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# Constitutive expression of lymphoma-associated NFKB-2/Lyt-10 proteins is tumorigenic in murine fibroblasts

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**The *NFKB-2* (Lyt-10) gene codes for an NF-κB-related transcription factor containing *rel-polyG-ankyrin* domains. Rearrangements of the *NFKB-2* locus leading to the production of 3′ truncated NFKB-2 proteins are recurrently found in lymphoid neoplasms, particularly cutaneous lymphomas. Such mutant NFKB-2 proteins have lost the ability to repress transcription that is typical of NFKB-2 subunit p52, and function as constitutive transcriptional activators. To verify whether the expression of abnormal NFKB-2 proteins can lead to malignant transformations in mammalian cells, we transfected human lymphoblastoid cell lines and murine fibroblasts (Balb/3T3) with expression vectors carrying the cDNAs coding for normal NFKB-2p52, Lyt-10Cα or LB40 proteins, which are representative of the abnormal types found in lymphoma cases. The expression of both normal and mutant NFKB-2 proteins has a lethal effect on lymphoblastoid cells and a cytotoxic effect was also observed in murine fibroblasts. The fibroblast cell lines expressing Lyt-10Cα or LB40, but not those expressing normal NFKB-2p52, were capable of forming colonies in soft agar. The analysis of individual clones revealed that cloning efficiency correlated with the expression levels of the abnormal proteins. Injection of the Lyt-10Cα-transfected Balb cells in SCID mice led to tumor formation in all of the animals, whereas no tumors were observed in the mice injected with control or NFKB-2p52-transfected cells, thus indicating that abnormal NFKB-2 protein expression is tumorigenic *in vivo*. Our results show that mutant NFKB-2 proteins can lead to the transformed phenotype, and support the hypothesis that alterations in *NFKB-2* genes may play a role in lymphomagenesis.**

**Keywords:** NF-κB; NFKB-2; transcription factor; transformation

## Introduction

The NF-κB proteins are a family of transcription factors that regulate the transcription of a wide variety of genes, including those involved in immune response and the control of cell growth (Grilli *et al*., 1992; Baeuerle and Henkel, 1994).

The *NFKB-2* gene homologous to *NFKB-1* (Bours *et al*., 1990; Ghosh *et al*., 1990) encodes a 100 Kd protein containing an N-terminal DNA binding domain (*rel*-domain), a poly(G) hinge as a site of proteolytic cleavage, and a carboxy-terminal domain containing *ankyrin* repeats (Neri *et al*., 1991; Schmid *et al*., 1991; Bours *et al*., 1992). NFKB-2p100 is the primary translational product and is localized in the cytoplasm. The active NFKB-2p52 subunit, which retains the *rel*-domain and loses the *ankyrin* regions, derives from the post-translational processing of p100 and, upon NF-κB activation, can be found in the nucleus as part of NF-κB complexes (Mercurio *et al*., 1993; Chang *et al*., 1994). NFKB-2p52 has no intrinsic transcription activity, but positively regulates NF-κB transcription when complexed with RelA or the IκB-like protein Bcl-3; the homodimers have inhibitory effect (Bours *et al*., 1993; Nolan *et al*., 1993; Mercurio *et al*., 1993; Chang *et al*., 1994). NFKB-2 activity is thus regulated at various integrated levels in both the cytoplasm and the nucleus, and is dependent on protein-protein interaction.

Accumulating evidence indicates that the members of the NF-κB family may be involved in oncogenesis. The retroviral variant of c-*rel*, v-*rel*, is a potent oncogene in avian cells (Beug *et al*., 1981; Gilmore and Temin, 1988), and chromosomal alterations involving c-*rel* (Lu *et al*., 1991) and the IκB-related protein, Bcl-3 (Ohno *et al*., 1990), have been found in lymphoid neoplasms. The transforming activity of a variant form of RelA called p65Δ has been reported, although this finding is controversial (Narayanan *et al*., 1992; Grimm and Baeuerle, 1994). Furthermore, the expression of antisense RelA RNA reduces tumorigenicity (Higgins *et al*., 1993) and the disruption of IκBα regulation by antisense RNA expression leads to malignant transformation (Beauparlant *et al*., 1994). With regard to NFKB-2, a number of observations support the hypothesis that structural alterations in the *NFKB-2* locus may play a role in lymphomagenesis. The *NFKB-2* (Lyt-10) gene was originally cloned from a chromosomal translocation t(10;14) (q24;q32) involving the IgHCα locus in a case of B-cell non Hodgkin's lymphoma (Neri *et al*., 1991); as an effect of the chromosomal rearrangement a truncated form of the NFKB-2 protein was encoded that lacks the *ankyrin* domain and is constitutively localized in the nucleus. Analysis of a large panel of different subtypes of human lymphoid tumors indicates that *NFKB-2* rearrangements are present in about 2% of cases, including non Hodgkin's lymphoma, chronic lymphocytic leukemia (B-CLL), multiple myeloma, and particularly in cutaneous lymphomas (10%) (Fracchiolla *et al*., 1993). Molecular analysis of the

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breakpoints within the *NFKB-2* gene from several cases showed that these rearrangements can occur as a consequence of translocations or internal deletions leading to the removal of variable portions of the *ankyrin* domain (Migliazza *et al*., 1994), thus suggesting that truncation within the *ankyrin* domain may be the general mechanism underlying the constitutive activation of NFKB-2 *in vivo*. Further and more convincing data supporting this hypothesis derive from the functional analysis of two abnormal NFKB-2 proteins found in B-cell lymphomas (Chang *et al*., 1995), whose functional abnormalities suggest that they can escape the physiological regulatory mechanisms of NFKB-2 proteins, such as proteolytic processing and retention by IκB molecules. In particular, they are localized in the nucleus and bind DNA as primary translational products. These proteins maintain their ability to heterodimerize with RelA and transactivate κB-dependent transcription, but have lost their transcription repressing activity as homodimers and, furthermore, may transactivate regardless of their association with RelA or Bcl-3.

Although structural and functional analyses of abnormal NFKB-2 proteins suggest that the *NFKB-2* gene may be a protoncogene, there is still no direct evidence of their ability to transform mammalian cells. We addressed this point by directly analysing the biological effects of the constitutive expression of two abnormal NFKB-2 proteins in different cell lines. We found that mouse fibroblasts expressing altered NFKB-2 proteins are capable of growing in soft agar and forming tumors in SCID mice, thus suggesting that altered NFKB-2 proteins may play a role in tumor transformation.

## Results

### *Generation of cell lines expressing mutant NFKB-2 proteins*

Our previous studies have shown that all of the rearrangements within the NFKB-2 gene involve the 3′ coding region corresponding to the *ankyrin* domain (Fracchiolla *et al*., 1993; Migliazza *et al*., 1994). The consequence of these rearrangements is the generation of 3′ truncated NFKB-2 proteins or, in a minority of cases, fusion proteins with heterologous tails of different lengths. Figure 1 schematically shows the three abnormal NFKB-2 proteins used in our experiments as representative models of the rearranged proteins generated in tumor cells *in vivo*. The characteristics of these proteins have already been extensively described. Briefly, the Lyt-10Cα variant deriving from a case of B-cell non-Hodgkin's lymphoma (RC685) is a fusion protein in which the first *ankyrin* repeat is linked to a tail of 174 aa generated by an off-frame Cα coding region (Neri *et al*., 1991; Chang *et al*., 1995). The HUT-78p85 protein derived from a T-cell lymphoma cell line (Zhang *et al*., 1994) is a truncated form that maintains five *ankyrin*-repeats. The LB40 protein was cloned from a case of B-CLL (Migliazza *et al*., 1994) and is the longest truncated protein we have found in the tumor cases analysed so far, having its breakpoint within the sixth *ankyrin* repeat. The transcriptional and DNA-binding

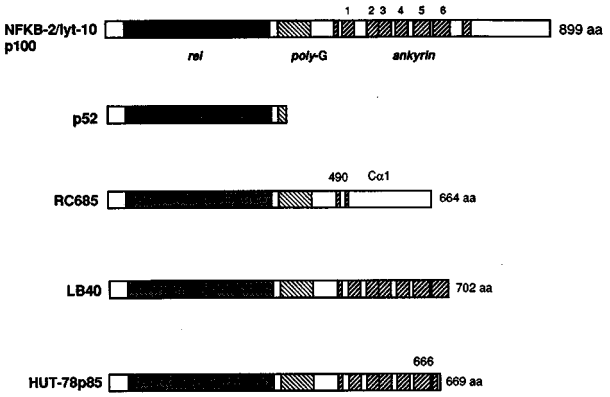


**Figure 1** Schematic representation of normal and abnormal NFKB-2 proteins, showing the *rel*, poly-G and *ankyrin* domains. 1–6 *ankyrin* repeats. The predicted number of amino acids (aa) of each protein is shown on the right. The number of amino acid residues of normal NFKB-2 protein is also indicated for each protein

activities of the Lyt-10Cα and HUT-78p85 proteins have been extensively analysed (Chang *et al*., 1995), and our unpublished data indicate that the LB40 variant has similarly abnormal functional activities. The involvement of the *NFKB-2* locus in the chromosomal rearrangements found in B-cell lymphoma prompted us to select human lymphoblastoid cell lines (LCL) immortalized by the Epstein-Barr virus as the specific targets for our experiments. Using vectors in which the cDNAs coding for the NFKB-2p52 and abnormal proteins were expressed under the control of the cytomegalovirus promoter (CMV), we could not select any survival clone in three different cell lines. Assuming that the constitutive expression of the abnormal proteins may be cytopathic for LCLs, we used the genomic clone of Lyt-10Cα including the 5′ promoter region, in the presence of the enhancer element of the IgH locus, in an attempt to obtain a more regulated expression of the mutant protein. We were able to obtain a few clones expressing Lyt-10Cα mRNA, but none expressing Lyt-10Cα protein. On the contrary, when transfected with CMV Lyt-10Cα the Burkitt cell line Daudi did express Lyt-10Cα protein (data not shown), thus suggesting that the possibility of expressing abnormal NFKB-2 proteins in B-cells may be related to the stage of differentiation or to the transformed phenotype.

Using CMV vectors, we transfected immortalized Balb/3T3 mouse fibroblasts. Although we were unable to obtain clones expressing HUT-78p85, we did obtain clones expressing NFKB-2p52, Lyt-10Cα or LB40 proteins. Significantly fewer survival clones were observed after selection in all of the transfected cell lines expressing the three proteins than in the control cells transfected with the vector alone (Table 1), which suggests that the constitutive expression of the three proteins has cytopathic effects on various cell lines. Figure 2 shows the mRNA and protein expression of the polyclonal transfected cells. Using the NFKB-2 cDNA fragment SmaI–HincII as a probe, RNAse protection analysis (Figure 2a) indicated that the NFKB-2p52, Lyt-10Cα and LB40 mRNAs have the expected size and a similar level of expression. Immunofluorescence analysis using anti-Lyt-10 antiser-

**Table 1** Toxicity of NFKB-2 proteins in mouse fibroblasts

| *Transfected cells* | *Exp 1** | *Exp2** |
|---|---|---|
| Balb-pCMV | 153.3±23.8 | 197.5±17.4 |
| Balb-pCMVp52 | 36.7±6.6 | 54.0±6.0 |
| Balb-pCMVLyt-10Cα | 23.5±5.7 | 37.8±4.6 |
| Balb-pCMVLB40 | 46.3±7.5 | 57.5±8.5 |

*The resistent clones were scored 14 days after the cells were seeded at $10^4$/cells per culture dish in hygromycin-containing medium. The average number was obtained from at least four examinations and is followed by the standard error

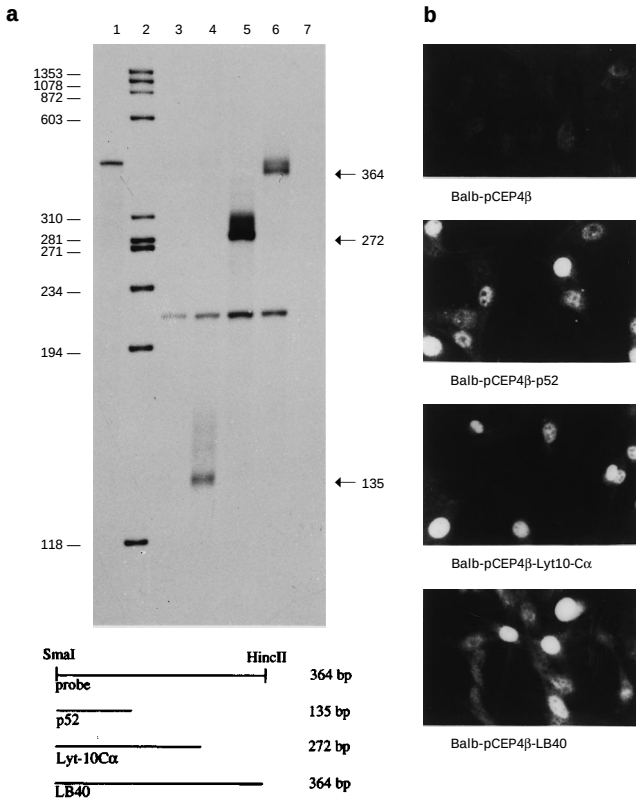


**Figure 2** Expression of NFKB-2 mRNAs and proteins in Balb/3T3 cells. (**a**) RNAse protection analysis: 20 μg of total RNA from fibroblasts transfected with plasmid pCEP4*β* as control (lane 3), pCEP4*β*-NFKB-2p52 (lane 4), pCEP4*β*-Lyt-10Cα (lane 5) and pCEP4*β*-LB40 (lane 6) were hybridized to $\alpha^{32}$P-labeled antisense riboprobe spanning sequences 1267–1631 of NFKB-2 cDNA. tRNA was used as a negative control (lane 7). The probe and the various protected fragments relating to NFKB-2 cDNA are shown at the bottom. The band between markers 234 and 194 bp, which is common to all of the transfected cells, derives from cross-hybridization with mouse NFKB-2 mRNA. Lane 1: Probe; lane 2: *ϕ*X174 DNA markers. (**b**) Immunofluorescence analysis. After selection in hygromycin, the various bulk transfected cells were analysed using an antiserum recognizing the N-terminal region of NFKB-2 protein

um (Neri *et al*., 1991) confirmed the nuclear localization of the transfected proteins and indicated that the selected bulk populations were representative of several clones with different levels of protein expression (Figure 2b). EMSA analysis using HLA-κB probe and nuclear extracts from transfected cells revealed increased DNA binding activity in the cells expressing Lyt-10Cα and LB-40 proteins (data not shown). Western-blot analysis to detect RelA, RelB, c-rel and NFKB-1 proteins indicated increased expression of NFKB-1 in the Lyt-10Cα and LB-40 transfected cells, whereas no relevant differences in RelA, RelB or c-rel levels were observed (data not shown). Experiments are currently being undertaken in order to investigate further the alterations within the NF-κB system in Lyt-10Cα and LB-40 expressing cells.

### *Fibroblasts expressing Lyt-10Cα or LB40 proteins are clonogenic in soft agar*

Analysis of the morphological phenotype and growth pattern of the transfected cells showed that the morphology and saturation densities of the control, NFKB-2p52, Lyt-10Cα and LB40 polyclonal cell populations were similar; we could not detect any differences in their growth curves at serum concentrations of 10% or even as low as 1–2% (data not shown). We then analysed the ability of the cells to grow in soft agar as an assay for the transformed phenotype *in vitro*. Despite the fact that a low background level was found in the control cells, the cloning efficiency of the Lyt-10Cα and LB40 cells was significantly greater than that of the NFKB-2p52 or control cells (Table 2). Moreover, the colonies in the LB40 and Lyt-10Cα populations were much larger than those observed in the control or NFKB-2p52 cells (data not shown). In order to verify directly whether this ability to form colonies in agar is related to the expression of abnormal NFKB-2 proteins, we isolated and analysed individual clones expressing Lyt-10Cα. The data in Table 3 clearly indicate that there is a direct correlation between mRNA expression and cloning efficiency.

### *Fibroblasts expressing Lyt-10Cα protein are tumorigenic* in vivo

We finally tested the ability of Lyt-10Cα cells to form tumors *in vivo* when injected subcutaneously into SCID mice. As shown in Table 2, only the mice injected with Lyt-10Cα cells developed tumors (100% of the injected mice), whereas no tumors were detected in the mice injected with control or NFKB-2p52 cells during the

**Table 2** Tumorigenicity of abnormal NFKB-2 proteins *in vitro* and *in vivo*

| *Cell lines* | *Cloning efficiency*[a] | *Tumors/ injections*[b] | *Tumorigenicity* in vivo *Latency time*[c] (>3 mm) | *Survival time*[c] (>10 mm) |
|---|---|---|---|---|
| Balb-pCMV | 0.046±0.007 | 0/4 | 0 | 0 |
| Balb-pCMVp52 | 0.023±0.003 | 0/4 | 0 | 0 |
| Balb-pCMVLyt-10Cα | 1.206±0.103* | 4/4 | 20.5±11 | 44.9±5.8 |
| Balb-pCMVLB40 | 1.330±0.418* | ND | ND | ND |

[a] Colonies were examined 14 days after the cells were seeded at $10^3$, $2\times10^3$, $10^4$ cells per culture dish. The data are the average of at least three experiments and are followed by the standard error. [b] Five-week-old female SCID mice were subcutaneously injected with $10^6$ transfected cells. The data show the numbers of tumors of 3 mm or more *versus* the number of mice injected [c] Number of days±s.d. *$P<0.01$ vs control cells. ND: not determined

**Table 3** Correlation between cloning efficiency and mRNA expression in Balb-pCMVLyt-10Cα cells

| | *Cloning efficiency*[a] | *mRNA expression*[b] |
|---|---|---|
| *Balb-pCMVLyt-10Cα* | | |
| Clone 1 | 0 | − |
| Clone 2 | 0.08 | + |
| Clone 3 | 0.33 | + + |
| Bulk | 1.19 | + + + |

[a] The data are the average of at least two experiments, performed in duplicate at $10^3$, $2 \times 10^3$, $10^4$ cells per culture dish. [b] RNA expression was determined by RNAase protection analysis

two months of observation. Two of the tumors were excised and found to express high levels of Lyt-10Cα mRNA (data not shown).

## Discussion

Structural and functional alterations in the NFKB-2 proteins associated with lymphoid neoplasms have led to the hypothesis that *NFKB-2* gene alterations may be involved in lymphomagenesis. In an attempt to substantiate this hypothesis, the aim of our study was to verify whether two lymphoma-derived abnormal NFKB-2 proteins were capable of transforming mammalian cells *in vitro* and being tumorigenic *in vivo*. Our data show that the constitutive expression of the two altered proteins in mouse fibroblasts allows growth in soft agar and tumor formation in immunodeficient mice. These data provide the first evidence of the potentially oncogenic effect of altered NFKB-2 proteins *in vivo* which implies that they do play a role in lymphomagenesis.

### *Cytopathic effect of NFKB-2p52, Lyt-10Cα and LB40 proteins*

Our attempt to express NFKB-2 proteins failed to generate transfected human LCLs and led to the poor clone recovery of mouse fibroblasts. The mechanisms of this cytopathic effect (slowed or arrested growth, or cell death) have not yet been elucidated, but they seem to be related to the cell context and/or to the level of protein expression, as has been observed in relation to other oncogenes such as c- and v-*rel* (Gelinas and Temin, 1988; Schwartz and Witte, 1988; Hannick and Temin, 1989; Lu *et al*., 1991; Abbadie *et al*., 1993), v-*src* (Tarpley and Temin, 1984), v-and c-*abl* (Ziegler *et al*., 1981; Renshaw *et al*., 1992; Sawers *et al*., 1994) and the gene encoding the Latent Membrane Protein of EBV (Hammerschmidt *et al*., 1989). The fact that both normal and mutant NFKB-2 proteins have a similar cytophatic effect suggests that this may not be relevant in terms of transformation.

### *Transforming activity of Lyt-10Cα and LB40 proteins, and role of altered NFKB-2 proteins in lymphomagenesis*

Our data show that fibroblasts expressing Lyt-10Cα and LB40 proteins, but not those expressing normal NFKB-2p52, are capable of growing in soft agar and forming tumors in inmmunodeficient mice. The mutant NFKB-2 proteins are functionally abnormal, having lost their transcription repressing activity and acquired constitutive transactivating activity (Chang *et al*., 1994). Although the direct consequences of these alterations are still unknown, it is conceivable that the expression of mutant NFKB-2 proteins may lead to changes in the composition of NF-κB complexes and alterations in NF-κB functions. It is also possible that these altered proteins may deregulate the expression of target genes that preferentially bind NFKB-2-containing complexes. Our data demonstrate that the overall effect of the functional alterations in mutant NFKB-2 proteins is the ability to induce transformed phenotype. On the other hand, the capacity of the cells to express these proteins seems to be related to the level of expression and/or to one or more possibly genetic events that silence the cytopathic effect (see Discussion above). In the light of this, it can be speculated that the expression of mutant NFKB-2 proteins play a role in the transformation process only if it occurs as a secondary or late event.

Among the NF-κB transcription factors, transforming activity has only been demonstrated in relation to v-*rel* (Beug *et al*., 1981; Gilmore and Temin, 1988), although controversial data has been published regarding the variant p65Δ (Narayanan *et al*., 1992; Grimm and Baeuerle, 1994). Our data provide new evidence that alterations in NF-κB factors may play a role in oncogenesis. More specifically, the recurrent associations betwen *NFKB-2* rearrangements and lymphoid neoplasms suggest that these alterations may be involved in the pathogenesis of these tumors. The finding that lymphoma-derived NFKB-2 proteins are capable of transforming mammalian cells strongly supports the hypothesis that the rearrangements in the NFKB-2 locus leading to the production of 3′ truncated NFKB-2 proteins may be the common mechanism of NFKB-2 activation as a genetic event involved in lymphomagenesis.

## Materials and methods

### *Expression vectors*

An EcoRI – XhoI fragment of NFKB-2 cDNA coding for a C-terminal truncated protein containing the *rel* domain and lacking the *ankyrin* repeats (Neri *et al*., 1991), and the EcoRI – EcoRI fragments containing the cDNA coding for Lyt-10Cα, LB40 and Hut-78p85 variants generated by chromosomal rearrangements (Neri *et al*., 1991; Migliazza *et al*., 1994; Zhang *et al*., 1994), were blunted and cloned into the PvuII restriction site of the expression vector pCEP4β (Invitrogen, San Diego, CA).

### *Cell cultures and transfections*

The Balb/3T3 fibroblast cell line was grown in Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The lymphoblastoid cell lines, CB33, UH-1 and UH-2, as well as the Burkitt cell line Daudi, were maintained in Iscove's medium supplemented with 10% FCS. To generate stable transfected cell lines, the Balb/3T3 cells were plated at $3 \times 10^5$ cells/100 mm Petri dish 24 h before the experiments; the cells were transfected according to a modified protocol of the $CaPO_4$ precipitation method (Gu *et al*., 1993). Forty-eight hours after transfection, $10^5$ cells were seeded in selective medium containing 300 μg/ml of hygromycin B (BMB) and incubated at 37°C in 5% $CO_2$ for 2 weeks; single clones

or pools of transfected cells were grown for further analysis. The lymphoid cells were transfected using the electroporation method (250 volts, 900 capacitance) and, 48 h after transfection, were grown in a selective medium containing 300 $\mu$g/ml hygromycin B for 3 weeks.

*RNA extraction and RNAse protection analysis*

RNA was extracted using the guanidine isothiocyanate method, and analysed by RNAse protection assay. An antisense riboprobe of 364 bp (SmaI – HincII fragment of NFKB-2 cDNA) was generated in the presence of $\alpha^{32}$ CTP. $5 \times 10^5$ c.p.m. of riboprobe were hybridized with 20 $\mu$g of total RNA for 16 h at 60°C, and digested with RNAseT1 and RNAseA for 7 min at RT. The protected fragments were resolved in a 6% denaturing polyacrylamide gel and analysed by autoradiography.

*Growth in soft agar*

The trypsinized cells were resuspended in Iscove's medium containing 20% FCS and 0.3% agar (Difco). The cell suspensions were plated at $10^3$, $2 \times 10^3$, $10^4$ on a layer of medium containing 20% FCS and 0.5% agar. Colony growth was scored after 14 days.

*Cytotoxicity assay*

Forty-eight hours after transfection, the cells were seeded at $10^4$ into selective medium. The number of hygromycin-resistant clones was scored after 14 days.

*Indirect immunofluorescence staining*

$3 \times 10^5$ cells were plated onto cover slips the day before the experiments. After fixation in 3% paraformaldehyde in PBS for 30 min, they were extensively washed in PBS and permeabilized with 0.2% Triton X-100 in PBS. After being blocked for 4 h in 3% bovine serum albumin in PBS at RT, the cells were incubated with polyclonal antiserum 8892 (Neri *et al*., 1991) (1 : 2000 dilution) for 16 h at 4°C. FITC-conjugated goat IgG directed against rabbit IgG (1 : 50 dilution) were added for 30 min at RT. The slides were extensively washed and mounted in 50% glycerol-PBS. The photographs were taken using a Leitz Dialux microscope (Leitz, Wetzlar, Germany).

In vivo *tumorigenicity assay*

Five-week-old female SCID mice (C.B-17™/IcrCrl-scidBR) were purchased from Charles River Laboratories (Calco, Italy) and treated in accordance with the European Community guidelines. They were fed and maintained under specific pathogen-free conditions, and received sterilized food pellets and tap water *ad libitum*. The mice were challenged subcutaneously in the left inguinal region with 0.3 ml of a single cell suspension containing $1 \times 10^6$ cells of the various Balb/3T3 cell lines. The cages were coded and the incidence and growth of tumors were evaluated twice weekly, with the investigators being blind as to the treatment the animals had received. The two perpendicular diameters of the neoplastic masses were measured with calipers for 60 days. The mice which were tumor-free at the end of this period were classified as survivors. The latency and survival times were respectively considered as the period (in days) between the challenge and growth of neoplastic masses with mean diameters $>3$ and $>10$ mm. Only the mice that eventually developed tumors were considered. The mice bearing neoplastic masses with a mean diameter $>10$ mm were killed, and the tumor masses were excised and routinely processed for histological evaluation.

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