Down-Regulation of Tropomyosin-2 Expression in c-Jun-transformed Rat Fibroblasts Involves Induction of a MEK1-dependent Autocrine Loop

Sofia Ljungdahl, Stig Linder, Bo Franzén, Bernard Binétruy, Gert Auer, and Maria C. Shoshan

Radiumhemmet's Research Laboratory [S. L., S. L., M. C. S.] and Unit of Cell and Molecular Analysis [B. F., G. A.], Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institute and Hospital, S-171 76 Stockholm, Sweden; and Institut de Recherche sur le Cancer, Center National de la Recherche Scientifique, 94801 Villejuif, France [B. B.]

Abstract

Overexpression of the c-Jun transcription factor in rodent fibroblasts may result in cell transformation or in apoptosis. The mechanisms whereby c-Jun induces transformation are unknown. We show here that the expression of high-molecular weight tropomyosin-2 (TM-2) is down-regulated in c-jun-transformed FR3T3 rat fibroblasts. However, down-regulation did not seem to be a direct effect of c-Jun on TM-2 gene expression. Thus, TM down-regulation in c-jun-transformed cells was alleviated by inhibitors of Ras (BZA-5B) or MEK1 (PD98059). Furthermore, medium conditioned by c-jun-transformed cells induced TM-2 down-regulation in untransformed cells by a mechanism requiring MEK1. Consistent with a central role for the MEK/ERK, but not SEK/JNK, pathway for TM down-regulation, constitutively active mutants of Raf induced TM down-regulation, whereas constitutively active Rac did not. We also show that anchorage-independent growth of c-jun-transformed cells requires MEK1. These findings suggest that indirect induction of the MEK/ERK pathway is central to c-Jun-induced transformation of rat fibroblasts.

Introduction

The c-Jun gene encodes the major component of the sequence-specific activator protein transcription factor. Its regulation has been extensively studied, and it seems that the c-Jun protein is a component of several signaling pathways, notably normal and oncogenic signaling via Ras. Expression of exogenous c-Jun can cooperate with an activated ras gene to transform primary cells (1). Overexpression of exogenous c-Jun may on its own induce transformation of chick embryo fibroblasts or established rat fibroblast cell lines (1, 2).

Although all immediate events initiated by c-Jun should be nuclear and pertain to gene regulation, activated Ras is known to activate a plethora of signaling pathways, some of which relay cytoplasmic events not immediately related to gene regulation. Experiments using dominant negative and activated forms of p21-Rac, which normally plays a role in cytoskeleton organization, have shown a role for Rac in transformation by Ras (3, 4). Rac has recently also been reported to be an intermediate in the UV-response, placing Rac downstream of Ras and upstream of Jun N-terminal kinase (5). The Raf-1 kinase immediately downstream of Ras does not seem to affect Rac, but instead mediates signaling to the p42/p44 MAP kinases which then act on nuclear substrates. Constitutively active Raf-1 may on its own induce transformation (6, 7). Cotransfection of activated Raf-1 and Rac affords the same transformational efficiency as activated Ras (4).

TM3s have been shown to be down-regulated in transformed cells and in tumors in a number of studies (8–10). The down-regulation of high-molecular weight TMs is likely involved in the morphological alterations, loss of density inhibition, and anchorage-independent growth which are typical for transformed cells (11, 12). The mechanisms leading to down-regulation of TMs in transformed cells are poorly understood, but may involve an epidermal growth factor signal transduction pathway (13). Down-regulation of 36 and 38 kDa forms of TM in fos-transformed rat fibroblasts has been suggested to be mediated by an indirect mechanism not directly involving Fos (9).

In some contrast to knowledge about Ras transformation, the mechanisms underlying transformation by c-Jun overexpression are not known. Previous work has shown that the genes encoding TSP-1, SPARC, and tenasin-C are repressed after stable transformation by c-Jun or Ras (14). interestingly, TSP-1 and SPARC repression was shown to be mediated by a secreted factor, but the mechanisms involved were not studied in detail. In the present study, 2-DE was used to screen for genes that are affected by stable overexpression of c-Jun. We identified TM1-3 as being repressed in c-jun transformed cells. Additional studies showed that down-regulation depended on Ras and MEK activity and suggested that c-Jun-induced transformation depends on autocrine mechanisms.

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2 To whom requests for reprints should be addressed, at Radiumhemmet's Research Laboratory, CCK R8:03, Karolinska Institute, S-171 76 Stockholm, Sweden. Phone: 46-8-51-77-29-49; Fax: 46-8-33-90-31; E-mail: minmi.shoshan@onkpat.ki.se.

3 The abbreviations used are: TM, tropomyosin; TSP-1, thrombospondrin-1; PCNA, proliferating cell nuclear antigen; TGF-α, transforming growth factor α.
**Results**

**Down-Regulation of TM1-3 in FR3T3 Cell Lines Transformed by c-Jun.** 2-D electrophoresis was used to screen for differences in protein expression between FR3T3, an immortalized rat fibroblast cell line with untransformed characteristics, and c-jun-transformed derivatives of FR3T3. Autoradiographs of [35S]-methionine-labeled polypeptides in extracts of each cell line were scanned, and the images analyzed with the PDQUEST software (15). Gel images of these cell types are presented in Fig. 1.

Two polypeptides with molecular weights of Mw 37,000 and Mw 36,000 were strongly down-regulated in FRcJun1 and FRcJun3 cells compared with FR3T3. A weaker down-regulation of a 39 kDa polypeptide was also observed. These spots were identified as TM1-3 by position (aligning with the REF52 and Biobase patterns), and by comigration with pu-
rified TMs. Results from quantitation of the gels using PDQUEST software are shown in Fig. 1. Several differences in gene expression were observed between FR3T3 and c-jun-transformed cells, e.g., increases in TM-5, PCNA and α-enolase in FRcJun1 and FRcJun3 cells (Fig. 1). Since the high-molecular-weight TMs have been causally linked to the transformed phenotype in rodent fibroblasts (11, 12, 16), we chose to further study the expression of these proteins.

TM expression in c-jun transformed cells was re-examined by Western blot analysis (Fig. 2) as the more accurate method for assessing individual protein levels. The main bands in Fig. 2A represent TM-1 and TM-2. A decrease in TM-2 to <one-tenth of the level in untransformed FR3T3 cells was found in both c-jun-transformed cell lines examined (Fig. 2B). The level of TM-1 expression varied, but was by Western blot seen to be reduced to at least half the FR3T3 level. The expression of TM-2 mRNA in four c-jun-transformed cell lines was examined also by Northern analysis (Fig. 2C) and was, again, found to be down-regulated.

Inhibition of Ras or MEK1 Alleviates Repression of TM-2. Repression of TSP-1 and SPARC in c-jun transformed cells has been suggested to be mediated by an autocrine loop (14, 17). Because such a loop may be expected to be mediated by cytokines requiring the Ras/Raf/MEK/ERK pathway, we examined whether down-regulation of TM expression in c-jun transformed cells is affected by PD98059, an inhibitor of MEK1, or by BZA-5B, an inhibitor of Ras farnesylation. As shown in Fig. 3, TM-2 expression in FRcJun1 cells increased after treatment with BZA-5B or PD98059. Subsequent 2DE analysis of PD98059 treated FRcJun1 cells showed that with total protein as reference, TM-2 was up-regulated whereas tubulin was not affected (data not shown). Because BZA-5B has been suggested to affect also the farnesylation of, e.g., RhoB (18) involved in cytoskeletal regulation, and because BZA-5B required a 2-day preincubation, we performed all subsequent inhibition experiments with the highly specific PD98059.

We next examined whether PD98059 would not only increase TM expression but also reverse the transformed characteristics of FRcJun1 cells. As shown in Fig. 4 and Table 1, PD98059 induces a reversal of the transformed morphology and inhibited anchorage independent growth of FRcJun1 cells. Most PD98059 treated FRcJun1 cells formed very small colonies in agar (data not shown). PD98059 also inhibited the growth of FRcJun1 cells in monolayer cultures (Table 1), but this decrease in proliferation could not on its own explain the strong inhibition of soft agar growth. PD98059 did not significantly affect the growth of FR3T3 cells.

TM Expression Is Down-Regulated by a Factor Secreted from c-jun-transformed Cells. To determine whether c-jun-transformed FR3T3 cells secrete factor(s) which down-regulate TM expression in untransformed cells, conditioned media were collected and added to FR3T3 cells. All cells were grown in 0.5% serum to preclude the effects of exogenous growth factors. As shown in Fig. 5A, TM-2 mRNA expression in FR3T3 cells decreased 24 h after addition of medium from FRcJun1 cells. The FR3T3 morphology also changed to a more elongated and refractile one (Fig. 6). The same experiment was conducted for protein analysis by Western blotting. Analysis of FR3T3 cells incubated with FRcJun1 medium shows a decrease in TM-2 (Fig. 5B and C).

When FR3T3 cells were treated with PD98059 before addition of conditioned medium from FRcJun1 cells, the secreted factor(s) therein was no longer able to repress TM-2 (Fig. 5C). Thus, the paracrine effect of the factor(s) on TM-2 expression in normal cells depends upon MEK function.

The data above suggest that c-Jun transformation involves the establishment of an auto-/paracrine loop. To directly
Table 1  Effect of PD98059 on the growth of FRcJun1 and FR3T3 cells

<table>
<thead>
<tr>
<th>Population doubling time in monolayer culturesa</th>
<th>-PD98059</th>
<th>+PD98059 (50 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRcJun1</td>
<td>14.5</td>
<td>24.1</td>
</tr>
<tr>
<td>FR3T3</td>
<td>22.9</td>
<td>23.7</td>
</tr>
<tr>
<td>Plating efficiency (%) in semi-solid mediumb</td>
<td>FRcJun1</td>
<td>45</td>
</tr>
<tr>
<td>FR3T3c</td>
<td>~</td>
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a The number of population doublings of monolayer cultures during exponential growth during 72 h was determined.
b Plating efficiency in soft agar. One hundred colonies were counted after 6 days. The fraction of colonies (%) with a diameter of >0.5 mm is shown. 
c Do not form colonies in soft agar.

TM Expression Is Down-Regulated in FR3T3 Cells Expressing 12V-H-Ras, but not 12V-Rac. Transformation by Ras has been shown to require the cognate p21-Rac G-protein (3, 4), which in turn has been suggested to mediate activation by JNK (19) and hence of c-Jun. Because of this putative pathway from Ras to c-Jun, 2-DE analysis of Ras3T3 cells, expressing 12V-H-Ras, and Rac3T3 cells, which express a Myc-tagged 12V-Rac protein (Fig. 9, top right) was performed. 12V-Ras transformed cells showed a transformed morphology, whereas 12V-Rac expressing cells showed a similar morphology to FR3T3 cells (data not shown). The 2-DE results show that TM1-3 levels were decreased in the 12V-Ras transformed cells, but not in 12V-Rac expressing cells (Fig. 9). Similar to c-jun transformed cells, PCNA levels were increased in ras-transformed cells, whereas 12V-Rac expressing cells did not show an increase in PCNA (Fig. 9).

Discussion

Although the regulation of c-Jun production and activity is well-documented, little is known about c-Jun target genes and the outcome of signal transduction pathways acting via c-Jun. By necessity, the mechanisms underlying transformation caused by overexpression of c-Jun are also unknown. We here show that high-molecular-weight TMs are down-regulated in c-jun-transformed FR3T3 cells. High-molecular-weight TMs have been found to be down-regulated in a number of transformed cell types (8, 22) and in human tumor cells (10). Re-expression of TM-1 or TM-2 in transformed cells restores contact-inhibited growth or reverses anchorage-independent growth capability (12, 16, 23–25). Alter-
Fig. 5. Down-regulation of TM-2 in FR3T3 cells by FRcJun1-conditioned medium. A. quantitated Northern blot analysis of TM-2/β-actin mRNA ratios in FR3T3 cells grown in medium conditioned by FRcJun1. B, representative Western blot of TM-1 and TM-2 in FR3T3 cells grown in medium conditioned by FR3T3 cells (control) and by FRcJun1 cells (FRcJun1-medium). C, quantitated Western blot analysis of TM-2/β-tubulin ratios in FR3T3 cells grown in medium conditioned by FRcJun1 in the presence or absence of PD98059. Conditioned medium was prepared as described in "Materials and Methods."

Regulation of TM expression thus seems to be central to the transformed phenotype, but the underlying mechanisms are incompletely understood.

The finding that BZA-5B or PD98059 derepressed TM expression in FRcJun1 cells suggested that c-Jun mediated transformation depends on the Ras-MEK-ERK pathway. Inhibition of MEK1 by PD98059 reversed the transformed morphology and inhibited anchorage independent growth of FRcJun1 cells, supporting this view. Whereas monolayer growth of FRcJun1 was inhibited by PD98059, proliferation of untransformed FR3T3 cells was not affected. These findings are similar to those of Kizaka-Kondoh et al. (26), who reported that the Raf pathway was involved in oncogenic transformation but not in monolayer growth of NRK cells.

Experiments using conditioned media directly suggested that c-jun transformed cells secrete a factor which induce down-regulation of TM-2. Because this effect was sensitive to PD98059, and because the conditioned medium increased ERK activity in FR3T3 cells, we conclude that down-regulation of TM-2 by the secreted factor was mediated by stimulation of MEK-ERK. The level of nuclear ERK activity induced by FRcJun1- and Raf-CAAX conditioned media was within the range observed in fibroblast cell lines stably transformed by ras (27). It is well-known that the oncogenic activation of signal pathways in transformed cells leads to secretion of autocrine factors which may further affect the phenotype. These factors are often growth-factor type, e.g., TGFs, insulin-like growth factors, or epidermal growth factor (22, 28, 29). More than one secreted factor may be responsible for autocrine down-regulation of TM in transformed cells. It has been reported that TGF-α has transforming activity on fibroblasts (28). Other workers have suggested that although TGF-α was able to confer suppression of TM synthesis, it could in this context likely be replaced by other growth factors or other oncogene-mediated events (22). We have attempted to block the autocrine loop in our system using anti-TGF-α antibodies, but this treatment had only marginal effects (data not shown), possibly reflecting a partial, but minor, involvement. Differently composed "cocktails" of secreted factors may, furthermore, explain cell-type specific differences in the experiments reported here.

We also show here that membrane-targeted Raf kinase could induce cell transformation of FR3T3 and concomitant down-regulation of TM expression, whereas an activated form of Rac had no effect. Thus, although activated forms of Rac have been reported to increase the transformation efficiency of Raf (4), they do not seem to cause down-regulation of TM expression on their own, at least not in FR3T3 cells. In contrast, 12V-Ras induced down-regulation of TM expression. Interestingly, down-regulation of TM by 12V-Ras did not necessarily require c-Jun. This finding is consistent with the view that down-regulation of TM in transformed cells is not due to stimulation of c-Jun via the Rac/JNK pathway, but is mediated by the MEK/ERK pathway.

Different roles for c-Jun have been described in various cell systems. Johnson et al. (30) reported that c-Jun is required for transformation of mouse embryo fibroblasts by Ras. By contrast, we have observed cdeletion of the c-jun gene together with CDKN2A in 12V-ras transfected REF cells (21). Transformation is not the only possible outcome of overexpression of c-Jun, since it was recently shown that overexpression can also trigger apoptosis (31). c-Jun may, thus, have various roles in cell physiology, possibly due to cell type, genetic context, and expression level.

We conclude from the present study that triggering of autocrine loops occurs in cells transformed by c-Jun and
MEK1-mediated Loop Supports c-jun Transformation

Fig. 6. Effect of FRcJun1-conditioned medium on morphology of FR3T3 cells. A, FR3T3 cells were incubated in low-serum medium (DMEM-0.5% FCS) for 96 h. B, FR3T3 cells were incubated in low-serum medium for 48 h, then received low-serum medium conditioned by FRcJun1 cells. The FR3T3 cells were then incubated for an additional 48 h. C, FRcJun1 cells incubated in low-serum medium for 96 h.

Fig. 7. Effect of FRcJun1-conditioned medium on nuclear ERK activity in FR3T3 cells. Starved FR3T3 cells were grown in FRcJun1-conditioned medium for the indicated time intervals in the presence or absence of PD98059 (50 μM; 18 h preincubation). Nuclear extracts were then prepared in duplicate and assayed for ERK activity towards a specific synthetic peptide. Results were normalized for protein content. Similar results were obtained when the experiment was repeated.

Fig. 8. TM expression in FR3T3 cells transformed by a membrane targeted Raf-kinase (RatCAAX). Shown are quantitated Western blots of three RatCAAX-transformed FR3T3 cell lines and nontransformed FR3T3 cells. ■, TM-1; □, TM-2.

that these loops are essential for down-regulation of TM expression and soft-agar growth. These findings are important for understanding the mechanism of c-Jun-induced transformation, since they suggest that with regard to cell
transformation c-Jun is "upstream" of Ras. These data underscore the general potential in auto- or paracrine loops as targets for anticancer therapies.

Materials and Methods

Cell Lines. FR3T3 is a clonal, immortalized rat fibroblast cell line (32). FrCJun1, FrCJun3, FrCJun4, and FrCJun8 were derived from FR3T3 by transfection with a human c-jun cDNA expression vector (14). Rac3T3 cells were isolated after transfection of FR3T3 with activated 12V-Rac. Ras3T3 and RafCAAX-3T3 cells were isolated after transfection with the T24-H-ras oncogene and dominant-positive RafCAAX, respectively. A14 and B25 cells are ras-transformed rat embryo fibroblast lines shown to harbor homozygous deletions of an area of chromosome 5q which includes the CDKN2A locus (21). In both lines, the c-jun gene, also located on 5q31-33, is codeleted with CDKN2A (20, 21). All cells were maintained in DMEM supplemented with 5% FCS.

Anchorage independent growth was assayed by plating cells in DMEM/5% FCS containing 0.33% soft agarose. After 6 days, colonies were examined under the inverted phase microscope and scored as larger or smaller than 0.5 mm in diameter. One hundred colonies in each culture were scored.

2-D Electrophoresis. Cells were metabolically labeled with 35S-methionine, and cell extracts were prepared as described (33). 2-DE was

Fig. 9. TM and PCNA expression in Ras3T3 and Rac3T3 cells. A, shows 20-50 kDa acidic polypeptides from Ras3T3 and Rac3T3 cells. C, PCNA: □, TM-1, -2, and -3 (see Fig. 1). A Western blot analysis of the expression of the Myc-tagged 12V-Rac protein in Rac3T3 cells is shown on the top right. B, autoradiograms of 2-D PAGE gels were analyzed by PDQUEST and individual polypeptide quantities were expressed as ppm of the total integrated optical density.
performed by standard procedures (34, 35). Resolyte (2%, pH 4–8, BOD) was used for isoelectric focusing, and 10% or 10–13% linear gradient SDS-polyacrylamide gels in the second dimension. Gels were dried and exposed to X-ray film.

**Scanning and Image Analysis.** Autoradiography films were scanned at 100 μm resolution using a Molecular Dynamics laser densitometer. Data were analyzed using the PDQUEST software (Ref. 15; Pharmacia Biotech AB). Background was subtracted, peaks located, and the individual polypeptide quantities were expressed as ppm of the total integrated optical density.

**Polypeptide Identification.** 2-DE maps were matched with the REFe2 pattern (36), and the mouse kidney IEF database (http://biobase.dk/cgi-bin/cells). The identity of TfMs was confirmed by coelectrophoresis with protein purified as described by Matsumura and Yamashiro-Matsumura (37).

**Northern Blot Analysis.** Total cellular RNA was prepared by extraction with acidic phenol (pH 5.2) and separated by electrophoresis in 1% agarose gels containing 2.2 w formaldehyde. RNA was transferred to Hybond-N+ membranes (Amerham Life Science, Amersham Sweden AB, Solna, Sweden) and hybridized to 32P-labeled probes for TM-2 and β-actin according to standard procedures.

**Drug Exposure.** Cells were exposed to 100 μM BZA-5B (Genentech Inc., South San Francisco, CA) for 48 h, or 50 μM MEK1 inhibitor PD98059 (New England Biolabs, Beverly, MA) for 18–24 h in medium containing 5% FCS.

**Conditioned Medium.** Conditioned medium, collected from transformed cells grown in 0.5% FCS for 2 days, was added with as little delay as possible to similarly serum-starved or drug-treated FR3T3 cells. Samples to be analyzed by Western or Northern blotting were collected 24 h later, whereas samples for ERK analysis were taken after the time intervals indicated in Fig. 7.

**Western Blot Analysis.** To obtain protein extracts, cells were rinsed and scraped into SDS-sample buffer (2.3% SDS, 0.065 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, and 10% glycerol) and boiled for 3 min. The samples were loaded on SDS-polyacrylamide gels and electrophoretically transferred to PVDF membranes. Probing with mouse anti-tropomyosin antibody T2780 (Sigma Chemical Co., Sigma-Aldrich, Sweden), and the subsequent detection with Enhanced Chemiluminescence substrates (Amersham) was performed according to manufacturer’s instructions. The relative intensities of the resulting bands on Hyperfilm-ECL (Amerham) were analyzed with a Molecular Dynamics laser densitometer. All membranes were probed also with anti-β-tubulin (Sigma Chemical Co.), the resulting bands of which were used to correct for variability in loading.

**ERK Activity Assay.** ERK1/ERK2 activity in nuclear extracts was assayed using a kit (Amersham). Briefly, samples were incubated in kinase buffer for 30 min at +34°C with γ-32P-ATP and a synthetic substrate peptide specific for ERK, and then spotted onto nitrocellulose filters. Phosphorylation of the substrate was assessed by scintillation counting of these filters. Results were normalized for protein content and represent duplicate samples.

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**References**


