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Regulation of major histocompatibility complex class I expression by NF-κB-related proteins in breast cancer cells

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Downregulation of MHC Class I antigens has been observed in many cancers and usually results from a decreased gene transcription. A reporter CAT gene dependent on the MHC Class I κB site or on a longer promoter is transactivated by NF-κB complexes containing p65 or RelB. p100 as well as IκB-α are potent inhibitors of this transcription and p100 sequesters RelB and p65 complexes in the cytoplasm of breast cancer cells. However, although p100 is highly expressed in a number of breast cancer cell lines, MHC Class I antigen expression was observed on all the cell lines we analysed and could be further induced by stimulation with the cytokines IFN-γ or TNF-α. Stable transfection of a unresponsive mutated IκB-α Ser 32-36 expression vector showed that TNF-α induced MHC Cl I expression in an NF-κB-dependent way while IFN-γ did it independently of any NF-κB activation.

Keywords: MHC class I; NF-κB, breast cancer

Introduction

The function of the Major Histocompatibility Complex (MHC) is critical for antigen presentation and immune response (Accolla *et al*., 1995). MHC Class I (MHC Cl I) antigens are required for cell recognition and subsequent destruction by cytotoxic T lymphocytes. Down-regulation or loss of MHC Cl I antigens have been described in many solid tumors and probably allow them to escape T cell-mediated immune surveillance (Blanchet *et al*., 1992; Garrido *et al*., 1995). Moreover, MHC Cl I expression is lower in metastases than in primary tumors or in metastatic compared to non metastatic cell lines (Cordon-Cardo *et al*., 1991; Plaskin *et al*., 1993).

Several mechanisms can account for a down-regulation of MHC Cl I antigens function in cancers: loss of MHC Cl I genes, mutation in the β2-microglobulin associated protein or defects in the assembling and cellular transport of MHC Cl I / β2-microglobulin/peptide antigen complexes (Garrido *et al*., 1995). However, the low expression of MHC Cl I antigens is very often associated with a decrease of gene transcription.

The promoter of the MHC Cl I gene contains three major regulatory elements: the enhancer A or Class I regulatory element (CRE), the sequence ICS (Interferon Consensus Sequence) and the enhancer B (Garrido *et al*., 1995). The enhancer A can be divided in two regions, I and II. Region I binds the transcription factors NF-κB, KBF1 and H2TF1 (Baldwin and Sharp, 1987, 1998; Israël *et al*., 1987).

NF-κB is an ubiquitous transcription factor regulating the expression of a large variety of genes and viruses (Siebenlist *et al*., 1994). It is made itself from a family of proteins which are characterized by a conserved Rel homology domain (RelHD) responsible for dimerization, nuclear translocation and specific DNA-binding. Among these proteins, p65 (RelA), RelB and c-Rel contain one or two transactivating domains (Siebenlist *et al*., 1994). Two other proteins belonging to the same family, p50 and p52 do not harbor any transactivating motif and can thus inhibit NF-κB-dependent transcription by binding to DNA as homodimers (Bours *et al*., 1990, 1992; Kieran *et al*., 1990; Neri *et al*., 1991; Franzoso *et al*., 1992).

NF-κB complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the IκB family (Beg and Baldwin, 1993; Baeuerle and Henkel, 1994; Siebenlist *et al*., 1994; Miyamoto and Verma, 1995). The members of the human IκB family are IκB-α, IκB-β, IκB-ε, p100 and p105 (Haskill *et al*., 1991; Rice *et al*., 1992; Beg and Baldwin, 1993; Mercurio *et al*., 1993; Whiteside *et al*., 1997). Bcl-3 is also a member of this family but, at least in some cellular types, is located in the nucleus and participates in NF-κB transactivating activity (Ohno *et al*., 1990; Bours *et al*., 1993; Nolan *et al*., 1993; Watanabe *et al*., 1997).

NF-κB has been shown to participate in the control of the MHC Cl I genes basal expression as well as in their transcriptional upregulation following treatment by retinoids and TNF-α (Israël *et al*., 1987, 1989; Logeat *et al*., 1991; Segars *et al*., 1993; van't Veer *et al*., 1993). Moreover, inhibition of NF-κB activity has been associated with the downregulation of MHC Cl I expression in tumor cell lines and in adenovirus 12-transformed cells (Blanchet *et al*., 1992; van't Veer *et al*., 1993; Schouten *et al*., 1995; Liu *et al*., 1996). KBF1 has been purified and is identical to transactively inactive p50 homodimers (Yano *et al*., 1987; Kieran *et al*., 1990). It indeed acts as a repressor of MHC Cl I expression in metastatic cancer cells (Plaskin *et al*., 1993).

The proteins p100 and p105, precursors of p52 and p50 respectively, function as IκB-like molecules (Rice *et al*., 1992; Mercurio *et al*., 1993; Dejardin *et al*., 1995) and thus sequester NF-κB complexes in the cytoplasm.

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A downregulation of p105 processing into p50 has been associated with decreased MHC Cl I expression in adenovirus 12-transformed cells (Schouten *et al*., 1995). The H2TF1 transcription factor was shown to contain the p100 protein which thus could bind *in vitro* the MHC Cl I κB site (Potter *et al*., 1993; Scheinman *et al*., 1993). However, several reports confirmed that p100 is mostly localized in the cytoplasm where it sequesters other NF-κB proteins (Mercurio *et al*., 1993; Potter *et al*., 1993; Scheinman *et al*., 1993; Dejardin *et al*., 1995). In a previous report, we demonstrated high expression of p100 in breast cancer cell lines and in primary tumors (Dejardin *et al*., 1995). In these cell lines, p100 is the major NF-κB inhibitor and sequesters most p65 protein in the cytoplasm.

The putative role of p100 in human cancer cells remains to be elucidated. The H2TF1 data indicate that p100 probably plays a role in the regulation of MHC Cl I expression in normal and maybe in cancer cells. It is not clear, however, whether p100 sequesters other NF-κB proteins in the cytoplasm and thus downregulates MHC Cl I expression or whether trace amounts of H2TF1 or p100 can translocate to the nucleus and participate in the basal expression of MHC Cl I proteins.

This study investigates the regulation of MHC Cl I expression in breast cancer cells by NF-κB and IκB proteins. It demonstrates that, in transient transfection assays, NF-κB complexes containing p65 or RelB can activate transcription of a reporter CAT-gene driven by the κB site from the MHC Cl I promoter or by a more complete promoter. We also show a novel trimeric cytoplasmic complex formed of p100, p50 and RelB in MDA-MB-231 breast adenocarcinoma cells. While we could not find any correlation between p100 expression and basal or induced MHC Cl I expression on the surface of breast cancer cell lines, stable expression of a unresponsive IκB-α mutant inhibited Tumor Necrosis Factor-α- but not Interferon-γ-induced MHC Cl I expression.

Results

NF-κB transactivates through the κB site from the MHC class I promoter

The NF-κB site of the MHC Cl I was inserted in position −56 of the minimal c-*fos* promoter of a CAT reporter plasmid in order to study the NF-κB transactivation efficiency through this particular site. This reporter plasmid, named MHC-κB-CAT, was transfected into the breast cancer derived cell line MCF7 A/Z together with expression vectors directing the production of various NF-κB-related proteins. The transactivating activities of p50/p65, p52/p65, p50/c-Rel, p52/c-Rel, p50/RelB and p52/RelB heterodimers were determined by measuring CAT activities in the transfected MCF7 A/Z cells (Figure 1a). The p65 and RelB-containing complexes induced significant CAT activity. The most important effect was observed with p50/p65 or p50/RelB (about a 10-fold induction over control CAT activity) whereas c-Rel-containing complexes did not transactivate the reporter plasmid. The same experiments were repeated in MDA-MB-435 cells and showed similarly that p50/p65 and RelB containing complexes were the most active for the transactivation of the MHC-κB-CAT plasmid while c-Rel complexes were only weak transactivators (data not shown).

This transactivating effect was dose-dependent since transfections with increasing amounts of p50/p65 or p50/RelB expression vectors led to progressively increased CAT activities (data not shown).

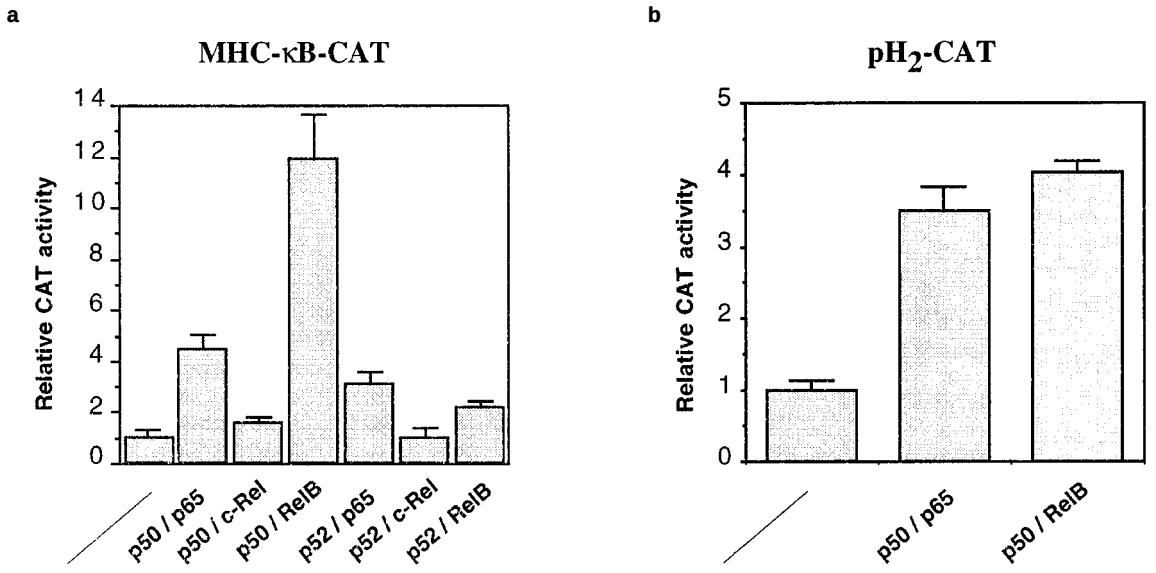

Figure 1 Various NF-κB complexes transactivate the MHC Class I promoter. MCF7 A/Z cells were transfected with expression vectors for various NF-κB-related proteins together with a CAT reporter plasmid containing a single κB site from the MHC Cl I gene promoter (MHC-κB-CAT) (**a**) or a longer MHC Cl I promoter (pH_2-CAT) (**b**). 0.5 μg of each expression vector were transfected as indicated in the figure together with 3 μg of the reporter plasmid. The figure shows the relative CAT activity over the activity observed with the CAT vector alone after normalization to the protein concentration of the extracts. Each column represents the mean of three independent experiments (± sd). The total amount of transfected DNA was kept constant throughout the experiment by adding appropriate amounts of the expression vector without insert

Experiments performed with a longer MHC Cl I promoter regulating CAT expression (pH_2-CAT) showed that the p50/p65 and p50/RelB complexes can also stimulate transcription through this promoter (Figure 1b).

Inhibition of NF-κB-dependent transactivation by p100 and IκB-α

As we had previously observed high p100 expression in human breast cancers (Dejardin *et al*., 1995), p100 and IκB-α-mediated inhibition of NF-κB-induced transactivation of the MHC-κB-CAT reporter plasmid were compared. MCF7 A/Z cells were transfected with fixed amounts of the p50 and p65 or p50 and RelB expression vectors together with increasing amounts of p100 or IκB-α expression vectors (Figure 2). In these conditions, a strong and dose-dependent inhibition of the CAT expression was observed with both inhibitory proteins. This inhibitory effect seemed to be more dramatic with IκB-α than with p100 as the transfection of 0.5 μg of the IκB-α expression vector already completely abolished the induced transcription. Interestingly, in the same experimental conditions, the IκB-like protein p105 did not produce any inhibition of the transactivation (data not shown).

p100 sequesters RelB in the cytoplasm of breast cancer cells

The expression of various NF-κB and IκB-related proteins in breast cancer cell lines was investigated. p100 expression can easily be detected in a number of breast adenocarcinoma cell lines (MDA-MB-231, MDA-MB-435, T47D and MCF7 A/Z) (Figure 3) as well as in primary breast cancers (Dejardin *et al*., 1995). Among these cell lines, the highest level of p100 expression was observed in MDA-MB-435 cells (Figure 3). Similarly, immunoblots demonstrated RelB expression in these four cell lines with the strongest signal observed in MDA-MB-231 cells and the weakest in MCF7 A/Z cells (Figure 3). The level of p65 expression was similar in the four cell lines (Figure 3).

We had previously shown that in MDA-MD-435 cells, p100 is the major NF-κB inhibitor and sequesters most p50/p65 complexes in the cytoplasm (Dejardin *et al*., 1995). To investigate how p100 and IκB-α form cytoplasmic complexes with p65 or RelB, cytoplasmic extracts from the same four breast cancer cell lines were immunoprecipitated with antibodies directed against p65 or RelB. The precipitated materials were analysed by immunoblots with specific antibodies recognizing IκB-α or p100 (Figure 4). p65 was sequestered in the cytoplasm by both IκB-α and p100 in all the cell lines with the exception of MDA-MB-435 while RelB coimmunoprecipitates only with p100 and not with IκB-α in all four cell lines. In MDA-MB-435 cells, highly expressed p100 sequesters all detected p65 and RelB in the cytoplasm as we could not observe any coimmunoprecitation of these two proteins with IκB-α. The specificity of the immunoprecipitations was verified by the addition of the peptides used to generate the antibodies (Figure 4). These experiments confirmed that p100 was the major inhibitor of RelB-containing NF-κB complexes as already demonstrated by others (Dobrzanski *et al*., 1995).

p100 can form trimeric complexes with p50 and p65 in Jurkat and in MDA-MB-435 cells (Kanno *et al*., 1994; Dejardin *et al*., 1995). Double immunoprecipitations were performed to determine whether p100/p50/RelB complexes were formed in breast cancer cells. In these experiments, cytoplasmic extracts were first immunoprecipitated with RelB antibodies and the supernatant was discarded. The immune complexes were then dissociated with an excess of RelB peptides and the supernatant was immunoprecipitated with anti-p50 antibodies. The material which had been immunoprecipitated successively by RelB and p50 antibodies was finally analysed on immunoblots with antibodies recognizing specifically p100 (Figure 5). In

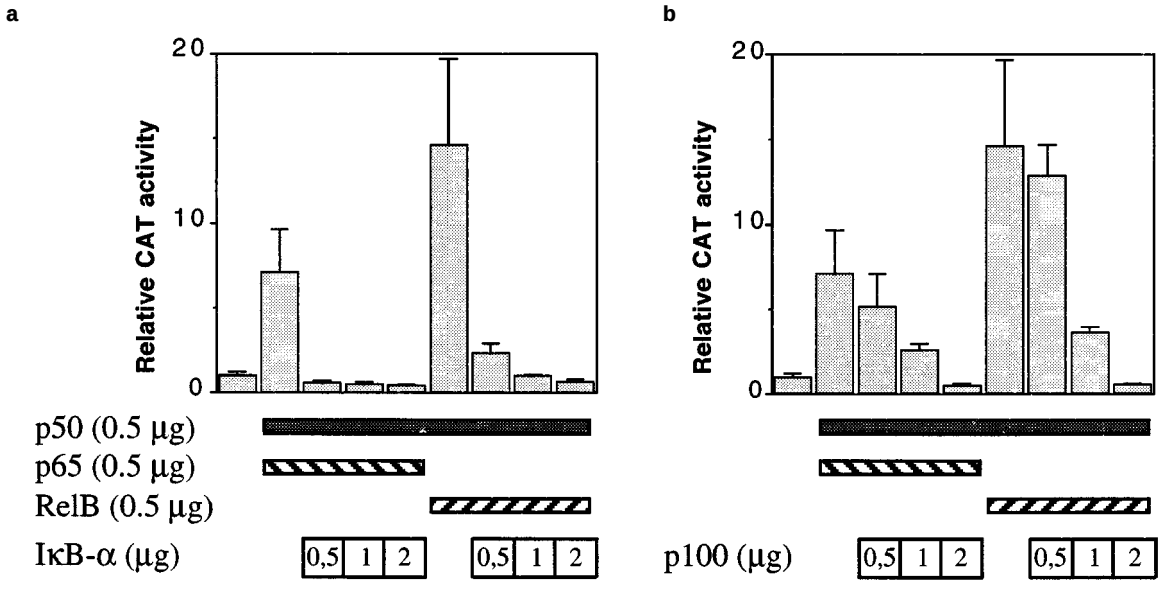

Figure 2 p100 and IκB-α inhibit NF-κB-dependent transactivation of the MHC-κB-CAT reporter plasmid. MCF7 A/Z cells were transfected with expression vectors for p50, p65 and RelB (0.5 μg each) together with the MHC-κB-CAT reporter plasmid (3 μg). Increasing amounts of expression vectors for IκB-α (**a**) or p100 (**b**) were cotransfected as indicated in the figure

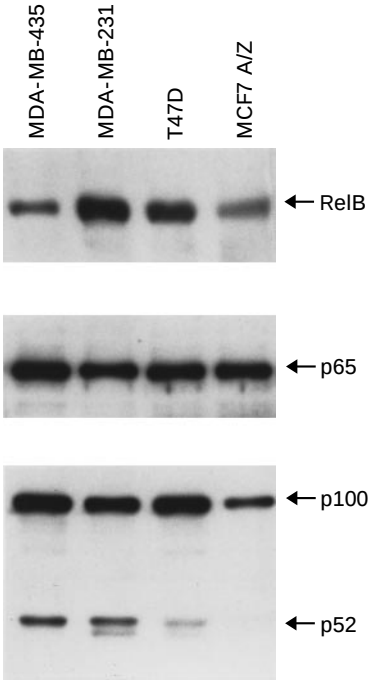

Figure 3 Expression of p100, p65 and RelB in breast cancer cell lines. Equal amounts of total cell extracts (10 μg) from four different breast cancer cell lines (MDA-MB-435, MDA-MB-231, T47D and MCF7 A/Z) were analysed by immunoblots for expression of RelB, p65 and p100. Specific bands are indicated in the figure. p52 refers to the processed form of p100

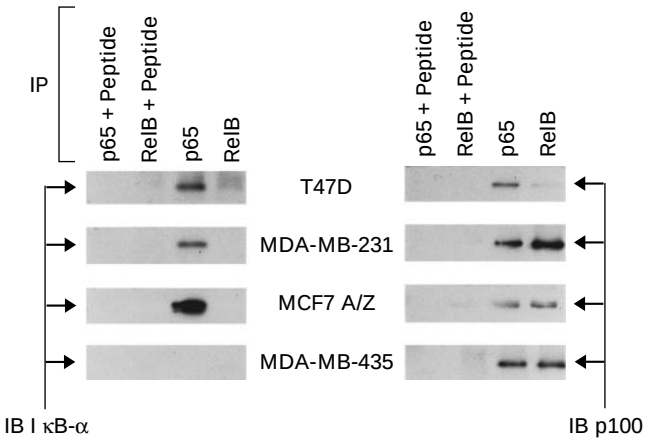

Figure 4 p100 and RelB are coimmunoprecipitated from cytoplasmic extracts. Cytoplasmic extracts from four breast cancer cell lines were immunoprecipitated (IP) with anti-p65 or anti-RelB antibodies. The immunoprecipitated material was then analysed on immunoblots (IB) for the presence of IκB-α (left panel) or p100 (right panel). As controls, the immunoprecipitations were also performed in the presence of the p65 and RelB peptides used to generate the antibodies

these experimental conditions, p100/p50/RelB complexes were only detected in the MDA-MB-231 cells. In the other cell lines, p100 forms a complex with RelB as shown in Figure 4 but it is apparently not engaged in multimeric complexes with p50 and RelB (Figure 5).

Induction of MHC Cl I expression by IFN-γ and TNF-α in breast cancer cells

The expression of MHC Cl I proteins on the surface of breast cancer cells was studied by flow cytometry in basal conditions and after stimulation with Interferon-γ (IFN-γ) and TNF-α (Figure 6). In basal conditions, the four cell lines investigated showed some expression of MHC Cl I proteins. The lowest expression was observed in MCF7 A/Z cells and the highest in MDA-MB-231 cells. There is no correlation between the basal level of MHC Cl I proteins expression and that of p100 (compare Figures 6 and 3).

Cells were then stimulated with the cytokines IFN-γ, TNF-α or a combination of them. IFN-γ (100 U/ml) induced MHC Cl I in the four cell lines and most significantly in cells demonstrating low basal MHC Cl I expression (Figure 6). Cell stimulation with TNF-α also induced MHC Cl I expression in three out of the four cell but the effect observed was not as strong as with IFN-γ (Figure 6). A combination of both cytokines generated in the MCF7 A/Z cells an increase of MHC Cl I expression corresponding at least to the addition of the effect obtained which each of them alone. In the three other cell lines, the stimulation observed with IFN-γ was not boosted by the addition of TNF-α.

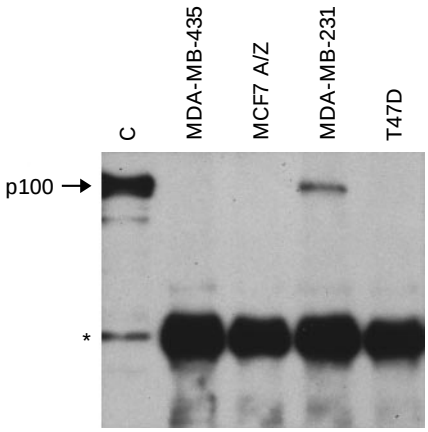

Figure 5 p100 forms a trimeric complex with p50 and RelB in MDA-MB-231 cells. Cytoplasmic extracts from the breast adenocarcinoma cell lines were first immunoprecipitated with anti-RelB antibodies. The immunoprecipitated complexes were then dissociated with an excess of the RelB peptide and the supernatants were re-immunoprecipitated with anti-p50 antibodies. The immunoprecipitated material was finally analysed on immunoblots for the presence of p100. The specific band is indicated. The broad band indicated by an asterisk corresponds to the reaction of the secondary antibody used for the immunoblot with the immunoprecipitating antibodies. The lane C shows protein extracts directly analysed by immunoblot without previous immunoprecipitation

To study whether this MHC Cl I induction could be related to cytokine-induced NF-κB activation, we performed Electrophoretic Mobility Shift assays with nuclear extracts from MDA-MB-435 and MCF7 A/Z cells. As previously demonstrated in a number of cell types, TNF-α rapidly induced nuclear NF-κB DNA-binding activity in both cell lines (data not shown). Conversely, IFN-γ stimulation did not induce any detectable NF-κB activity in MCF7 A/Z cells and generated only a very weak and delayed (24 h) NF-κB activity in MDA-MB-435 cells (Figure 7, lane 14).

Regulation of MHC Class I expression by NF-κB proteins

Mutations of the serines 32 and 36 of IκB-α phosphorylation sites abolish IκB-α degradation following a number of external stimuli and thus prevent NF-κB activation (Brown *et al*., 1995; Traeckner *et al*., 1995; Whiteside *et al*., 1995). Basal and induced MHC Cl I expression was thus compared in MCF7 cells stably transfected with the pcDNA3 expression vector containing or not the mutant IκB-α gene. It has been previously shown that induction of NF-κB DNA-binding activity was abolish in these stably transfected MCF7 MAD cells (Cai *et al*., 1997). The basal expression of MHC Cl I proteins, as measured by FACS analysis, was significantly lower in MCF7 MAD cells than in control cells (Figure 8, basal expression). In the MCF7 MAD cells, the fluorescence intensity was reduced to the background level suggesting a complete inhition of MHC Cl I expression. However, a significant induction of MHC Cl I expression could still be observed in these cells after IFN-γ stimulation while TNF-α treatment was without effect (Figure 8). The same experiment was then reproduced with MCF7 A/Z cells. Again, stable transfection of the mutated IκB-α vector completely abolished NF-κB activation as demonstrated by EMSAs (data not shown). In the MCF7 A/Z MAD cells, as compared with cells transfected with an empty expression vector, the basal MHC Cl I expression was not significantly decreased and remained higher that the background level (Figure 8). Again, cells that expressed the mutated IκB-α protein did not show any induction of MHC Cl I expression following TNF-α stimulation (Figure 8). Similar observations were also made with stably transfected HCT116 colon carcinoma cells and OVCAR-3 ovarian carcinoma cells expressing the mutated IκB-α protein (data not shown).

Discussion

Investigating the mechanisms regulating MHC Cl I expression is most important for our understanding

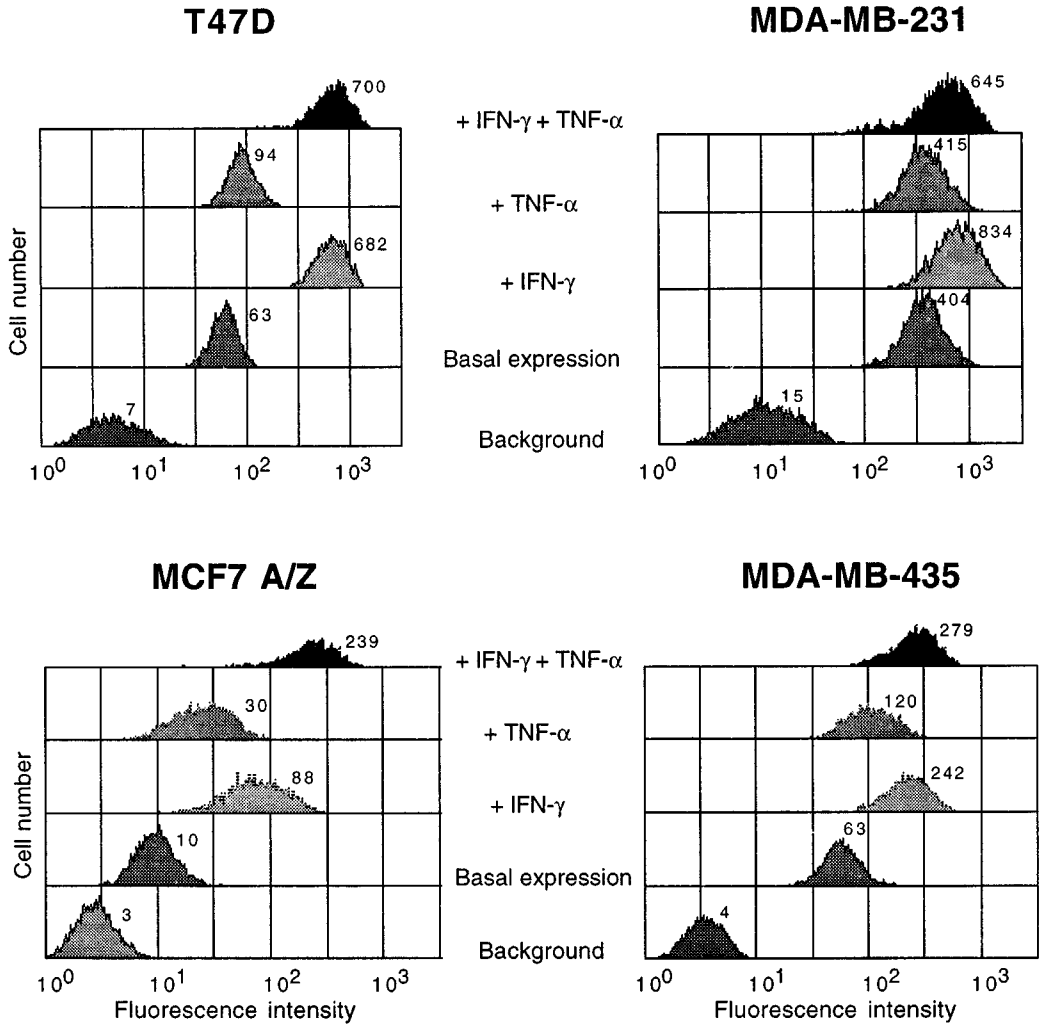

Figure 6 Expression of MHC Class I proteins in breast cancer cell lines. Four breast cancer cell lines were analysed by flow cytometry for basal and stimulated expression of MHC Cl I proteins. Background lanes correspond to the signal obtained in the presence of an irrelevant first antibody (purified mouse IgG1). Control lanes refer to the basal expression of MHC Class I proteins in unstimulated cells. The cell lines T47D, MDA-MB-231, MCF7 A/Z and MDA-MB-435 were stimulated for 48 h with IFN-γ (100 U/ml) alone, with TNF-α (100 U/ml) alone or with both cytokines at the same time. The relative fluoresence intensity is indicated next to each peak. This experiment was performed independently twice

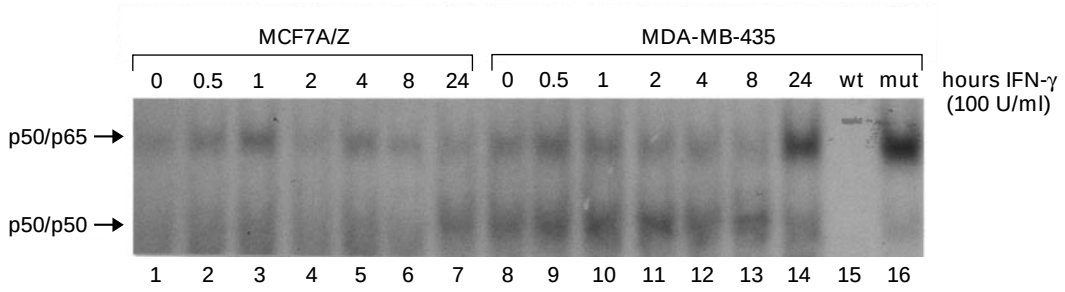

Figure 7 Analysis of NF-κB DNA-binding after interferon-γ stimulation of breast cancer cell lines MCF7 A/Z and MDA-MB-435. Nuclear extracts were prepared following IFN-γ stimulation for various times as indicated. 5 μg of nuclear proteins from MCF7 A/Z (lanes 1–7) and MDA-MB-435 (lanes 8–14) were mixed with a labelled probe corresponding to the κB site from the human MHC Cl I promoter. For competition assays, a 20 × molar excess of unlabelled wild type (wt) or mutated (mut) MHC Cl I probe was added to the binding reactions containing nuclear extract from MDA-MB-435 stimulated for 24 h. Supershifting experiments confirmed that the faster migrating complex was the p50/p50 homodimer while the slower one was the p50/p65 heterodimer (data not shown). Same results were obtained with the palindromic κB probe

of immune response and carcinogenesis. The NF-κB transcription factor certainly plays a key role in this regulation but its precise effect on the MHC Cl I promoters has yet to be determined. In this report, we confirmed that, as already shown by others in various cell types (Drew *et al*., 1993, 1995; Scheinman *et al*., 1993; Segars *et al*., 1993), NF-κB complexes activated transcription of the MHC Class I antigens in breast cancer cells.

Stable transfections of the uninducible IκB-α mutant abolished basal MHC Cl I expression in MCF7 breast adenocarcinoma cells but not in the other cells we analysed. It is not surprising that such a mutant does not influence basal MHC Cl I expression as serines 32 and 36 had been shown to be phosphorylated in response to stimuli such as proinflammatory cytokines (Brown *et al*., 1995; Traeckner *et al*., 1995; Whiteside *et al*., 1995). In other words, basal NF-κB activity is not influenced by phosphorylation of these IκB-α two serine residues but rather by the IκB-α or IκB-β PEST sequence or by the IκB-α ankyrin repeat domain (Krappmann *et al*., 1996; Van Antwerp *et al*., 1996; McKinsey *et al*., 1996; Good and Sun, 1996).

The mutated IκB-α inhibitor completely blocked MHC Cl I induction by TNF-α in MCF7 and MCF7 A/Z cells as well as in the colon carcinoma HCT116 and ovarian carcinoma OVCAR-3 cells. We could thus conclude that TNF-α-induced MHC Cl I expression is regulated by NF-κB. However, complete inhibition of NF-κB activation does not prevent MHC Cl I induction by IFN-γ. Indeed, we observed that IFN-γ rapidly activated the transcription factor IRF-1 in breast cancer cells while it induced NF-κB DNA-binding only very faintly (Figure 7 and data not shown). IFN-γ-induced MHC Cl I expression in these breast cancer cell lines is thus NF-κB-independent.

RelB-containing NF-κB complexes transactivate MHC Class I promoters in breast cancer cells. Moreover, flow cytometry analysis indicated high basal expression of MHC Cl I in the MDA-MB-231 cells and a much lower expression in MCF7 A/Z cells. In these cells, MHC Cl I expression might be correlated with the level of RelB expression although such a correlation should be confirmed on a larger number of cell lines. These observations confirm that the RelB protein might be a major regulator of antigen presentation. It was indeed reported that RelB is expressed in dendritic cells and is required for the differentiation of these antigen presenting cells (Burkly *et al*., 1995; Weih *et al*., 1995). Moreover, RelB is implicated in the constitutive expression of κB-dependent genes (Dobrzanski *et al*., 1994; Weih *et al*., 1996).

p100 acts as an IκB molecule and sequesters NF-κB complexes in the cytoplasm of various cell types. It was indeed demonstrated that p100 can form cytoplasmic complexes with p65, p50 and RelB (Mercurio *et al*., 1993; Scheinman *et al*., 1993; Kanno *et al*., 1994; Dejardin *et al*., 1995). Previous reports indicated that RelB complexes poorly interact with IκB-α, p105 or Bcl-3 and are preferentially inhibited by p100 in B lymphocytes (Lernbecher *et al*., 1994; Dobrzanski *et al*., 1994, 1995). Our data confirm that, in breast cancer cells, RelB-containing complexes are not inhibited by IκB-α but by p100, indicating a preferential interaction between RelB and the IκB-like molecule p100. In transient transfections however, as there is no possible competition with p100, overexpressed IκB-α interact with p50/RelB complexes and block transcription of the MHC-κB-CAT (Figure 2).

It was also shown that p100 can form trimeric complexes with p50 and p65 in the cytoplasm of Jurkat and MDA-MB-435 cells, presumably through an interaction of the p100 ankyrin repeats with p50 or p65 nuclear translocation signal (Kanno *et al*., 1994; Dejardin *et al*., 1995). In this report, we immunoprecipitated from MDA-MB-231 cells a novel form of multimeric complex which associates RelB, p50 and p100. This observation thus confirms that triple complexes are formed with p100. However, p100 is much more stable than IκB-α and p105 and does not respond to the same stimuli as IκB-α (Dejardin *et al*., unpublished data). Although a previous study showed p100 processing into p52 after PMA stimulation of Hela cells (Mercurio *et al*., 1993), our data indicate that, in breast cancer cells, these p100-containing multimeric complexes constitutes a separate pool of NF-κB factors which are released after specific, yet to be identified, stimuli.

Despite our experiments and previous data on p100 and RelB interaction, we did not observe any correlation between basal or TNF-α-induced MHC Cl I expression and the level of p100 expression in the studied cell lines. Indeed, the basal MHC Cl I

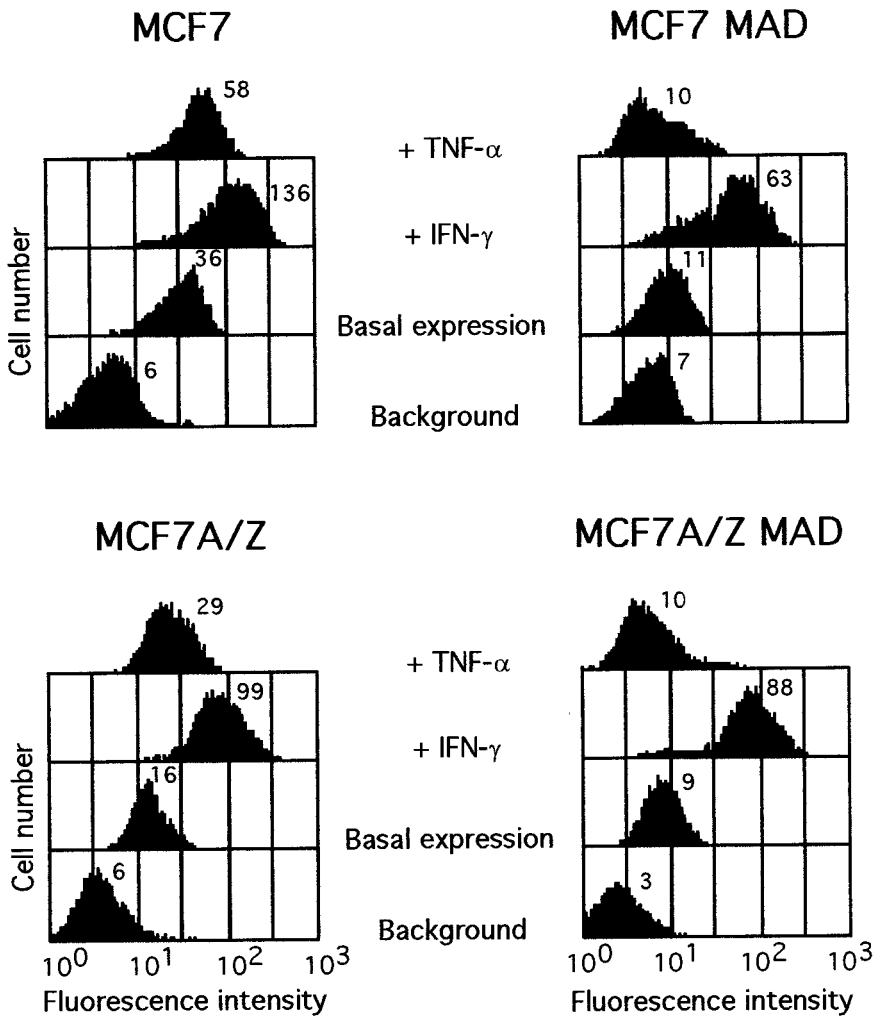

Figure 8 MHC Cl I expression in MCF7 and MCF7 A/Z cells stably transfected with an IκB-α mutant expression vector. The expression of MHC Cl I was measured by FACS analysis in MCF7 and MCF7 A/Z cells transfected with a pcDNA3 empty expression vector (left panels) or with an expression vector coding for an IκB-α protein mutated at serines 32 and 36 (MCF7 MAD and MCF7 A/Z MAD). Background lanes correspond to the signal obtained in the presence of an irrelevant first antibody (purified mouse IgG1-FITC). Basal expression refers to the expression of MHC Class I proteins in unstimulated cells. The cells were stimulated either with IFN-γ or TNF-α for 48 h as indicated in the figure. The relative fluorescence intensity is indicated next to each peak. This experiment was performed independently twice

expression is higher in MDA-MB-435 cells than in MCF7 A/Z cells which express p100 at a much lower level.

This observation indicates that, although highly expressed p100 forms stable cytoplasmic complexes with all detectable p65 and RelB proteins in MDA-MB-435 cells, it does not completely block NF-κB activity as the IκB-α mutant does. Two hypotheses could explain such an observation. Either, p100 allows small amounts of NF-κB to translocate to the nucleus and to regulate basal and TNF-α-induced transcription of the MHC Cl I gene; this hypothesis is supported by the observation that p100 is not as efficient as IκB-α in inhibiting NF-κB-dependent transcription (Figure 2) and that stable expression of p100 in MCF7 A/Z cells does not completely block NF-κB nuclear translocation and does not modify basal or induced MHC Cl I expression (data not show). Alternatively, p100 itself can bind the MHC Cl I promoter as a part of the H2TF1 complex and this complex would thus be responsible for uninduced MHC Cl I expression (Potter *et al*., 1993; Scheinman *et al*., 1993). We do not favor this last hypothesis as we never observed any p100 expression in the nucleus of breast cancer cells.

In summary, the present report demonstrates that NF-κB proteins regulate basal and TNF-α-induced but not IFN-γ-induced MHC Cl I expression in breast cancer cells and that highly expressed p100 probably does not inhibit this expression.

Materials and methods

Cell culture and biological reagents

The human breast cancer cell lines MCF7, MCF7 A/Z, MDA-MB-435, MDA-MB-231 and T47D were grown in RPMI 1640 medium (Gibco BRL) supplemented with 1% antibiotics, 1% glutamine and 10% fetal bovine serum (FBS). The cancer cell line MCF7 A/Z is a generous gift from Professor Mareel (University of Ghent, Belgium).

In the experiment described in Figures 6 and 9, the cells were treated with 100 U/ml of TNF-α (Boehringer

Mannheim, Germany) or 100 U/ml of IFN-γ (Boehringer Mannheim) for 48 h.

Transient transfections and CAT assays

MDA-MB-435 and MCF7 A/Z cells were transfected using liposomes (DOTAP system, Boehringer Mannheim, Germany) following the manufacturer's instructions. CAT assays were performed as described previously (Neumann *et a*., 1987; Bours *et al*., 1992).

The MHC-κB-CAT reporter plasmid was constructed by inserting the κB site from the H-2K^b promoter (5′-GATCTCAACGGCAGGCGGGGATTCCCTC-3′) at position −56 of a minimal c-*fos* promoter (Yano *et al*., 1987; Bours *et al*., 1992). A longer construct (pH_2-CAT: a gift from Dr A Israel, Institut Pasteur, Paris, France) containing a 2027 bp *Hind*III–*Nru*I fragment from the mouse H-2K^b promoter was also used (Daniel-Vedele *et al*., 1985). The expression vectors contained the cDNAs for p50, p65 (RelA), c-Rel, RelB, p52, IκB-α or p100 cloned into the PMT2T expression vector at the *Eco*RI site (Bours *et al*., 1992).

Immunoblots, immunoprecipitations and protein quantification

Cellular extracts, immunoblots, immunoprecipitations and protein quantification were performed as described (Dejardin *et al*., 1995; Bonizzi *et al*., 1996). Immunoblots were analysed by chemiluminescence (ECL, Amersham). The antibodies used were: an anti-RelB anti-peptide antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-p100 monoclonal antibody (a gift from Dr Siebenlist, NIH, Bethesda, MD), and an anti-p65 anti-peptide antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Double immunoprecipitations

Cytoplasmic extracts were first immunoprecipitated with a specific anti-RelB-peptide antibody and protein A-sepharose beads. The supernatant was discarded and the beads were incubated in TNT buffer (Tris 20 mM pH 7.5, NaCl 200 mM, Triton-X-100 1%) in the presence of an excess (5 μg of peptide for 5 μl of antibody) of the specific RelB peptide for 16 h at 4°C. The resulting supernatant containing the released multimeric RelB complexes was then immunopreciptated with anti-p50 antibodies and protein A-sepharose beads to isolate complexes containing both p50 and RelB. The final immune complexes were separated by SDS–PAGE and examined by immunoblot analysis with anti-p100/p52 monoclonal antibody.

Electrophoretic mobility shift assays

Nuclear extracts were prepared as previously described (Dejardin *et al*., 1995). The oligonucleotide probe used was the palindromic κB site (Bonizzi *et al*., 1996) or the MHC Cl I κB site with the sequence 5′-TTGGAAGGA-GAGGGGATTCCCCCTGCCGTTG-3′. A 20-fold excess of unlabelled wild type or mutated probe (5′-TTGGAAG-GAGATCTATTCCCCCTGCCGTTG-3′) was used for competition experiments.

Flow cytometry

Adherent cells were first treated with dispase II (Boehringer Mannheim, Germany) and washed twice in PBS. 5×10^5 cells were then incubated for 30 min at 4°C with a monoclonal antibody directed against the proteins HLA A, B, C (Pharmingen, San Diego, CA) coupled with fluorescein isothiocyanate (FITC). After three washes in PBS, the cells were analysed using a flow cell sorter (FACStar Plus, Becton Dickinson, San Jose, CA) with a 100 mW air-cooled argon laser (Spinnaker 1161; Spectra Physics, Mountain View, CA) and the CellQuest software (Macintosh, Facstation; Becton Dickinson, San Jose, CA). For each sample, 3000 cells were analysed and fluorescence histograms (1024 channels, log scale) were constructed.

Stable transfections

The MCF7 cells stably transfected with the pcDNA3 empty vector (pcN-183) or with the mutated IκB-α expression vector (MCF7 MAD) were previously described (Cai *et al*., 1997).

The MCF7 A/Z cells were transfected with the linearized pcDNA3 empty vector or with the mutated IκB-α expression vector (MCF7 A/Z MAD) using the DOTAP system (Boehringer Mannheim). Forty-eight hours after transfection, transfected cells were selected in a medium containing G418 at 0.5 mg/ml. After 15 days of selection, 10 resistant colonies were isolated and grown separetly. Positive clones were selected for their ability to prevent nuclear NF-κB translocation following TNF-α stimulation.

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