



# High constitutive level of NF- $\kappa$ B is crucial for viability of adenocarcinoma cells

AS Smirnov<sup>1</sup>, AS Ruzov<sup>1</sup>, AV Budanov<sup>2</sup>, AV Prokhortchouk<sup>1</sup>, AV Ivanov<sup>2</sup> and EB Prokhortchouk<sup>\*1</sup>

<sup>1</sup> Group of Transcriptional Control and Oncogenesis, Institute of Gene Biology, Russian Academy of Sciences, Vavilova 34/5, 117334 Moscow, Russia

<sup>2</sup> Cell Proliferation Laboratory, Engelhardt Institute of Molecular Biology, Vavilova 32, 117339 Moscow, Russia

\* Corresponding author: B Prokhortchouk, Institute of Cell and Molecular Biology, The University of Edinburgh, Kings Buildings, Swan Building 426, UK. Tel: +44-131-6507079; Fax: +44-131-6505379, E-mail: Egor.Prokhortchouk@ed.ac.uk

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## Abstract

**Most of cells exhibit low nuclear level of NF- $\kappa$ B. However, in some cell lines and tissues aberrantly activated NF- $\kappa$ B is playing an important role in cell motility, growth control and survival. Here we describe the result of decrease of constitutive NF- $\kappa$ B level in different adenocarcinoma cell lines. Treatment of mouse adenocarcinoma cell line CSML-100 with both synthetic (TPCK or PDTC) or natural (I $\kappa$ B- $\alpha$ ) NF- $\kappa$ B inhibitors caused apoptotic death. Low doses of TPCK were harmless for CSML100 cells but sensitized them to TNF-induced apoptosis. Death of CSML100 cells in the presence of high concentration TPCK was not accompanied with significant changes in *c-myc* activity but strongly correlated with rapid decrease in p53 level. Thus, mutual behavior p53 and NF- $\kappa$ B represented a unique feature of TPCK-induced apoptosis in CSML-100 adenocarcinoma cells. *Cell Death and Differentiation* (2001) 8, 621–630.**

**Keywords:** NF- $\kappa$ B constitutive level; apoptosis; p53; adenocarcinoma cell lines

**Abbreviations:** EMSA, electrophoretic mobility shift assay (gel shift); PDTC-pyrrolidine ithiocarbamate; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone

## Introduction

NF- $\kappa$ B is a multifunctional transcription factor that plays a crucial role in a variety of cellular processes such as immune and inflammatory response, proliferation and survival, invasiveness and motility, transformation (for review see<sup>1</sup>). Dimers that consist of polypeptides named p50, p52, p65 (RelA), p68 (RelB) and c-Rel represent a repertoire of NF- $\kappa$ B in particular cell. In most cells NF- $\kappa$ B is sequestered to the

cytoplasm where it is bound to inhibitors molecules, I $\kappa$ B's. In response to different stimuli IKK kinase complex modifies I $\kappa$ B- $\alpha$ , the most studied among I $\kappa$ B's, on 32 and 36 Serines.<sup>2–5</sup> Phosphorylation is essential step in this cascade since only thereafter I $\kappa$ B is ubiquitinated and degraded by 26S proteasome<sup>6</sup> allowing NF- $\kappa$ B translocation to a nucleus (reviewed in<sup>7–9</sup>). In nucleus NF- $\kappa$ B may activate expression of plenty of genes some of which have antiapoptotic functions (c-IAPs, TRAF2 etc).<sup>10,11</sup> Several researchers have used a super-repressor form of I $\kappa$ B which had mutations or deletions on its N-terminal phosphorylation sites to inhibit nuclear translocation of NF- $\kappa$ B.<sup>3,12,13</sup> Inhibition is often resulted in acquiring TNF sensitive phenotype giving a good basis to treat accumulation of NF- $\kappa$ B in a nucleus as a protective, antiapoptotic event, although, in some systems NF- $\kappa$ B may carry out proapoptotic function.<sup>14,15</sup>

High constitutive level of NF- $\kappa$ B was reported in neurons,<sup>16</sup> B lymphocytes,<sup>17</sup> nontransformed mouse hepatocytes,<sup>18</sup> mammary tumors<sup>19</sup> and cells specific for Hodgkin disease.<sup>20,21</sup> It was shown that decrease of constitutive level of NF- $\kappa$ B in most of these cell types led to cell death or growth arrest.<sup>17–19,22</sup>

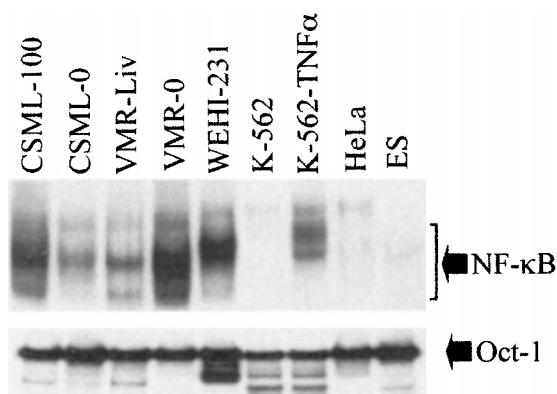
Oncogene *c-myc* and antioncogene p53, both are NF- $\kappa$ B transcriptional targets,<sup>23,24</sup> were reported to be involved in cell growth, transformation and death. Transcriptional cross talk between p53 and NF- $\kappa$ B has been established recently. It was shown<sup>25,26</sup> that these proteins competed for a limiting pool of p300/CBP transcriptional coactivator complexes. Drop of *c-myc* paralleled behavior of NF- $\kappa$ B in TPCK-induced apoptosis in WEHI 231 cells suggesting its antiapoptotic role.<sup>17</sup> Moreover, ectopic expression of *c-myc* rescued WEHI231 cells from TPCK-induced death.<sup>17</sup> Some indirect data converge the roles of p53 and NF- $\kappa$ B in the process of cellular transformation.<sup>27</sup>

In our experiments we used mouse adenocarcinoma cell lines CSML-100, CSML-0, VMR-0 and VMR-Liver.<sup>28</sup> While CSML-0 and VMR-0 are highly tumorigenic, however, they cannot form metastases when injected subcutaneously. In contrast, CSML-100 and VMR-Liver cells are highly malignant. CSML-100 and VMR-Liver cells were resistant to TNF- $\alpha$  induced apoptosis, but CSML-0 and VMR-0 were highly susceptible.<sup>29</sup> We found previously, that these cells are characterized by elevated constitutive level of NF- $\kappa$ B.<sup>29</sup> This work was directed towards understanding the importance of maintaining NF- $\kappa$ B in the nucleus of the cells.

## Results

### CSML100 and CSML0 cells exhibited high constitutive nuclear level of NF- $\kappa$ B

Initially we have tested nuclear extracts derived from different cell lines and tissues for the binding to NF- $\kappa$ B consensus oligonucleotide (Figure 1). The amount of nuclear proteins



**Figure 1** Gel-Shift analysis of NF- $\kappa$ B in different cell lines. Shifted bands that corresponded to NF- $\kappa$ B and Oct-1 binding are depicted. Complexes responsible for NF- $\kappa$ B binding migrated with different mobility (p50-p50, p50-p65, p50-p68, for details see text) and are pointed by the bracket. Following cell lines were used for nuclear extracts isolation: lane 1, CSML-100 cells; lane 2, CSML-0 cells; lane 3, VMR-Liv cells; lane 4, VMR-0 cells; lane 5, WEHI231 cells; lane 6, K562 cells; lane 7, K562 cells were pretreated for 30' with 1 nM concentration of TNF- $\alpha$ ; lane 8, HeLa cells; lane 9, mouse embryonic stem cells (strain 129)

from different sources was normalized by Oct-1 binding. Electronic images of the gels were used to measure intensities of the bands that corresponded to NF- $\kappa$ B and Oct-1 containing complexes. We found that CSML100 (lane 1) cells exhibit abnormally high NF- $\kappa$ B constitutive nuclear level while in CSML0 (lane 2) it is comparable with WEHI231 (lane 5) or TNF- $\alpha$  induced K562 (lane 6) human erythroleukemia cells. WEHI231 cells contain a lot of nuclear NF- $\kappa$ B and are known to be a model system to study high constitutive NF- $\kappa$ B level. TNF- $\alpha$  is a potent NF- $\kappa$ B activator. Noninduced K562 cells showed nondetectable level of NF- $\kappa$ B, but after 15 min TNF- $\alpha$  treatment it dramatically (we estimated it as 50 times) increased to the level that could be compared with that of WEHI231 or CSML0 cells. In the further analysis we used HeLa cells. As for noninduced K562 cells NF- $\kappa$ B level in HeLa cells was barely detectable (lane 6). Identification of the complexes that were formed in CSML100 and CSML0 cells has been made previously.<sup>30</sup> They contain abundant p50-p50 homodimer and p50-p65 heterodimer and minor fraction of p50-RelB heterodimer. Highly metastatic VMR-Liv and nonmetastatic VMR-0 adenocarcinoma cell lines were used to obtain additional information on the level of constitutive nuclear NF- $\kappa$ B in adenocarcinomas. However, no correlation between malignant phenotype and NF- $\kappa$ B level, still elevated, was observed: in VMR-Liv it was lower than in VMR-0 cells.

### TPCK and PDTC treatment reduces NF- $\kappa$ B factor binding in CSML-100 and CSML-0

The serine/threonine protease inhibitor TPCK, which has been found to inhibit the normally rapid turnover of I $\kappa$ B- $\alpha$  in WEHI 231 cells was examined for its effects on NF- $\kappa$ B binding in CSML-100 and CSML-0 cells. Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts prepared after 1, 3 or 7 h of treatment with either 25 or

50  $\mu$ M TPCK (Figure 2A). As a probe we have used NF- $\kappa$ B consensus oligonucleotide and Oct-1 binding site in control experiments. A decline in formation of NF- $\kappa$ B complexes was detected with extracts isolated after 1 h TPCK treatment using both concentration (25 and 50  $\mu$ M). After 3 and 7 h of treatment NF- $\kappa$ B slightly increased (Figure 2B). These effects were dependent on a dose of TPCK. More profound effect was observed with 50  $\mu$ M TPCK: 1 h, 8% of untreated CSML100 and CSML-0 NF- $\kappa$ B level; 3 h, 10%; 7 h, 15%, while using 25  $\mu$ M of TPCK the general profile was the same but with quantitative differences: 1 h, 20%; 3 h, 35%; 7 h, 45%. In contrast, Oct-1 factor complexes were unaffected as judged by EMSA with an Oct-1 oligonucleotide, indicating that the drop in NF- $\kappa$ B activity was not due to a general inhibition of factor binding. The same experiment using PDTC showed quite different profile in NF- $\kappa$ B behavior. PDTC has been found to inhibit NF- $\kappa$ B factor by inactivating reactive oxygen species (ROS).<sup>31</sup> Treatment of CSML100 cells with 15  $\mu$ M PDTC showed slow prolonged decrease in NF- $\kappa$ B (Figure 2C and D): 1 h, 75%; 3 h, 50% and 7 h, 25%. The same profiles of decline were observed with CSML0, VMR-0 and VMR-Liv cells (data not shown).

### TPCK has not changed the whole-cell level of NF- $\kappa$ B subunits

We have monitored by Western blot analysis the level of p50, RelA, RelB, I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , I $\kappa$ B- $\gamma$  and bcl-3 in untreated and TPCK-treated CSML-100 cells. We have observed that (i) steady state level of all tested molecules was equal in CSML and VMR cell lines as well as in HeLa cells, and (ii) no significant changes in the protein level of NF- $\kappa$ B/I $\kappa$ B molecules were detected upon TPCK treatment of CSML100 cells (these data not shown). These data meant that nuclear export, but not degradation of NF- $\kappa$ B subunits was responsible for the observed drop.

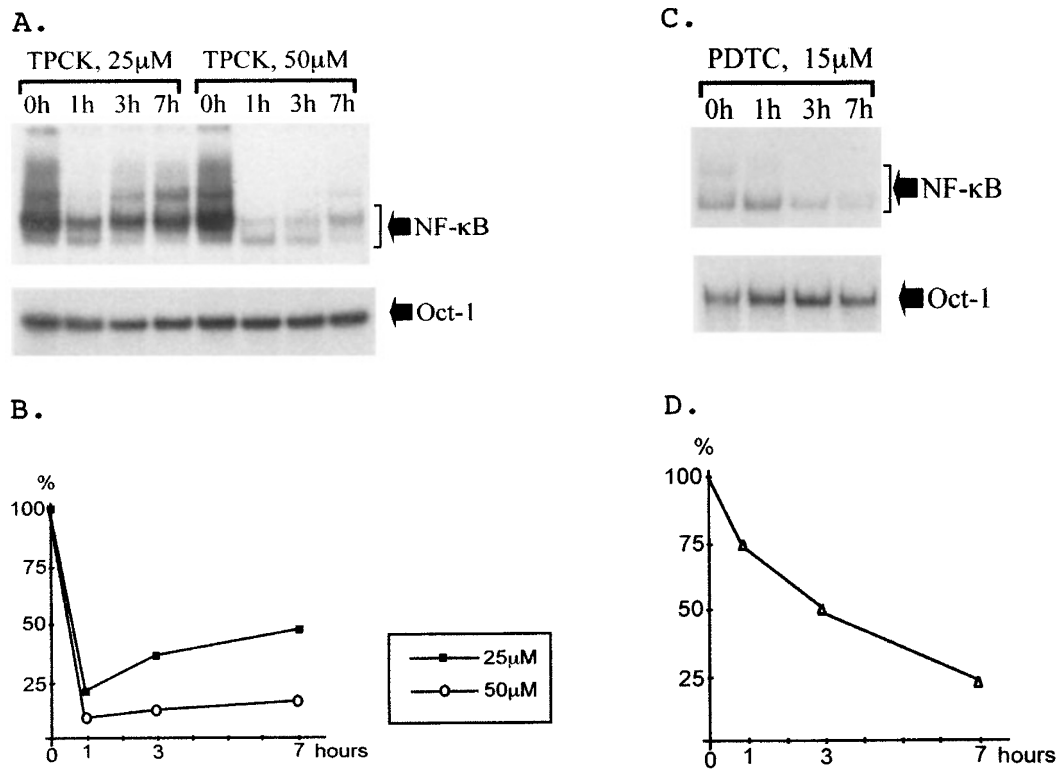
### I $\kappa$ B- $\alpha$ and I $\kappa$ B- $\beta$ are stable in CSML-100 cells

In many cases drop in NF- $\kappa$ B activity is caused by increased life time of the inhibitor molecule I- $\kappa$ B. We have confirmed that the high constitutive level of NF- $\kappa$ B in CSML-100 cells is not due to rapid turnover of I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\beta$ . We measured the stability of the inhibitors by monitoring their decay by Western blot-hybridization upon cycloheximide stopped protein synthesis. The half-life time of I $\kappa$ B- $\alpha$  molecule in CSML-100 cells was approximately equal to 3 h, and I $\kappa$ B- $\beta$  had a half-life time of 8 h (Figure 3). Thus, high speed drop of nuclear NF- $\kappa$ B that takes place in 60 min after TPCK addition cannot be explained by the stabilization of the inhibitor molecules.

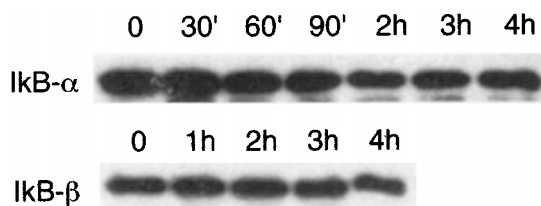
### TPCK induced apoptosis in CSML100 cells in dose-dependent manner

To investigate the effects of reduced NF- $\kappa$ B activity on induction of apoptosis in CSML100 cells we performed double staining with AnnexinV-FLUOS and propidium iodide.

We analyzed an effect of TPCK treatment on CSML-100 cell viability by flow cytometry. For analyzing AnnexinV staining only those cells that have not been stained with PI



**Figure 2** Gel-Shift analysis of NF- $\kappa$ B in drug treated CSML100 cells. (A) CSML100 cells were treated with 25  $\mu$ M (lanes 1–4) and 50  $\mu$ M (lanes 5–8) for indicated time interval (1, 3 and 7 h). 0 h treatment indicates nontreated CSML-100 cells. (B) Digital representation of data obtained by gel shifts in A. 100% coincides with basal NF- $\kappa$ B level in CSML100 cells. (C) CSML100 cells were treated with 15  $\mu$ M PDTC for indicated time intervals (1, 3 and 7 h). 0 h treatment indicates nontreated CSML-100 cells. (D) Digital representation of data obtained in C by gel shifts



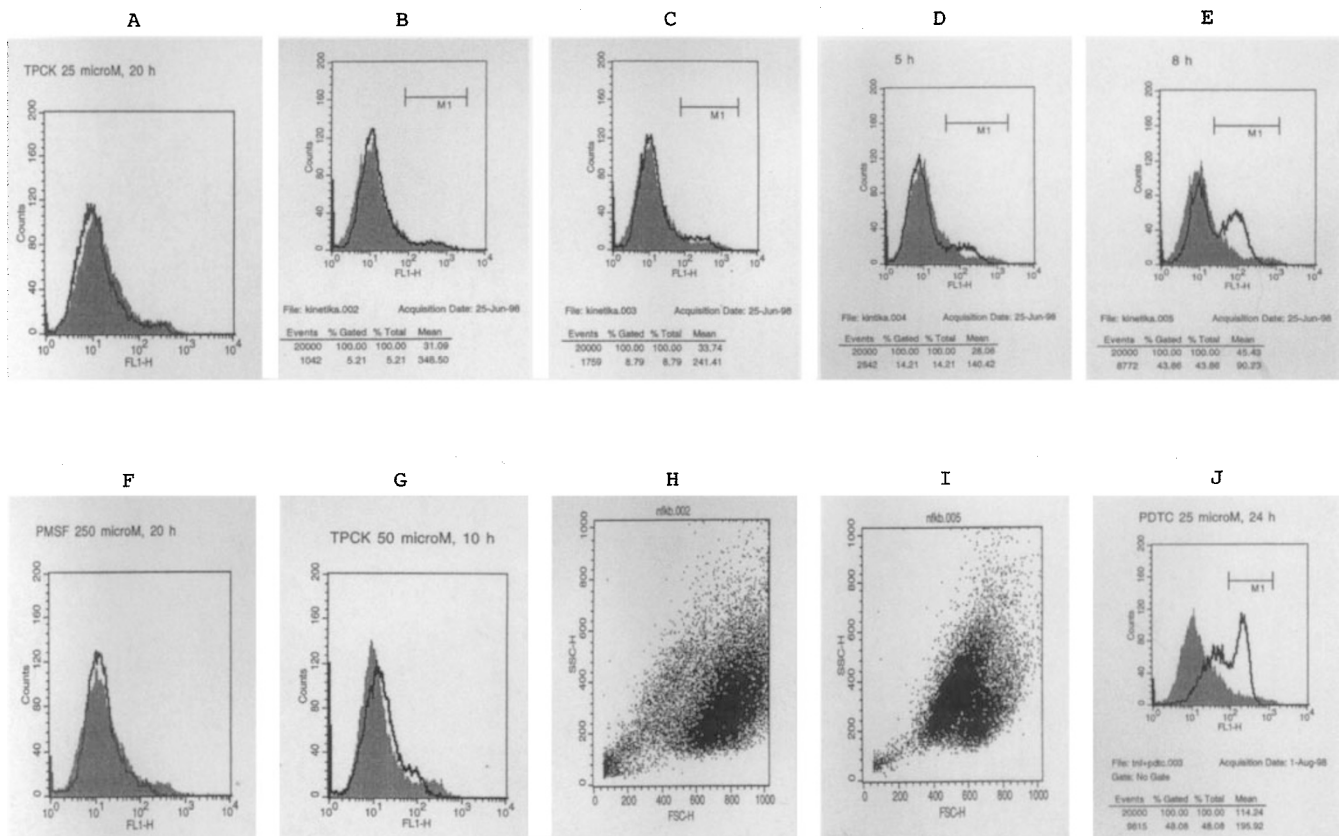
**Figure 3** I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  have relatively long half-life time in CSML100 cells. After addition of cycloheximide, cells were lysed and total extracts were prepared. I $\kappa$ B's content was analyzed by Western blot-hybridization with anti-I $\kappa$ B- $\alpha$  (top panel) or anti-I $\kappa$ B- $\beta$  (bottom panel) antibodies

were gated. Lower dose of TPCK (25  $\mu$ M) has not produced any shifts in annexinV staining intensity. After 20 h of 25  $\mu$ M TPCK treatment CSML100 cells were still viable (Figure 4A). However, 50  $\mu$ M TPCK produced annexinV staining. Amount of stained cells was estimated by gating M1. Cells staining obeyed the following kinetics (Figure 4B–E): 1 h,  $5 \pm 2\%$ ; 3 h,  $8 \pm 3\%$ ; 5 h,  $14 \pm 5\%$  and 8 h,  $45 \pm 14\%$ . On the basis of these results we concluded that CSML100 cells underwent apoptosis upon TPCK treatment in dose-dependent manner, i.e. 25  $\mu$ M has not, but 50  $\mu$ M has induced apoptosis. Both concentration of TPCK (25 and 50  $\mu$ M) killed CSML0 cells in 10 and 16 h

respectively. Since TPCK was reported to be a protease inhibitor we used another protease inhibitor PMSF to test specificity of TPCK activity. However, PMSF has not induced any changes in cells staining (Figure 4F).

Thus, we found that drop in NF- $\kappa$ B nuclear level below critical value in CSML100 cells was associated with apoptotic death. In order to exclude the possibility that cell death was a consequence of nonspecific toxicity of TPCK sample we treated HeLa cells with different concentrations of TPCK. HeLa cells appeared to be resistant to TPCK since after 20 h incubation even with 75  $\mu$ M of TPCK there were no annexinV intensity shifts (Figure 4G). HeLa cells contain only low level of nuclear NF- $\kappa$ B (Figure 1, lane 8), the level that was already low and was not subjected at all or just slightly to further decrease by TPCK.

Apoptotic mechanism for TPCK-induced cell death manifested in changes of cell shape. Normally, CSML100 cells resembled adherent fibroblasts with irregular shape, but after 1 h incubation with 50  $\mu$ M TPCK CSML100 cells changed its morphology and became round. This fact is reflected in changes in forward-side scattering that is dependent on cell shape (Figure 4H and I). However, in spite of dramatic changes in morphology cell were still adherent and detached from the layer and flew in the medium only after 6–8 h of TPCK treatment.



**Figure 4** FACS analysis. Cells were treated in different ways prior to the analysis. Shaded area represents FACS distribution of nontreated CSML-100 cells. Bold line represents experimental FACS distribution of: (A) CSML-100 cells treated with 25  $\mu$ M TPCK for 20 h; (B) CSML-100 cells treated with 50  $\mu$ M TPCK for 1 h; (C) for 3 h; (D) for 5 h; (E) for 8 h; (F) CSML-100 cells treated with 250  $\mu$ M PMSF for 20 h; (G) HeLa cells treated with 50  $\mu$ M TPCK for 20 h; (H) side/forward scattering of CSML100 cells; (I) side/forward scattering of CSML-100 cells treated with 50  $\mu$ M TPCK for 1 h; (J) CSML-100 cells treated with 25  $\mu$ M PDTC for 24 h. Gate is depicted by M1 interval

PDTC (15  $\mu$ M) effect on CSML100 cells was obvious only upon long term treatment. In 24 h we observed around 50% annexinV stained cells (Figure 4J).

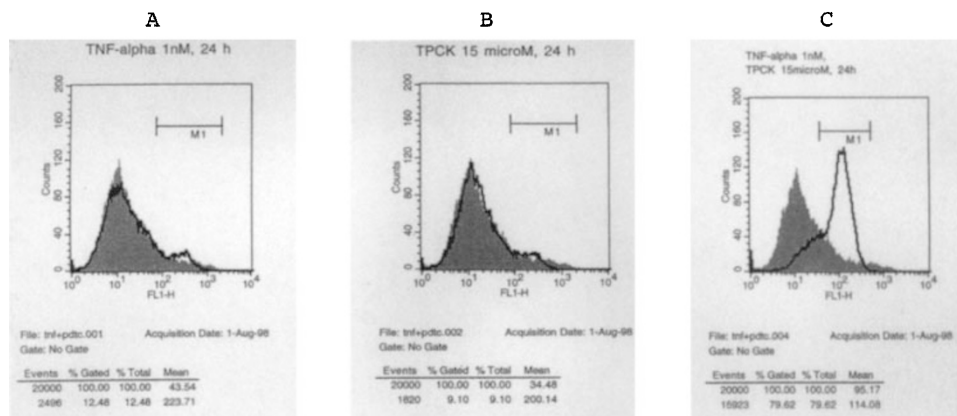
### CSML100 cells were sensitized to TNF- $\alpha$ -induced apoptosis by low doses of TPCK

Previously, we found that CSML100 cells were resistant to TNF- $\alpha$  action.<sup>29</sup> Here, we treated CSML100 cells with 1 nM concentration of TNF- $\alpha$  for different time intervals (from 15 min to 5 h) and observed maximum 50% relative increase of nuclear NF- $\kappa$ B in EMSA experiments (data not shown). In parallel, the half-life time of I $\kappa$ B- $\alpha$  molecule reduced substantially and consisted approximately 30 min (data not shown). To test a hypothesis that constitutive NF- $\kappa$ B protects CSML100 cells from TNF-induced apoptosis we used the property of low doses of TPCK (10–20  $\mu$ M) to reduce nuclear NF- $\kappa$ B binding to harmless level. As we expected 24 h of treatment 1 nM concentration of human recombinant TNF- $\alpha$  did not produce any shifts in annexinV staining (Figure 5A). TPCK in concentration 15  $\mu$ M failed to produce staining shifts in 24 h as well (Figure 5B). However, dual effect of TPCK and TNF induced strong apoptosis in CSML100 cells as it was

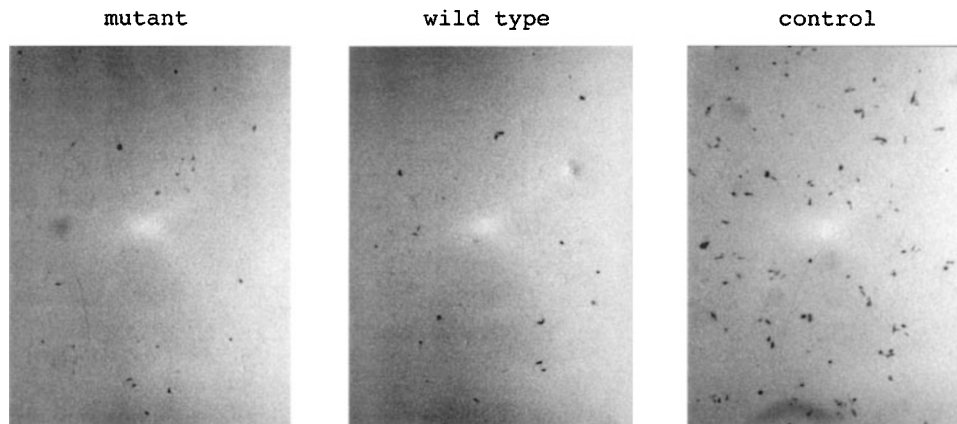
measured by flow cytometry (Figure 5C). More than 75% of cells were M1 gated, suggesting profound cellular death.

### Transient transfection of CSML100 cells with I $\kappa$ B- $\alpha$ super repressor eukaryotic expression vector influenced cell viability

Having TPCK treatment results as a basis we specifically inhibited NF- $\kappa$ B nuclear activity by natural inhibitor I $\kappa$ B. For this we used eukaryotic expression vector with I $\kappa$ B- $\alpha$  super repressor cDNA. Enhanced NF- $\kappa$ B repression is associated with the abnormal stability of this form of I $\kappa$ B molecule that is due to point substitutions of 32 and 36 Serines on Alanines. We have transiently transfected CSML100 cells with combination of two eukaryotic expression vectors. One of them harbored bacterial lacZ gene under CMV promoter, another was either human I $\kappa$ B- $\alpha$  wild-type (I $\kappa$ B-*wt*) or 32,36-mutated molecule (I $\kappa$ B-*mut*) or empty vector. Two days after transfection we stained the cells *in situ* for lacZ expression. We found that amount of lacZ positive cells decreased fivefold in the case of I $\kappa$ B-*wt* (32  $\pm$  7 per field) and I $\kappa$ B-*mut* (29  $\pm$  4) as compared to the empty vector (153  $\pm$  17) (Figure 6). No difference was observed using *wt* and *mut* constructs, most



**Figure 5** FACS analysis. Cells were treated in different ways prior to the analysis. Shaded area represents FACS distribution of nontreated CSML-100 cells. Bold line represents experimental FACS distribution of: (A) CSML-100 cells treated with 1 nM of TNF $\alpha$  for 24 h; (B) CSML100 cells treated with 15  $\mu$ M of TPCK for 24 h; (C) dual action of 1 nM of TNF $\alpha$  and 15  $\mu$ M of TPCK for 24 h



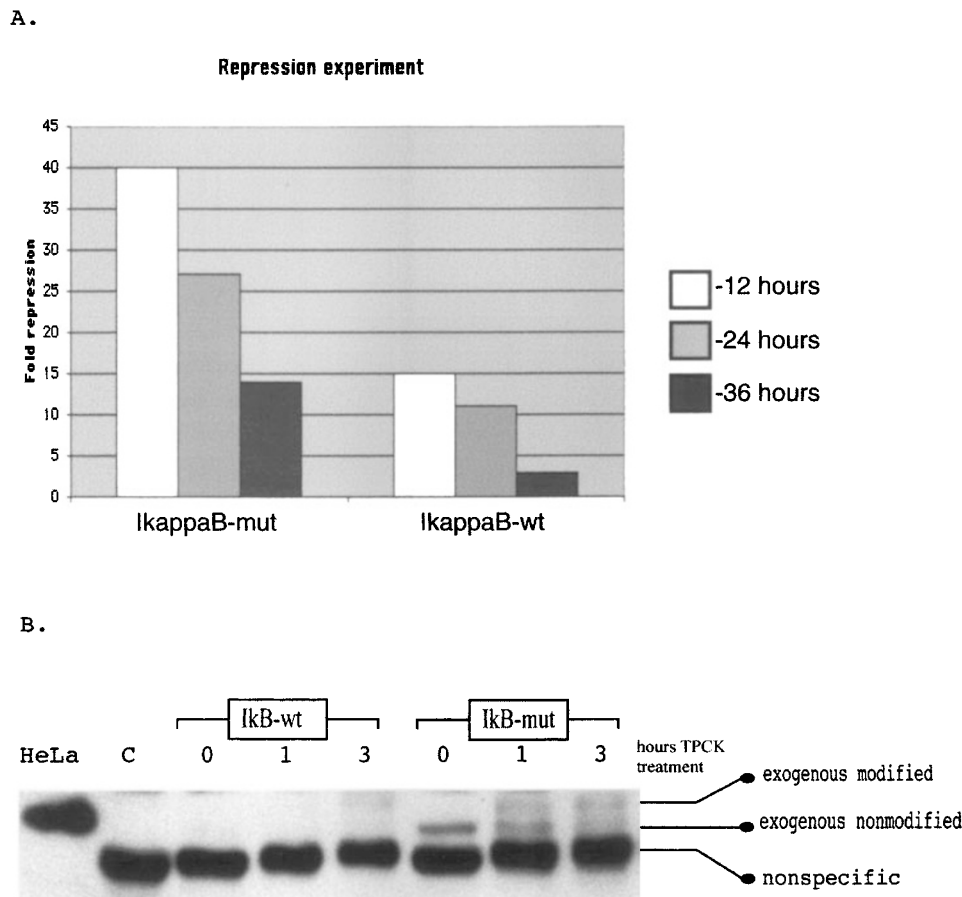
**Figure 6** *In situ* X-gal/ $\beta$ -gal staining of CSML100 cells transiently transfected with  $\beta$ -gal expression vector along with: (mutant)- pI $\kappa$ B-mut; (wild type)- pI $\kappa$ B-wt; control-pRcCMV

probably because I $\kappa$ B-*wt* construct still decreased NF- $\kappa$ B level below the critical level.

To test this we measured NF- $\kappa$ B activity upon transfection of CSML100 cells with I $\kappa$ B *wt* and *mut* constructs. As a reporter we used pN5 plasmid that contained five consensus NF- $\kappa$ B binding sites downstream from CAT gene under SV40 minimal promoter. Plasmid pH6 had the same structure but lacked NF- $\kappa$ B binding sites. Plasmids pH6 and pN5 were transiently cotransfected with either pI $\kappa$ B-*mut* or pI $\kappa$ B-*wt* or empty vector into CSML100 cells. CAT activity was examined after different time intervals: 12, 24 and 36 h posttransfection. Results with pH6 plasmid showed slight decrease in fold of background activation of heterologous promoter during time course with no general respect either pI $\kappa$ B-*mut* or pI $\kappa$ B-*wt* vector was cotransfected. Figures from pH6 experiment were used to normalize pN5 data. We found that cotransfection of pI $\kappa$ B-*mut* efficiently blocked NF- $\kappa$ B dependent transcription. The repression was time-depen-

dent and varied from 40-fold in 12 h to 25-fold in 24 h and to 15-fold in 36 h (Figure 7A). Cotransfection of pI $\kappa$ B-*wt* had less profound repression effect: 12 h, sevenfold; 24 h, fivefold; 36 h, threefold. Drop in repression probably was a consequence of death of those cells that efficiently transcribed effector plasmid and survival of those that did not. Thus, I $\kappa$ B-*wt* negatively affected NF- $\kappa$ B dependent transcription, but its effect is not that profound as for I $\kappa$ B-*mut*.

We detected exogenous I $\kappa$ B $\alpha$ -*mut* (Figure 7B) but not exogenous I $\kappa$ B $\alpha$ -*wt* molecules in transiently transfected CSML100 cells. Interestingly, after 1 and 3 h of treatment of the transfected (20 h posttransfection) cells with 50  $\mu$ M TPCK we have observed accumulation of modified exogenous I $\kappa$ B $\alpha$ -*mut* but not I $\kappa$ B $\alpha$ -*wt* molecule suggesting (i) posttranslational modifications (presumably ubiquitination) of the mutated I $\kappa$ B- $\alpha$  molecule (ii) enhanced stability of the mutated I $\kappa$ B- $\alpha$  molecule. Enhanced stability of the exogenous I $\kappa$ B $\alpha$ -*mut* molecule most probably caused that



**Figure 7** (A) Repression of NF- $\kappa$ B dependent transcription by I $\kappa$ B-mut and I $\kappa$ B-wt. CSML-100 cells were transiently transfected with pI $\kappa$ B-mut or pI $\kappa$ B-wt expression vectors. Bars represented fold of repression of NF- $\kappa$ B driven transcription in pN5 plasmid. Results were normalized by pH6 data (see text); (B) CSML-100 cells were transiently transfected with either I $\kappa$ B-wt or I $\kappa$ B-mut vector and 24 h post-transfection cells were treated with 50  $\mu$ M TPCK for indicated time interval (1 or 3 h). As a positive control HeLa total extracts were loaded (lane 1), nontransfected CSML-100 cells were loaded on lane 2; The blot was developed using rabbit anti-human I $\kappa$ B- $\alpha$  SantaCruz antibodies sc-203. Exogenous nonmodified, exogenous modified and nonspecific bands are arrowed

dramatically decreased NF- $\kappa$ B dependent transcription in repression experiments and, on the other hand, unstable, easily degraded I $\kappa$ B-wt molecule had a minor effect on NF- $\kappa$ B.

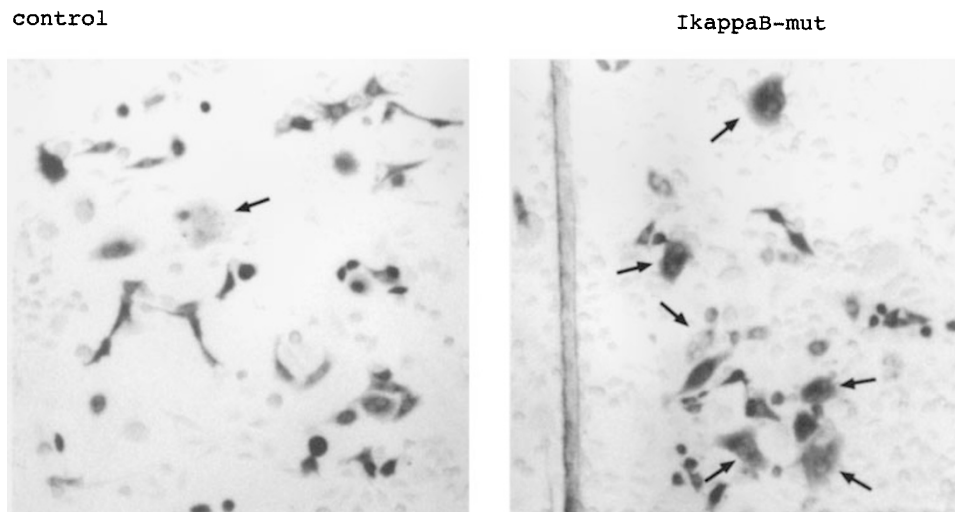
### Microinjection of CSML100 cells with I $\kappa$ B- $\alpha$ super repressor eukaryotic expression vector cause appearance of multinuclei cells

To verify that TPCK induced drop in NF- $\kappa$ B nuclear activity was the primary event that triggered apoptosis we microinjected CSML100 cells with either pI $\kappa$ B-mut or pRc-CMV vectors. Efficiency of microinjection was monitored by coinjection of  $\beta$ -galactosidase expression vector. Approximately 200 of cells were microinjected in each experiment. Expression of  $\beta$ -galactosidase was assayed in 24 h by *in situ* staining with X-Gal. Cell viability after microinjection was equal to 70%. We found that around 30% of cells microinjected with I $\kappa$ B-mut construct had nuclear fragmentation as was detected by morphologic assessment (Figure 8, panel I $\kappa$ B-mut). Multinucleinization in the case of use of pRc-

CMV vector was not obvious and has not exceeded it of noninjected CSML100 cells (Figure 8, control panel). In parallel, we stained microinjected cells with X-Gal and than with Propidium Iodide to address the same question: whether I $\kappa$ B-mut microinjected cells exhibited signs of apoptosis that could be detected by chromatin condensation. Stained cells were analyzed by fluorescence microscopy. We observed that around 20% of X-Gal stained cells had a characteristically condensed chromatin (data not shown). In contrast, mock injection has not produced apoptotic chromatin condensation. Thus, specific inhibition of constitutive NF- $\kappa$ B activity resulted in apoptotic death of CSML100 adenocarcinoma cells.

### Tumor suppressor p53 but not c-myc was downregulated with both TPCK and I $\kappa$ B- $\alpha$

Oncogene c-myc and p53 are implicated in different processes that concern NF- $\kappa$ B as well. Thus, we tested the kinetics of changes of c-myc and p53 level in CSML100 cells upon TPCK treatment. Nuclear extracts derived from TPCK-treated cells (TPCK concentration, 50  $\mu$ M) were subjected to



**Figure 8** CSML-100 cells were microinjected with either pRc-CMV or  $\text{I}\kappa\text{B}$ -mut vectors along with  $\beta$ -gal expression vector. Cells were stained *in situ* with X-Gal. Multinucleic cells are arrowed

EMSA using *c-myc* and p53 consensus oligonucleotides (Figure 9A). In contrast to previous reports we found that *c-myc* DNA-binding was almost stable, although, a slight temporary increase after 1 h was detected. DNA-binding of p53 dramatically decreased after 1 h and stayed extremely low after 3 and 7 h. Amounts of nuclear proteins were normalized by Oct-1 binding. By Western-blot analysis using total cell extracts from TPCK-treated cells we have detected no changes in *c-myc* (data not shown) but dramatic decrease of p53 (Figure 9C). Biochemical fractionation of cytosolic and nuclear extracts showed that p53 resided in nuclei of CSML100 cells (Figure 9B). As a control for fractionation we have used antibodies against S100A4 gene product<sup>33</sup> and TATA-binding protein (TBP) for cytoplasmic and nuclear component correspondingly. To exclude possible nonspecific effect of TPCK on p53 we transiently transfected p53-responsive CAT reporter construct in combination with either  $\text{I}\kappa\text{B}\alpha$ -mut or  $\text{I}\kappa\text{B}\alpha$ -wt or empty vector. Analysis of CAT activity in transfected cells has shown that p53-dependent transcription decreased sixfold and 2.5-fold when super repressor and wild type form of  $\text{I}\kappa\text{B}$  was used correspondingly (Figure 9C). These results suggested that drop in NF- $\kappa$ B nuclear activity caused in parallel significant decrease of p53 protein level.

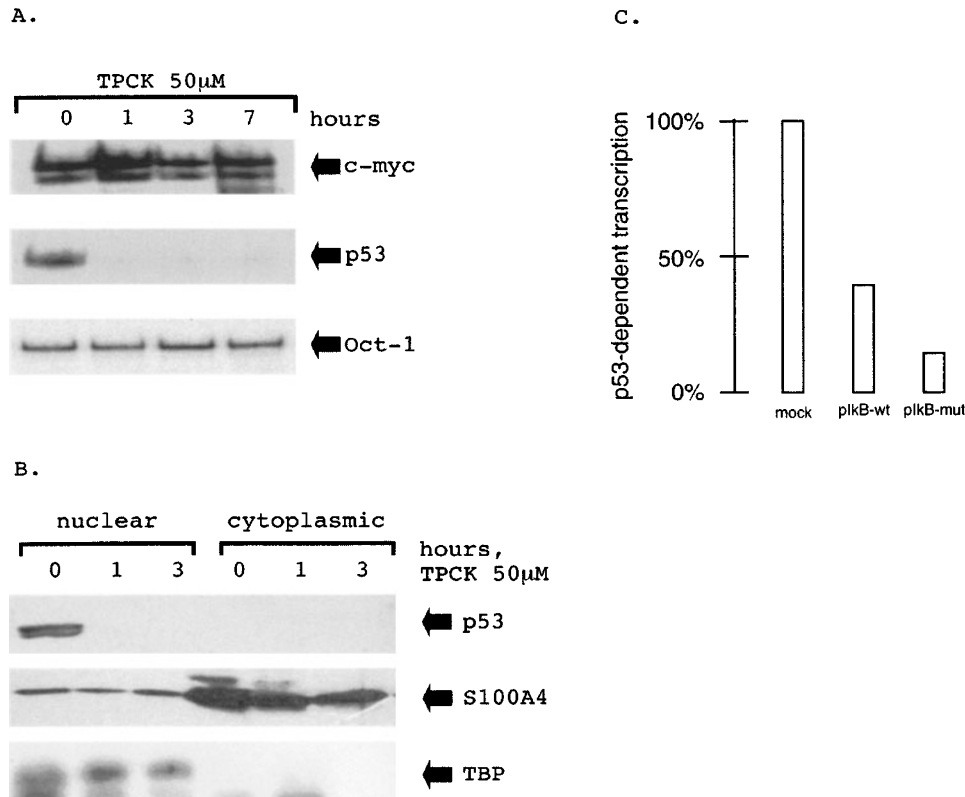
## Discussion

In this study we have shown that decrease of active NF- $\kappa$ B in adenocarcinoma cell lines led to rapid cell death. Growing data suggest that this behavior is common for cells with high level of nuclear NF- $\kappa$ B. When NF- $\kappa$ B level drops below a critical parameter (i.e. high TPCK doses) cells undergo apoptosis, however, they can overcome cell death if NF- $\kappa$ B does not drop less than 20% of the original level (low TPCK doses). In agreement with this statement, transfection of both  $\text{I}\kappa\text{B}$ -mut or  $\text{I}\kappa\text{B}$ -wt was harmful for CSML-100 cells. The constructs repressed NF- $\kappa$ B dependent transfection not

equally, but, probably, still strongly enough (20-fold for  $\text{I}\kappa\text{B}$ -mut; fourfold for  $\text{I}\kappa\text{B}$ -wt) to induce apoptosis.

Korobko *et al*<sup>29</sup> insisted that cell resistance to TNF- $\alpha$  correlated with high metastatic potential. We provided more direct evidences on relation between NF- $\kappa$ B level and resistance to TNF- $\alpha$ -induced apoptosis in CSML-100 cells. Low doses of TPCK which caused particular decrease in NF- $\kappa$ B level but didn't kill CSML-100 cells made them sensitive to TNF- $\alpha$ . It is for the first time that TPCK sensitized but not adenocarcinoma cells. Similar observation has been made by Giri *et al*<sup>34</sup> who reported that pretreatment of HuT-78 human T cell line with PDTC inhibited constitutive NF- $\kappa$ B activation and sensitized the cells to TNF-induced apoptosis, thus suggesting a critical role of ROS. PDTC is a potent inhibitor of inducible NF- $\kappa$ B activation in most cells.<sup>31,35</sup> Generation of ROS has been proposed as an important mechanism to mediate apoptotic and gene regulatory effects of TNF. Apoptotic death of CSML100 and other adenocarcinoma cells upon PDTC treatment, however, indicate that ROS are also involved in protection of cells from spontaneous apoptosis by activating NF- $\kappa$ B.

Which molecular changes occurred during TPCK-induced apoptosis in CSML100 cells? It was reported that *c-myc*, that is a transcriptional target of NF- $\kappa$ B, had a parallel decrease upon TPCK-induced apoptosis in WEHI231 cells.<sup>36</sup> On this ground its antiapoptotic role has been proposed. However, in our experiments no significant changes in *c-myc* level was observed. Instead of *c-myc* changes we found fast and significant drop of p53 level. Only once before such a parallel behavior of p53 and NF- $\kappa$ B was reported in the system where NF- $\kappa$ B was essential for p53 dependent apoptosis.<sup>37</sup> While p53 is functionally active and abundant in CSML-100 cells its role in TPCK-induced apoptosis in CSML-100 cells is unclear. Previous studies have shown that p53 is a transcriptional target of NF- $\kappa$ B and have suggested that p53 expression depends,



**Figure 9** (A) Gel-Shift analysis of c-myc and p53. Nuclear extracts were derived from CSML-100 cells treated with 50  $\mu$ M TPCK for indicated time intervals. (B) CSML-100 cells were lysed and nuclear and cytoplasmic proteins were isolated according to Schreiber *et al*<sup>40</sup>. Western blot analysis of p53 in nuclear and cytoplasmic fraction of CSML-100 cells treated with 50  $\mu$ M TPCK. Fractionation quality was controlled by Western analysis of S100A4 and TBP. (C) Effects on p53 driven transcription of pCAT-Waf plasmid exerted by cotransfected expression vectors: pPS (mock), plκB-mut and plκB-wt. CAT results were normalized by  $\beta$ -galactosidase monitoring using pCMV-gal plasmid as an internal control

at least partially, on NF- $\kappa$ B activity.<sup>24</sup> Mutual negative regulation of p53 and NF- $\kappa$ B, where each transcription factor inhibits the activity of the other by competing for a limiting pool of the co-activator p300/CBP, has also been described.<sup>25,26</sup> Probably, in CSML-100 cells p53 under the tight positive control of NF- $\kappa$ B. However, p53 was shown to bind DNA efficiently and has no mutations in its coding region (our unpublished data). Thus, cell with such a high level of p53 (that is comparable with DNA-damage induced level of p53 in REF52 fibroblasts, A Smirnov unpublished results) would undergo apoptosis or cell cycle arrest. Perhaps, some compensatory mechanisms that inactivates p53 in CSML-100 cells exist. As a possibility we admit that NF- $\kappa$ B compete for CBP with p53 and thus abolishes its capacity for transcriptional activation.

Constitutively high level of the active NF- $\kappa$ B in metastatic cells may serve to ensure protection of tumor cells from enhanced level of pro-apoptotic cytokines secreted by the cells of immune system. The mechanisms which ensure such preactivated NF- $\kappa$ B state are quite different in distinct cell lines. For instance, we have discussed below T cell lymphoma model of HuT-78 cells and additional example are WEHI231 immature B cell lymphoma cells that maintain high NF- $\kappa$ B level due to the fast 26 proteasome independent Ca<sup>2+</sup>-dependent turnover of I $\kappa$ B- $\alpha$  mole-

cule.<sup>38</sup> Our data implicated NF- $\kappa$ B in processes of survival of tumor cells, in particular malignant adenocarcinoma cells, suggesting the general rule with no respect to a particular mechanism of its realization: the more aggressive cells are the higher constitutive NF- $\kappa$ B level is.

## Materials and Methods

### Cell lines and transfections

The mouse cell lines CSMLO, CSML100 (mammary adenocarcinomas with low and high metastatic potential, respectively) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 50  $\mu$ g/ml each of streptomycin and penicillin.<sup>28</sup> Normally we used  $2 \times 10^5$  cells for transient transfection by LIPOFECTIN reagent (Gibco-BRL) according to the manufacturer's protocol. Two days post-transfection (if the time course is not specifically stated in the text body) cells were lysed, and CAT activity was measured by standard thin layer chromatography.<sup>39</sup> Alternatively, cells were stained with X-gal to visualize  $\beta$ -gal activity *in situ*. The staining protocol was as follows. Cells were rinsed with PBS, fixed at 4°C for 5 min in fix solution (2% formaldehyde, 0.2% (v/v) glutaraldehyde in PBS), washed with PBS three times, stained with a staining solution (20 mM potassium ferricyanide, 20 mM potassium



ferrocyanide, 2 mM MgCl<sub>2</sub> in PBS; X-Gal (in N,N-dimethylformamide) were added to final concentration 1 mg/ml) and incubated at 37°C overnight. Stained cells were visualized in light microscope.

### Microinjection

CSML100 cells were microinjected with I $\kappa$ B- $\alpha$  (mutated 32 and 36 Serines) eukaryotic expression vector together with  $\beta$ -gal eukaryotic expression vector at concentration 0.1  $\mu$ g/ml each in H<sub>2</sub>O using a Narishige micromanipulator and glass capillaries (tip diameter 0.1  $\mu$ m) under conditions of constant nitrogen flow at a pressure of 0.4 p.s.i. Each experiment approximately 200 cells were microinjected. Successful microinjection was estimated to occur greater than 70% of the time. After microinjection, the culture was washed five times with sterile PBS to minimize potential contamination during microinjection. After 48 h cells were stained *in situ* with X-gal for  $\beta$ -gal activity and visualized using light microscope. Additional step for staining with propidium iodide was used to assess chromatin condensation of  $\beta$ -gal positive cells. In this case cells were visualized in fluorescence microscope with the length of excitation 470 nm.

### Plasmids

The plasmid pN5 and pH6 contained five copies of  $\kappa$ B oligonucleotide, AGTTGAGGGGACTTCCAGGC, from the mouse Igk enhancer, and mutated NF- $\kappa$ B binding site AGTTGAGGTTTTTCCAGGC downstream of the CAT gene in the pCAT-Promoter plasmid (Promega). These plasmids were described by Tulchinsky *et al.*<sup>30</sup> Human MAD3 cDNA was inserted into pRc-CMV vector (Invitrogen) to create pI $\kappa$ B $\alpha$ -wt plasmid. Plasmid pI $\kappa$ B $\alpha$ -mut contained point mutations that converted 32 and 36 Ser to Ala. Dr. Nancy Rice kindly provided both plasmids. All plasmids were purified (two times) by centrifugation in CsCl continuous gradient prior to transfection or microinjection into eukaryotic cells. Reporter plasmid pCAT-Waf contained three p53 binding sites from p21<sup>WAF</sup> promoter in pCAT-Promoter vector (Promega) cloned through blunted *Bam*HI site. Eukaryotic expression vector for  $\beta$ -galactosidase (pCMV-gal) was purchased from Invitrogen.

### Apoptosis assays

For the fluorescence assay of apoptosis, cells were detached by Versen solution, washed with PBS and incubated for 15 min on ice with AnnexinV-FLUOS/Propidium Iodid Staining Solution according to the manufacturer's recommendations (Boehringer Mannheim). Cell suspensions were analyzed by flow cytometry. Flow cytometry was performed on a dual laser Becton/Dickinson Vantage flow cytometer using 488 nm excitation and a 515 bandpass filter for fluorescein detection and a filter >560 nm for PI detection. Recombinant human TNF- $\alpha$  was purchased from Sigma.

### Nuclear extract preparation, EMSA

For nuclear extract preparation 1  $\times$  10<sup>6</sup> cells were lysed with mild detergent NP40 and nuclear proteins were released from the nuclei by high salt concentration.<sup>40</sup> For fractionation of cytoplasmic component supernatant of lysed cells (buffer A 10 mM Hepes KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA supplemented with 1% of NP-40) was used. Binding reactions were settled using standard protocol.<sup>39</sup> For NF- $\kappa$ B binding we used <sup>32</sup>P-labeled consensus oligonucleotide 5'-AGTTGAGGGGACTTCCAGGC-3', for Oct 5'-TGTCGAATGCAAATCACTAGAA-3'; for *c-myc* 5'-GGAAGCAGACCACGTGTC-TGCTTCC-3'. We used 4% PAAG on 0.3  $\times$  TBE to resolve DNA-

protein complexes. Gels were dried and exposed to either autoradiography film for 16 h or used for electronic imaging in Packard-Canberra Company Laser based imaging system.

### Western blots

Total cell extracts were performed lysis of cells in RIPA buffer as described in<sup>39</sup>. Western blots were developed using ECL-Plus System (Amersham-Pharmacia). Following antibodies were purchased from SantaCruz Inc.: anti-p50 (H119) sc7178, anti-p65 (C20) sc372, anti-RelB (C19) sc226, anti-I $\kappa$ B- $\alpha$  (C15) sc203 or (C21) sc371, anti-I $\kappa$ B- $\beta$  (C20) sc945, anti-I $\kappa$ B- $\epsilon$  (M364) sc7155, anti-I $\kappa$ B- $\gamma$  (5177C) sc201, anti-*c-myc* (N-262) sc764, anti-bcl3 (C-14) sc185. Anti-p53 antibodies (#421) were kindly provided by Dr. Peter Chumakov.

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