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13. ABSTRACT *(Maximum 200 Words)*

During the second year of this postdoctoral training award, I continued to characterize the involvement of two proteins, NF-kB and BRCA1 that were identified in the first year of training, in androgen receptor transcription activity. Because they regulate expression of the PSA gene that is tightly regulated by androgen receptor, they are potential transcriptional intermediary factors for androgen receptor. I have successfully identified BRCA1 as a transcriptional co-repressor for androgen receptor. I demonstrated that NF-kB regulates expression of the PSA gene by direct binding to an enhancer of the PSA gene and ruled out the original hypothesis that NF-kB is a transcriptional intermediary factor for androgen receptor.

14. SUBJECT TERMS
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Introduction

The goal of this research is to identify and characterize transcriptional intermediary factors (TIF) that participate in the progression of prostate cancer from androgen-dependence (AD) to androgen-independence (AI). The rationale is that androgen receptor (AR), one of the nuclear receptor superfamily, is believed to play an important role in the progression. Because the TIFs regulate transcriptional function of nuclear receptors, changing the activity of these factors may contribute to the development of AI tumors. The strategy is to use a series of established human prostate cancer xenografts (LAPC4, LAPC9, and LNCaP) to identify TIFs that change expression levels between AD and AI tumors. During the first year of training, I identified NF-kB and BRCA1 as potential TIFs for AR after extensive screening by RT-PCR, western blot analysis, and reporter assay. This screening is the aim of task 1 (identification of genes). During the second year of training, I continues to characterize these two proteins in the regulation of AR transcription activity and to study their roles in prostate cancer progression. I demonstrated that BRCA1 interacts with AR, which is the aim of task 2 (characterization of interaction). I also demonstrated that BRCA1 inhibits AR function and NF-kB directly regulates the expression of prostate specific antigen (PSA), which is the aim of task 3 (functional analysis of the genes).

Body

Task 1 Identification of TIFs that change expression or post-translational modification in AI xenografts or Her-2/neu expressing cells (months 1-12)

After extensive screening on established human prostate cancer xenografts using RT-PCR, western blot analysis, and reporter assay, we identified NF-kB and BRCA1 as potential TIFs for AR because they affect expression of an AR-regulated reporter. We also demonstrated that an AI prostate cancer xenograft has higher constitutive NF-κB binding activity than its AD counterpart and that BRCA1 is down-regulated in AI prostate cancer xenograft.

Task 2. Biochemical characterization of interactions between AR and the candidate TIFs (months 7-18)

With the help of a technician in the lab, we demonstrated that BRCA1 interacts with AR. In these experiments, LNCaP cells were lysed in non-ionized detergent buffer and subjected to immunoprecipitation using an antibody against BRCA1. The precipitate was resolved in SDS-PAGE gel and probed with an antibody against AR. AR was detected in the precipitate, suggesting that AR associates with BRCA1. This data is consistent with a then newly published data demonstrating that BRCA1 interacts with AR in GST pull-down assay. We did not try to do the reversed experiment, i.e., to immunoprecipitate AR and probe the precipitate with BRCA1 antibody, because the association between AR and BRCA1 was already published when we were working on this aspect.

We did not try to examine interactions between NF-kB and AR because we concluded from the experiments described in Task 3 that NF-kB did not activate PSA expression through regulating AR transcription activity.

Task 3. Functional analysis of the candidate TIFs in AR signaling and in the progression of AI prostae cancer

-generate constructs for transfection and infection

In order to determine whether NF-kB is directly involved in the regulation of prostate specific antigen expression, I obtained expression vectors for p50 and p65 of NF-kB, and a dominant negative mutant IkBα (IkBα 32/36AA) from Genhong Cheng's lab at the University of California at Los Angeles (UCLA). The IkBα 32/36AA mutant, in which both serine 32 and 36 were mutated to alanine, prevents phosphorylation and degradation of IkBα. This mutant inhibits the activation of NF-kB by blocking the translocation of NF-kB to the nucleus.

I obtained a wild-type BRCA1 expression vector from Tim Lane at UCLA. In order to determine the mechanism that BRCA1 inhibition on AR transcription function, I generated two BRCA1 mutants. One has a cytosine (C) insertion at 5382 position (BRCA1 Cins). This is a frameshift mutation occurred commonly in human breast, ovary, and prostate cancers. The other one expresses the C-terminal portion of BRCA1 (FLAG-BRCT). This portion of protein was demonstrated to interact with the histone diacetylase complexes (HDAC).

-transfect into LNCaP or BHK cells, monitor PSA expression or detect luciferase activity

To determine whether NF-kB regulates AR transcription function, p50 and p65 of NF-kB were co-transfected into LNCaP or DU145 cells with a reporter containing the transcription regulatory element of PSA. NF-kB activated the transcription of the PSA promoter in LNCaP cells in the absence of androgen, suggesting that NF-kB activates the transcription of the reporter independent of AR because androgen is required for the activation of AR. This conclusion was confirmed by the result that NF-kB activated the reporter in DU145 cells because these cells do not express AR.

To determine how NF-kB regulates PSA expression, footprint analysis was performed on the core enhancer of PSA. Four NF-kB binding sites were identified in this region. Mutation of two binding sites partially abolished the activation of NF-kB on the reporter, indicating NF-kB regulates PSA expression by direct binding to the PSA enhancer.

To determine if BRCA1 directly inhibits AR transcription function, an artificial promoter consisting of a concatemer of AR response element (ARE) was used as a reporter. AR activates the expression of this reporter, and the activation was abolished by co-transfection of BRCA1 in a dose-dependent fashion. BRCA1 inhibition on AR transcriptional activity is specific because it did not affect NF-kB activating its cognate response element. These results indicates that BRCA1 is a TIF for AR. Because the

inhibition was reversed when a specific inhibitor of the HDAC was present, or when a Flag-BRCT that mediates the interaction between BRCA1 and AR was co-transfected, we concluded that BRCA1 inhibition on AR transcription activity is mediated by the HDAC complex.

-infect LNCaP and LAPC4, measure growth by cell number and MTT level
-inject infected LNCaP and LAPC4 to male, castraed, and female mice, monitor serum PSA level and tumor size

We have been successfully using a lentivirus system that was developed in the Inder Vermus Lab at the Salk Institute in San Diego to transfer target genes into mammalian cells. The transduction efficiency of this system is virtually 100%. We would like to use this system to do these two experiments.

Key Research Accomplishments

1. I identified two genes, NF-kB and BRCA1, that may be important for prostate cancer progression
2. I demonstrated that NF-kB activates expression of the PSA gene.
3. I demonstrated that BRCA1 inhibits transcription activity of AR through the HDAC complexes.

Reportable Outcomes

Meeting presentation:

1. Chen, CD, Abreu, M, and Sawyers CL. 2000. MEKK1 activates transcription activity of androgen receptor (AR) through NF-kB. Poster presentation, American Association of Cancer Research, 91th Annual Meeting, San Francisco, California, 2000
2. Chen, CD, Abreu, M, and Sawyers CL. 2001. Evidence implicating the NF-kB pathway in androgen-independent prostate cancer progressison. Poster presentation, American Association of Cancer Research, 92th Annual Meeting, New Orleans, Louisiana, 2001

Conclusions

This study is to identify transcriptional intermeidiary factors that may participate in the progression of prostate cancer by regulating AR function. We have successfully identified BRCA1 as a corepressor for AR and demonstrated that NF-kB regulates PSA expression.

Reference

Chen CD, Abreu M, and Sawyers CL. MEKK1 activates transcription activity of androgen receptor (AR) through NF-KB. March 2000, the Proceedings of the American Association for Cancer Research.

Chen CD, Abreu M, and Sawyers CL. Evidence Implicating NF-kB in Androgen-Independent Prostate Cancer Progression, March 2001, the Proceedings of the American Association for Cancer Research.

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

March 20, 2001

Research Data Management

SUBJECT: Review of Annual Summary Report Dated July 2000, Award Number DAMD17-99-1-9514

Charlie D. Chen, Ph.D.
Departments of Medicine/Hematology and Oncology
University of California, Los Angeles
10833 Le Conte Avenue
Los Angeles, California 90095-1678

Dear Doctor Chen:

Subject report has been reviewed and is acceptable as written. A copy of your report has been forwarded to the Defense Technical Information Center's Technical Reports database.

To assist you in preparing future reports under the subject award, we have enclosed the reviewer's comments. We ask that you review these comments and incorporate any recommended changes into future reports.

Point of contact for this action is Ms. Judy Pawlus at 301-619-7322 or by email at judy.pawlus@det.amedd.army.mil.

Sincerely,

Judy Pawlus
Technical Editor

Enclosure

AACR American Association for Cancer Research

92nd Annual Meeting

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Ukrain 10 mg every second day x 10 (21 patients), or to vitamin C 5.4 g every second day x 10 and normal saline 10 mL (control group, 21 patients). The measures of efficacy were overall survival, body weight change, pain intensity (measured by analgesic consumption), Karnofsky performance status, and tumor response. 12-month survival was 81% in the Ukrain group compared to 14% in the control group, and 19-month survival was 48% in the Ukrain group compared to 5% in the control group. Both pain and Karnofsky status improved in ten Ukrain patients. Three Ukrain patients had an improvement in pain and no worsening of Karnofsky performance status. The longest survival in the Ukrain group was 50 months after the start of therapy (from January 1996 to March 2000). Median survival duration was 17.17 months for Ukrain treated patients and 6.97 months for the control group (p=0.05). Three Ukrain patients (14.3%) achieved partial tumour response with median duration of 14 months, 9 patients (42.9%) had stable disease for 13 months (median value). Ukrain treatment was well tolerated. Blood and urine examinations revealed no negative or toxic influence of Ukrain, and moreover, showed an improvement in the immune profile in Ukrain treated patients. We conclude that Ukrain not only improves the quality of life of pancreas cancer patients, but also prolongs survival in this disease. Whether and to what extent this drug can be used as standard therapy in pancreas cancer should be the object of a phase III study.

#3347 Oxaliplatin, 5-Fluorouracil, and Radiation in Cancer of the Esophagus: A Pharmacokinetic (PK)/Molecular Correlates Study. Lakshmi Pendyala, Cynthia Gail Leichman, Kimberly Clark, Hector Nava, Enriqueta Nava, Patrick Smith, Brent M. Booker, William Greco, and Lawrence Leichman. *Roswell Park Cancer Institute, Buffalo, NY.*

Oxaliplatin (OXP), with a manageable toxicity profile, activity in gastro-intestinal cancers and reported synergy with 5-fluorouracil (5FU) is evaluated in a clinical trial at RPCI as a potentially favorable substitute for cisplatin in the treatment of locally advanced cancer of the esophagus. Our previous *in vitro* and clinical molecular correlates studies indicate that a) OXP resistance is multi-factorial and b) an inverse relationship exists between the mRNA expression of thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) or the excision repair cross complementing gene-1 (ERCC-1) and response to 5FU (colon cancer) or 5FU/cisplatin (gastric cancer). Based on these, we hypothesized that a) molecular markers within a primary esophageal tumor will predict sensitivity or resistance to chemotherapy, b) OXP may improve 5FU therapy by down-regulating TS and c) PK of OXP will influence the changes in gene expression. Patients receive OXP on day 1 (85 mg/m^2), day 15 and day 29 of a 6wk cycle, continuous infusion 5FU (180mg/m^2) beginning day 8 for 5 wks, along with external beam radiation (180 cGY/day) for 25 days (5wks). Endoscopic biopsies are obtained prior to therapy, 1 wk after OXP alone and after 1 cycle of combination therapy. Intratumoral gene expression for TS, DPD, γ-glutamylcysteine synthetase (γGCS), γ-glutamyltranspeptidase (γGT) and ERCC-1 are determined with β-actin as the internal standard using real time quantitative RT-PCR (Taqman® assays). PK of ultrafilterable platinum (Pt) after OXP alone on day1 and again on day15 after combination with 5FU and radiation are determined using Atomic Absorption Spectrophotometry. Nine of 10 patients showed a reduction in the expression of TS and γGCS genes from day 1 to day 8 after OXP alone. Other genes showed an inconsistent pattern. The median % change (range) in gene expression in tumor biopsies of 10 patients one wk after OXP for TS, DPD, ERCC-1, γGCS and γGT are ↓45 (-28 to 100)*, ↑9(-144 to 99), ↓14 (-44 to 91), ↓60 (-55 to 75)* and ↑8(-77 to 85) respectively (*p<0.05, Wilcoxon signed rank test). In 5 patients studied to date at the end of 6wks, the median TS expression has decreased by 67%. PK of OXP showed no differences when given alone or after combining with 5FU, radiation and OXP exposure may be associated with the observed changes in TS and γGCS expression. The data suggest that OXP either down regulates TS and γGCS or that a change in the populations of cells with differential expression of these genes occurs during the chemotherapy. The significance of changes in gene expression with respect to response is being evaluated. The study is ongoing. (Supported in part by CA16056)

#3348 Operative Indication of Advanced Gallbladder Cancer with Hepatoduodenal Ligament Invasion. Itaru Endo, Hiroshi Shimada, Shinji Togo, Kuniya Tanaka, Hitoshi Sekido, Yoshiro Fujii, and Masahiko Matsumoto. *Matsumoto Clinic, Omiya, Japan, and Yokohama City Univ. Med., Yokohama, Japan.*

The hepatoduodenal ligament invasion(HDLi) is a inhibitiong factor for curative resection for advanced gallbladder cancer. In this study, it was aimed to be clarified the significance and the indication of surgical resection for the patients with the HDLi, from the analysis of outcome of gallbladder cancer. This study was conducted on 58 patients with advanced gallbladder cancer who underwent aggressive resection with extended lymphadenectomy and 20 non-resection cases who were diagnosed as positive HDLi. The existence of stromal cancerous infiltration at 6 points in the HDL were examined. According to the tumor occupying area, HDLi was classified as follows: types I, Stromal cancer infiltration detected in the middle and upper part of the HDL; type II, Stromal cancer infiltration detected in the middle and lower part of the HDL. Cumulative 5 year-survival rate of the patients with HDLi was 10.9%, significantly lower than 46.6% without HDLi(P<0.01). Outcome of type II patient was significantly worse than type I(P<0.05), and it was equivalent to the non-resection cases. The type I patients with paraaortic lymph node metastasis annihilated within 1 year. Cumulative 5 year-survival rates after curative resection were significantly better than that of non-curative resection (P<0.05). Our results indicate that aggresive surgical resection for curative operation should be restricted to the type 1 patients without paraaortic lymph node metastasis.

#3349 Usefulness of Transcatheter Arterial Infusion of Anti-Cancer Agents with Lipiodol in Prevention of the Recurrence After Hepatic Resection for Hepatocellular Carcinoma. Shinji Togo, Kuniya Tanaka, Itaru Endo, Kouichiro Misuta, Hitoshi Sekido, Kaoru Nagahori, and Hiroshi Shimada. *Yokohama City University, Yokohama, Japan.*

Residual liver recurrence rate after hepatic resection for hepatocellular carcinoma (HCC) remains high. To ensure a further improvement in the prognosis after hepatectomy for HCC, a chemotherapy using the postoperative transcatheter arterial infusion of lipiodol in combination (TAI) was repeated. This study is to evaluate the efficacy of this treatment for prevention of residual liver recurrence after hepatectomy. Material and method: The TAI after hepatectomy was performed in 30 (TAI group) out of the 68 cases with positive tumor invasiveness such as infiltration to capsule, intraportal spread, and intrahepatic metastasis. The TAI procedure uses injection of a mixed of MMC and ADM with lipiodol via the hepatic artery. Two groups comparing TAI (n=30) and non-TAI (n=38) were compared from the recurrence rate and survival rate to evaluate the efficacy of TAI after hepatectomy. Result: The cumulative residual liver recurrence rate in TAI group was lower than that in non-TAI group (p<0.05). When the residual liver recurrent cases divided into two groups by terms of the duration of recurrence, the rate of recurrence within 1 year after hepatectomy was lower in TAI group than in non-TAI group (p<0.05). The cumulative survival rate in TAI group was significantly higher than in non-TAI group (p<0.05). Morbidity rate was 10%. In 2 cases, non infected bilomas occurred, and 1 case was complicated liver abscess. Conclusion: It is suggested that TAI would be effective as a surgical adjuvant for prevention of residual liver recurrence by paying full attention to a development of complications.

MOLECULAR BIOLOGY 23: Gene Expression II: Prostate and Breast Cancer

#3350 TBP May Activate a Mutant Form of the Human SRD5A2 Gene Promoter: Implications for Prostatic Disease. Nick M. Makridakis and Juergen KV Reichardt. *University of Southern California, Los Angeles, CA.*

Both prostate cancer and Benign Prostatic Hyperplasia (BPH) are prevalent diseases of largely unknown etiology that are believed to be androgen dependent. The SRD5A2 gene encodes the prostatic steroid 5α-reductase (type 2), which converts testosterone into dihydrotestosterone (DHT), the main androgen in the prostate gland. We have recently shown that an SRD5A2 missense mutation predisposes men to develop prostate cancer by activating the gene product. We then screened the entire genomic sequence of the SRD5A2 gene for polymorphisms with direct sequencing analysis. We utilized lymphocyte DNA from mostly Caucasian-American patients whose prostate glands were evaluated for the presence of BPH or prostate cancer, after bladder cancer surgery. The most common polymorphism out of 14 identified, C682G (cytosine-682 to guanine), is located in the 5' UTR (untranslated region), 12 nucleotides downstream of the transcription start, and is significantly more common in men with early-onset BPH. In order to characterize the biochemical effect of some of those polymorphisms, we have subcloned a 750-bp fragment containing the promoter and 5' UTR of the SRD5A2 gene in front of the luciferase gene on the pGL3 expression vector. After appropriate site-directed mutagenesis construction, we transfected *cos* cells with either the wild type (682C) or the mutant (682G) construct, and measured the luciferase activity of the isolated cell lysates. The C682G polymorphism resulted in significantly higher luciferase expression in *cos* cells, suggesting that it has functional consequences *in vivo* . This polymorphism is predicted to improve a putative TATA box binding consensus in the SRD5A2 gene. In prostatic PC3 cells, the C682G polymorphism resulted in slightly higher luciferase expression, but co-transfection of human TATA box binding protein (TBP) significantly increased luciferase expression of the mutant (682G) but not the wild-type (682C) construct. Thus, at least one of the SRD5A2 polymorphisms identified (the C682G) is predicted to increase 5α-reductase enzyme levels *in vivo*, and therefore may increase the risk for BPH and/or prostate cancer, and TBP appears to be at least partially responsible for that increase. These findings also suggest that the common C682G mutation, although in the 5' UTR, may lie in a region that is part of the SRD5A2 gene promoter, and that it is activated by TBP.

#3351 Evidence Implicating the Nf-Kb Pathway in Androgen-Independent Prostate Cancer Progression. Charlie Degui Chen, Maria Abreu-Martin, and Charles L. Sawyers. *Cedars-Sinai Medical Center, LA, CA, and UCLA, Los Angeles, CA.*

Previously we showed that the mitogen-activated protein kinase kinase kinase 1 (MEKK1) activates the promoter and enhancer of prostate specific antigen (PSA), a marker for prostate cancer development and progression. Because we were unable to detect changes of phosphorylation of AR, we hypothesized that

the transcription activity results from the activation of AP-1 and/or NF-KB, two notable MEKK1 downstream effectors involved in transcription regulation. To further characterize the mechanism of activation, we carried out reporter assays that read out each pathway independently by transient transfection. The transcription activity does not result from AP-1 activation because JBD, a dominant negative mutant of JNK interacting protein (JIP) that blocks signal transduction from MEKK1 to AP-1, did not repress MEKK1-activating PSA transcription. However, the activation was abolished by a dominant negative mutant of IkBa, of which both ser32 and 36 were substituted by alanine. This result suggests that MEKK1 activates the PSA enhancer through NF-kB activation. This conclusion was confirmed by two other observations. First, TNFa, a stimulus that leads to NF-kB activation, activates the PSA enhancer. Second, overexpression of the p65 subunit of NF-kB was sufficient to activate the PSA enhancer. The IkBa mutant abrogates the activation by both TNFa and overexpression of p65. By DNA footprinting analysis, we identified four NF-kB binding sites in the PSA enhancer. We also observed that androgen–independent LAPC4 tumor cells have a higher constitutive activity of NF-KB than the LAPC4 androgen–dependent tumors, consistent with other reports showing that androgen irresponsive cell lines, DU145 and PC3, have a higher constitutive activity of NF-kB than the androgen responsive LNCaP cell line. These studies support a potential role for the NF-kB pathway in the transition of some prostate cancers to an androgen-independent state. We are now in the process of directly testing this hypothesis by engineering constitutive high NF-kB activity in androgen-dependent prostate cancer models.

#3352 Signal Transduction Mediated Regulation of Urokinase Gene Expression in Human Prostate Cancer. Joy C. Yang, Jonathan A. Eandi, and Christopher P. Evans. *Department of Urology, University of California, Davis, School of Medicine, Sacramento, CA.*

Urokinase-type plasminogen activator (u-PA or urokinase) contributes to tumor progression in prostate cancer (CaP). We have previously shown that expression of u-PA is enhanced by a 5'AP-1 transcriptional factor binding site and repressed by androgen through its binding to the androgen receptor on a hormone response element in its promoter region. To delineate signal transduction pathways that mediate transcriptional factors and u-PA gene expression we studied specific ERK, JNK, and P38-MAPK pathway mutant constructs and inhibitors in vitro. Human, androgen insensitive PC3 prostate adenocarcinoma cells stably transfected with the androgen receptor ($PC3(AR)_2$) and one mock transfected cell line (PC3(M)) were used. Transient transfection in $PC3(AR)_2$ cells using a u-PA promoter regulated CAT (u-PA-CAT) reporter construct was used to study signaling regulation for u-PA. Cotransfection of u-PA-CAT with the mutant constructs CL100, K97M, and PC4 raf, for ERK1/ERK2. MEK1, and c-raf respectively, and dominant negative mutant constructs for JNK and P38-MAPK were used to elucidate the u-PA signaling pathways. All of these mutants when co-expressed drastically reduced activation of u-PA promoter in $PC3(AR)_2$; this demonstrated a role for ERK, JNK, and P38-MAPK mediated AP-1 transcriptional activation of u-PA. Futhermore, inhibitors LY294002 and Wortmannin for PI3K, an upstream regulator in the JNK/SAPK pathway, were instituted and also resulted in a decrease in u-PA promoter transcription. Collectively, these results show that MAPK signal transduction pathways ERK, JNK/SAPK, and P38-MAPK represent a significant component in the regulation of u-PA expression in human prostate cancer. We also investigated whether the 5'AP-1 binding is androgen regulated. Electrophoretic mobility shift assays were performed using PC3 nuclear extract incubated with end-labeled (^{32}P-ATP) oligonucleotide. AP-1 protein was bound to this site only in $PC3(AR)_2$ and LNCaP cell lines in the presence of the synthetic androgen mibolerone. This binding was competitively inhibited with anti-c-*jun* and anti-c-*fos* proteins. These studies show that both c-*jun* and c-*fos* proteins are involved in binding to the 5'AP-1 site and that this binding is androgen dependent.

#3353 p53-Independent Regulation of Mdm-2 Transcription in Estrogen Receptor-Positive Breast Cancer Cells. Jeremy Paul Blaydes and Matthew Darley. *University of Southampton, Southampton, UK.*

The onco-protein mdm-2 is an essential regulator of the cell cycle, and is currently under investigation as a target for tumor therapy. Despite this relatively little is known about how mdm-2 is regulated in normal and tumor cells, particularly the activation of its transcription by tissue-specific, p53-independent factors. Clinical and experimental data has identified a role for mdm-2 in a high percentage of breast cancers, and mdm-2 expression is upregulated in ER^{+ve} breast tumors. Here we have made use of a panel of breast cancer cell lines in order to examine the mechanisms regulating mdm-2 expression in these cells. We demonstrate that transcription from the p53-dependent P2 promoter of mdm-2 is upregulated in ER^{+ve}, but not ER^{-ve}, cell lines, and that this transcription is independent of p53. We have used reporter vectors containing deletions of this promoter region to map the sequence elements which control this transcription. We also demonstrate how transcription from this promoter is regulated by agents which control breast cancer cell growth and differentiation. Constitutively elevated levels of mdm-2 expression in ER^{-ve} breast cancer cells may explain the relatively low frequency at which p53 mutations occur in these tumors.

#3354 Triiodothyronine (T3) Sustains the Serum Free Proliferation of Prostate, Breast and Myeloid Tumor Cell Lines. Quang Trong Luong, Moray Campbell, Sarah Fenton, and Christopher Bunce. *University of Birmingham, Birmingham, UK.*

We have demonstrated that replacement of serum with ITS^- (commercial serum-free medium supplement) results in growth arrest and neutrophil differentiation of HL-60 acute myeloid leukaemia (AML) cells. However, HL-60 cells remained in cycle and did not differentiate when serum was replaced with 1nM T_3 or the RARα antagonist Ro 41-5235. Here, we report that T_3 also sustains the proliferation of prostate cancer (including PC-3 and DU-145) and breast cancer (including MCF7 and Hs578t) cell lines when in serum-free medium containing ITS^+. These observations suggest that T_3 exerts a survival/pro-proliferative signal to a broad spectrum of malignant cells. DNA array analyses to identify genes regulated by T_3, that are associated with the capacity of the hormone to preserve HL-60 proliferation, have identified *Nm23 H1* and *Nm23 H2*. Real time PCR confirmed the elevated expression of both *Nm23* genes in T_3-treated serum-free HL-60 and PC-3 cells when compared to non-treated cells. *Nm23* genes belong to a highly conserved gene family that regulate prokaryote, plant and animal development. In rodents and humans these two genes have been associated with the metastatic behaviour of solid tumour cells and with development and prognosis of AML. Over-expression of either *Nm23 H1* or *H2* blocks the in vitro differentiation of several myeloid cell lines. Clearly therefore an understanding of the regulation of *Nm23* gene expression will contribute significantly to our understanding of cancer as a whole. We are interested in the possibility that *all-trans* retinoic acid and T_3 interact directly to differentially regulate *Nm23 H1* and *H2*. The *Nm23 H1* and *H2* genes lie in tandem at 17q21. We have identified a complex hormone response element upstream of the *Nm23 H1* promoter that has a central DR4 with each consensus half-site also contributing to two flanking DR2 elements. A near identical element regulates the expression of human myeloperoxidase. In this case the DR4 has been shown to be a functional thyroid hormone response element (TRE) and the proximal DR2 a retinoic acid response element (RARE). We are using gel mobility shifts and transient reporter assays to establish if this element can activate or repress *Nm23* expression and whether retinoids and thyroid hormones do differentially regulate these activities.

#3355 TMEFF2 is a New Member of the EGF-Like Family Regulated by Androgen and Having Antiproliferative Effects in Prostate Cancer Cells. Sigal Gery, Vijay Vegesna, Charles L. Sawyers, David B. Agus, and Phillip H. Koeffler. *Cedars-Sinai Medical Center/UCLA School of Medicine, Los Angeles, CA, and UCLA School of Medicine, Los Angeles, CA.*

Using subtractive hybridization, we identified a gene that was highly expressed in the androgen dependent (AD) prostate cancer cell line, LNCaP and undetectable in the androgen independent (AI) PC3 and DU145 cell lines. Sequence analysis revealed that it was identical to a newly cloned gene designated TMEFF2. TMEFF2 encodes a transmembrane protein containing an epidermal growth factor (EGF)-like motiff and two follistatin domains. We mapped TMEFF2 to human chromosome 2q32.1. Extensive examination of normal and cancer human tissues for TMEFF2 mRNA expression by Northern blot demonstrated that it is expressed almost exclusively in prostate and brain. TMEFF2 mRNA was low in LNCaP cells grown in the absence of hormones, and markedly increased in a dose-dependent manner by dihydrotestosterone and 17β-estradiol. Furthermore, TMEFF2 expression was higher in the AD human prostate cancer xenografts, LAPC4-AD, LAPC9-AD and CWR22 than in their AI sublines (LAPC4-AI, LAPC9-AI and CWR22R). In addition, TMEFF2 levels decreased by day 10 post castration in the AD CWR22 xenografts. TMEFF2 expression vector was stably placed into DU145. These cells grew slowly in liquid culture and were much less tumorigenic as compared to wild type cells in nude mice. Our results demonstrate that TMEFF2 is an androgen responsive gene expressed in normal prostate cells as well as in AD prostate cancer cells and its forced expression inhibits tumor growth. Loss of expression of TMEFF2 occurs with transformation to AI suggesting it may act as a suppressor of prostate cancer progression to AI.

#3356 Gene Expression in the ACI Rat Prostate. Niradiz Reyes, Michael Iatropoulos, Abraham Mittelman, and Jan Geliebter. *New York Medical College, Valhalla, NY.*

The natural history of CaP is a multistage process that involves the transition from normal tissue to sub-clinical CaP, with progression to carcinoma in situ and eventually metastatic disease. Evidence indicates that a high fat diet plays a critical role in the biology and progression of the disease. ACI rats were maintained for 2 generations on high fat and control diets for 6, 12 and 18 months (high corn oil diet - 23.52% corn oil; high beef fat diet- 22.52% beef fat/1% corn oil; control diet 5% corn oil, by weight). Rats on the high beef fat diets had higher plasma triglyceride levels than rats on high corn oil diets, which, in turn, had higher levels than control rats ($p<.05$). We are currently analyzing the expression levels of various genes in the ventral and dorsolateral prostates of rats at different ages, on different diets. Results from these studies will lead to a better understanding of the effect of diet and age on gene expression in the prostate and facilitate the rational design and assessment of potential dietary programs for prostate cancer prevention. Supported by AICR grant #97A072 and the Zita Spiss Foundation.

AACR

American Association for Cancer Research

91st ANNUAL MEETING

April 1-5, 2000 San Francisco, CA

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organization. Interestingly, very similar morphological changes have been observed by others after INFγ treatment. This effect of HOXC8 downregulation on the morphology of DU145 cells possibly suggest a role in their differentiation.

#127 TRANSCRIPTION FACTORS REGULATION BY 12(S) HETE IN PC-3 PROSTATE CANCER CELLS. Mustapha Kandouz, and K. V Honn, *Wayne State Univ, Detroit, MI*

12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE], a 12-lipoxygenase metabolite of arachidonic acid has proven to be affecting a plethora of cellular activities of importance in tumor growth, metastasis and angiogenesis, including adhesion, spreading, motility and invasion. In order to investigate the signaling pathways involved in these functions, we sought to study the effect of 12(S) HETE and other arachidonic acid metabolites on the activation of various proteins involved in the regulation of the cellular transcriptional machinery. We particularly studied the Nuclear Factor-kappa B (NF-κB) and the cAMP-response element binding protein (CREB) pathways, both being key mediators of the transcriptional regulation of a large number of genes and playing an important role in various cellular contexts, such as inflammation or cancer. PC-3 cells stably transfected with the cDNA of platelet-type 12-Lipoxygenase, show an increase in the binding activity of different transcription factors. We show that exogenous treatment with 12(S)-HETE increases DNA binding activity and nuclear translocation of NF-κB. As evidenced by the use of proteasome inhibitors and transfection with a dominant negative mutant, this effect is I-κB-α (NF-κB inhibitor-α)-dependent. Concomittantly to its effect on NF-κB, 12(S)HETE induces CREB binding activity. Data will be presented regarding the effect of 12(S)HETE and other lipoxygenase metabolites and inhibitors, as well as the signaling pathways leading to the activation of NF-κB and CREB in prostate cancer cells.

#128 MEKK1 ACTIVATES TRANSCRIPTION ACTIVITY OF ANDROGEN RECEPTOR (AR) THROUGH NF-KB. Charlie Degui Chen, Maria Abreu, and Charles L Sawyers, *Cedars-Sinai Med Ctr, LA, CA, and UCLA, LA, CA*

We have previously demonstrated that the signal transducer MEKK1 promotes apoptosis of prostate cancer cells in an AR dependent manner and activates the transcription activity of AR. But the involved mechanism was unknown. MEKK1 may transduce signals through different pathways. To delineate the mechanism responsible for the activation of AR transcription activity by MEKK1, we carried out a series of transfection experiments. Because we were unable to detect changes of phosphorylation of AR, we hypothesized that the transcription activity results from the activation of AP-1 and/or NF-KB, two notable downstream effectors involved in transcription regulation. The transcription activity does not result from AP-1 activation because JBD, a dominant active mutant of JNK interacting protein (JIP) that blocks signal transduction from MEKK1 to AP-1, did not repress the activation. However, a dominant active mutant of IkBa, of which serine 32 and 36 were mutated, abolished transcription activation of AR by MEKK1. This suggests that NF-KB is involved in this process. We are examining the relationship between the transcription activity of AR and the activation of NF-KB.

#129 DIFFERENTIAL GSK3β RESPONSES TO TNF-α IN PROSTATE CELLS. T Salas, S M Lippman, and D G Menter, *Univ of Texas, MD Anderson Cancer Ctr, Houston, TX*

We compared the responses of prostate epithelial cells (PrEC) to LNCaP, PC-3 and DU145 prostate tumor cells after TNF-α treatment. These cells were examined for differences in glycogen synthase kinase 3β (GSK3β) activity involving the phosphorylation and activation of the cyclic AMP response element binding protein (CREB). GSK3β protein that was immunoprecipitated from all cells increased CREB peptide phosphorylation on S129 in PrEC cells but not in LNCaP cells and PC-3 cells after TNF-α treatment. Immunofluorescence analysis revealed an increase in the perinuclear distribution of P-Tyr 216, GSK3β and nuclear P-CREB only in the PrECs after TNF-α. Furthermore, gel shift analysis with the cAMP response element (CRE) confirmed that treated PrEC's had a greater pool of P-CREB in DNA binding complexes than that found in the tumor cell lines. Transfection of a CRE-luciferase construct into PrECs and PC-3 cells confirmed that transcriptional activation was modulated differentially by TNF-α treatment. These findings suggest that GSK3β responds differentially to TNF-α, depending on the cell type and context. Supported by a Predoctoral Fellowship from the M.D.Anderson Program in Cancer Prevention, R25-CA57730 from the NCI and ACS grant TPRN-99-240-01-CNE-1.

#130 CHARACTERIZING THE ANDROGEN REGULATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN GENE. A Vlahou, T L Bullock, P F Schellhammer, and G L Wright, Jr., *Eastern Virginia Med Sch, Norfolk, VA*

The high expression of the Prostate Specific Membrane Antigen (PSMA) gene in prostate cancer cells as well as the neovasculature of a variety of solid tumors has turned it into a target of novel diagnostic and therapeutic strategies. The mechanism of regulation of its expression, however, remains largely unknown. *In vitro* studies have previously shown that PSMA is downregulated by androgens. The objective of this study is the characterization of the mechanism of androgen responsiveness of PSMA in prostate. Experiments from our lab and others support that this regulation is not mediated through a 2kb genomic region that encompasses the promoter of the gene. Using prostate cancer LNCaP cells which express endogenous PSMA we tested whether androgens affect PSMA at a post-transcriptional level. Determination of PSMA mRNA levels was carried out after inhibition of transcription by Northern blot analysis. Our results support that in the presence of androgens there is a significant increase in the degradation rate of PSMA mRNA compared to the rate observed in cells cultured in media devoid of steroids. RNA-protein interaction assays are currently under way to further explore this effect. With this study we found a post-transcriptional effect of androgens at the level of the PSMA mRNA stability, as a possible mechanism for the observed androgen-induced decrease of PSMA steady state levels. Supported by a fellowship from the American Foundation of Urologic Disease and Hoechst Marion Roussel (AV) and a grant from the Virginia Prostate Center.

#131 DYSREGULATION OF HOXC GENE EXPRESSION IN PROSTATIC CARCINOMA. Gary J Miller, and Heidi L Miller, *Univ of Colorado Health Sci Ctr, Denver, CO*

The malignant phenotype including the process of metastasis is known to involve coordinated changes in the expression of numerous genes. The HOX genes encode a family of helix-turn-helix transcription factors that represent "master regulatory" genes known to coordinate developmental processes such as proliferation, apoptosis, motility, adhesion and differentiation. Evidence also indicates that dysregulation of HOX gene expression plays a role in establishing the malignant phenotype of leukemias and solid tumors. We have studied the expression of HOX genes in prostatic cancer cells using a degenerate RT-PCR technique. cDNAs from 8 malignant cell lines (ALVA-31, DU145, JCA-1, LAPC4, LNCaP, PC-3, PPC-1 and TSU-Pr1) 1 primary epithelial cell culture, 4 normal prostate specimens, 1 BPH nodule and 2 lymph node metastases were examined. The PCR products were cloned and 484 inserts, 192 from benign sources and 292 from malignant ones, were sequenced. Expression of homeoboxes from the HOXC cluster was almost exclusively limited to malignant cells. Only 2 HOXC6 inserts were found in the BPH PCR products. Conversely, 40% (116/292) of the inserts from malignant cells were derived from the HOXC cluster. HOXC transcripts were detected in each of the 8 malignant cell lines and both of the lymph node metastases. No HOXC transcripts were found in normal prostate RNA. The expression pattern of selected HOXC genes was also confirmed in benign and malignant RNA sources using specific RT-PCR. These results indicate that a primary dysregulation of HOXC expression may accompany or cause the malignant phenotype of prostate carcinoma cells.

#132 ANTISENSE EXPRESSION FOR AMPHIREGULIN SUPPRESSES TUMORIGENICITY AND ANGIOGENESIS OF A TRANSFORMED HUMAN BREAST EPITHELIAL CELL LINE. Lin Ma, C. Gauville, Y. Berthois, G. Millot, G. R Johnson, and F. Calvo, *Faculty de Medicine Nord, Marselle, France, FDA, Bethesda, MD, and INSERM EPI-9932, Paris, France*

The epidermal growth factor (EGF) family of receptors and their respective ligands play a major role in breast cancer progression and are the targets of new therapeutic approaches. Following immortalization with SV40 T antigen of normal human breast epithelial cells, a transformed variant cell line (NS2T2A1) was selected for its increased tumorigenicity in nude mice. This cell line was shown to have a higher expression of EGF receptors (EGFR) and amphiregulin (AR) when compared to their normal counterparts or less aggressive transformed cells. Dual staining of EGFR and AR was observed in 50-60% of NS2T2A1 cells, while 30-40% cells expressed AR only. To explore the potential tumorigenic role of AR, a 1.1 kb AR cDNA in an antisense orientation was transfected in NS2T2A1 cells. Three clones, selected by hygromycin B, expressed AR antisense RNA (AR AS1, AR AS2 and AR AS3 cell lines) in which AR protein expression was reduced (ranging from about 50% to <5%). The anchorage-independent growth of AR AS cell lines was reduced to levels ranging from 32.4% to 6.8% relative to the control cell line transfected with the vector alone. The clones expressing AR antisense RNA showed a reversion of the malignant phenotype when injected in nude mice, since a significant reduction of tumor intake was observed coincident with a significant tumor mass reduction (>96%). Moreover, intra-tumoral vascularization decreased significantly in tumors derived from AR AS cells (26.7, 70.7 and 50.4% of control). These *in vitro* and *in vivo* data reveal the oncogenic nature of AR in transformed breast epithelial cells and imply a role for AR in tumor angiogenesis.

#133 DOMINANT NEGATIVE KNOCKOUT OF P53 PERMITS GROWTH ACCELERATION IN ERBB2-EXPRESSING HUMAN BREAST CANCER CELLS. G. C Huang, S. Hobbes, M. Walton, and R. J Epstein, *Imperial Coll Sch of Medicine, London, Uk, and National Cancer Ctr, Singapore, Singapore*

We previously showed that the ErbB2 oncoprotein prolongs and amplifies ambient growth factor signalling by impairing downregulation of heterooligomerized receptors. Here we show that the length and intensity of ligand-dependent ErbB2 tyrosine phosphorylation parallels the induction of apoptosis and growth inhibition in human A431 squamous cancer cells containing wild-type p53. Apoptosis is also induced by ErbB2 transfection of p53-expressing MCF7 human breast cancer cells; in these cells reduced growth and increased p53 expression accompany increased protein levels of epidermal growth factor receptor and Myc. ErbB2 transfectants lacking functional p53 exhibit reduced apoptosis and enhanced growth, on the other hand, implying that p53 mutation plays a permissive role in ErbB2 overexpression. These data suggest a model of cell signal sensing in which the length and intensity of nuclear signals evoke different outcomes depending upon the integrity of the cell-cycle control machinery.

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NF-κB Activates Prostate Specific Antigen Expression and Is Upregulated in Androgen-Independent Prostate Cancer

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Running title: Activation of NF-κB in AI prostate cancer

ABSTRACT

The transcription factor NF-κB regulates gene expression involved in cell growth and apoptosis, and has been implicated in progression of hormone-independent breast cancer. By expressing a dominant-active form of MEKK1, by exposure to TNFα, or by overexpression of p50/p65, we show NF-κB activates transcription regulatory element of prostate specific antigen (PSA), a marker for prostate cancer development, treatment, and progression. Furthermore, endogenous PSA expression was stimulated by the NF-κB inducer PMA. By DNase I footprinting, we identified four NF-κB binding sites in the PSA core enhancer. We also demonstrate that androgen independent (AI) prostate cancer has higher constitutive NF-κB binding activity than its androgen dependent (AD) counterpart. The elevated activity correlates with decreased activity of IκBβ and increased protein level of NF-κB in nucleus. These results suggest a role of NF-κB in prostate cancer progression.

INTRODUCTION

Prostate cancer begins as an androgen-dependent (AD) tumor and regresses in response to androgen ablation therapy. This therapy eventually fails, and the tumor progresses despite castrated levels of androgen. This stage of the disease is referred to as androgen-independent (AI) or hormone refractory prostate cancer. PSA is the most commonly used marker for prostate cancer. Serum PSA level correlates with prostate cancer development and effectiveness of treatment. Rising PSA levels can signal the progression from AD to AI, suggesting that regulation of PSA expression may correlate with disease progression. Biochemical and genetic analyses have identified two regulatory regions responsible for androgen-regulated tissue-specific PSA expression, the proximal promoter that contains a core TATA box and an enhancer region located at approximately 4.2 kb upstream of transcription start site (7, 19, 31). We and others have used these regions as a tool to identify molecular mechanisms responsible for PSA expression in the absence of androgen, in order to gain insight into mechanisms of prostate cancer progression (2, 9).

The NF-κB/Rel genes encode a family of heterodimeric transcription factors that share a 300 amino acid Rel-homology domain (RHD) (16, 25). Classical NF-κB is a heterodimer composed of a 50 kD (p50) and a 65 kD (p65 or RelA) subunit and was discovered as a κ-immunoglobulin enhancer DNA binding protein (36). c-Rel is another member of this family originally identified as a proto-oncogene. The activity of these proteins is primarily regulated through post-translational modification. In unstimulated cells, NF-κB/Rel proteins are sequestered as inactive forms in the cytoplasm by association with

inhibitory proteins, termed inhibitor of NF-κB (IκBα and IκBβ). Upon phosphorylation by IκB kinase (IKKs), IκB is modified by ubiquitination and degraded, allowing NF-κB to translocate into the nucleus and to activate target gene transcription (16).

Breast cancer studies have proposed a role for NF-κB in progression of hormone-dependent cancers to hormone-independence. Constitutive activation of NF-κB was found in estrogen receptor (ER) negative breast cancer cell lines and poorly differentiated primary tumors (27). Progression of the rat mammary carcinoma cell line RM22-F5 from an ER-positive, nonmalignant phenotype to an ER-negative, malignant phenotype was also accompanied by constitutive activation of NF-κB (27). The current data in prostate cancer is less certain. In the androgen-independent prostate cell lines PC-3 and DU-145, the DNA-binding activity of NF-κB is constitutively activated and IκBα level is decreased, whereas the androgen-sensitive prostate cancer cell LNCaP has a low level of NF-κB binding activity (29). However, PC-3 and DU-145 do not express androgen receptor (AR) or PSA. therefore, the relevance of this finding to clinical AI prostate cancer progression is not clear. In a different set of studies, the RelA subunit of NF-κB was shown to inhibit AR function through competing for the coactivator, CREB-binding protein (CBP) (1, 30). This result suggests that NF-κB may play a negative role in AI progression, since AR activation (not suppression) is associated with prostate cancer progression.

We have examined this issue in a xenograft model of AD to AI prostate cancer progression in which AR and PSA expression are retained (8, 22). We showed previously

that MEKK1 activates PSA expression in prostate cancer cells (2). This activation is only partially abolished by the AR inhibitor bicalutamide (casodex), suggesting that pathway(s) other than AR may be involved in this activation. MEKK1 can activate Jun N-terminal Kinase (JNK) or IκB kinase (IKK), resulting in activation of the AP-1 or NF-κB transcription factor complex respectively. In this report, we demonstrate that NF-κB, but not AP-1, is required for MEKK1 to fully activate PSA transcription. We also identify previously unrecognized NF-κB binding sites in the PSA enhancer and show that progression to AI is associated with increased constitutive NF-κB binding activity.

MATERIALS AND METHODS

Abbreviation. AD, androgen-dependent; AI, androgen-independent; AR, androgen receptor; EMSA, electrophoretic mobility shift assay; GFP, green fluorescence protein; IκB, inhibitor of NF-κB; IKK, IκB kinase; JBD, JNK binding domain of JIP-1; JNK, jun N-terminal kinase; LAPC, Los Angeles Prostate Cancer; MEKK1, mitogen-activated protein kinase kinase kinase 1; MEKK1-DA, dominant active MEKK1; PMA, phorbol 12-myristate 13-acetate; PSA, prostate specific antigen; PSA-P/E-Luc, luciferase reporter driven by PSA enhancer promoter.

Cell culture and xenograft. The human prostate cancer cell line LNCaP was obtained from ATCC and maintained in RPMI1640 supplemented with 10% FBS. For phorbol 12-myristate 13-acetate (PMA) treatment, LNCaP cells were grown in the presence of 10% FBS overnight, and then in the absence of serum for 2 days. The serum-starved LNCaP cells were challenged with 5% charcoal-stripped FBS with or without indicated amount of PMA (2 or 10 ng/ml) for 24 hours, and the media were subjected to ELISA (American

Qualex) to determine PSA expression. The LAPC-4 xenograft model was established as described (9, 22) and maintained subcutaneously in mice. Progression to AI was modeled by castration of mice bearing AD tumors.

Plasmids. The cDNA of MEKK1-DA (MEKK1 dominant active) was subcloned into pcDNA3 as described (2). MEKK1-DA is a truncated form of MEKK1 in which the N-terminal 351 amino acids were deleted. The PSA promoter enhancer luciferase reporter (PSA-P/E-Luc) was constructed by inserting a 600-bp fragment of the PSA promoter and a 2.4-kb enhancer sequence into upstream of luciferase (pSE) as described (2, 9, 31). Mutation of NF-κB binding sites on PSA-P/E-Luc were kindly provided by Michael Carey (UCLA) (19). The IκBα mutant contains amino acid substitutions of serine 32 and 36 to alanine. Plasmids encoding the IκBα mutant, p50, p65, and c-Rel were kindly provided by Genhong Cheng (UCLA).

Transfection. LNCaP cells were plated at 40-50% confluency and transfected one day later according to the manufacturer's instructions using TFX-50 (Promega). To minimize interference from androgen, transfected cells were maintained in RPMI1640 medium supplemented with 5% charcoal-stripped serum. Two days after transfection, cells were harvested and luciferase activity of the reporter constructs was measured using luciferase assay kit or dual luciferase assay kit (Promega). Luciferase activity was normalized to the percentage of GFP positive cells in transfections that included pcDNA3 EGFP, or to *renilla* luciferase when the pRL-TK plasmid was used as the normalization control.

DNase I footprinting. Probes were created by polymerase chain reaction (PCR) using the reporter plasmid PSA-P/E-Luc that contains the PSA core enhancer as a template. The 3' primers are: ACCAGCTCAATCAGTCAC, CATGTTCACATTAGTACACC, AACCTGAGA-TTAGGAATCC, and TGAGAGAGATATCATCTTGC. The common 5' primer is: CGTTGAGACTGTCCTGCAG. The 3' primers were end-labeled by T4 polymerase kinase with [γ-^{32}P]ATP, and each 3' primer was combined with the common 5' primer to PCR amplify DNA probe for footprinting. The binding reactions for DNase footprinting were as described (13). The DNA was precipitated and resolved on a 6-8% polyacrylamide-7 M urea sequencing gel. Recombinant human p50 was purchased from Promega.

Electrophoretic mobility shift assays (EMSAs). Concentrated nuclear extracts for EMSAs were prepared as described (6). Nuclear extracts were diluted in buffer containing 2 mM EDTA, 25 mM HEPES pH7.5, 150 mM NaCl, 1% Triton X-100, and 10% glycerol. The wild type and mutant double strand probes were purchased from Operon and they are: GCCATGGGGGGATCCCCG-AAGTCC and GCCATGGGCCGATCCCCGAAGTCC. The probes were end-labeled by T4 polymerase kinase with [γ-^{32}P]ATP. Diluted nuclear extracts were incubated with indicated probe in the same buffer as for DNase I footprinting (13), except glycerol was added to a final concentration of 1%. Antibodies against NF-κB p50 (SC-114 X), p65 (SC-372-G), and androgen receptor (SC-816) were also incubated in supershift experiments. After one hour incubation, the reaction was resolved in a 6% native PAGE gel in buffer containing 0.5X TBE and 1% glycerol.

Western blot analysis. Nuclear extracts and cytoplasmic fractions of xenograft tumors were prepared as described (6). Western blot analysis was performed according to standard procedures (33). The antibodies used in this report are: SC-1643 against IκBα, SC-945 against IκBβ, SC-372-G against NF-κB p65, SC-1190 against NF-κB p50 (Santa Cruz Biotechnology) and AC-15 against β-actin (Sigma).

RESULTS

NF-κB, but not AP-1, is required for MEKK1-mediated activation of the PSA promoter enhancer (PSA-P/E). Previously we demonstrated that a constitutively active form of MEKK1 (MEKK1-DA) induces AR-dependent activation of the PSA-P/E (2). The MEKK1-induced activation was partially blocked by casodex and augmented by the co-expression of MEKK1-DA and AR in an AR-negative cell line. However, this activation was only partially dependent on AR in that casodex did not fully block the effect and MEKK1-DA alone resulted in partial activation. To identify the AR-independent component, we used dominant negative mutants to examine downstream pathways of MEKK1.

An AP-1 binding site was previously identified in the PSA enhancer by sequence inspection (34). Because MEKK1 activates AP-1 through JNK, we asked if JNK is required for MEKK1-mediated induction of PSA. LNCaP cells were cotransfected with MEKK1-DA and JBD, a truncated form of the selective JNK inhibitor JIP-1 (11). In a titration experiment, the minimal dose of MEKK1-DA plasmid required to fully activate

the PSA-P/E was defined (data not shown). Transfected cells were maintained in 5% charcoal-stripped medium to minimize interference from androgen. As expected, MEKK1 stimulated transcription of a c-Jun responsive element, and the activation was inhibited by JBD (Figure 1A). MEKK1-DA also activated the PSA-P/E, but this activation could not be blocked by JBD (Figure 1B). This result is consistent with our previous finding that JBD does not impair the effect of MEKK1 on prostate cancer cell survival (2) and suggests that AP-1 is not involved in MEKK1-mediated activation of PSA.

Another target of MEKK1 is IKK. MEKK1 activates IKK, which phosphorylates IκB, leading to NF-κB activation. To determine if NF-κB is involved in MEKK1-mediated activation of PSA, LNCaP cells were transfected with MEKK1-DA and a dominant active form of IκBα containing alanine substitution at serine 32 and 36. These mutations prevent phosphorylation by IKK, thereby preventing NF-κB translocation to the nucleus to activate transcription. Cotransfection of the IκBα mutant blocked the effect of MEKK-DA on NF-κB responsive element (Figure 2A) and on the PSA-P/E (Figure 2B) in a dose dependent manner. While high doses of IκBα mutant completely abolished the effect of MEKK1-DA on an NF-κB responsive element (Figure 2A), the maximal effect on the PSA-P/E was a reduction by 65% (Figure 2B). However, PSA activation was completely abolished when both casodex and the IκBα mutant were used (Figure 2C), consistent with our previous observation that MEKK1-DA also activates AR (2).

NF-κB activates the PSA-P/E. If NF-κB is required for transcriptional activation of PSA by MEKK1-DA, we reasoned that other signals that lead to activation of NF-κB may also activate the PSA-P/E. Indeed, TNFα, a cytokine that activates NF-κB, was sufficient to activate transcription of the PSA-P/E in LNCaP cells, and this activation was completely blocked by the IκBα mutant (Figure 3). To provide more direct evidence that NF-κB plays a regulatory role in PSA transcription, we asked if NF-κB is sufficient to increase the transcriptional activity of the PSA-P/E. Overexpression of p50, the NF-κB subunit without a transcriptional activation domain, did not activate PSA-P/E (Figure 4). However, overexpression of p65, or the combination of p50 and p65, did activate the PSA-P/E, and this activation was abolished by cotransfection of the IκBα mutant (Figure 4). Of note, c-Rel, another member of the p65 family, did not activate the PSA-P/E (Figure 4). This result indicates that NF-κB is sufficient to activate the PSA-P/E in the absence of androgen and suggests that this activation may be specific to the classical p50/p65 NF-κB complex.

NF-κB directly binds the PSA enhancer. To determine whether activation of PSA transcription by NF-κB was a direct or indirect effect, DNase footprinting analysis was performed. We first analyzed a core enhancer between –4366 to –3824 because this region was previously shown to be important for androgen-induced PSA expression (7, 19, 31). The ^{32}P-labeled PSA core enhancer was incubated with recombinant p50 protein, and the reaction was subjected to DNase I digestion. p50 protected four regions in the PSA core enhancer with an order of binding affinity: I > II > IV > III (Figure 5A). One half of a gel shift unit (defined as the amount of p50 required to shift 0.38 pmol of the

NF-κB oligonucleotide), completely protected site I from DNase digestion, and one gel shift unit greatly reduced DNase digestion of site II. The four protected sequences (Figure 5B) in the PSA core enhancer resemble a κB consensus sequence GGGRNNYYCC (25), and the order of NF-κB binding affinity is consistent with the prediction from a comparative analysis (40). This result indicates that NF-κB directly binds to the PSA enhancer.

To examine if NF-κB is directly involved in the regulation of the PSA enhancer, two of the binding sites were mutated (19). Either mutation abolished the binding of p50 (data not shown) and partially lose the response to NF-κB (Figure 5C).

PMA, an NF-κB inducer, stimulates endogenous PSA expression. To determine whether NF-κB activates endogenous PSA expression, LNCaP cells were maintained in plain RPMI1640 medium for two days to deplete PSA (data not shown). The serum-starved cells were then challenged with PMA, a chemical that activates NF-κB, in 5% charcoal-stripped FBS, and the secreted PSA was determined by ELISA (25, 35). PMA stimulated the expression of PSA in a dose-dependent fashion (Figure 6), suggesting that NF-κB activation alone is sufficient to stimulate PSA expression.

Increased NF-κB binding activity in AI prostate cancer. To determine if NF-κB activity correlates with ligand-independent PSA expression during AI prostate cancer progression, we measured NF-κB binding activity in the LAPC-4 prostate cancer xenograft model using electrophoretic mobility gel shift analysis (EMSA). A strong band

shift was detected when two micrograms of nuclear extract from AI tumors were used (Figure 7A, lane 8). The band shift was also seen when lower quantities of nuclear extracts from LAPC-4 AI tumors were used (Figure 7A, lanes 6, 7). However, nuclear extracts from LAPC-4 AD tumors formed barely detectable shifted complexes, even at high concentration (Figure 7A, lanes 3-5).

The pattern of the band shift was similar to previous reports describing p65/p50 heterodimer and p50/p50 homodimer complexes (10, 27, 39). Nevertheless, the identity of the shifted complexes were examined by antibody supershift experiment. Addition of p65 antibody caused the upper band to shift (Figure 7B, lanes 5-7), and addition of p50 antibody shifted both complexes to a higher position in a dose-dependent fashion (Figure 7B, lanes 9-11). The supershift was specific because a non-specific antibody did not alter the mobility of the shifted complexes (Figure 7B, lanes 2-4). These results indicated that the upper complex contains p65/p50 heterodimer and the lower band contains p50.

The binding specificity was determined using nuclear extracts from LAPC-4 AI tumor. One half to two micrograms of nuclear extracts produced the band shift with the wild-type probe (Figure 7C, lanes 14-17) but not a mutant probe (Figure 7C, lanes 17-19). The shifted complex was competed away by 5-80 fold excess of unlabeled wild-type probe (Figure 7B, lanes 8-10) but not by unlabeled mutant probe (Figure 7C, lanes 11-13). The wild-type, but not the mutant, competitor also competed away the shifted complex from recombinant p50 (Figure 7C, lanes 2-6) with comparable competition efficiency. These

results demonstrate an increase in constitutive NF-κB binding activity during progression to androgen independence.

Nuclear accumulation of NF-κB correlates with its binding activity. To determine the mechanism for the elevated NF-κB binding activity in the AI xenograft, we measured the levels of p50, p65, and IκB in nuclear and cytoplasmic extracts of LAPC-4 AD and AI tumors. p50 and p65 protein levels were higher in nuclear extracts and lower in cytoplasmic extracts from AI tumors (Figure 8A and 8B). Therefore, the higher NF-κB binding activity in LAPC-4 AI tumor is associated with increased concentration of NF-κB proteins in nucleus. To determine if this change in NF-κB is a consequence of reduced concentration of IκB, we measured the protein levels of IκBα and IκBβ in cytoplasmic extracts. IκBβ, but not IκBα, was lower in AI tumors (Figure 8C). This result supports the hypothesis that increased level of NF-κB in nucleus of AI tumor results from decreased level of IκBβ in cytoplasm.

DISCUSSION

Our data from analysis of prostate cancer cell lines and xenografts supports a role for NF-κB in prostate cancer progression. We demonstrate that NF-κB regulates the expression of PSA, an important clinical marker of prostate cancer progression. We also show that NF-κB directly binds to the PSA core enhancer. Using matched AD and AI prostate cancers derived from a xenograft model, we demonstrate that NF-κB binding activity is upregulated in AI tumors, and this upregulation correlates with the downregulation of IκBβ.

Previous work has shown that NF-κB negatively regulates AR function. This conclusion is based on cotransfection studies showing that NF-κB and AR are mutually inhibitory due to competition for a common transcriptional coactivator CBP (1, 30). Because AR is activated in AI prostate cancer (9, 17, 24), these findings imply an inhibitory role for NF-κB in prostate cancer progression. However, NF-κB inhibition of AR was observed using artificial promoters containing either consensus ARE or NF-κB binding sites. Natural promoters are more complex and typically contain cis-acting elements for different transcription factors. The PSA core enhancer studied here contains multiple low affinity AREs that act synergistically (19). Among the four NF-κB binding sites we identified (Figure 5B), three of them (I, II, and IV) are adjacent to AREs, and NF-κB binding site III overlaps with an ARE (19). These observations raise the possibility of cooperative NF-κB and AR binding contributory to PSA transcriptional regulation. Cooperation between nuclear receptors and NF-κB has been demonstrated in other systems (1, 5, 20, 30). For example, retinoid receptors and NF-κB synergistically induce ICAM-1 expression, which promotes metastasis potential (5). NF-κB and the aryl hydrocarbon receptor (AhR) cooperate to activate c-myc expression in mammary cells (20).

The transition of prostate cancer from AD to AI is a two-step process (8). AI cells must first survive androgen deprivation then grow to become AI tumors. NF-κB may participate in both processes. NF-κB is known to be a critical regulator of apoptosis (3). p65/RelA knockout mice die during embryogenesis from extensive liver apoptosis (4). NF-κB also functions as a survival factor in neurons in response to cell injury through the

upregulation of antiapoptotic genes (26). Accumulating evidence indicates that NF-κB also promotes proliferation. Inhibiting NF-κB activation by dominant negative p65 blocks cell cycle progression in human glioma cells (28). Lymphocytes lacking p50, p65, or c-Rel show defective responses to mitogenic stimulation (12, 23, 37, 38). Inhibiting NF-κB activation by IκBα expression, or by phenylarsine oxide blocks focus formation in NIH3T3 cells (15) and colony-forming cell proliferation of acute myelogenous leukemia cells (14). The proliferation promoting effect of NF-κB may result from its ability to activate c-myc expression (21), and/or cyclin D1 expression (18, 32).

The link between NF-κB activation and prostate cancer androgen-independence may provide an opportunity for drug development for this deadly disease. A therapeutic intervention might target upstream pathways that lead to activation of NF-κB, NF-κB itself, or downstream effectors that participate in cancer progression. Because NF-κB is involved in multiple signal transduction pathways, identification of the pathway(s) responsible for increased activity of NF-κB in AI progression will be of great interest.

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FIGURE LEGENDS

Figure 1. JBD does not inhibit MEKK1-mediated activation of PSA. LNCaP cells were transfected with an empty vector pcDNA3, JBD (500 ng), MEKK1-DA (400 ng), or JBD with MEKK1-DA. together with the reporter construct driven either (A) by Jun responsive element (Jun-Luc) (200 ng) or (B) by PSA promoter enhancer (PSA-P/E-Luc) (200 ng). Luciferase activity was measured two days after transfection and normalized by transfection efficiency, which was determined by GFP cotransfection. The data represents three experiments.

Figure 2. Dominant active IκBα mutant inhibits MEKK1-mediated activation of PSA. LNCaP cells were transfected with an empty vector pcDNA3, IκBα (32/36AA) mutant (800 ng), MEKK1-DA (300 ng), or MEKK1-DA with increasing amount of IκBα (32/36AA) mutant (100, 200, 400, 800 ng), together with the reporter construct driven either (A) by NF-κB responsive element (NF-κB-Luc) (200 ng), or (B) and (C) by PSA promoter enhancer (PSA-P/E-Luc) (200 ng). Casodex was used in (C) as indicated. Luciferase activity was measured two days after transfection and normalized by transfection efficiency, which was determined by GFP cotransfection. The data represents three experiments.

Figure 3. TNFα activates PSA expression through the NF-κB pathway. LNCaP cells were transfected with the reporter constuct PSA-P/E-Luc (200 ng) with or without IκBα (32/36AA) mutant (800 ng) as indicated. After transfection, cells were treated with or without TNFα (10 or 50 ng/ml) as indicated. Luciferase activity was measured two days

after transfection and normalized to *renilla* luciferase. The data represents two experiments.

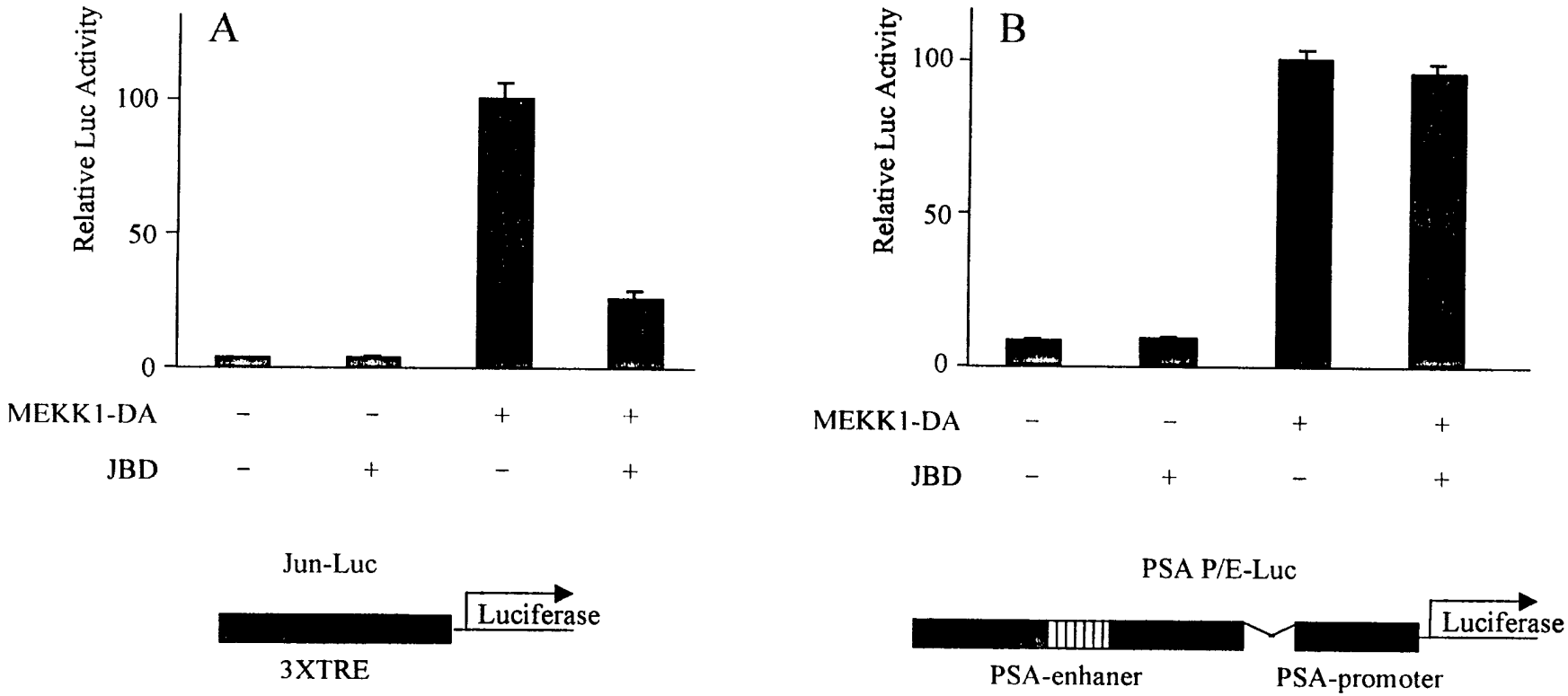
Figure 4. Overexpression of NF-κB activated PSA expression and the activation was abolished by IκBα mutant. LNCaP cells were transfected with the reporter constructs PSA-P/E-Luc (200 ng) with increased amount of human p50 (p50) (25, 50, 100 ng), human p65 (p65) (25, 50, 100 ng), combination of p50 and p65, c-Rel (25, 50, 100 ng), or combination of p50 and c-Rel, and with or without IκBα (32/36AA) mutant (800 ng) as indicated. Luciferase activity was measured two days after transfection and normalized to *renilla* luciferase. The data represents two experiments.

Figure 5. Identification of NF-κB binding sites in PSA core enhancer. A, increasing amounts of recombinant human p50 protein (0, 0.5, 1.0, 2.0, 4.0 gel shift units in lane 1, 2, 3, 4, 5) were incubated with ^{32}P-labeled enhancer fragment, from –4366 to –3824, to detect footprints. The sites identified are denoted as I, II, III, IV in sequence order. Sequencing ladders are included alongside the footprints to localize the protected sites. The order of binding affinity are: I > II > III > IV. B, sequence of the PSA core enhancer. Footprinted regions are underlined and compiled below the consensus. The sequence of the site I is reversed.

Figure 6. PMA induces endogenous PSA expression. Serum-starved LNCaP cells were grown in 5% charcoal-stripped FBS with or without indicated amount of PMA (2 or 10 ng/ml) overnight, and the media were subjected to ELISA to determine PSA expression.

Figure 7. AI tumors have a higher constitutive NF-κB binding activity than AD tumors. A, increasing amounts (0.5, 1.0, 2.0 microgram) of nuclear extracts from both AD and AI tumors of LAPC-4 were incubated with ^{32}P-labeled NF-κB binding sequence to detect NF-κB binding by gel shift analysis. The shifted complex and free probe are indicated. B, identity of the shifted complexes. Two microgram of nuclear extracts from LAPC-4 AI tumor were incubated with ^{32}P-labeled NF-κB binding sequence with or without increasing amount of antibodies against androgen receptor, p65, or p50. C, specificity of the shifted complexes. Shifted complexes (lane 2) formed by the recombinant p50 protein can be efficiently competed away by increasing amounts of the unlabeled wild type probe (lanes 3 and 4), but not by the unlabeled mutant probe (lanes 5 and 6). Nuclear extracts (0.5, 1.0, 2.0 microgram) from LAPC-4 AI tumor formed band shift with the wild type probe (lanes 14-16) and but not with the mutant probe (lanes 17-19), and the band shift can be competed away by increasing amounts of the unlabeled wild-type probe (lanes 8-10), but not by the unlabeled mutant probe (lanes 11-13).

Figure 8. Western blot analysis on NF-κB and IκB protein levels in LAPC-4 xenograft tumors. A, nuclear extracts were subjected to western blot analysis using antibodies against p50 and p65. B, cytoplasmic fractions were subjected to western blot analysis using antibodies against p50 and p65. C, cytoplasmic fractions were subjected to western blot analysis using antibodies against IκBα and IκBβ.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JBD - + - +
Jun-Luc
Luciferase
3XTRE
B
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MEKK1-DA - - + +
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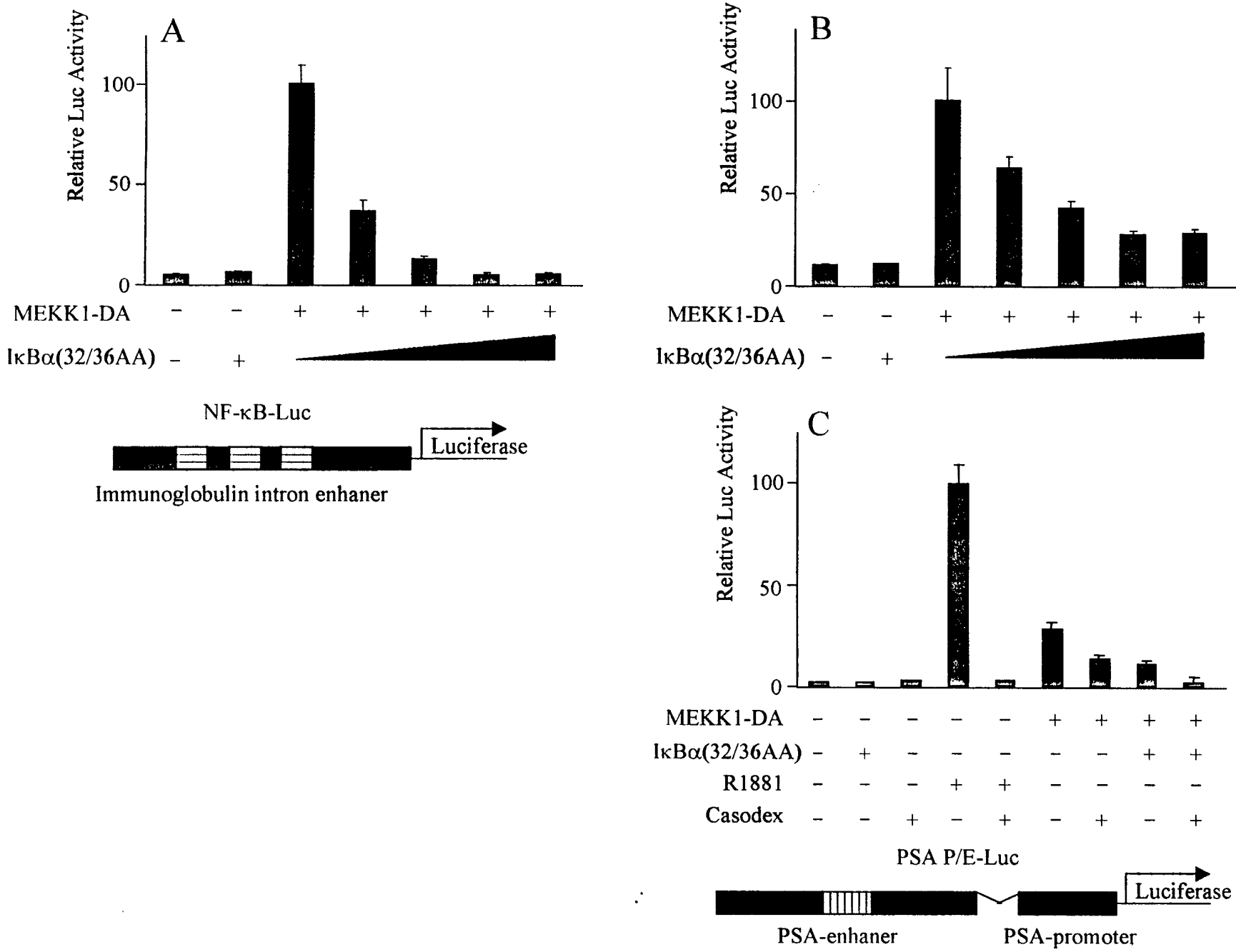
PSA P/E-Luc
Luciferase
PSA-enhaner
PSA-promoter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Relative Luc Activity
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IκBα(32/36AA) - +
NF-κB-Luc
Luciferase
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B
Relative Luc Activity
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MEKK1-DA - - + + + + +
IκBα(32/36AA) - +
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100
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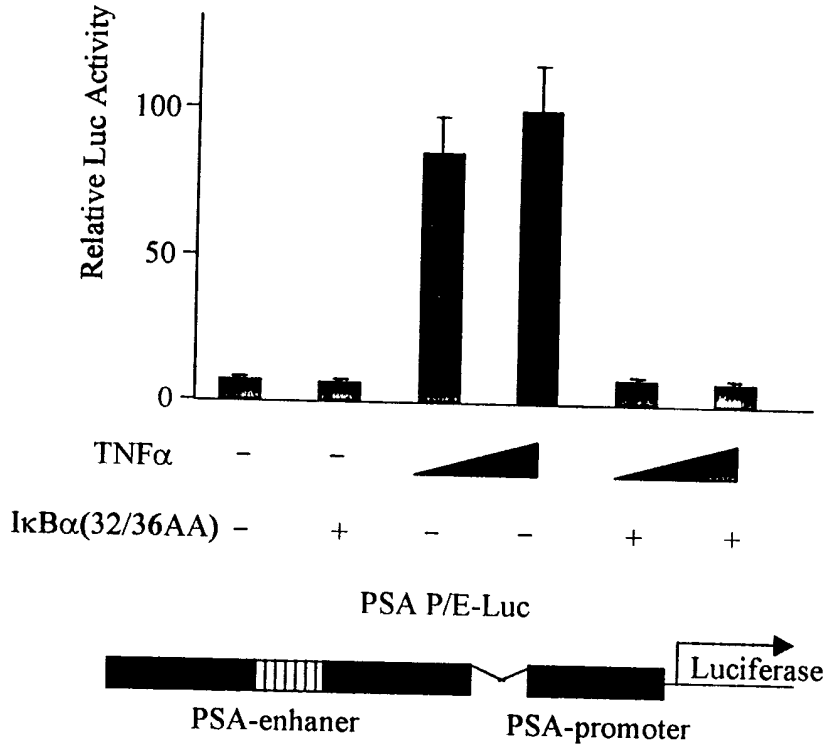
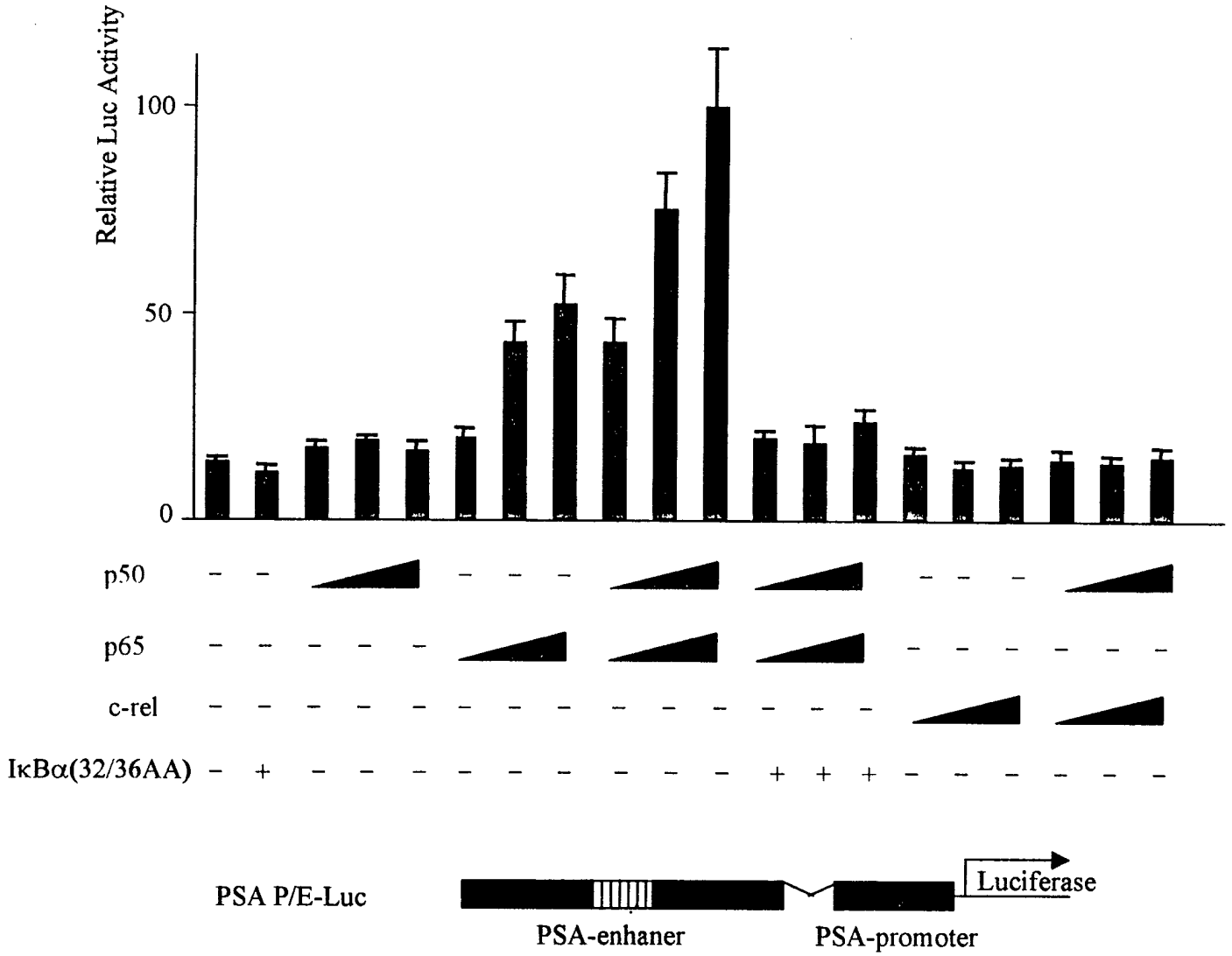
PSA-promoter


Relative Luc Activity
100
50
0
TNFα - - + + + +
IκBα(32/36AA) - + - - + +
PSA P/E-Luc
Luciferase
PSA-enhaner
PSA-promoter


Relative Luc Activity
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50
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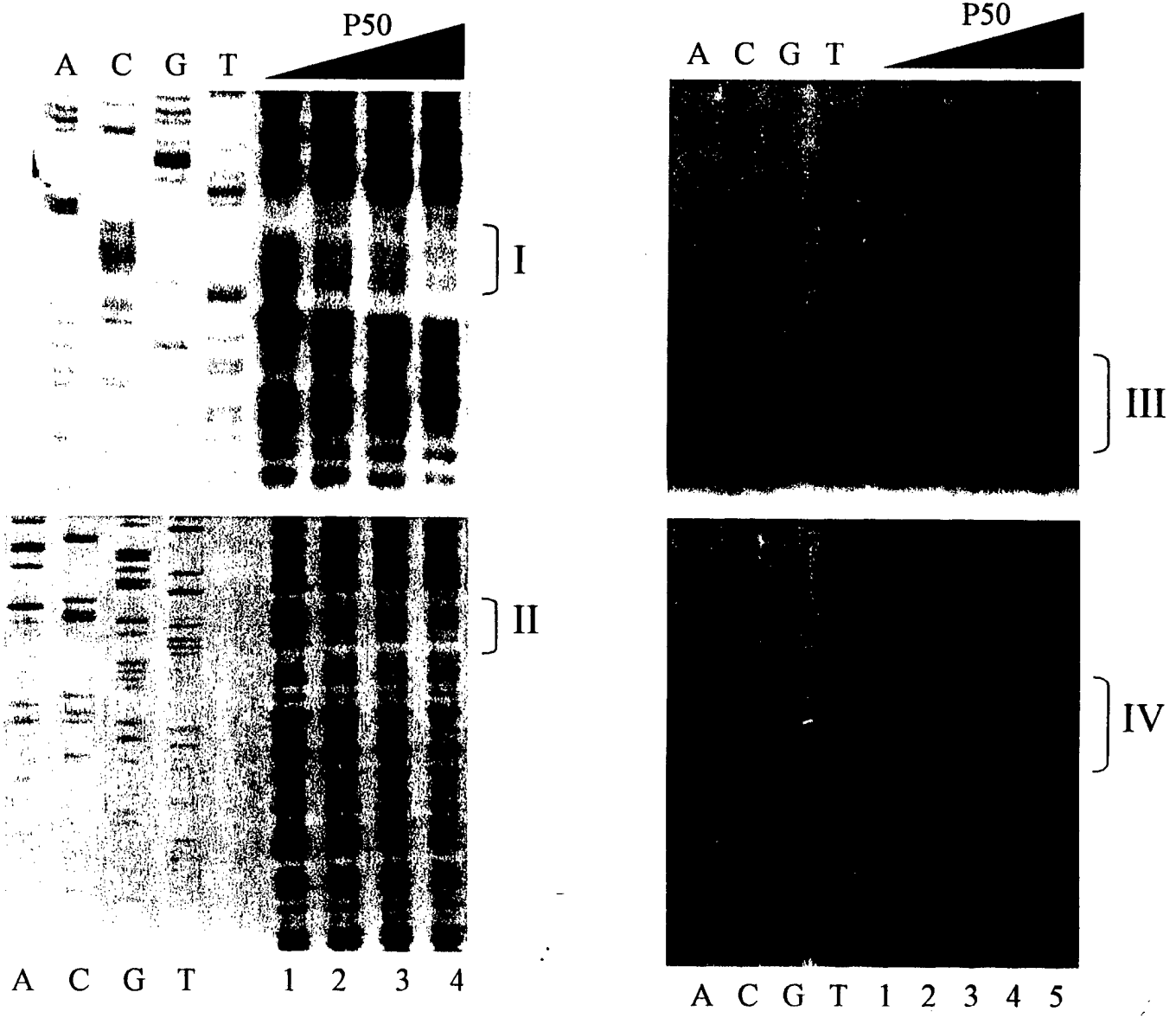
p50
p65
c-rel
IκBα(32/36AA)
PSA P/E-Luc
Luciferase
PSA-enhaner
PSA-promoter

A



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NF-KB consensus sequence	**GGGRNNYYCC**	
Protected sites	**GGGGGGggCa**	I (reversed)
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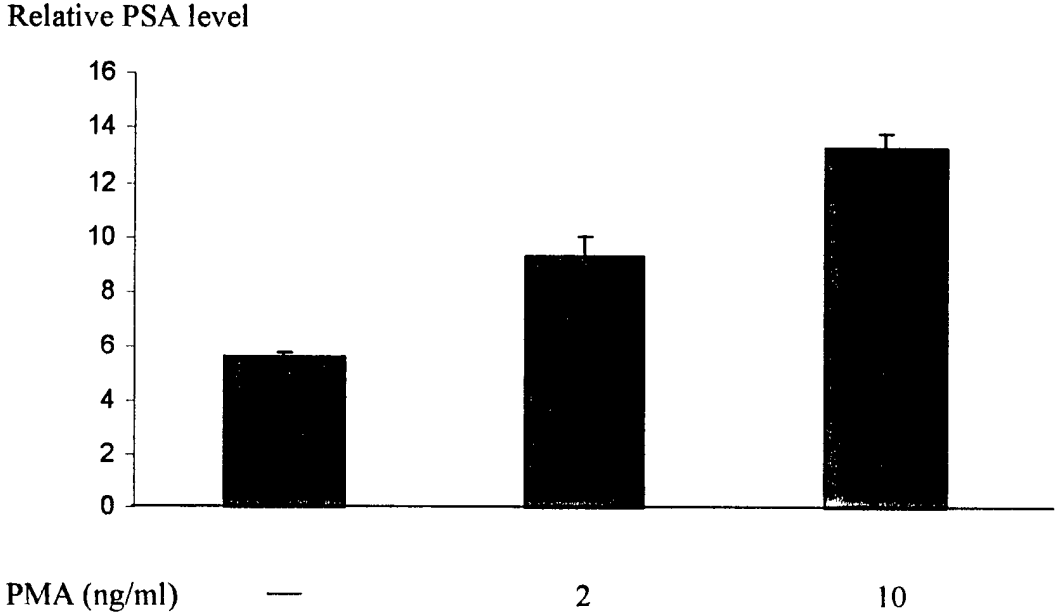
	GGGACAaCtt	III
	GGtGTTgCtg	IV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6
4
2
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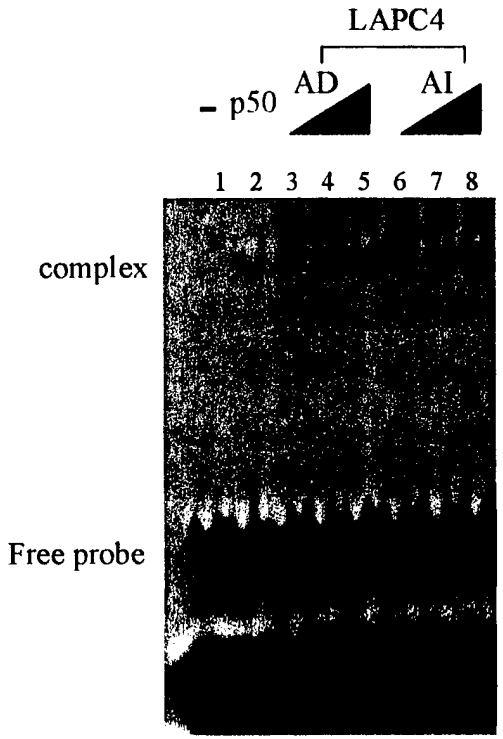
PMA (ng/ml)
—
2
10

A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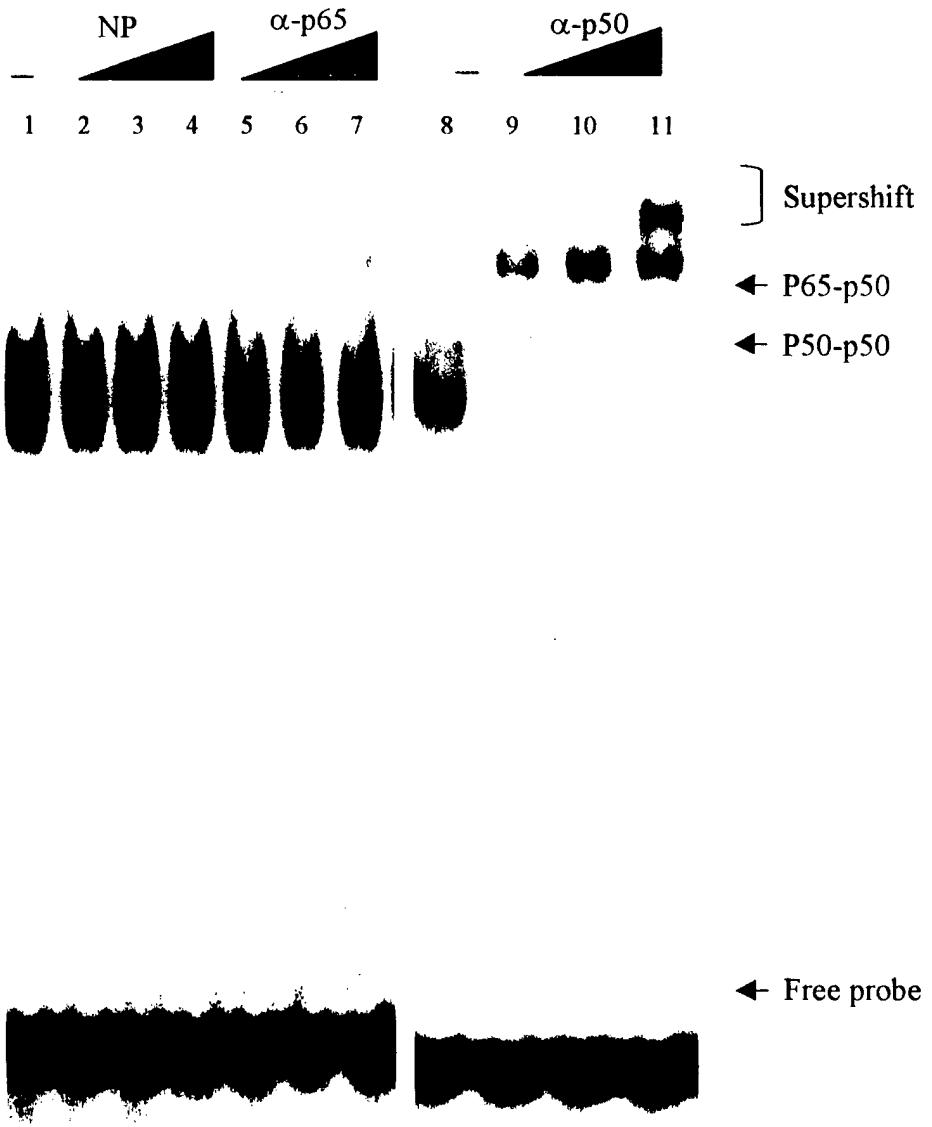
complex
Free probe

B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α-p50
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Supershift
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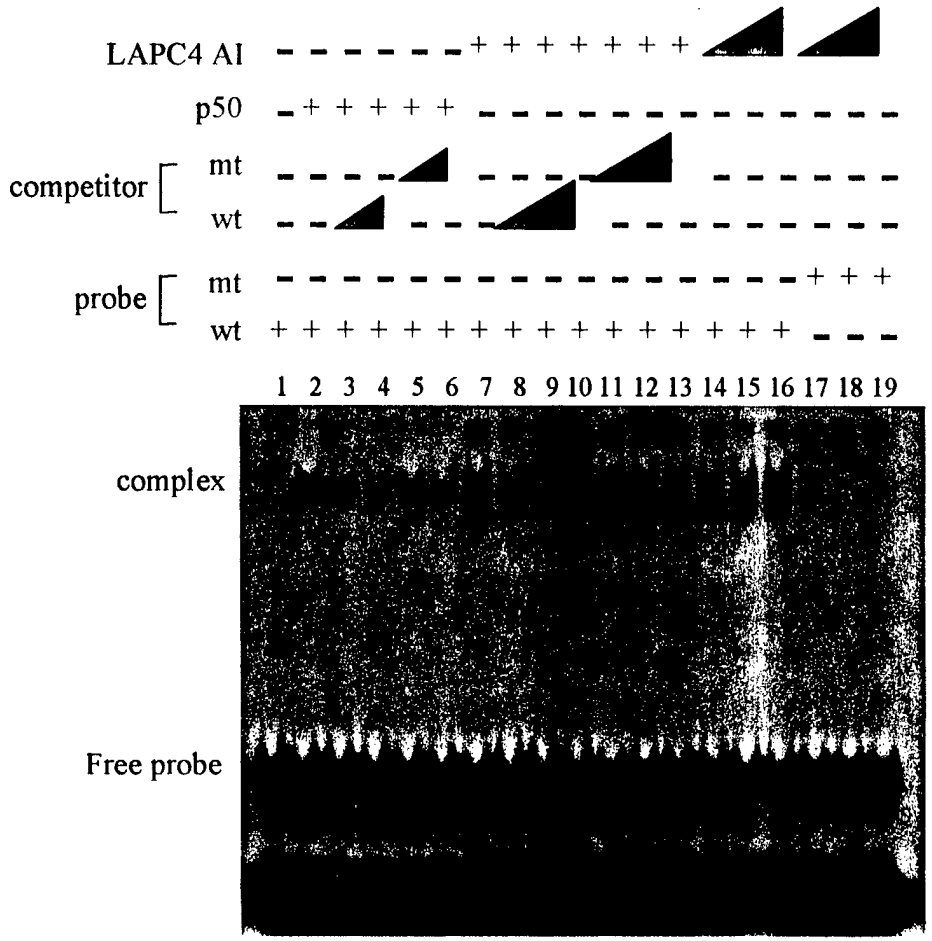
Free probe

C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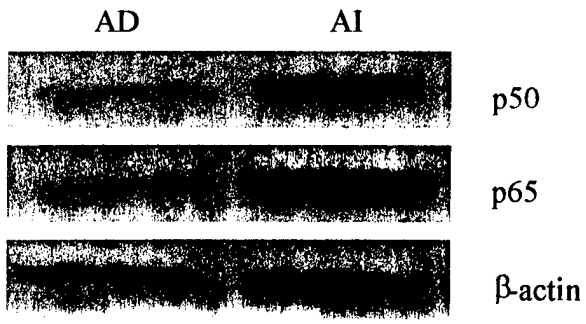
A



Nuclear extracts

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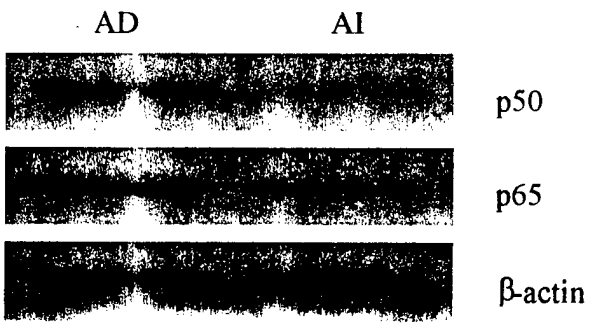
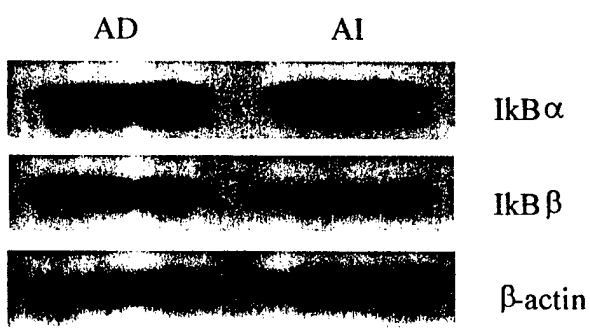


Cytoplasmic fractions

C



Cytoplasmic fractions