Loss of Net as repressor leads to constitutive increased $c\text{-}fos$

transcription in cervical cancer cells

Jan van Riggelen$^1$, Gilles Buchwalter$^2$, Ubaldo Soto$^3$, Johanna De-Castro Arce$^1$, Harald zur Hausen$^1$, Bohdan Wasylyk$^2$ and Frank Rösl$^1$$^*$

$^1$ Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

$^2$ Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, 1 Rue Laurent Fries, 67404 Illkirch Cedex, France

$^3$ Department of Biochemistry, School of Medicine, Loma Linda University, Loma Linda, California 92350, U.S.A.

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*Corresponding author:
Prof. Dr. rer. nat. Frank Rösl
Deutsches Krebsforschungszentrum
Forschungsschwerpunkt Angewandte Tumorvirologie,
Im Neuenheimer Feld 242
69120 Heidelberg, Germany
Phone: +49-6221-424900
Fax: +49-6221-424902
e-mail: F.Roesl@dkfz.de
SUMMARY

We have investigated the expression of c-fos in cervical carcinoma cells and in somatic cell hybrids derived there from. In malignant cells, c-fos was constitutively expressed even after serum starvation. Dissection of the c-fos promoter showed that expression was mainly controlled by the SRE-motif, which was active in malignant cells, but repressed in their non-malignant counterparts. Constitutive SRE activity was not mediated by sustained MAP kinase activity but due to inefficient expression of the ternary complex factor (TCF) Net, which was either very low or even barely discernible. Chromatin-immunoprecipitation (ChIP) assays revealed that Net directly binds to the SRE nucleoprotein complex in non-tumorigenic cells, but not in malignant segregants. siRNA targeted against Net resulted in enhanced c-fos transcription, clearly illustrating its repressor function. Conversely, stable ectopic expression of Net in malignant cells negatively regulated endogenous c-fos, resulting in a disappearance of the c-Fos protein from the AP-1 transcription complex. These data indicate that loss of Net and constitutive c-fos expression appear to be a key event in the transformation of cervical cancer cells.
INTRODUCTION

Development of cervical cancer is a multi-step process that is initiated by infection with “high risk” human papillomaviruses (HPVs). The viral oncoproteins E6 and E7 are indispensable for proliferation of cervical carcinoma cells. They cause unlimited growth, which eventually leads to accumulation of cell damage, chromosomal instability, aneuploidy and loss of tumor suppressor genes. However, expression of E6/E7 in primary human keratinocytes merely results in immortalization without further progression to tumorigenicity, showing that further events are important (1). Moreover, HPV-induced cancer can be considered as a recessive genetic trait that can be complemented by somatic cell hybridization with primary human fibroblasts or keratinocytes. A genetically well defined in vitro model is provided by fusion of HPV18-positive HeLa cells with primary human fibroblasts, which results in hybrids that still express HPV18 E6/E7, but are non-tumorigenic in nude mice (2). Long-term in vitro cultivation generates segregants with morphological features of tumorigenic cells (3).

The transcription factor AP-1, which is composed of heterodimers between Jun and Fos family members plays a crucial role in various cellular processes such as enhanced proliferation, differentiation and neoplastic transformation (4). In the case of HPV-positive cells, there is substantial evidence that AP-1 composition determines their in vivo behaviour in nude mice, their sensitivity against growth-inhibitory cytokines, as well as their ability to express certain chemokines that are necessary to maintain immunological surveillance of persisting HPV infections (5). While in asynchronous growing primary human fibroblasts, keratinocytes and HPV16-immortalized human cells AP-1 mainly consists of Jun family members heterodimerized with Fra-1, c-Fos was found to be almost completely absent. Conversely, in cervical carcinoma cell lines such as HeLa, SW756, SiHa or tumorigenic segregants derived from non-malignant hybrids, increased amounts of c-Fos and low levels of
Fra-1 can be discerned, resulting in a prevalent Jun/c-Fos dimerization pattern (6, 7, 8, 9). Moreover, in view of the fact that ectopic expression of c-fos induces tumorigenicity in non-malignant hybrids by changing the Jun/Fra-1 ratio in favor of Jun/c-Fos (7), it is reasonable to assume that c-Fos regulation plays a fundamental role in the conversion of HPV-positive cells towards malignancy (1).

c-fos transcription is tightly controlled in normal cells and inappropriate expression contributes to various phenotypic changes (4). c-fos transcription is normally very low but is transiently induced in response to a wide range of extracellular stimuli, including serum, growth factors, cytokines and tumor promoters (10). Impaired serum inducibility of c-fos is a feature of senescent human fibroblasts (11), whereas its over-expression is a marker for progression of skin tumors and in general related to tumorigenicity (12). Transient induction of c-fos expression is mainly mediated by four promoter elements: the sis-inducible element (SIE), the serum response element (SRE), the cAMP response element (CRE) and the c-fos AP-1 site (FAP-1) (13, 14). The majority of the signals regulate the SRE (15), which is constitutively occupied by the serum response factor (SRF) and a ternary complex factor (TCF) (16). Three members of the TCF family have been identified: Elk-1 (17, 18), Sap1 (19) and Sap2/Erp/Net (20, 21). Net and to a lesser extent Elk-1 are repressors and all three TCFs are activators when phosphorylated by mitogen-activated protein kinases (MAPKs) (22). The SRE is inactive or repressive in the absence of positive signalling (23, 24) but can be induced by two pathways: TCF phosphorylation by MAPKs (25, 26) and Rho-actin signalling to SRF (27, 28). Moreover, SRE-mediated c-fos transcription is modulated by the exchange of TCFs and the recruitment of co-regulatory complexes such as histone acetylases (HAT) (29, 24) or histone deacetylases (HDAC) (30). Less is known about the transcriptional repression processes that control basal activity and the transient and self-terminating expression pattern of the c-fos gene (31).
In the present study we have unravelled the mechanism underlying deregulated \textit{c-fos} expression in cervical carcinoma cells. Tumorigenic cells express relatively low levels of \textit{net} and high levels of \textit{c-fos}. Re-introduction of Net, in either a transient or a stable fashion, suppresses \textit{c-fos} promoter directed transcription and triggers the disappearance of the c-Fos protein from the AP-1 complex. In contrast, non-tumorigenic HPV-positive cells express relatively high levels of \textit{net} and low levels of \textit{c-fos}. Within these cells, Net can be detected bound to the endogenous \textit{c-fos} promoter and decreasing Net levels with siRNA increases \textit{c-fos} expression. These results support the notion that deregulation of Net, and in turn of \textit{c-fos} expression, is an important event in the multi-step progression to cervical cancer, the second leading cancer in women worldwide.
EXPERIMENTAL PROCEDURES

Cell lines and somatic cell hybrids

The cervical carcinoma cell line HeLa, SiHa, CaSki, SW756, human lung fibroblasts (IMR-90), the non-tumorigenic somatic cell hybrid (“444”) between HeLa and IMR-90, and the tumorigenic segregant (“CGL3”) (2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% FCS (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). Stable CGL3-Net clones were grown in the same medium containing 1.2 mg/ml G418 (Gibco BRL).

Cell treatment

Serum: subconfluent cells were starved for 24 h in DMEM without FCS followed by incubation with 10% FCS for 1 to 8 h. TNF-α: asynchronously growing cells were incubated with 500 U/ml TNF-α (Strathman Biotech GmbH, Hannover, Germany) for 15 min. U0126 (Calbiochem): 10 or 20 µM in the culture medium for 5 h. Latrunculin B: 0.5 or 2 µM for 5 h (32).

Plasmid constructs

pc-fos-luc, containing the -400/+50 region of the human c-fos promoter, was kindly provided by G.T. Bowden (14). The pSRE5-Luc contains 5 tandem copies of the c-fos SRE, the pCRE4-Luc four copies of the CRE-binding sequence fused to the TATA minimal promoter (Stratagene). pCI-neo and the SV40 early promoter driven Renilla-luciferase construct (pRL-SV40) were purchased from Promega. pTL2-hNet encodes the human Net cDNA driven by the SV40 early promoter (23). The corresponding empty vector construct (pTL2) was derived from pTL2-hNet by SmaI excision of the 1.4 kbp cDNA insert.
Transient and stable transfections

The Effectene™ transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer’s instructions. For reporter gene assays, 0.7 µg firefly-luciferase reporter plasmid was transiently co-transfected with various constructs. Transfection efficiencies were normalized with pRL-SV40. Stable CGL3-Net cell lines were generated by co-transfection of CGL3 cells with pTL2-hNet and pCI-neo. Clones were isolated by G418 selection and maintained in DMEM with 10% FCS, 1% penicillin/streptomycin and 1.2 mg/ml G418.

Luciferase reporter assays

Cell extracts were prepared 24 h after transfection. Firefly-luciferase activity was measured in 25 mM glycylglycine, 15 mM MgSO₄, 5 mM ATP, pH 7.8 and 7 µg/ml luciferin with a luminometer (Lumat 9501, Berthold). Renilla-luciferase activity was examined using Renilla Luciferase Assay System (Promega) according to the manufacturer’s instructions. Experiments were done in triplicate and normalized luciferase activity is shown as x-fold increase relative to the basal activity.

RNA interference

Net targeted siRNAs (Dharmacon, Inc.) were designed according to published criteria (33). Sequences: 5’-ACGAUGGUGAAUUAAGCU-3’ region (Net1) and the 5’-CUUGUGGUGAAUUAAGCU-3’ region (Net2). The siRNA sequence targeting GL2 luciferase was already described (34). 3 µg of the siRNA duplexes were transfected into 444 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s guidelines. Cells were harvested 48 h after transfection and total RNA was extracted with RNASolv (Omega Bio-tek). 1 µg of total RNA was used for first-strand cDNA synthesis using SuperScript II reverse Transcriptase (Invitrogen). 10% of the first strand reaction was used for
PCR to analyse the expression of net (5'-AGACCAAGTCTCCATCTCTTC-3' and 5'-GACTAAGGCTGCTCCAGAAAT-3'); c-fos: (5'-AGTGGAAACCTGTCAGACCGTCAAGAGCAT-3' and 5'-GCTCCCAGTCTGCTGCATAGA-3'); and 28S: (5'-GGCGGCCAAGCGTTCATAGC-3' and 5'-GCCAAGCACATACACCAAAT-3'). PCR conditions were 94°C for 5 min, 94°C for 30 sec; 61°C for 30 sec, 72°C for 30 sec (25 cycles for net, 25 cycles for c-fos and 15 cycles for 28S) and 72°C for 5 min.

Cell extracts and Western blot analysis

Nuclear extracts for EMSA and Western blot analysis were prepared as described previously (35). Protein concentration was determined by the Bradford method (Biorad) using BSA as a standard. Extracts were separated in 10% SDS-PAGE, electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA) and probed with the following antibodies: c-Fos (catalogue no. 06-341, Upstate Biotechnology), c-Jun (sc-1694 X), Fra-1 (sc-605 X) and SRF (sc-335 X) from Santa Cruz Biotechnology; phospho-Ser103 SRF (#4261), Elk-1 (#9182), phospho-Ser383 Elk-1 (#9181) from New England Biolabs (Frankfurt, Germany) and polyclonal anti-Net sera #2005 (23). For MAPK analyses, total cellular extracts were prepared using a lysis buffer containing 50 mM Tris, 2% SDS, 10% glycerol, 0.74 M β-mercaptoethanol, sonicated on ice using a Sonifier 250 (Branson Ultrasonics, Geneva, Switzerland), and finally heated for 5 min at 99°C. Protein concentrations were determined using the BioRad DC Protein Assay Kit (Biorad). Antibodies used for detection of ERK1/2 (#9102), phospho-Thr202/Tyr204 ERK1/2 (#9101S), JNK (#9252), phospho-Thr183/Tyr185 JNK (#9251S), p38-MAPK (#9212) and phospho-Thr180/Tyr182 p38-MAPK (#9211S) were obtained from New England Biolabs (Frankfurt, Germany). For Western blot analysis to monitor Net after siRNA delivery, total cellular extracts were prepared using a lysis buffer containing 0.4 M KCl, 20 mM Tris-HCl pH 7.5, 20
% glycerol, 5 mM DTT, 1 mM PMSF, 10 mM NaF and proteinase inhibitor cocktail Complete (Roche Diagnostics, Penzberg, Germany). Cellular lysis was performed by 3 cycles of freezing/thawing and finally heating for 10 min at 99°C. Membranes were incubated overnight in Tris buffered saline (TBS) supplemented with 5% skim milk powder (Roth) and 0.1% Tween 20 (Sigma). The bands were visualized with horseradish peroxidase conjugated anti-rabbit IgG or an anti-mouse IgG (Promega) using the ECL (enhanced chemiluminescence) detection system (PerkinElmer Life Sciences, Inc). Loading was confirmed by re-incubating the membranes with a monoclonal actin-specific antibody (ICN Biomedicals, Costa Mesa, CA). For re-incubation with additional antibodies, the filters were stripped for 5 min in 0.2 M NaOH.

RNA analysis and RT-PCR

Cytoplasmic RNA was isolated with the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. To check RNA quality, 5 µg of RNA was separated on 1% agarose gels in the presence of ethidium bromide under non-denaturing conditions (36). For reverse transcription (RT) 1-3 µg RNA was mixed with 0.2 µg of random primers (Roche), heated at 70°C for 10 min, and chilled on ice. The mixture was supplemented with reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 10 mM DTT, 500 µM deoxynucleoside triphosphate mix (Roche Diagnostic, Mannheim, Germany) and incubated at 25°C for 10 min. 100U of reverse transcriptase SuperScript II (Gibco BRL) was added, the reaction was incubated at 42°C for 50 min, heated to 70°C for 15 min, and chilled on ice. PCR was performed in a total volume of 50 µl containing 10 mM Tris-HCl pH 8.3, 200 µM deoxynucleoside triphosphate mix (Roche Diagnostic, Mannheim, Germany), 500 nM of forward and reverse primers, 5U of Taq polymerase (Invitrogen) and 1-3 µl of RT product, using an MJ Research PTC-200 thermal cycler. The conditions were: c-fos, primers 5'–
AACTCATTTCCACGGTGAC-3’ and 5’-CCTTCTCCTTCAGCAGGTTG-3’, 35 cycles of 30 sec at 94°C, 45 sec at 55°C and 30 sec at 72°C (the last extension time was 10 min) and rapid cooled to 4°C; fra-1, primers 5’-GCGCCTAGGCCTTGTATCTCCCTTTCCCC-3’ and 5’-CCGCCTAGGGCGAGGGGTTGGAGGAGCC-3’, 35 cycles and annealing temperature 65°C; net, primers 5’-AACTACGACAAGCTGAGCAGAGC-3’ and 5’-AGCGGTCTCAGGATGGAAGG-3’, annealing temperature 55° (23); GAPDH internal control, primers 5’-TGGATATTGTGTCATGAATGACC-3’ and 5’-GATGGCATGGGACTGAGTCATG-3’ (37), 35 cycles, annealing temperature 65°C. The PCR products were analyzed with 1-2% agarose gels containing ethidium bromide.

EMSA

The AP-1 consensus sequence 5’-CGCTTGATGACTCAGCAGGGAA-3’ from the human collagenase promoter (38) was used. Oligonucleotides were made with an Applied Biosystems (Foster City, CA) synthesizer and purified by HPLC. The annealed oligonucleotides were labelled with 3000 Ci/mmol [γ-32P] ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany), and purified with a 15% polyacrylamide gel. Binding reactions were performed in 20 µl containing 10% glycerol, 12 mM HEPES pH 7.9, 4 mM Tris-HCl pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.6 mg/ml BSA, 2 µg poly(dI-dC) and 2 µg nuclear extract. After 5 min, 10000 cpm of the probe was added and incubation continued for 30 min at room temperature. For super-shift assays, 2 µg of c-Fos antibody (sc-52 X, Santa Cruz Biotechnology) was added and further incubated for 1 h at 4°C. The complexes were resolved with 5.5% non-denaturing polyacrylamide gels (29:1 cross-linking ratio). The gels were dried and exposed to X-ray films (Amersham) or analysed using a Phosphor-Imager Storm 820 and the Image-Quant software (Molecular Dynamics, Amersham).
**ChIP assay**

The ChIP Assay Kit (#17-295) from Upstate Cell Signaling Solutions (Lake Placid, NY, USA) was used according to the manufacturer’s instructions. Subconfluent cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed and scraped in ice-cold PBS containing proteinase inhibitor cocktail Complete (Roche Diagnostics, Penzberg, Germany). After lysis of 1 x 10^6 cells, the DNA was sheared into 200-1000 bp fragments by sonification (4 times for 10 sec, Sonifier 250, Branson Ultrasonics, Geneva, Switzerland) and immunoprecipitated with 4 µl of polyclonal anti-Net sera #2005 or #2007 (23) or anti-SRF (sc-335 X, Santa Cruz Biotechnology). The co-precipitated DNA was analysed by semi-quantitative PCR as described above. The conditions for PCR and the primers were as followed: c-fos SRE (position -300) 5’-GAGCAGTTCCGTCATCC-3’ and 5’-CCCCAAGATGAGGGTTT-3’, 35 cycles, annealing temperature 57°C. c-fos promoter (position -600), 5’-CAAACGCAGGAACAGTGCTA-3’ and 5’-GAAGGAATGCACCCCTAC-3’, 35 cycles, annealing temperature 57°C. GAPDH was used as an additional control sequence located on a heterologous chromosome (39). The PCR products were analysed on 1.5% agarose gels in the presence of ethidium bromide.
RESULTS

Inverse correlation of c-fos and fra-1 expression in tumorigenic and non-tumorigenic HPV-positive cell lines

To monitor the regulation of the transcription factor AP-1 in cells differing in their capability to form tumors in immunocompromised animals, we used HPV 18-positive HeLa cells and derived somatic cell hybrids as an experimental model (2). This allows the examination of AP-1 in a cellular environment harbouring identical viral transcription cassettes (36), but where the oncoproteins are expressed in a different genetic background (35). As depicted in Fig. 1, asynchronously growing tumorigenic (HeLa, CGL3) and non-tumorigenic hybrids (“444”) varied considerably in their c-Fos and Fra-1 content. Both proteins can be resolved as broad bands by SDS-PAGE due to differential post-translational phosphorylation (40). Similar to normal human lung fibroblasts (IMR-90), nuclear extracts obtained from 444 cells showed a complete absence of the c-Fos protein and only a marginal detection of c-fos specific RNA after semiquantitative RT-PCR. In contrast, fra-1 was highly expressed both on mRNA and protein levels. Examining malignant HeLa and CGL3 cells, both inherently express more c-fos mRNA and corresponding protein than their non-malignant counterparts.

Serum inducibility of Fos expression in non-malignant and malignant cells

To determine whether increased mRNA and protein levels in tumorigenic cells remain constitutive after serum depletion, cells were starved for 24 hours one day after seeding. Expression was monitored by Western blot and RT-PCR analyses (Fig. 2A). Considering both parental HeLa and CGL3 cells, starvation was not accompanied with any changes of c-fos expression. However, when the cells were incubated for 1 hour with fresh serum, c-fos could be induced. On the other hand, in asynchronized as well as in serum deprived non-malignant
cells, \textit{c-fos} expression was barely detectable while serum addition led to a striking accumulation of c-Fos both on protein and RNA level. Next we examined binding affinity and composition by electrophoretic mobility shift assays (EMSAs). As shown in Fig. 2B, serum starvation of non-malignant cells resulted in a substantial reduction of AP-1 binding affinity, whereby AP-1 completely lacked c-Fos. Nonetheless, 1 hour of serum stimulation was sufficient to incorporate c-Fos into AP-1, leading to the appearance of lower migrating bands after antibody addition. In malignant counterparts, omission of serum did not reveal any reduction of binding. Here, AP-1 inherently contained c-Fos that was further elevated in its ratio upon serum stimulation. In kinetic experiments (Fig. 2C), c-Fos was initially undetectable in serum starved non-tumorigenic cells, but appeared within 1 hour after serum addition, increased up to 5 hours and returned to baseline level approximately 8 hours after supplementation. However, within CGL3 cells, stimulation resulted in a prolonged increase of c-Fos, even after several hours. Hence, only in non-tumorigenic cells, c-Fos behaves as typical immediately early gene product, where the extent and the temporal range of its transcription are tightly controlled (41).

The SRE mainly contributes to an increased \textit{c-fos} promoter activity in tumorigenic cells

To test whether \textit{c-fos} is differentially regulated at the level of transcriptional initiation, transient transfection assays were performed. Comparing the relative luciferase activity, where the absolute counts for the 444 hybrids were arbitrarily set as 1, the \textit{c-fos} promoter was found to be 2 to 4-fold more active in malignant than in non-tumorigenic cells (Fig. 3). In another set of experiments, we analyzed the SRE and the CRE, known to be critical in spatial and temporal gene response (42, 14). While CRE-controlled reporter plasmids did not reveal any notable differences, SRE-driven luciferase constructs showed a 5-fold higher activity in HeLa cells and an about 7-fold higher activity in tumorigenic segregants when compared with
444 cells. This suggests that the SRE acts as the major cis-regulatory sequence in normal cells, mediating both induction and successive repression of the gene.

**ERK1/2, JNK and p38 MAPKs are not responsible for constitutive SRE-activity in tumorigenic cells**

Since the SRE is activated by MAP kinases (25), we next examined whether tumorigenic cells have enhanced activities of three major types of MAPKs, the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and p38 MAPK. TNF-α was used to stimulate these pathways (43). As shown in Fig. 4A, there was no detectable activated JNK or p38 MAPK in tumorigenic or non-tumorigenic cells, but phosphorylation was induced after TNF-α application. However, as depicted for ERK1/2, little but still visible phosphorylation in untreated HeLa and CGL3 cells could be discerned. Differential phosphorylation could be not attributed to quantitative changes of the proteins, since control incubations with non-phosphorylation specific antibodies revealed that the net amount of the corresponding proteins was the same. The higher pre-existing phosphorylation of ERK1/2 in 444 cells that have lower SRE activity reinforces the notion that increased MAP kinase activity does not account for the greater SRE activity in tumorigenic cell lines.

In response to extracellular stimuli, ERK1/2 are normally activated by phosphorylation by the dual-specific MAPK kinases MEK1/2, which in turn activates c-fos transcription via phosphorylation of TCF family members at the SRE (15). Hence, one can anticipate that inhibition of ERK1/2 by the MEK1/2-inhibitor U0126 should prevent induction of SRE-mediated c-fos transcription (24). As shown in Fig. 4B, U0126 treatment resulted in a dose-dependent decline of ERK1/2 phosphorylation, while the amount of the protein remained constant. Inspecting the steady state level of the c-fos mRNA, no difference was detectable. One can therefore conclude that constitutive c-fos transcription in tumorigenic cells was not
depending on ERK1/2 or JNK/p38-MAPKs, even though the gene was still sensitive to serum stimulation. This raised the question, whether phosphorylation or the expression of SRE-activator proteins were elevated in a MAPK-independent manner.

Expression and phosphorylation levels of the SRE-activator proteins Elk-1 and SRF

In response to stimulation, c-fos SRE is activated by phosphorylation of TCFs such as Elk-1 via the Ras-ERK pathway (44). In addition, c-fos can be also induced via Rho-actin signalling, targeting SRF in a TCF-independent manner (27, 28). To investigate the potential contribution of these two signalling routes, we examined the basal phosphorylation levels of both Elk-1 and SRF. Treatment with TNF-α again served as positive control for a temporal defined MAPK activation. Western blot analyses (Fig. 5A) revealed similar basal levels of Elk-1 and SRF phosphorylation in tumorigenic and non-tumorigenic cells, which could be further stimulated by TNF-α. In addition, inhibition of Rho-acting signalling by Latrunculin B (28) did not revealed any effect of c-fos expression in CGL3 cells (Fig. 5B), also excluding this pathway being responsible for enhanced c-fos transcription. Taken together, these data argue against the concept that increased SRE-activity was due to constitutive hyper-phosphorylation of Elk-1 or SRF.

Diminished Net expression in tumorigenic cells: Loss of repressor function at the c-fos promoter

Because none of the classical activators we re found to be involved in differential c-fos expression, we reasoned that the loss of a repressor at the SRE might be responsible for constitutive transcription in tumorigenic cells. Within the TCF family, two members can principally act as repressors. In the absence of MAP kinase signalling, Net and to less extent Elk-1, have been demonstrated to repress transcription (23, 45). Since there were no quantitative and qualitative differences of Elk-1, Net expression was monitored. Interestingly,
high levels of Net were only detected in normal human fibroblasts (IMR-90) and in non-tumorigenic hybrids (444), while Net was significantly diminished in HeLa cells and almost absent in tumorigenic segregants both on RNA and protein level (Fig. 6A). Moreover, as depicted in Fig. 6B, we also found an inverse correlation of Net and c-fos transcription in other cervical carcinoma cells. Both HPV16-positive SiHa as well as HPV18-positive SW756 cells exhibited low levels of Net, while c-fos transcription was highly up-regulated. Exceptional for cervical carcinoma cells, c-fos was weakly transcribed in HPV16-positive CaSki cells (8). Notably, examining these cells by RT-PCR and Western blot analysis, a high amount of Net could be discerned (Fig. 6B). Since it has been postulated that Net significantly contributes to the repression of basal c-fos SRE-activity (22), these data may provide the first direct mechanistic link between its diminished expression and increased c-fos transcription in a highly clinical relevant form of human cancer.

**In vivo binding of Net to the c-fos SRE: Loss of repressor function in tumorigenic cells**

In order to demonstrate that Net is differentially recruited at the c-fos SRE nucleoprotein complex in vivo, chromatin-immunoprecipitation assays (ChIPs) were carried out (Fig. 7). Formaldehyde cross-linked protein-DNA complexes were immunoprecipitated using two different polyclonal antibodies against Net. Co-precipitated DNA fragments were amplified using specific primers surrounding the area of the c-fos SRE. The specificity of the ChIP reaction was confirmed using a randomly selected DNA sequence of the c-fos upstream region (position -600) and for a coding stretch within the GAPDH gene. Equal amounts of sheared genomic DNA were used as template. SRF was found to be constitutively associated with the SRE in both cell lines. In contrast, Net binding at the c-fos SRE occurred exclusively in 444 cells, but was absent in CGL3. These data suggest that the loss of Net leads to increased c-fos transcription in malignant HPV-positive cells.
Exogenous Net suppresses \( c-fos \) in tumorigenic cells while Net siRNA induces \( c-fos \) transcription in the non-malignant counterparts

To prove a causal relationship between the lack of repressor function at the SRE and increased \( c-fos \) transcription, we first examined the effect of ectopically expressed Net in transient transfection assays. As shown for CGL3 cells, Net expression inhibited SRE and \( c-fos \) promoter activity, but had no effect on the CRE (Fig. 8A). To exclude potential squelching of general transcription factors due to the high copy number of the effector plasmid under transient transfection conditions, we established stable cell lines of CGL3 expressing a net cDNA under a heterologous promoter. In comparison with parental cells, transfected clones showed distinct net expression levels which inversely correlated with endogenous amount of \( c-fos \) mRNA and protein (Fig. 8B). In addition, supershift EMSA demonstrated that the amount of c-Fos associated with AP-1 was reduced (Fig. 8C). To further corroborate the repressor function, non-malignant cells were transfected with siRNA directed against Net. As shown in Fig. 8D, in the same way as endogenous Net mRNA and protein was diminished, \( c-fos \) transcription increased. Delivery of a non-specific siRNA showed had no effect on both genes. These results provide strong evidence that constitutive expression of \( c-fos \) transcription in tumorigenic cells is due to the loss of Net repressor activity.
DISCUSSION

The dimeric transcription factor AP-1 acts as a junction point for many regulatory mechanisms associated with proliferation, apoptosis and tumorigenicity (4). AP-1 becomes activated upon a whole variety of extra/intracellular stimuli including growth factors, cytokines, tumor promoters and oncogenes (46). The decision, however, which target genes finally turns out to be induced, is apparently depending on AP-1 composition (47). For HPV-positive human cells, AP-1 is part of an intracellular surveillance network determining not only the \textit{in vivo} phenotype but also the sensitivity/response against growth-inhibitory cytokines and chemokines (1, 7, 8).

Raising the question how \textit{c-fos} is deregulated in HPV18-positive cervical carcinoma cells, we took advantage of a model system where fusion of malignant and normal cells resulted in somatic hybrids that are non-tumorigenic in nude mice (2). We found that only malignant cells transcribed high levels of \textit{c-fos} under asynchronized growth conditions, while there was almost no detectable signal when non-tumorigenic cells were examined (Fig. 1). Although the intracellular availability of c-Fos can be also regulated in a post-translational manner (48), the amount of protein in Western blot closely matched with the steady state level of the corresponding mRNA (Fig. 1). Moreover, since actinomycin D treatment (49) also did not reveal any differences of the mRNA half-life (data not shown), \textit{c-fos} expression was evidently controlled at the level of initiation of transcription. Although both viral oncoproteins can stimulate the \textit{c-fos} promoter in rodent cells (50), transcriptional regulation of \textit{c-fos} in the human system was actually independent of E6/E7 expression. This supports previous data demonstrating that the induction of particular cytokines/chemokines was determined by the \textit{in vivo} phenotype, rather then by the expression of the viral oncoproteins \textit{per se} (35, 51).

We found that non-tumorigenic hybrids behave similar to normal cycling cells (IMR-90), where both the extent and the temporal range of \textit{c-fos} expression were tightly controlled.
Such a negative regulatory loop is obviously missing in tumorigenic cells, where \( c-fos \) remained expressed both under asynchronous growth conditions and during serum starvation (Fig. 2). Remarkably, although not followed up in molecular terms at the time, an earlier study has already provided initial clues for a constitutive re-expression of higher \( c-fos \) levels after transition of non-tumorigenic HPV-positive somatic hybrids towards malignancy (52). Accordingly, ectopic expression of \( c-fos \) in 444 hybrids provoked rapid tumor formation by shifting the AP-1 composition from Jun/Fra-1 to Jun/c-Fos. Hence, a potential linkage between \( c-fos \) deregulation and E6/E7 expression during human tumor progression has been predicted (7).

In fact, inappropriate transcriptional control of \( c-fos \) in malignant cells could be further substantiated in transient transfections (Fig. 3). Using either the entire \( c-fos \) promoter or individual cis-regulatory elements, luciferase reporter activity was consistently higher in malignant cells than in their non-malignant counterparts. This property was apparently mediated by the SRE, known to be the major target sequence responsive to extracellular signal transduction (53).

Signal transduction is controlled both by the strength and duration of MAPK activation (54). Since increased MAPK activity is normally considered as a hallmark for cancer cells (55), we reasoned that sustained activation of this pathway might account for enhanced SRE activity. However, neither JNK nor p38 MAPK was affected in its basal activity (Fig. 4). Only ERK1/2 showed constitutive phosphorylation in non-tumorigenic hybrids (“444”), arguing for a stronger functionality of the upstream kinases MEK1 and 2 (56). Elevated ERK1/2 activity, however, could not be responsible for constitutive \( c-fos \) expression, because treatment with the MEK1/2 inhibitor U0126 (55) abrogates ERK1/2 phosphorylation without any consequences on the steady state level of the \( c-fos \) specific mRNA (Fig. 4B). Notably, even though enhanced ERK1/2 activity has been described for many other human malignancies as the result of Ha-ras proto-oncogene activation (57), HeLa cells as well as primary tumor
specimens directly obtained from cervical cancer patients apparently lack mutated H-ras allele expression (58).

Although c-fos expression was maintained at an elevated level through a mechanism independent of increased MAPK activity, constitutive SRE-activity could be either due to deficient antagonistic phosphatase function (59, 60) or by stimuli-independent phosphorylation by other kinases (61). Alternatively, SRE-activity could be also regulated by a pathway involving the Rho family GTPases (62). These options, however, could be excluded, because both the steady-state and phosphorylation levels of potential target proteins such as Elk1 and SRF (54) were the same (Fig. 5).

In the absence of active MAPK signalling, the TCF family members Net and to a lesser extent Elk-1 have been implicated in repression of transcription (reviewed in Buchwalter et al., 2004). Moreover, there is also experimental evidence that silencing of Net in NIH3T3 cells was followed by an increase of basal c-fos expression (20, 63). By using RT-PCR and Western blot analyses, we found considerable differences in Net expression in malignant and non-malignant HPV-positive cells. While net was constitutively expressed in fibroblasts and in non-tumorigenic 444 cells, the corresponding mRNA level was significantly diminished in three out of four cervical carcinoma cells (HeLa, SiHa, SW756) and almost undetectable in tumorigenic CGL3 hybrids (Fig. 6). Notably, similar to non-malignant cells, HPV16-positive CaSki cells expressed high/low levels of Net/c-Fos. This was interesting in the context of our previous observation that in contrast to SiHa or SW756, CaSki cells were able to complement highly tumorigenic HeLa cells to a non-malignant phenotype after somatic cell hybridization (8). Moreover, a potential link between Net and the nucleosomal organisation of the c-fos promoter was provided by the fact that its binding to native chromatin could be exclusively detected in non-malignant cells when formaldehyde-cross-linked lysates were analysed (Fig. 7). Intriguingly, Net harbours a repression domain, which is not conserved in other TCFs, but is capable of suppressing transcription by interacting with CtBP as co-repressor. This
connection may provide a possible mechanism for inhibition, since CtBP is involved in the recruitment of histone deacetylase (HDAC) and in turn in silencing of chromatin (64). Interestingly, when asynchronized 444 cells were incubated in the presence of the HDAC inhibitor trichostatin A (TSA), c-fos transcription could be induced (data not shown). This reinforces the notion that Net and probably also CtBP mediate both transcriptional repression and chromatin remodelling.

In any case, ectopically expressed net cDNA resulted almost in a complete suppression of SRE- and c-fos-promoter-directed reporter constructs under transient transfection conditions (Fig. 8A). Moreover, stable reconstitution of net expression in tumorigenic cells significantly reduced endogenous c-fos transcription (Fig. 8B), whereby c-Fos was diminished from the AP-1 transcription complex (Fig. 8C). However, the amount of ectopically expressed net was apparently not sufficient to completely suppress c-fos in CGL3 cells (Fig. 8B). Hence, we currently cannot answer the question, whether Net is acting as tumor suppressor, since its simple over-expression in malignant cells did not block cellular growth in nude mice (data not shown). However, transient delivery of siRNA against net resulted in increased c-fos transcription in asynchronized 444 hybrids, unequivocally demonstrating a causal relationship between Net and c-fos expression in non-tumorigenic cells (Fig. 8D). It will be worthwhile in future experiments whether a stably introduced Net siRNA transcription cassette leads to similar phenotypic changes (e.g. tumorigenicity) in 444 cells as previously reported after ectopical expression of c-fos (7).

In conclusion, our data show a direct functional link between the loss of Net, its absence at the SRE in a repressive chromatin constellation and the constitutive c-fos transcription in tumorigenic HPV-positive cells. This has important implications for the role of Net function and regulation during HPV-induced carcinogenesis. Interestingly, loss of Net (Elk3) expression has recently been observed in malignant mesothelioma compared to normal mesothelial cells (65), suggesting that Net as a repressor may be important in other tumours.
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FIGURE LEGENDS

Figure 1. Inverse correlation of c-fos and fra-1 expression. IMR-90, HeLa, 444 and CGL3 were harvested 1 day after seeding. **Upper part:** RNA quality monitored by agarose gel electrophoresis, and amounts of c-fos, fra-1 and GAPDH estimated by semi-quantitative RT-PCR. 28S and 18S ribosomal RNAs and sizes in base-pairs (bp) are indicated. **Lower part:** Western blots of nuclear extracts (20 µg per lane) using 10% SDS-PAGE gels and antibodies against c-Fos, Fra-1, and Actin as loading control. Molecular weights are indicated in kDa.

Figure 2. Constitutive, but still inducible c-fos expression in tumorigenic cell lines. (A) c-fos expression in IMR-90, 444, HeLa and CGL3 cell lines cultivated for 24 h in DMEM containing 10% FCS (untreated), for 24 h in DMEM without FCS (starved) or for 1 hour with 10% FCS (1 h serum) after 24 h of depletion. RT-PCR and Western blot analyses were carried out as indicated in Fig. 1. (B) EMSAs using 32P-labeled AP-1 consensus oligonucleotides and 2 µg of a c-Fos antibody for the “super-shifts”. The AP-1 and retarded complexes are marked by arrows. Serum omission and re-addition was performed as above. (C) Kinetics of c-Fos induction. 444 and CGL3 cells, starved of FCS for 24 h, were stimulated with 10% FCS for 1-8 h. Western blot analyses were performed using 40 µg nuclear protein extract per lane and antibodies raised against c-Fos and Actin as loading control.

Figure 3. Analysis of c-fos promoter activity. (A) Luciferase activity in HeLa, 444, CGL3 cells using a c-fos promoter (-400/+50 region) luciferase reporter (left), a 5xSRE-luciferase construct (middle) or a 4xCRE-luciferase vector (right). A co-transfected SV40-driven Renilla-luciferase construct was used for normalization. Ordinate: normalized luciferase activity relative to 444. All experiments were done in triplicate. (B) Schematic overview of the cis-regulatory elements of the c-fos promoter and the regulatory element specific
luciferase reporters. SIE: *sis*-inducible element; SRE: serum responsive element; FAP-1: c-Fos AP-1-like sequence; CRE: cyclic AMP responsive element. TATA: TATA-like sequences.

**Figure 4. Activities of MAP kinase pathways.** (A) Western blots of total cellular extracts (20 µg per lane). HeLa, 444 and CGL3 cells were cultivated for 24 h in DMEM, 10% FCS (untreated) or stimulated for 15 min with TNF-α (500 U/ml). Filters were first incubated with antibodies recognizing the phosphorylated forms of the ERK1/2, JNK or p38, or total non-phosphorylated ERK1/2, JNK or p38. (B) Effect of ERK1/2-inhibition on *c-fos* expression. 444 and CGL3 cells were cultivated for 24 h in DMEM including 10% FCS (untreated) and incubated for 5 h with 10 or 20 µM of the MEK1/2-inhibitor U0126. **Upper part:** semi-quantitative RT-PCR for *c-fos* and GAPDH using cytoplasmic RNA. **Lower part:** Western blot analysis of phosphorylated and non-phosphorylated ERK1/2. The sizes of the PCR products and proteins are indicated.

**Figure 5. Analysis of Elk-1 and SRF.** (A) Western blot analysis of phospho-serine 383 Elk-1 (P-Elk-1), phospho-serine 103 SRF (P-SRF) and non-phosphorylated Elk-1 and SRF. The cells were cultivated for 24 h in DMEM including 10% FCS (untreated) and stimulated for 15 min with TNF-α (500 U/ml), as indicated. Nuclear proteins (20 µg per lane) were separated by 10% SDS-PAGE. Actin was the loading control. (B) Semi-quantitative RT-PCR of *c-fos* and GAPDH in CGL3 cells after 5 h treatment with 0.5 and 2 µM Latrunculin B. The positions of the 28S and 18S rRNAs as well as the sizes of the PCR products are indicated.

**Figure 6. Semi-quantitative RT-PCR and Western blot analysis of Net expression.** IMR-90, HeLa, 444, CGL3, CaSki, SiHa and SW756 cells were harvested 1 day after seeding. (A)
Upper part: RNA quality monitored by agarose gel electrophoresis. The amounts of *net* and GAPDH was estimated by semi-quantitative RT-PCR. 28S and 18S ribosomal RNAs and sizes in base-pairs (bp) are indicated. Lower part: Western blots of nuclear extracts (40 µg per lane) using 10% SDS-PAGE gels and antibodies against Net and Actin. Molecular weights are indicated in kDa. (B) Upper part: Semi-quantitative RT-PCR for *net*, *c-fos* and GAPDH. Lower part: Western blot as described in panel A.

**Figure 7. ChIP detection of Net bound to the c-fos SRE.** (A) Two different polyclonal antibodies recognizing Net (IP: anti-Net 05 and 07) and a polyclonal antibody directed against SRF (IP: anti-SRF) were used. The co-precipitated DNA fragments were analyzed by PCR using specific primers for the *c-fos* SRE, the -600 region of the *c-fos* transcriptional start (*c-fos* -600 bp), and the coding region of the GAPDH gene. PCR products were analyzed in 1.5% agarose gels. (B) Schematic overview of the *c-fos* promoter, showing the location of the primers (indicated by arrows).

**Figure 8. Effects of modulation of Net expression on c-fos promoter activity and expression** (A) Effect of Net over-expression on SRE-, CRE- and *c-fos*-directed transcription (see Fig. 3 for details). Co-transfected SV40 driven renilla-luciferase was used for normalization. Ordinate: relative luciferase activity of transfections with the Net expression vector compared to the empty vector. Abscissa: activity of SRE-, CRE- and *c-fos*-reporter plasmids. (B) Effects of ectopic Net expression on endogenous *c-fos* transcription. Semi-quantitative RT-PCR was used to monitor the levels of *net*, *c-fos* and GAPDH mRNA in 444, CGL3 and the stable clones (CGL3-Net clone 1-3). c-Fos and Actin were detected by Western blotting as indicated above. (C) EMSA showing the disappearance of c-Fos from the AP-1 complex in “clone 1”, expressing the highest amount of Net. 2 µg of an anti-c-Fos antibody was used for the “super-shifts”. Arrows mark the AP-1 and the retarded complexes. (D)
Down-regulation of endogenous Net by siRNA increases c-fos expression in 444 cells. Control: non-transfected cells; siRNA luc: luciferase siRNA; siRNA net 01 and 02: siRNA targeting two distinct region of net mRNA. Total RNA was purified 48 hours post-transfection and semi-quantitative RT PCR was used to measure the amounts of net, c-fos, and the internal control 28S rRNA. Net and Actin were detected by Western blot analysis of 50 µg total lysates per lane.
Fig. 3

A

Relative luciferase units
A

b

HeLa 444 CGL3
HeLa 444 CGL3
HeLa 444 CGL3

SRE

CRE

B

c-fos

SRE

FAP-1

CRE

TATA

5x SRE

4x CRE

TATA

luciferase

luciferase

luciferase

luciferase
Fig. 5

A

|          | IMR-90 | HeLa | 444 | CGL3 |
|----------|--------|------|-----|------|
| untreated|        |      |     |      |
| TNF-α    |        |      |     |      |
| TNF-β    |        |      |     |      |
| JNK-α    |        |      |     |      |

Western blot

- Elk-1: ~62 kDa
- Elk-1: ~62 kDa
- SRF: ~56 kDa
- SRF: ~56 kDa
- Actin: ~42 kDa

B

|          | CGL3 |
|----------|------|
| untreated|      |
| 0.5 µM  |      |
| 2 µM    |      |

Lat B

- RNA: 28S rRNA, 18S rRNA
- c-fos: 400 bp
- GAPDH: 500 bp
Fig. 6

(A) Western blot

- RNA
- net
- GAPDH
- Net
- Actin

- 28S rRNA
- 18S rRNA
- 200 bp
- 500 bp
- 47 kDa
- 42 kDa

(B) Western blot

- RNA
- net
- c-fos
- GAPDH
- Net
- Actin

- 28S rRNA
- 18S rRNA
- 200 bp
- 400 bp
- 500 bp
- 47 kDa
- 42 kDa

RNA, net, GAPDH, and c-fos were analyzed by Western blotting. The blots were probed with antibodies specific to the respective proteins. RT-PCR was also performed to confirm the RNA expression levels. The results are consistent with the Western blot data.
Fig. 7

A

|          | 444          |          |
|----------|--------------|----------|
| Input    | IP control   | IP anti-Net 05 |
| c-fos SRE|              |           |
| c-fos -600 bp |            |           |
| GAPDH    |              |           |
|          |              | IP anti-Net 07 |

|          | CGL3         |          |
|----------|--------------|----------|
| Input    | IP control   | IP anti-Net 05 |
| c-fos SRE|              |           |
| c-fos -600 bp |            |           |
| GAPDH    |              |           |
|          |              | IP anti-Net 07 |

B

- c-fos SRE
- SRE
- TCF SRE
- FAP-1
- -200 bp
- CRE
- TATA

- c-fos -600 bp
Fig. 8

A

Relative luciferase units fold difference

SRE

25  
20  
15  
10  
5  
0  
-  +  -  +  Net empty

CRE

c-fos

15  
10  
5  
0  
-  +  -  +  Net empty

B

CGL3-Net

444  CGL3  Clone 1  Clone 2  Clone 3

RNA
net
28S rRNA 18S rRNA

c-fos
200 bp
400 bp
500 bp

GAPDH

C

CGL3

supershift antibody

c-Fos  c-Fos

AP-1

D

444

control

RNA
net
28S

siRNA luc

siRNA net 01

siRNA net 02

RT-PCR

Western blot

Actin

55 kDa 42 kDa

400 bp

200 bp

240 bp

47 kDa 42 kDa
Loss of Net as repressor leads to constitutive increased c-fos transcription in cervical cancer cells
Jan van Riggelen, Gilles Buchwalter, Ubaldo Soto, Johanna De-Castro Arce, Harald zur Hausen, Bohdan Wasylyk and Frank Rösl

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