starved overnight at 24 h post-transfection and treated with IL6 (200 ng/ml) for 15 min. Pim1 kinase was immunoprecipitated by anti-Flag and IVK kinase assays were performed using Histone1 as a substrate. As shown in Figure 2a, Pim1 kinase activity was dramatically increased by IL6 treatment while the kinase-inactive Pim1KM showed virtually no activity. To investigate what signaling molecules may be involved in Pim1 activation induced by IL6, we examined the effects of tyrosine kinase Etk on Pim1 activation because Etk is known to be activated by IL6 in prostate cancer cells. Pim1 and Etk were cotransfected into LNCaP cells. Figure 2b showed that cotransfection of Etk with Pim1 dramatically increased the kinase activity of Pim1. Interestingly, tyrosine phosphorylation of Pim1 is also significantly increased in the presence of Etk (Figure 2c). However, the kinase-inactive Etk (EtkKQ) or another tyrosine kinase FAK has little effect on tyrosine phosphorylation of Pim1, suggesting that Etk may be responsible to induce tyrosine phosphorylation of Pim1 which is correlated with activation of Pim1 kinase activity. To confirm that tyrosine phosphorylation of Pim1 also occurs under physiological conditions, we examined whether IL6 induces the phosphorylation of Pim1 in 22Rv1 and PC3 cells. As shown in Figure 2d, tyrosine phosphorylation of the endogenous Pim1 was detected at 15 min after IL6 treatment in both types of cells. The phosphorylation seemed to be transient because the phosphorylation disappeared at 30 min after the treatment. To investigate whether tyrosine phosphorylation of Pim1 plays a role in the activation of its kinase activity, we mutated the tyrosine residue Y218 that is located in the activation loop of the kinase domain and conserved among PIM family kinases (Figure 3a). Figure 3b showed that the substitution of Y218 to phenylalanine dramatically reduced both tyrosine phosphorylation and kinase activity of Pim1 induced by Etk. These data suggest that tyrosine phosphorylation of Y218 by Etk may be important for the activation of Pim1 kinase activity.

We also examined the effects of Pim1 on Etk by contransfecting Etk and Pim1 into LNCaP cells. As shown in Figure 4, tyrosine phosphorylation of Etk is dramatically increased by Pim1 but not the kinase-

inactive Pim1 (Pim1KM). The kinase activity of Etk is coordinarily increased as evidenced by its autophosphorylation activity in an IVK assay. Taken together, these data suggest that there exists a positive feedback loop between Pim1 and Etk.

To test whether Etk is required for Pim1 activation induced by IL6, Flag-tagged Pim1 was cotransfected with the vector control or EtkKQ into LNCaP cells. The cells were serum starved for 24 h followed by IL6 treatment. Figure 5a shows that coexpression of the kinase inactive EtkKQ significantly inhibited IL6-induced activation of Pim1 kinase activity. On the other hand, the kinase-inactive Pim1KM also blocked IL6-induced activation of Etk in these cells (Figure 5b). These data suggest that Etk activity for Pim1 activation and the same is true *vice versa*.

It has been shown that IL6 can induce ligand-independent activation of androgen receptor-mediated transcriptional activity (Hobisch *et al*., 1998). We like to know whether Pim1 and Etk are involved in IL6-induced AR activation. Kinase-inactive Etk (EtkKQ) or

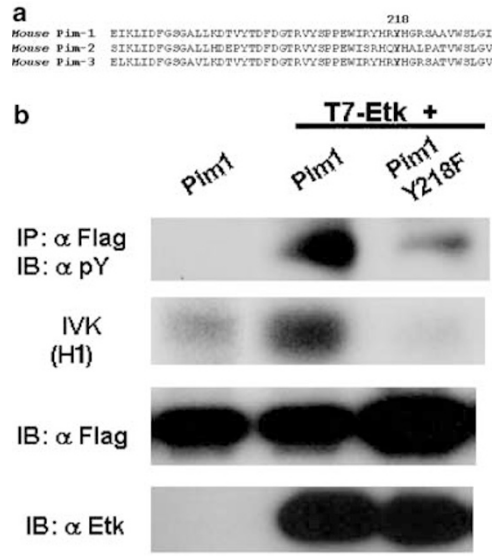

Figure 3 Effects of Etk on Pim1 kinase activity. (**a**) Alignments of PIM family kinases. The alignments of the subdomains VII and VIII of PIM family kinases are shown. The conserved Tyrosine residue (Y218) is in bold. (**b**) Tyrosine phosphorylation of Y218 of Piml is important for its activity. 22Rv1 cells were transfected with Pim1 wild-type and Pim1 Y218F mutant in the presence of anit-T7 tagged Etk wild type. At 24 h post-transfection, the cells were serum starved for 24 h. The cell lysates were immunoprecipitated with anti-flag antibody, followed by immunoblotting with anti-pY antibody. IVK was performed as a Figure 2b. The immunoblotting of total cell lysates with anti-Flag and anti-Etk were included to monitor the protein levels of Pim1 and Etk in these samples

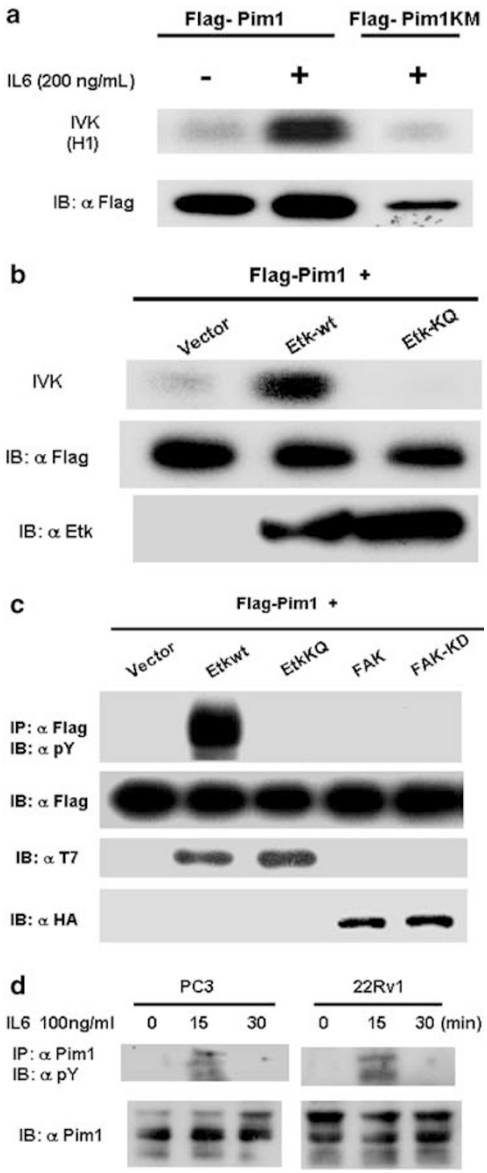

Figure 2 IL6 induces Pim1 kinase activity in PCA cells. (**a**) Effects of IL6 on Pim1 kinase activity. LNCaP cells were transfected with Flag-tagged Pim1 or kinase-deficient Pim1(PimKM). At 24 h post-transfection, the cells were serum starved for 6 h and treated with IL6 (200 ng/ml) for 15 min. The cell lysates were subjected to immunoprecipitation with anti-Flag, followed by IVK using H1 as a substrate. The amount of Pim1 in each sample was determined by immunoblotting with anti-Flag (bottom). (**b**) The effects of Etk on Pim1 kinase activity. Flag-tagged Pim1 was cotransfected with vector, T7-Etk or kinase-inactive T7-EtkKQ into LNCaP cells. At 24 h post-transfection, the cells were serum starved for another 24 h before they were lysed. Pim1 IVK was performed as above. The level of Pim1 and Etk in the cells were monitored by immunoblotting with anti-Flag and anti-Etk, respectively. (**c**) Etk induces tyrosine phosphorylation of Pim1. Flag-tagged Pim1 was cotransfected with vector control, T7-Etkwt, kinase-inactive T7-EtkKQ, HA-FAK and kinase-inactive HA-FAK-KD into LNCaP cells, respectively. At 24 h post-transfection, the cells were serum starved for 24 h. The cell lysates were subjected to immunoprecipitation with anti-Flag, followed by immunoblotting with antiphosphotyrosine (pY). The protein levels of Pim1, Etk and FAK in the cells were determined by immunoblotting of total cell lysates with anti-Flag, anti-T7 or anti-HA antibodies. (**d**) IL6 induces tyrosine phosphorylation of endogenous Pim1 in PCA cells. Subconfluent PC3 and 22Rv1 cells were serum starved for 6 h, followed by treatment with IL6 (100 ng/ml) for the times indicated. The cell lysates were subjected to immunoprecipitation with anti-Pim1, followed by immunoblotting with antiphosphotyrosine antibody. The amount of Pim1 in each sample was monitored by immunoblotting with anti-Pim1

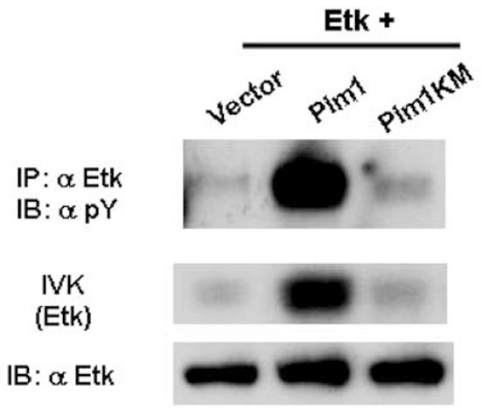

Figure 4 Effects of Piml on Etk kinase activity. T7-Etk was cotransfected with vector control, Pim1 or kinase inactive Pim1KM into 22Rv1 cells. At 24 h post-transfection, the cells were serum starved for 24 h. The cell lysates were immunoprecipitated with anti-Etk antibody, followed by immunoblotting with anti-pY antibody (top). The autophosphorylation activity of Etk was determined by IVK (middle). The protein level of Etk was monitored by immunoblotting with anti-Etk (bottom)

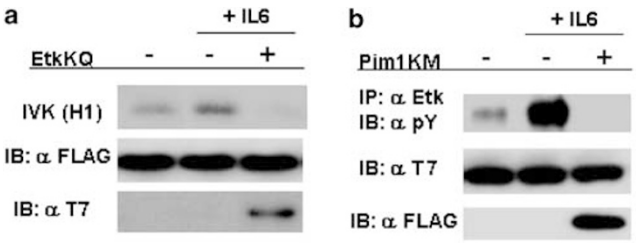

Figure 5 Requirement of both Etk and Pim1 for IL6 signaling in PCA cells. (**a**) Etk kinase activity is required for IL6-induced Pim1 activation. FLAG-Pim1 was cotransfected with EtkKQ (+) or vector (−) into LNCaP cells. At 24 h post-transfection, the cells were serum starved for 6 h and followed by treatment with IL6 for 15 min. Pim1 kinase activity was determined by IVK assay as described in Figure 2. (**b**) Pim1 kinase activity is required for IL6-induced Etk activation. T7-Etk was cotransfected with Pim1KM (+) or vector (−) into LNCaP cells. The cells were then treated as above. Etk kinase activity was determined as described in Figure 4

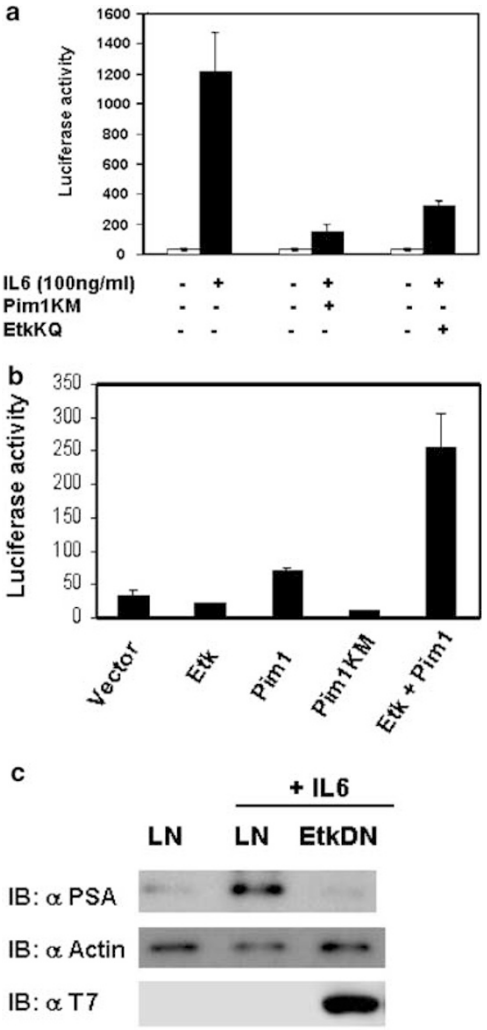

Figure 6 Effects of Piml and Etk on androgen receptor-mediated transcriptional activity. (**a**) Kinase activity of Pim1 and Etk are required for IL-6-induced ARE activity. ARR2-Luc and promoter-less-RL were transfected with the vector control, kinase inactive Pim1KM or Etk KQ into LNCaP cells grown in the complete medium containing 10% of charcoal-stripped FBS. At 24 h post-transfection, cells were subjected to incubation with fresh phenol-red free serum-free medium with or without IL6 (10 ng/ml) for 12 h. Luciferase assays were performed according to the manufacturer's instruction (Promega). The data were expressed as the relative luciferase activity. The error bars represent the mean ± s.d. of three independent experiments. (**b**) Synergistic effects of Piml and Etk on ARE-containing promoter activity. ARR2-Luc and promoter-less-RL were transfected with the indicated plasmids into LNCaP cells as above. At 24 h post-transfection, the cells were subjected to serum starvation for 12 h. Luciferase assays were performed as above. (**c**) Requirement of Etk kinase activity in IL6-induced PSA expression. LNCaP(LN) and its derivative EtkDN were cultured in the phenol red-free medium containing 10% charcoal-stripped FBS for 3 days. The cells were then treated with or without 50 ng/ml of IL6 in serum-free medium for 24 h. The levels of PSA in these cells were determined by immunoblotting with anti-PSA. The same blot was also probed with anti-actin or anti-T7 antibodies to monitor the sample loading (anti-actin) or the expression of the dominant-negative Etk (anti-T7)

Pim1(Pim1KM) was transfected into LNCaP with a Luciferase reporter under the control of the minimal probasin promoter (ARR2). At 24 h post-transfection, the cells were treated with IL6. Figure 6a shows that, in vector-transfected control cells, IL6 induced about 20-fold increase of transcription activity of the androgen receptor-dependent activity. However, in the presence of kinase-inactive Pim1 or Etk, the IL6 effects were dramatically blocked, suggesting that kinase activities of both Pim1 and Etk seem to be required for IL6-induced AR activation. In the meantime, overexpressing Pim1 or Etk alone had very minimal effect on AR-mediated transcription (Figure 6b). However, coexpression of Pim1 and Etk resulted in significant increase of the transcription activity. These data suggest the synergistic effects of Etk and Pim1 on activation of ARE-containing promoter. Furthermore, we examined the expression level of PSA in EtkDN cells, which is a LNCaP derivative stably expressing a dominant-negative Etk mutant (Xue *et al*., 1999), in response to IL6.

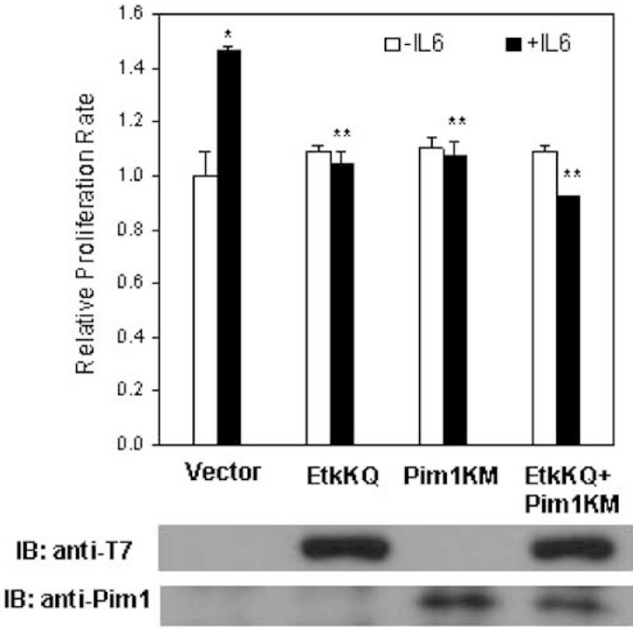

Figure 7 Requirement of Pim1 and Etk for IL6-induced proliferation of 22Rv1 cells. 22Rv1 cells were transfected with the vector control, T7-EtkKQ, FLAG-Pim1KM or both kinase-dead mutants. After 24 h, the cells were seeded on 96-well plates and the proliferation rate was assayed using WST-1 reagent. The data are given as mean $\pm$s.d. of three independent experiments. Error bar represent the s.d. $*P<0.01$, compared with the untreated vector control; $**P<0.005$, compared with the IL6-treated vector control. A fraction of these cells were lysed and subjected to immunoblotting with anti-T7 or anti-Pim1 to monitor the expression level of transfected EtkKQ or Pim1KM

Consistent with previous report, IL6 induces PSA expression in LNCaP cells. However, no elevation of PSA expression in IL6-treated EtkDN cells was detected (Figure 6c). These data suggest that Etk kinase activity is required for IL6-induced PSA expression.

To test whether Etk and Pim1 are required for IL6-induced proliferation of PCA cells. The vector control, Pim1KM or EtkKQ was transfected into 22Rv1 cells. The effects of IL6 on the proliferation of these cells were determined by the WST assay. As shown in Figure 7, IL6 treatment resulted in increased cell proliferation of vector-transfected cells by about 45%. This effect was diminished in the cells transfected with either EtkKQ or PimKM. Cotransfection of two kinase-dead mutants together further inhibited the cell proliferation. These results suggest that both Etk and Pim1 are required for IL6 promoted proliferation of 22Rv1 cells.

Discussion

Prostate cancer is the most commonly diagnosed cancer among men. Understanding the biological mechanisms involved in androgen-independent growth, tumor progression and metastasis has emerged as a fundamental and urgent issue in prostate cancer research. Mounting evidence suggests that IL6 may play an important role in ligand-independent activation of androgen receptor in prostate cancer cells. In this report, we presented the evidence of the synergistic effect of cytoplasmic kinases Pim1 and Etk on ligand-independent activation of androgen receptor induced by IL6. We showed that Pim1 is expressed in all PCA cell lines tested. The expression level of Pim1 in LNCaP cells is relatively lower than that in 22Rv1 and PC3. It is noteworthy that 22Rv1 and PC3 can usually form tumors when they are subcutaneously injected into male nude mice while LNCaP can hardly form any detectable tumors. These data suggest a possible correlation between the expression level of Pim1 and the tumorigenicity of these prostate cancer cells. Furthermore, the expression of Pim1 in PC3 cells appears to be regulated by IL6 autocrine loop because the neutralizing antibody against IL6 significantly diminished Pim1 expression in PC3 cells. IL6 not only regulates Pim1 expression at the transcriptional level, but also modulates the Pim1 kinase activity through its downstream tyrosine kinase Etk. We showed that Etk can induce tyrosine phosphorylation of Pim1 on Y218 in its activation loop, which is required for the activation of Pim1 kinase activity. Interestingly, Pim1 can also in turn enhance Etk kinase activity. Our preliminary data showed that Pim1 and Etk can form a complex which is detectable in a coimmunoprecipitation experiment. However, we were unable to detect the direct interaction of these two kinases in the *In vitro* GST-pulldown assay, implying that Pim1 might be associated with Etk through other protein(s). Identification of the intermediate protein(s) would help us to understand how these kinases activate each other. We also showed that the kinase activity of both Etk and Pim1 seem to be required for IL6-induced ligand-independent activation of AR transcriptional activity. However, overexpression of Etk or Pim1 alone is not sufficient for AR activation, suggesting a synergism between these two kinases is essential for AR activation. We were unable to detect phosphorylation of AR in neither Etk nor Pim1 IVK assay, implying that other protein(s) regulated by these kinases are responsible for the direct activation of AR. Further investigation on the downstream effectors of Etk and Pim1 will be required to reveal how Etk and Pim1 exert their influence on AR. Our work suggests that androgen-independent activation of AR in PCA cells is a complicated process which requires the involvement of multiple factors. Understanding the mechanisms by which the key factors regulate the downstream events leading to activation of AR will be essential for developing more effective intervention against hormone refractory prostate cancer.

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