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During Human Melanoma Progression AP-1 Binding Pairs are Altered with Loss of c-Jun In Vitro

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We demonstrated previously that c-Jun, JunB and c-Fos RNA were dysregulated in metastatic melanoma cells compared with normal human melanocytes. The purpose of this study was to evaluate the distribution in composition of AP-1 dimers in human melanoma pathogenesis. We investigated AP-1 dimer pairing in radial growth phase-like (RGP) (w3211) and vertical growth phase-like (VGP) (w1205) human melanoma cells and metastatic cell lines (cloned from patients, c83-2c, c81-46A, A375, respectively) compared with melanocytes using electrophoretic mobility shift assay (EMSA), Western blot and transfection analyses. There are progressive variations in AP-1 composition in different melanoma cell lines compared with normal melanocytes, in which c-Jun, JunD and FosB were involved in AP-1 complexes. In w3211, c-Jun, JunD and Fra-1 were involved in AP-1 binding, while in w1205, overall AP-1 binding activity was decreased significantly and supershift binding was detected only with JunD antibodies. In metastatic c81-46A and A375 cells, only JunD was involved in AP-1 binding activity, but in a third (c83-2c) c-Jun, JunD and Fra-1

were present. Western blot evaluation detected c-Jun in melanocytes and w3211, but this component was decreased significantly or was not detectable in w1205, c81-46A and A375 cells. In contrast, JunD protein was elevated in c81-46A and c83-2c cells compared with melanocytes and RGP and VGP cell lines. Normal melanocytes and c83-2c cells (which have c-Jun involved in AP-1 binding), transfected with c-Jun antisense and treated with cisplatin, showed higher viability compared with untransfected cells, while in c81-46A cells (in which only JunD is detectable) no change in cell viability was observed following treatment with cisplatin and c-jun antisense transfection. A dominant-negative c-Jun mutant (TAM67) significantly increased the soft agar colony formation of w3211 and c83-2c cells. These results suggest that components of AP-1, especially c-Jun, may offer a new target for the prevention or treatment of human melanoma progression.

Key words: Ap-1, Melanoma, Melanocyte, c-Jun, JunD

INTRODUCTION

Human melanoma, a malignancy that develops through the malignant transformation of melanocytes, is a disease characterized by a rapidly growing incidence and mortality and is highly resistant to traditional cancer treatments including radiation and chemotherapy (1). Although various mechanisms for therapeutic resistance, such as altered transport, detoxification, DNA repair, and drug-target interaction have been demonstrated in selected cell lines, the biological basis

for the general pharmacological resistance of melanoma is still poorly understood.

Activating protein-1 (AP-1) is a family of transcription factors that specifically regulate transcription of tetradecanoylphorbol 13-acetate (TPA) responsive element (TRE)-containing genes by acting on their promoters (2). Modulation of AP-1 activity is one of the critical pathways in the control of cell proliferation and transformation; however, little is

Abbreviations – AP-1, activating protein-1; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C; RGP, radial growth phase-like; ROS, reactive oxygen species; TPA, 12-O-tetradecanoylphorbol 13-acetate; VGP, vertical growth phase-like

known about its role in melanoma. Proteins of AP-1 transcription factors (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, Fra-2) contain a leucine zipper and form either homodimers or heterodimers through this domain. This transcription factor reacts to changes in growth and environmental conditions to adjust the gene expression profile in a way that allows the cell to adapt to the new environment (3). Although AP-1 was identified almost 15 yr ago and retroviral homologues of some of its components were found even earlier, the biological relevance and physiological functions of AP-1 and its components are still being elucidated. Recent progress has begun to illuminate how AP-1 transcription factors control cell proliferation and growth. AP-1 target genes are differentially regulated by distinct AP-1 dimers. The dynamic changes in Jun and Fos composition after stress-stimuli balance discrete signals that play a key role in defining whether cells undergo apoptosis, survival or senescence (4). Many compounds can induce apoptosis in tumor cells by interfering with AP-1. These include the microtubule inhibitors vinblastine (5), RAR γ agonist CD 437(6), and the alkylator cisplatin (7).

Our previous studies revealed an increase in JunB and c-Fos RNA and a decrease in c-Jun RNA expression in metastatic melanomas compared with normal melanocytes, suggesting a potential role of AP-1 genes in cell transformation (8). Additionally, it has been demonstrated that the AP-1 binding site is a key regulatory element necessary for full stimulation of mda-7 gene transcription, which exhibits reduced expression as melanocytes progress to metastatic melanomas (9). Recently we demonstrated that the association of reactive oxygen species (most strongly for superoxide anion) with AP-1 binding was preserved in melanoma cells, but that the process was uncoupled from the anticipated apoptotic response (10). These observations raised the important issue of whether this association was related to a quantitative or qualitative difference in AP-1 signaling. In this study, we measured AP-1 composition and activity in normal melanocytes and primary and metastatic melanoma cells using electrophoretic mobility shift assay (EMSA), Western blot analysis and antisense transfection to evaluate in more detail the contribution of AP-1 to melanoma pathogenesis.

MATERIALS AND METHODS

Cell Cultures

Human neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. Following this incubation the tissue was scraped to recover the melanocytes and cultured in MCDB153 (Sigma, St Louis, MO, USA) medium containing 2% fetal calf serum, 0.3% bovine pituitary extract (Cambrex, Walkersville, MD, USA), 10 ng/ml TPA, 2 mM CaCl₂, 5 μ g/ml insulin and 0.1 mM IBMX (Sigma). Early stage primary melanoma cells (w3211) were cultured in RPMI 1640 with 5% fetal calf serum, 5% calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and insulin. Late stage primary melanoma cells (w1205) were cultured in L15/MCDB medium with 5% fetal calf serum, 5% calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml). Radial growth phase (RGP)-like w3211 and vertical growth phase (VGP)-

like w1205 primary melanoma cells were a generous gift from Dr M. Herlyn (11). Metastatic melanoma cell strains (c81-46A, c81-61, c83-2c) were cultured in F-10 with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and designated as melanoma complete medium. Cell strains c81-46A, c81-61, c83-2c have been previously described and were initially cloned through soft agar and form tumors in nude mice (12). The passage number for the various cell strains used in these experiments was < 8.

Nuclear Protein Extraction

Nuclear extracts from cells were prepared as described previously with minor changes (13, 14). Briefly, cells were seeded in a 100-mm tissue culture dish and allowed to attach overnight. On the next day, the media were aspirated and cells were scraped and suspended in 1.5 ml of ice-cold phosphate-buffered saline (PBS). After 10 s centrifugation in a microcentrifuge, PBS was aspirated completely. Cells were suspended in 400 μ l of ice-cold buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.9, 10 mM KCl, 0.150 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed to swell on ice for 10 min. 25 μ l of a 10% NP-40 solution was added and cells were lysed by vortexing for 10 s. After 30 s centrifugation in a microcentrifuge, the buffer was completely aspirated and the pelleted nuclei suspended in 25 μ l of buffer C (20 mM HEPES, 20% glycerol, 0.42 M NaCl, 0.15 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice with shaking for 15 min. After centrifugation for 30 min at 14 000 *g* the extract was removed and diluted with 75 μ l buffer D (20 mM HEPES, 20% glycerol, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). Using a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), the protein concentration of each sample was precisely measured three times and the correlation coefficient of standard curve was at least 0.99. Nuclear extracts were stored at -80°C.

Electrophoretic Mobility Shift Assay of AP-1 DNA-Binding Activity

AP-1 specific binding activity was determined by EMSA using the Gel Shift Assay Systems (Promega, Madison, WI, USA). Briefly, 10 μ g of nuclear extract was incubated in a final volume of 10 μ l containing 2 μ l Gel shift binding buffer (5 \times), 1 μ l ³²P-labeled AP-1 consensus oligos (approximately 6 \times 10⁵ cpm). Variable additions to the reaction mixture include 2 μ l of antibody to individual AP-1 proteins for supershift assay. For oligonucleotide competition experiments, the reaction mixture was preincubated with a 50-fold excess unlabeled oligonucleotide probes for 20 min before the addition of radioactive probes. The reaction mixture was separated on 4% non-denaturing polyacrylamide gels, autoradiographed, and shifted bands were quantified by densitometry. All the antibodies used in EMSA were the products of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) (c-Jun, sc-44X; JunB, sc-46X; JunD, sc-74X, c-Fos, sc-52X; FosB, sc-48X; Fra-1, sc-605X; Fra-2, sc-171X).

Following the instruction manual we also performed non-radioactive EMSA analysis using a DIG Gel shift kit (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, AP-1 oligonucleotides were labeled with digoxigenin-11-ddUTP and incubated with nuclear extract proteins. After electrophoretic separation, the oligonucleotide-protein complexes were transferred to a Nylon membrane for further immunological detection using an anti-Digoxigenin-AP conjugate antibody. The generated chemiluminescent signals were recorded on X-ray film.

Western Immunoblot Analysis

Equal amounts of nuclear protein (20 µg) were loaded per well. Electrophoresis was performed in a 10% polyacrylamide separating gel and a 5% stacking gel. Proteins were transferred to nitrocellulose membrane, and incubated in blocking solution for 2 h. The membrane was incubated for 1 h at room temperature with primary rabbit antibodies (anti-c-Jun, anti-JunB, anti-JunD, anti-c-Fos, anti-FosB, anti-Fra-1, anti-Fra-2 respectively, 1:200). Blots were washed in TBST and incubated with second antibody (peroxidase-conjugated anti-rabbit IgG, 1:500) for 1 h at room temperature. After extensive washing, the second antibody was visualized by chemiluminescence reagents.

Transient Transfection Studies

To study AP-1-dependent gene transcription, we used a reporter vector system containing the secreted alkaline phosphatase (SEAP) gene (Clontech Laboratories, Inc., Palo Alto, CA, USA). The reporter construct pAP1-SEAP is designed to measure the binding of transcription factors to AP1, providing a direct measurement of activation for this pathway. After transcription factors bind AP1, transcription is induced and the reporter gene is activated. The secreted SEAP enzyme is assayed directly from the culture medium using Great EscAPe Chemiluminescence Detection Kits (Clontech Laboratories, Inc.). For the transient transfection, we plated different melanoma cells at a density of 1×10^5 /well in a 24-well plate. After 24 h, the cells were transfected with 1 µg of the reporter constructs with the use of the LipofectAMINE PlusTM reagent kit (Invitrogen, Life Technologies, Inc., Rockville, MD, USA), according to the manufacturer's directions. Sixteen hours later cells were treated with TPA (50 ng/ml) for 8 h and then changed to fresh media. Forty-eight hours later, the media was collected for SEAP activity assay, which was performed according to the manufacturer's instructions (Clontech, BD Biosciences, Palo Alto, CA, USA) using a plate illuminometer.

The c-Jun and JunD antisense sequences (15) were chosen as 15-mer sequences containing the AUG translation initiation codon because this is short enough to penetrate living cells but long enough for specificity (5'-CATCTTTGAGT-CAT-3' and 5'-GAAGGGCGTTTCCAT-3', respectively); the control oligonucleotide used was (5'-ATGACTGCAA-AGATG-3'). Phosphorothioate-modified oligonucleotides were synthesized and purified by Invitrogen Inc., San Diego, CA, USA. For the oligonucleotide treatment, all the cells were suspended in medium containing only 0.5% fetal bovine

serum (FBS) to avoid degradation of oligonucleotides in the presence of high concentration serum, and then were seeded onto 60 mm plates at the density of 3×10^4 cells/ml. Cells were transfected with 1 µM antisense or control sense oligonucleotide using an oligofectamineTM reagent (Invitrogen, Life Technologies, Inc.) according to the manufacturer directions. Transfection was followed by medium change with presence or absence of 20 µM cisplatin. Serum was then added to give a final concentration of 10% until harvesting.

Cells were transfected with TAM67 (1 µg) using Lipofectamine PlusTM reagent kit (Invitrogen, Life Technologies, Inc.) according to the manufacturer's recommendations. Forty-eight hours after the start of transfection, the cells were passaged into fresh culture media and soft agar colony formation assays were performed. TAM67 was kindly provided by Dr Powell Brown (16).

Semiquantitative PCR amplification

To determine the c-Jun mRNA expression, cells grown in a monolayer were incubated with or without antisense. Following the manufacturer's protocol (Invitrogen, Inc.), mRNA was prepared using a Micro-FastTrack 2.0 kit and the concentration of each sample was determined by spectrophotometry (17). Equal amounts of mRNA (0.1 µg) were amplified in 50 µl reaction volumes containing 1 µM of the appropriate primers (forward primer: 5'-TCC TAT GAC GAT GCC CTC AAC-3'; backward primer: 5'-GTG TTC TGG CTG TGC AGT TC-3'), 200 µM of each of the dNTPs, 1 µl of RT/Platinum Taq mix and 1× Reaction Mix [SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen, Inc.)]. A 55°C incubation for 30 min was then followed with 94°C 2 min. Twenty-five cycles of PCR were carried out as: denaturation at 94°C for 30 s; annealing at 61°C for 30 s; followed by extension at 72°C for 60 s. The final extension cycle was 72°C for 10 min. The PCR product amplified with c-Jun primers had a total length of 353 bp and were separated by gel electrophoresis in 1.5% agarose and were visualized by ethidium bromide staining on an UV trans-illuminator.

Measurement of Cell Viability

Cell viability was measured by a colorimetric MTT assay. Briefly, cells were incubated in normal tissue culture medium as described above. Subsequently, 10 µl MTT reagent (10 mg/ml in PBS) was added and allowed to react for 2 h at 37°C prior to the addition of solubilization reagent dimethyl sulfoxide (DMSO) (100 µl). Substrate cleavage was monitored at 490 nm by use of a microplate reader and analyzed using related software. Control cells were untreated.

RESULTS

AP-1 Composition in Melanocytes and Melanoma Cells

Melanocytes and melanoma cells were cultured as described. Twenty-four hours before collection of the nuclear extracts, cells were changed to plain media to avoid AP-1 induction by serum. Using EMSA and supershift analysis, we determined

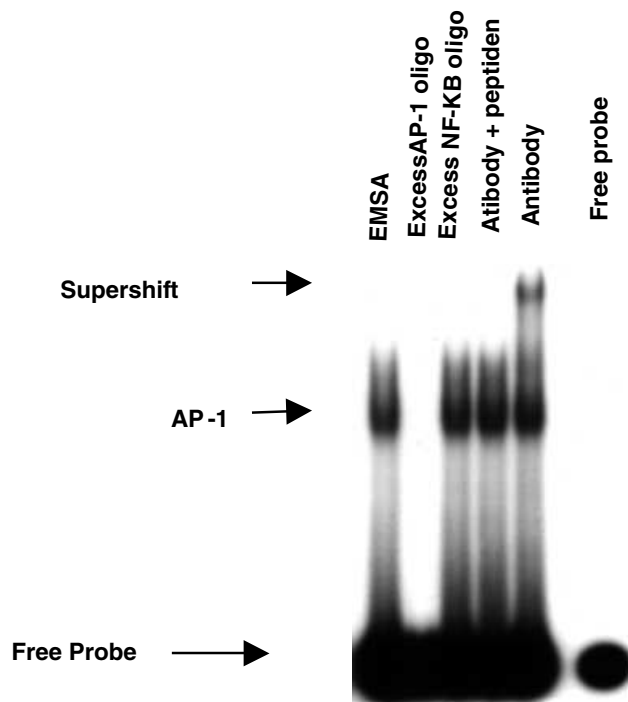


Fig. 1. The EMSA specificity of the AP-1 DNA-binding using nuclear extract from NIH/3T3 cells. Lane 1: no addition; lane 2: 50× excess of unlabeled AP-1 oligonucleotide probe; lane 3: 50× excess of unlabeled NF-κB oligonucleotide probe; lane 4: c-Jun antibody with corresponding peptide antigen; lane 5: c-Jun antibody.

the AP-1 components in normal melanocytes and different primary and metastatic melanomas.

Initially, we confirmed the specificity of the AP-1 DNA-binding complex by making use of a nuclear extract from NIH/3T3 cells. The mobility of the labeled AP-1 probe was shifted in the presence of nuclear extract, and a 50-fold molar excess of wild-type unlabeled AP-1 probe competed with binding while an excess unlabeled NF-κB probe did not (Fig. 1, lane 1–3). c-Jun antibody included in the binding reaction created a supershift in mobility (lane 5) and the supershift was no longer formed if the antibody was first neutralized with the corresponding peptide antigen (lane 4). These results demonstrate the specificity of AP-1 complexes for evaluating AP-1 binding and composition.

In melanocytes, c-Jun, JunD and FosB were involved in AP-1 complexes as demonstrated by supershift analysis (Fig. 2A). In the early stage primary melanoma cell line w3211, we observed that nuclear protein extracts formed supershifted complexes with c-Jun, JunD and Fra-1 antibodies, but not with FosB antibody (Fig. 2B). In VGP-like w1205 melanoma cells (Fig. 2C) and two metastatic melanoma cell lines c81-46A, A375 (Fig. 2E and F, respectively) only JunD was involved in AP-1 DNA-binding complexes, while in a third melanoma line (c83-2c) JunD and Fra-1 were also identified in addition to a supershift with the c-Jun antibody (Fig. 2D). Also as shown in Fig. 3, AP-1 DNA-binding activity of VGP-like w1205 and metastatic c83-2c, c81-46A, and A375 melanoma cells were all decreased remarkably compared with that of normal melanocytes and RGP-like w3211 cells.

To measure if the differences in medium might affect the AP-1 composition and activity, we also cultured w3211, c83-2c and c81-46A melanoma cells in MCDB+ medium (melanocyte medium containing TPA) for 3 d, collected nuclear extracts, and performed EMSA and supershift assays. No significant changes in AP-1 composition and activity in w3211, c83-2c and c81-46A human melanoma cells were observed by switching to MCDB/TPA+ medium (melanocyte medium containing TPA) for 3 d as shown in Fig. 2(B, D, E).

Loss of c-Jun/AP-1 Proteins in the Nuclear Compartment as Measured by Western Blot

c-Jun is critically important for the normal function of the AP-1 transcription factor, which responds to a variety of extracellular stimuli by changing expression levels, altering its phosphorylation status, or by forming homodimeric or heterodimeric complexes with Fos proteins. Immunoblotting changes in the relative levels of c-Jun, JunD and Fra-1 exhibited patterns (Fig. 4) similar to those observed by EMSA. c-Jun is present in normal melanocytes and early stage primary melanoma w3211 cells with a high-level expression, but was decreased significantly in late stage primary w1205 and metastatic c81-46A and A375 melanoma cells. In contrast, JunD protein was elevated in c81-46A and c83-2c cells compared with melanocytes and primary melanomas. Using specific anti-Fra-1 antibody, Fra-1 was only observed in RGP-like w3211 (at a high level) and c83-2c cells (much diminished), which is also consistent with our previous supershift data.

AP-1 Transcriptional Transactivation Activity

The AP-1-binding site is known as the TPA response element (TRE), and AP-1 DNA-binding activity does not necessarily reflect transcriptional activity. To determine whether AP-1 DNA-binding activity is a reflection of its ability to promote transcription in this system, we performed transfection with an AP-1 reporter vector system containing synthetic AP-1 DNA-binding sites (5'ATGAGT-CAG 3') and a SEAP gene. TPA induced SEAP activity in melanoma cells in vitro was measured. As shown in Fig. 5, in the early primary melanoma cells (w3211) the AP-1 transcriptional transactivation activity was significantly increased after TPA induction (more than 10-fold), while in VGP-like cells TPA induction only produced mild increases (w1205, 1.2-fold; c81-46A, 2.8-fold) or even a decrease in SEAP secretion (A375, 69.2% of basal).

Effects of the Alkylating Agent Cisplatin on Viability in Different Cell Lines

Experiments were performed with different melanoma cell lines to determine whether there were differences in the response to cisplatin. The cells were treated with different doses of cisplatin for 48 h, followed by co-incubation with MTT agent for 2 h. As shown (Fig. 6), c81-46A metastatic melanoma cells demonstrated higher resistance to cisplatin treatment than metastatic c83-2c cells and primary melanoma w3211 cells, both of which have c-Jun involved in AP-1 binding.

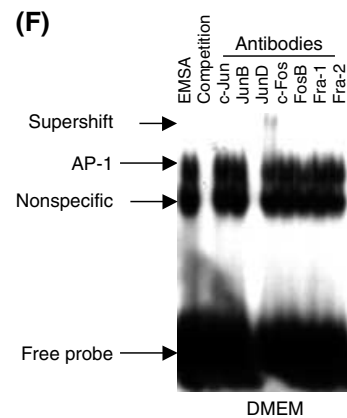
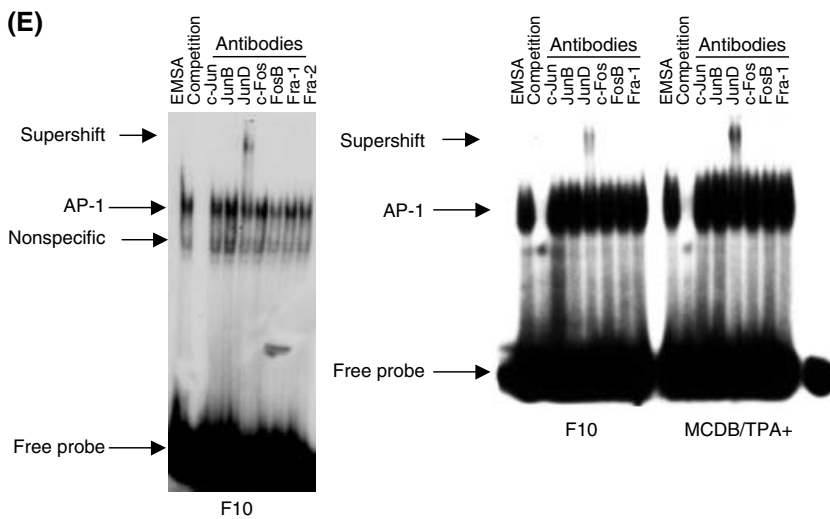
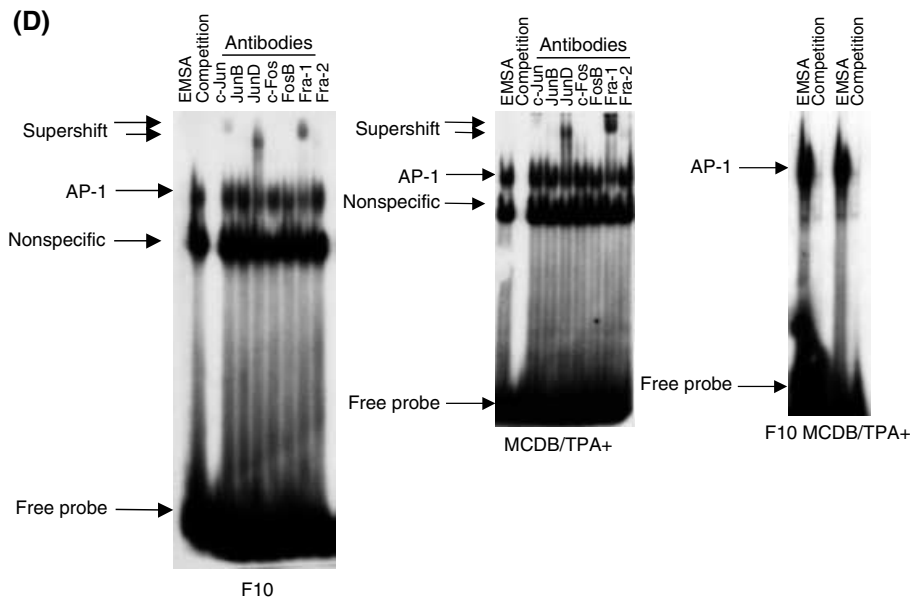
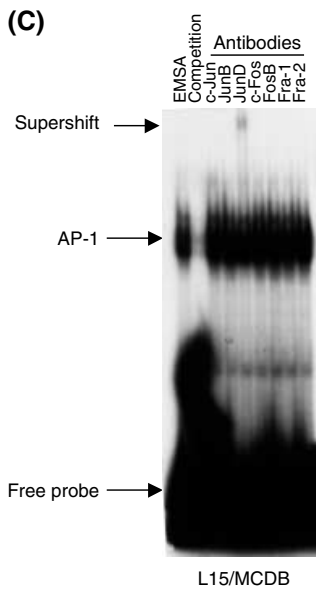
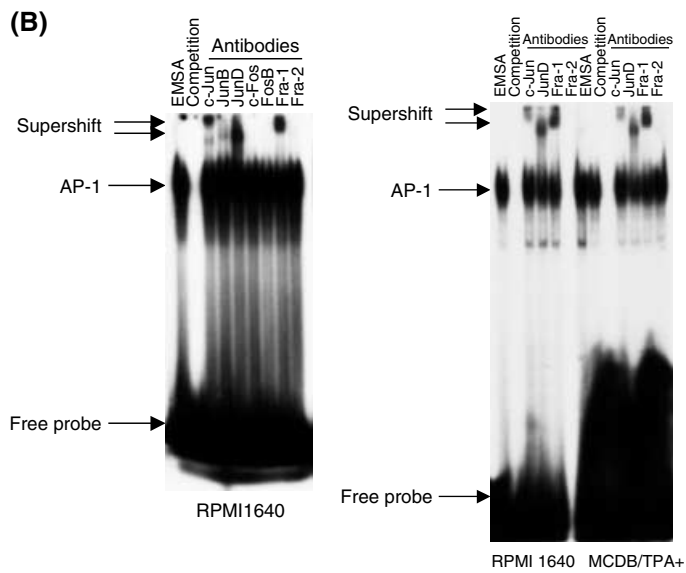
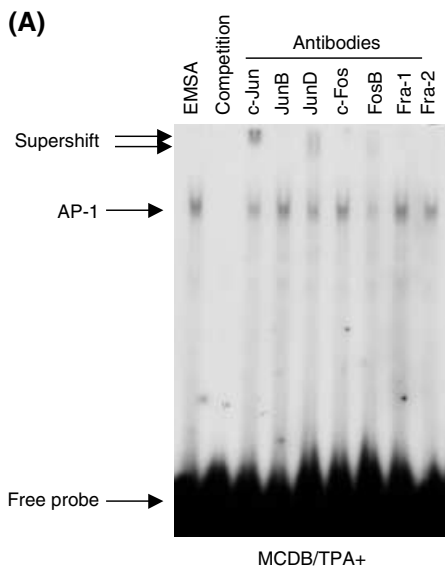


Fig. 2. The EMSA and supershift analysis of AP-1 DNA-binding activity and composition in normal melanocytes, primary melanoma (w3211, w1205) and metastatic melanoma cells (c81-46A and c83-2c). EMSA with ³²P-labeled AP-1 oligonucleotide probe was performed as described in 'Materials and Methods' and 50x excess unlabeled AP-1 was added for the competition. The nuclear extracts were subjected to EMSA in absence or presence of antibodies to the indicated AP-1 proteins. Melanoma lines were grown in their medium (w3211, RPMI 1640; c81-46A and c83-2c, F10) or melanocyte medium MCDB/TPA+ as detailed in the Materials and Methods. (A) normal melanocytes; (B) early/primary melanoma w3211; (C) late/primary melanoma w1205; (D), (E) and (F): metastatic melanomas c83-2c, c81-46A and A375, respectively.

Effects of AP-1 Antisense Transfection on Cell Survival

c-Jun antisense oligonucleotides were transfected into normal melanocytes and different metastatic melanoma cells (c83-2c and c81-46A). RT-PCR data confirmed that anti-c-Jun decreased the mRNA level of c-Jun in melanocytes, c83-2c and c81-46A cells with 48 h transfection (Fig. 7D). Normal melanocytes were sensitive to the replacement of normal MCDB+ media with oligofectamine-containing MCDB plain media, and 3 d later the viability of oligofectamine-treated melanocytes was 74.2% of untreated controls. As shown in Fig. 7A, with anti-c-Jun transfection normal melanocytes showed higher viability (98.1%) compared with control oligos (72.3%, untreated cells as 100%). The viability of melanocytes was also elevated from 26.4 to 50.0% when cisplatin treated cells had been transfected ($P < 0.05$). In metastatic melanoma c83-2c cells (Fig. 7B), which also have c-Jun involved in AP-1 binding, we did not observe a significant effect of transfection on cell viability; however, the c-jun antisense transfected cells showed relatively higher resistance to cisplatin and the viability of cells increased from 33.9 to 44.9% compared with control oligos ($P < 0.05$). Also, we investigated the effect of c-Jun antisense on c81-46A cells in which only JunD was detectable in AP-1 binding complexes. The inhibition of c-Jun by antisense had no effect on cell viability, nor did it affect the response to cisplatin treatment (Fig. 7C). Although anti-JunD reduced the expression level of JunD in c81-46A cells as measured by Western blot assay (Fig. 8B), it produced only a

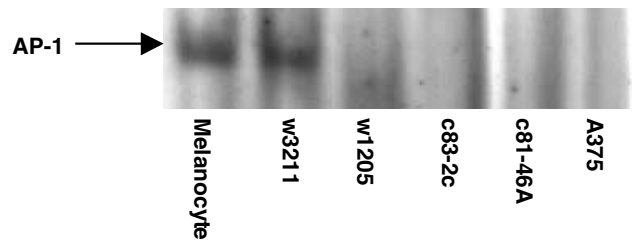


Fig. 3. AP-1 DNA-binding activities in different human melanoma cell lines by non-radioactive EMSA assay. EMSA with DIG-labeled AP-1 oligonucleotide probe was performed following the manufacturer directions. Equal amount of nuclear protein (7 μ g) were subjected to each EMSA reaction.

mild non-significant ($P > 0.05$) decrease in cell viability either alone or in combination with cisplatin (Fig. 8A).

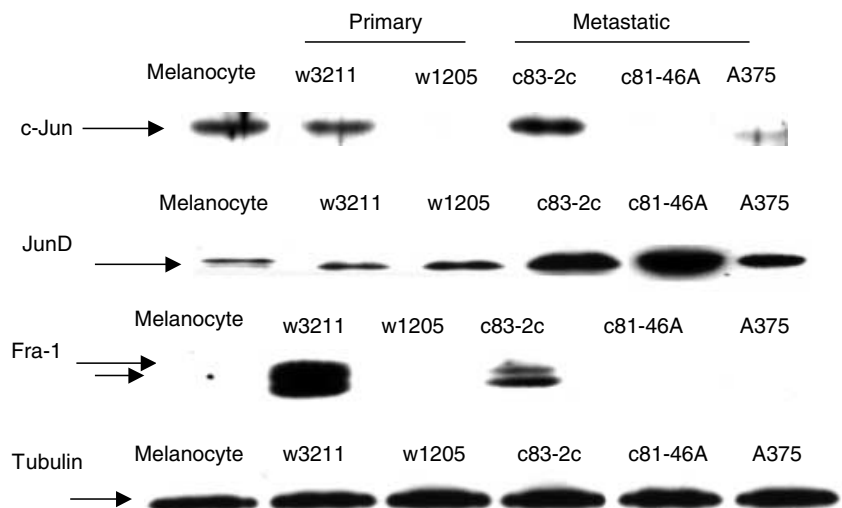
A Dominant-Negative c-Jun Mutant (TAM67) Increased Soft Agar Colony Formation of Melanoma Cells

To directly investigate whether loss of c-Jun affects cell malignancy, we have used the transcription dominant-negative c-Jun mutant, TAM67 (Fig. 9A) (16), to block c-Jun both in primary and metastatic human melanoma cells. First, we tested the effect of TAM67 on cell morphology. As can be seen (Fig. 9C) w3211 cells transfected with TAM67 lost contact inhibition and grew vertically compared with control cells (Fig. 9B). Soft agar colony formation (Fig. 10) was also significantly increased by transfection with TAM67 in w3211 and c83-2c cells (from 7.50 and 2.89 colonies per high-power field in controls to 10.04 and 6.44 colonies, respectively).

DISCUSSION

Development of malignant melanoma is considered to be a progressive process involving evolution of a normal melanocyte through phases: a nevus or a dysplastic nevus, a radial growth (primary, early stage), a vertical growth (primary, late stage), and eventually an aggressive metastatic phenotype (18).

Fig. 4. c-Jun, JunD and Fra-1 levels are altered in different melanoma cells compared with normal melanocytes by Western immunoblotting assay. Cells were collected and nuclear protein extracts were extracted as described in 'Material and Methods'. Protein expression was determined by Western blotting with antibodies specific for c-Jun (top), JunD (middle) and Fra-1 (bottom). Arrows indicate specific bands.



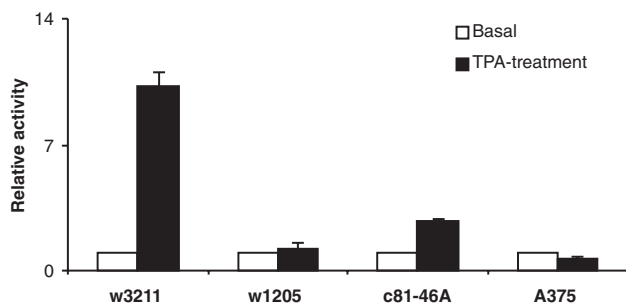


Fig. 5. Transcriptional activity of activating protein-1 (AP-1) in human primary and metastatic melanoma cells using AP-1/SEAP reporter system. Different melanoma cells were transiently transfected with AP-1/SEAP reporter vectors containing the consensus AP-1 site. AP-1 binding and transcription transactivation results in activation of the SEAP gene. This SEAP activity was quantified on a luminometer. The relative SEAP activity was calculated relative to that of non-TPA-treated cells.

In this study, we present evidence from EMSA and Western blot analysis that during melanoma progression the composition and activity of AP-1 dimers are distinctly altered (summary, Table 1). As these studies were done in cell lines derived from melanoma tissues, with the limitations therein, confirmation of these results in primary tissue will be required to confirm their clinical relevance.

The EMSA and antibody supershift showed that AP-1 DNA-binding activity in normal melanocytes and early primary melanoma cells (w3211) was much higher than in late primary and metastatic melanoma cells. c-Jun was involved in the AP-1 complexes in melanocytes and RGP-like cells, while in most melanoma cells that we tested c-Jun was absent in the AP-1 complexes and there was decreased AP-1 binding activity. Switching the melanoma cells to MCDB/

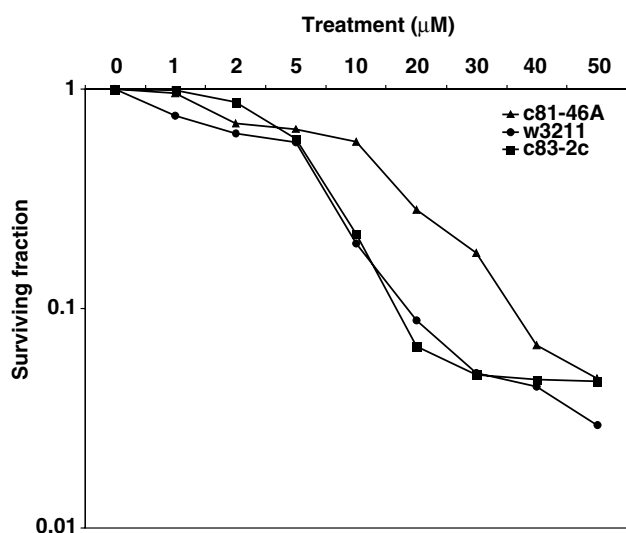


Fig. 6. Effect of cisplatin on viability of primary melanoma w3211, metastatic melanoma c81-46A and c83-2c cells. The assay was performed as described in 'Materials and Methods'. After 48 h of treatment MTT reagent was added. Substrate cleavage was monitored at 490 nm with a Bio-Rad microplate reader and analyzed using Microplate software. Values represent the mean of three separate experiments.

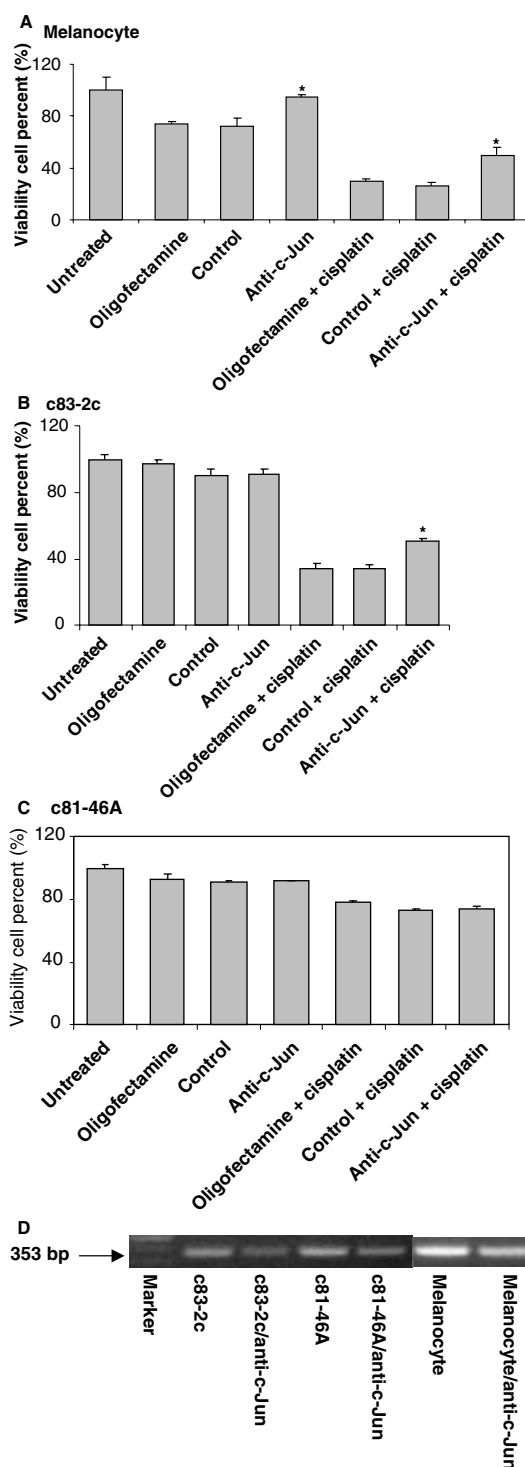


Fig. 7. Effects of anti-c-Jun oligonucleotides on viability of normal melanocytes and metastatic melanoma cells alone or in combination with cisplatin. Cells were transfected with anti-c-Jun oligonucleotide, followed by subsequent 20 μM cisplatin treatment prior to analysis. Cell viability was measured using MTT assay as described in 'Material and Methods'. The depicted viability is relative to the viability of the respective untreated cells. Values represent the mean of three separate experiments; bars ± SD. *P < 0.05 compared with control. (A) Normal melanocytes; (B) and (C): Metastatic melanoma c83-2c and c81-46A cells respectively; (D): RT-PCR amplification of c-Jun mRNA from melanocytes, c83-2c and c81-46A cells as described in 'Material and Methods'.

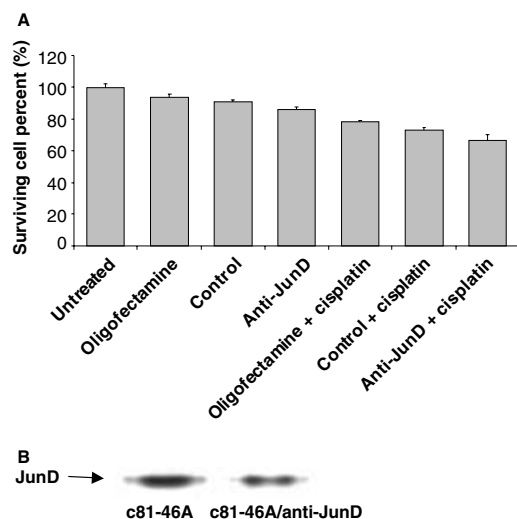


Fig. 8. Effects of anti-JunD oligonucleotide on viability of metastatic melanoma c81-46A cells. Cells were transfected with anti-JunD oligonucleotide for 48 h, followed by 20 μ M cisplatin treatment prior to analysis. Cell viability was measured by MTT assay. The depicted viability is relative to the viability of the respective untreated cells. (A) Graphic of mean \pm SD from three separate experiments. (B) Western blot analysis of JunD protein expression levels as described in 'Material and Methods'.

TPA + melanocyte medium for 3 d produced no significant changes in AP-1 composition and DNA-binding activities indicating that the differences in AP-1 among the different cell lines was an intrinsic feature.

Compared with normal melanocytes, FosB is lost in AP-1 complexes in the w3211 and c83-2c cells and is replaced by Fra-1. We are not sure if this change is critical to the initiation of malignant progression in human melanoma as the switch was only noted in w3211 (high level) and c83-2c (low level); this observation needs further investigation before a definitive comment can be offered.

We have shown in previous studies that melanoma cells produce large amounts of intracellular superoxide anion, which suggest that these cells are under constant oxidative stress from a high level of intracellular generation of ROS (10). We hypothesize that the alterations in AP-1 function might result from such chronic oxidative stress. An alternative hypothesis is that the genetic changes in melanoma, which are secondary to ROS or other causes, have led to the dysfunction of AP-1. As has been shown by Thompson (18), chromosome structural abnormalities in melanoma cells with deletions or clustering of breakpoints are common, and some of these are at the chromosomal locations of c-Jun (1p31-32) and FosB (19q13.32).

In metastatic melanoma cells TPA-induced AP-1 transcriptional activity was decreased significantly (Fig. 5). One possible explanation might relate to the altered composition of AP-1 dimers with a decreased AP-1 DNA-binding activity as discussed above, which does not allow effective transcription of certain target genes. An alternative explanation is that metastatic melanoma cells fail to respond to TPA secondary to abnormalities of PKC activation. In a prior study, we observed that TPA, a traditional tumor promoter, was not mitogenic for melanoma cells (19) and

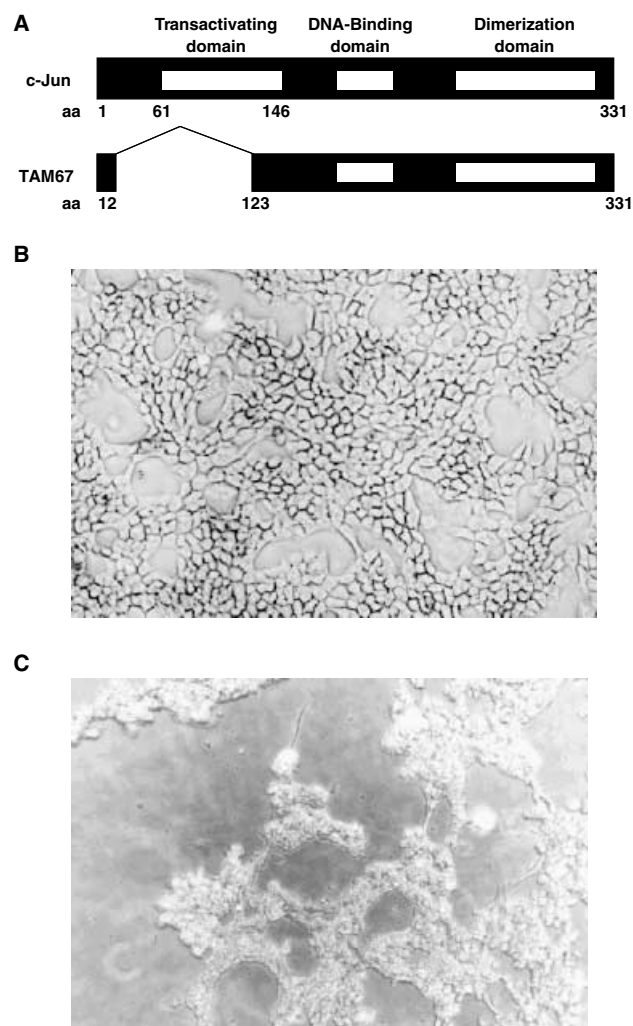


Fig. 9. A dominant-negative c-Jun mutant (TAM67) induced morphologic changes in early stage primary w3211 melanoma cells. (A) Schematic diagram of the c-Jun and TAM67 proteins; (B) Control w3211 cells ($\times 40$); (C) With TAM67 transfection ($\times 40$).

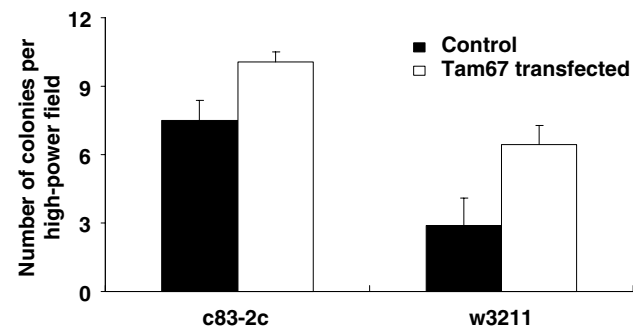


Fig. 10. TAM67 increased soft agar colony formation of w3211 and c83-2c melanoma cells. Cells were transfected with TAM67 for 48 h, passaged into fresh whole medium and seeded into soft agar. Effects on colony formation with TAM67 are expressed as number of colonies per high-power field, mean values \pm SD, of 20 independent high-power fields. Significant difference from control is $P < 0.05$ (*).

Table 1. Alterations in AP-1 composition during human melanoma progression

Cell lines	Composition of AP-1 complexes
Melanocytes	c-Jun/JunD/FosB
Melanoma	
Primary*	
w3211 (Early)	c-Jun/JunD/Fra-1
w1205 (Late)*	JunD
Metastatic	
c83-2c	c-Jun/JunD/Fra-1
C81-46A	JunD
A375	JunD
c81-61	JunD

* Although derived from a VGP melanoma, w1205 is highly metastatic in nude mice (M. Herlyn, pers. comm.). In contrast c81-46A was developed by cloning through agar from a metastatic deposit, but is of low metastatic potential and has biological features of a nearly normal phenotype.

that there was a decreased expression level of PKC β in metastatic melanoma cells (20), which is required for TPA-induced ROS production. PKC β -deficient human HL-525 cells are also resistant to TPA-induced differentiation with attenuated expression of c-Fos, c-Jun and JunB (21). The dysfunction of AP-1 during melanoma progression might be related, at least in part, to the deficiency of PKC expression or the differential expression of PKC subtypes, perhaps accounting for the failure of metastatic melanomas to respond to TPA induction.

Many studies have revealed that the JNK/c-Jun/AP-1 pathway is critical for certain forms of stress-induced apoptosis. For example, overexpression of TAM 67, a dominant negative c-Jun mutant has been found to be protective of U937 monoblastic leukemia cells from several forms of stress, including H₂O₂, X-rays, UV-C and heat shock (22) and to protect neurons from apoptosis induced by neuronal growth factor withdrawal (5). Moreover, c-Jun^{-/-} fibroblasts are relatively resistant to apoptosis induced by alkylating agents (23) and UV (24), and show impaired FasL expression. But in some other cases the conclusions are exactly opposite to these findings (25). These results imply that the c-Jun/AP-1 response depends on the type of cells and stresses. Our antisense c-Jun transfection experiments have shown that human melanocytes and c83-2c melanoma cells exhibit increased cell viability during cisplatin treatment. Our results also revealed that TAM67 significantly increased soft agar colony formation of human melanoma cells. These data are consistent with the conclusions by others that c-Jun plays an important role in cell survival (22).

JunD can have either positive or negative effects on cell survival and proliferation depending on the cellular and genetic context. In some cases JunD^{-/-} fibroblasts exhibited increased p53-dependent apoptosis after UV and were sensitive to the cytotoxic effect of tumor necrosis factor (TNF)- α (26), which suggests that JunD protected cells from responses to stress stimuli. However, in metastatic c81-46A cells, which only have JunD involved in the AP-1 complex, anti-JunD antisense did not produce a significant effect on cell viability. Either JunD/AP-1 in c81-46A cells does not play a critical role in control of gene transcription or other transcription factor signaling pathways are involved. We and

others have shown that NF- κ B is constitutively active in human melanoma cells (27, 28) suggesting that the transcription factor NF- κ B, plays a dominant role in unregulated cellular proliferation of melanoma cells.

Strategies for manipulation of abnormalities in transcription factor regulation for therapeutic benefit have been recently summarized by Darrell (29). Identification of target molecules conferring enhanced viability and a better understanding of the specific signal transduction controls should allow the development of more rational strategies to treat malignant melanoma. We propose that loss of c-Jun in AP-1 complexes during human melanoma cell progression may lead to a change in AP-1 complex composition and transactivation, resulting in expression of different sets of genes that are involved in cell viability or survival.

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