JunB negatively regulates AP-1 activity and cell proliferation of malignant mouse keratinocytes

Received: 3 August 2001 / Accepted: 12 September 2001 / Published online: 6 November 2001
© Springer-Verlag 2001

Abstract Objective: Previously we have shown that a malignant mouse keratinocyte cell line, 10Gy5, has elevated AP-1 transactivation and reduced JunB protein levels compared to its parental benign cell line, 308, and that the tumorigenicity in the 10Gy5 cells could be blocked by a dominant negative c-Jun mutant protein. We wished to determine whether the change in JunB protein levels could account for the elevated AP-1 activity and whether re-expression of JunB in malignant 10Gy5 cells altered their proliferative capacity. Design: In the current study, we reduced JunB expression in benign 308 cells with antisense oligonucleotides and increased JunB expression in malignant 10Gy5 cells by stable transfection of a JunB expression vector. Results: Increased AP-1 activity was detected after treatment of the benign 308 cell line with JunB antisense oligonucleotides that reduced JunB protein levels. Stably JunB-transfected clones of malignant 10Gy5 cells showed decreased AP-1 activity, slowed in vitro cell proliferation and reduced tumor growth when xenografted to athymic nude mice. Conclusion: These findings suggest that expression of JunB protein has a negative effect on malignant tumor cell proliferation in part through its ability to inhibit AP-1 transactivation.

Keywords Squamous cell carcinoma · Cell proliferation · AP-1 transactivation

Introduction

The transcription factor complex, activator protein-1, (AP-1), plays a functional role in mouse skin carcinogenesis. Convincing in vivo evidence for a role of AP-1 in mouse skin tumor promotion comes from work using a transgenic mouse where a dominant negative c-Jun mutant protein, TAM-67, is targeted for expression in the epidermis (Young et al. 1999). These transgenic mice are resistant to phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion. There is evidence that constitutive AP-1 activity is required for the maintenance of the malignant phenotype of some mouse squamous cell carcinoma cell lines (Domann et al. 1994a; Domann et al. 1994b). In addition, studies with c-fos knockout mice have suggested that c-fos, an AP-1 family member, is involved in benign-to-malignant tumor progression (Saez et al. 1995). In an in vitro model of skin tumor promotion using murine epidermal cells, elevated AP-1 activity has been correlated with increased neoplastic transformation (Bernstein et al. 1991). In this model system using JB6 cells, AP-1 activity increases with progression from a tumor promotion-resistant to a tumor promotion-sensitive phenotype. Tumor promoters stimulate AP-1 expression and modulate gene expression regulated by AP-1 in cultured mouse keratinocytes and in mouse skin (Bernstein et al. 1989; Holladay et al. 1992). Direct evidence for a causative role of AP-1 activation in tumor promotion comes from the finding that acquisition of a tumor promotion-resistant phenotype is consistent with the loss of responsiveness to tumor promoter-induced AP-1 activation (Dong et al. 1995). It has also been shown that down-regulation of AP-1 by “anti-tumor promoters” such as aspirin (Dong et al. 1997b) or retinoids (Huang et al. 1997) correlates with inhibition of tumor promoter-induced transformation and tumor development. The same effect has been found using a dominant-negative c-Jun mutant protein, TAM67 (Dong et al. 1997a), whereas overexpression of c-Jun protein leads to increased neoplastic transformation (Watts et al. 1995).
The AP-1 complex is a dimer, formed by Jun or Jun and Fos proteins, which binds to a promoter enhancer element called TPA-responsive element (TRE) (Karin et al. 1997). There are three different Jun proteins, c-Jun, JunB, and JunD and four different Fos proteins including c-Fos, FosB, Fra-1, and Fra-2. Regulation of AP-1 activity takes place at various levels including transcriptional and post-transcriptional mechanisms. Changes in AP-1 family member expression and post-translational modifications, such as phosphorylation and oxidation/reduction, alter both DNA binding affinity and transactivation potential. Since transactivation potential and DNA binding affinity are different for the various AP-1 proteins, AP-1 activity is also determined by its composition. In addition, not only the sequence of the TRE, but also the sequence of the adjacent bases (Ryseck et al. 1991), determines binding affinity for a particular heterodimer.

In previously published work we had demonstrated in a cell culture model of malignant progression that AP-1 was constitutively upregulated in a malignant variant, 10Gy5, of a benign papilloma cell line called 308. The malignant 10Gy5 cell line was generated from 308 cells that had been exposed to gamma radiation. We showed that the malignant phenotype seen in vivo with the 10Gy5 cells could be inhibited by suppressing AP-1 activity with a dominant negative c-Jun mutant protein (Domann et al. 1994a). When we examined AP-1 regulation in 308 and 10Gy5 cells, the one difference observed was that the malignant 10Gy5 have reduced basal levels of JunB protein in comparison to benign 308 cells (Joseloff et al. 1997). This decrease in JunB protein appeared to be the result of a defect in JunB protein synthesis in 10Gy5 cells as no differences in JunB transcription, mRNA stability or protein degradation were observed between the two cell lines. In the present study we used the 308 and 10Gy5 keratinocytes to determine whether changes in JunB protein levels could account for the elevated AP-1 activity in the 10Gy5 cells compared to 308 cells as well as the enhanced proliferation of the 10Gy5 when injected subcutaneously into athymic nude mice. We manipulated JunB steady state protein levels by reducing JunB expression in the benign 308 cells with an antisense oligonucleotide. In addition we increased JunB expression in the malignant 10Gy5 cells by stable transfection with a JunB expression plasmid. The results of these experiments suggest that JunB protein can negatively regulate malignant tumor cell proliferation through decreased AP-1 transactivation.

Stable transfections by electroporation

To generate 10Gy5-JunB overexpressing stable cell lines, 10Gy5 cells were trypsinized and rinsed twice with HBS buffer (25 mM HEPES, 70 mM NaCl, 0.75 mM Na3PO4) before 1x106 cells were resuspended in 1 ml of HBS buffer. Cells were incubated on ice for 5 min with 10 μg of pLLJunB DNA vector prior to electroporation at 250 V/250 μF with a Bio-Rad Gene Pulser. Transfected cultures were grown in media with 800 μg/ml of G418 for selection. Cells were trypsinized and seeded with approximately a single cell per well in 48-well dishes and those wells that had a single colony of cells were expanded under G418 selection. 1A5 cells were generated by stably cotransfecting 308 cells with a human collagenase TRE luciferase reporter construct, AP-1LUC, and a neomycin expressing plasmid, LneoL, by electroporation and plating at clonal density as above.

Transient transfections

One hundred thousand cells/well were plated in 6-well tissue culture dishes and grown to 50% confluency. Six micrometers per well of reporter DNA was transiently transfected according to the protocol with DOTAP transfection reagent (Boehringer Mannheim). Cells were transiently transfected for 18 h under serum-free conditions. Cells recovered in 2% serum-containing MEM for 8 h and then serum starved for 30 h for luciferase assays and 48 h for CAT assays. For experiments with 308 cells transiently transfected with JunB antisense phosphorothioate oligonucleotides, 308 cells were initially transfected with the luciferase reporter construct as above for 8 h and then subsequently transfected with DOTAP and the antisense oligonucleotides for 8 h at a concentration of 0.8 μM. A scrambled phosphorothioate oligonucleotide with a similar GC content (Schlingensiepen et al. 1993) was used as a negative control.

Western analysis

Western analysis for JunB steady state protein levels was performed in a similar manner as previously described (Joseloff et al. 1997). Twenty micrograms of protein were separated on 12.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Millipore). The membrane was blocked in 3% milk-TBST (0.05% Tween 20, 20 mM Tris-HCL pH 7.4, 0.9% NaCl), incubated with a primary JunB rabbit polyclonal antibody (SC-46, Santa Cruz Biotechnology) at a dilution of 1:2,500 for 3 h. The secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) was incubated at a dilution of 1:35,000 for 1 h and the blot treated with ECL western blotting detection reagent (Amersham Life Science) before exposure to X-ray film.

Luciferase assay

Transfected cells were lysed by freeze/thawing in 100 μl of luciferase lysis buffer (1% TritonX-100, 25 mM glycyglycine, 50 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.1 mM DTT). Total protein was measured. Luciferase activity was assayed using the Promega luciferase assay system.