Oncogene (2006) 25, 1367–1377

www.nature.com/onc

ORIGINAL ARTICLE

TNF-α enhances estrogen-induced cell proliferation of estrogen-dependent breast tumor cells through a complex containing nuclear factor-kappa B

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Breast tumors are usually classified according to their response to estrogens as hormone-dependent or -independent. In this work, we investigated the role of the proinflammatory cytokine TNF-α on the estrogen-receptor-positive T47D breast ductal tumor cells. We have found that TNF-α exerts a mitogenic effect, inducing cyclin D1 expression and activation of the transcription factor NF-κB. Importantly, activation of NF-κB was required for estrogen-induced proliferation and cyclin D1 expression. TNF-α enhanced the estrogen response by increasing the levels and availability of NF-κB. Chromatin immunoprecipitation analysis suggested that the action of estrogens is mediated by a protein complex that contains the activated estrogen receptor, the nuclear receptor coactivator RAC3 and a member of the NF-κB family. Finally, our results demonstrate that activation of this transcription factor could be one of the key signals for estrogen-mediated response.
Oncogene (2006) **25,** 1367–1377. doi:10.1038/sj.onc.1209176; published online 12 December 2005

Keywords: NF-κB; nuclear receptor coactivators; breast tumor; TNF-α; estrogen receptor

Introduction

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine that plays different biological roles depending on the activated receptor type (i.e. p55 or type I vs p75 or II), the cell type involved and the physiological context. Particularly, its proapoptotic role, the signaling cascade from each receptor type and its potential as an antineoplastic weapon have been widely studied (Larrick and Wright, 1990; Zornig *et al*., 2001).

The proapoptotic role of TNF-α has been mainly associated with the TNF p55 receptor activation, which belongs to a family of dead receptors that recruits additional intracytoplasmatic proteins with death domains, such as TNF receptor-associated death domain (TRADD) and FAS-associated death domain (FADD) (Hsu *et al*., 1995, 1996a, b). It has been demonstrated that these molecules are involved in the caspase activation that leads to cell death (Liu *et al*., 1996; Hengartner, 2000).

High levels of TNF-α were found in patients with cancer, and endogenous TNF-α production was linked to protection against the cytotoxicity of exogenous TNF-α (Partanen *et al*., 1995; Chouchane *et al*., 1997). Although this cytokine was initially identified as a macrophage-derived serum protein that induces necrosis in mouse solid tumors in mice (Larrick and Wright, 1990), only a few cell types are naturally sensitive to its proapoptotic activity. In fact, most cells require the TNF-α activation of the NF-κB protective pathway to be blocked before apoptosis can occur (Beg and Baltimore, 1996; Costas *et al*., 1996, 2000; Liu *et al*., 1996; Wang *et al*., 1998; Franco *et al*., 2002).

NF-κB is a transcription factor formed by protein dimers containing the Rel dimerization domain, the p65 (Rel-A)/p50 hetero-complex being the best characterized at present. Inactive NF-κB is associated with IκB inhibitor proteins. Phosphorylation of IκB by specific kinases, which are activated by extracellular signals, mark IκB for degradation, allowing activation of the NF-κB complex (Ghosh and Karin, 2002). In addition to its pivotal role in immune response and inflammation, NF-κB regulates the expression of genes that control cell cycle (Hsu *et al*., 1995; Chen *et al*., 2001; Joyce *et al*., 2001) and cell viability (Hsu *et al*., 1996a, b; Liu *et al*., 1996).

Breast tumors are the most frequent cause of cancer-related death in occidental women. In some cases,

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Received 8 December 2004; revised 17 August 2005; accepted 2 September 2005; published online 12 December 2005

growth of estrogen-receptor-positive tumors (ER-positive) can be controlled by hormone therapy. On the other hand, ER-negative tumors that do not respond to this kind of treatment (Biswas *et al*., 2000, 2001) are the focus for new drugs discovery.

ER belongs to a family of nuclear receptors that share some common coactivators with NF-κB, such as RAC3 (Webb *et al*., 1998; Klinge, 2000; Mora and Brown, 2000; Werbajh *et al*., 2000). Interestingly, it was found that this coactivator is amplified in some breast tumors (Anzick *et al*., 1997).

The data showed herein indicate that TNF-α could contribute to breast tumor development instead of inducing cancer cell death. We demonstrate that this inflammatory cytokine induces proliferation of ER-positive human breast ductal tumor cells, T47D, through the activation of NF-κB. Furthermore, this transcription factor is required for the mitogenic action of estrogens.

Results

TNF-α induces T47D cell proliferation and enhances estrogen mitogenic response

The human breast tumor T47D cells normally respond to ER stimulation by enhancing the proliferative response (Biswas *et al*., 2000). In order to analyse the effect of TNF-α on T47D breast cancer cell proliferation, cells were stimulated with different doses of this cytokine (5–20 ng/ml) in the presence or absence of 17-β-estradiol (17E). Cell proliferation was evaluated after 24, 48, 72, 96 h or 1 week by thymidine uptake or by staining with crystal violet. As expected, 17E induced proliferation of these cells (Figure 1a). However, the results concerning TNF-α effect were particularly surprising, because no proapoptotic activity was observed. Furthermore, this cytokine (at 10 ng/ml) showed mitogenic activity on T47D cells (Figure 1a). A TNF-α effect similar to the T47D cells was observed in another ER-positive mammary tumor cell line (Figure 1b). No mitogenic action of this cytokine was observed in the ER-positive MCF7 human breast tumor cell line (data not shown), which, as previously described, is sensitive to TNF-α-induced cell death (Zyad *et al*., 1994).

In agreement with its own mitogenic activity, TNF-α did not antagonize the 17E-induced mitogenic activity. Moreover, an increased proliferative response was obtained in the presence of both stimuli (Figure 1a). The TNF-α-plus-17E-induced cell proliferation was not more than the sum of each one of the stimuli alone, indicating that there is no synergism between these signals. Similar results were obtained when cell number was analysed at 48, 72, 96 h and 1 week.

NF-κB activation is required for cell proliferation in the estrogen-dependent and -independent pathways

In addition to the activation of several MAP kinases, TNF-α activates the transcription factor NF-κB, which is involved in the control of cell cycle and survival

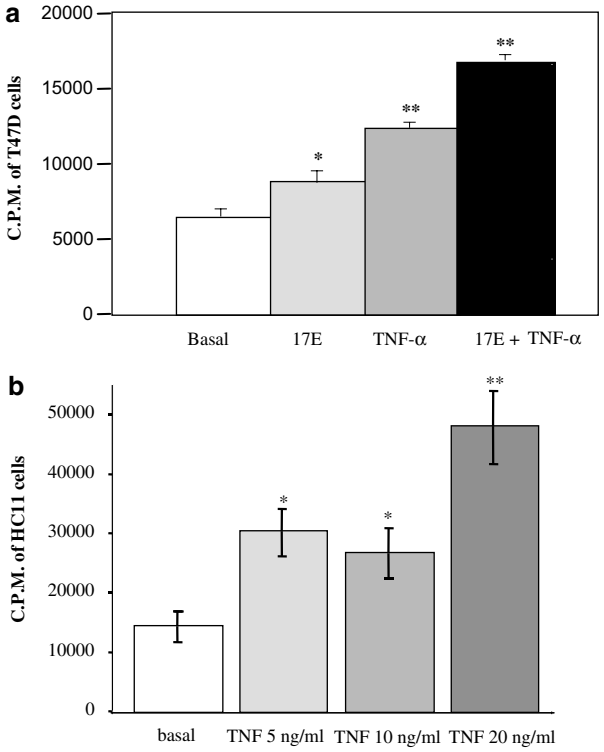

Figure 1 Effect of 17E and TNF-α on cell proliferation. (**a**) Diagram bars corresponds to the average of triplicate ± s.d. of [^{3}H]thymidine uptake during the last 8 h of culture, where T47D cells were stimulated for 48 h with 10 ng/ml of TNF-α and 100 nM of 17E. $^*P<0.01$; $^{**}P<0.001$ with respect to the basal conditions (Tukey' test). (**b**) Diagram bars correspond to the average of triplicate ± s.d. of [^{3}H]thymidine uptake during the last 8 h of culture, where HC11 cells were stimulated for 48 h. $^*P<0.014$, $^{**}P<0.0001$, both with respect to the basal condition (Scheffe's test).

(Franco *et al*., 2002). In order to determine whether NF-κB regulates the proliferative response of TNF-α in ER-positive cells, we analysed the effect of the NF-κB inhibitor sulfasalazine (SZ) (Wahl *et al*., 1998; Costas *et al*., 2000). Figure 2a shows that SZ significantly inhibits the mitogenic action of TNF-α, as well as the simultaneous treatment with 17E effect. Surprisingly, SZ was also able to inhibit 17E mitogenic activity, even treatment, in the absence of TNF-α. Similar results were obtained on HC11 cells (Figure 2b).

As it was previously shown that NF-κB inhibition during TNF-α stimulation induces apoptosis in several cell lines (Beg and Baltimore, 1996; Liu *et al*., 1996; Franco *et al*., 2002), the effect of SZ on the proliferative response could be a consequence of apoptosis induction. However, estrogen-dependent proliferative response and basal proliferation were similarly affected by SZ addition (Figure 1a), while cell viability remained unaffected at all the SZ doses employed, as determined by trypan blue exclusion staining and microscopy observation (Figure 2c), as well as by using the Apoptosis Biocolor kit.

It has been previously shown that, in addition to IκB, the nuclear receptor coactivator RAC3, which is also a

coactivator for ER, is also a substrate for the IκB kinase (IKK) (Wu *et al*., 2002). Next, we determined whether the SZ effects were due to either the specific NF-κB inhibition or the absence of RAC3 phosphorylation, which could affect estrogen-induced proliferation. As shown in Figure 2d, results similar to those observed under SZ treatment were obtained by the transfection of NF-κB decoy target DNA-specific oligomers, as well as by the transduction with an adenoviral vector expressing a dominant super-repressor IκB-α (Costas *et al*., 2000; Cafferata *et al*., 2001) (Figure 2e). These results clearly demonstrate that NF-κB plays a key role in breast tumor cell proliferation, even though a phosphorylated RAC3 could still be required for ER-mediated proliferation.

When cellular cycle was analysed by flow cytometry, SZ treatment resulted in an increased number of G0 cells and a lower number of cells in G2/M, together with a minor inhibition of S-phase induced by 17E at 24 h post-stimulation (Figure 3a).

Since cyclin D1 expression is associated to cell proliferation, we asked whether the stimuli described above could alter this protein levels.

In correlation to the observed proliferative response, cyclin D1 levels of T47D cells were significantly increased after treatment with TNF-α, 17E or both simultaneously for 24 or 48 h (Figure 3b). Similar results were obtained when T47D cell proliferation and cyclin D1 expression were evaluated at different TNF-α doses (data not shown).

These results indicate that proliferative response of ER-positive cells to TNF-α, which occurs even in the absence of estrogens, could be at least partially due to the enhancement of cyclin D1 expression.

It has been previously shown that cyclin D1 gene promoter contains κB elements and NF-κB binding to these sequences activates its transcription (Guttridge *et al*., 1999; Hinz *et al*., 1999). Accordingly, we have found that SZ inhibited TNF-α stimulation of cyclin D1 expression (Figure 3b). As observed on the proliferative response, all the stimuli are similarly affected by the inhibition of NF-κB, indicating that this transcription factor is necessary for cyclin D1 gene expression, even under ER activation, which correlates with its key role in the proliferative response.

In order to confirm the requirement of NF-κB on cyclin D1 gene expression control by TNF-α and 17E, we performed additional experiments of reporter assays,

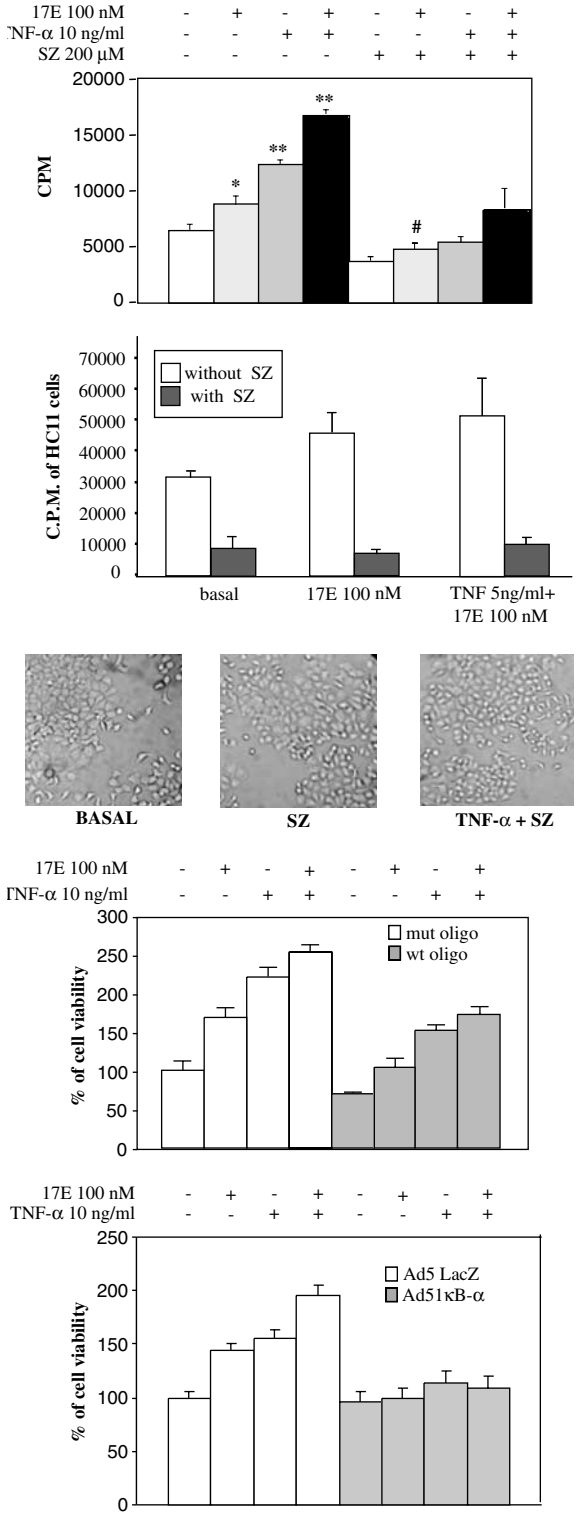

Figure 2 Effect of NF-κB inhibition on TNF-α- and 17E-mediated responses. (**a**) Diagram bars correspond to the average of triplicate ± s.d. of [^{3}H]thymidine uptake during the last 8 h of culture, where T47D cells were stimulated for 48 h. $^*P<0.01$; $^{**}P<0.001$ with respect to the basal conditions. $^{\#}P<0.001$ with respect to 17E treatment (Tukey' test). (**b**) Diagram bars correspond to the average of triplicate ± s.d. of [^{3}H]thymidine uptake during the last 8 h of culture, where HC11 cells were stimulated for 48 h. The dose of SZ was not apoptotic. (**c**) T47D cells after 24 h of stimulation with TNF-α in the presence or not of SZ (200 μM). (**d**) T47D cell viability was determined by crystal violet staining after 1 week of stimulation. Wild-type and mutated NF-κB decoy oligonucleotides are indicated as wt or mut, respectively. Each point corresponds to the average of triplicates. (**e**) The viability of T47D cells transduced with Ad5 IκB-α or the control Ad5 LacZ gene was determined by crystal violet staining after 24 h of stimulation with TNF-α, 17E or the combination of both.

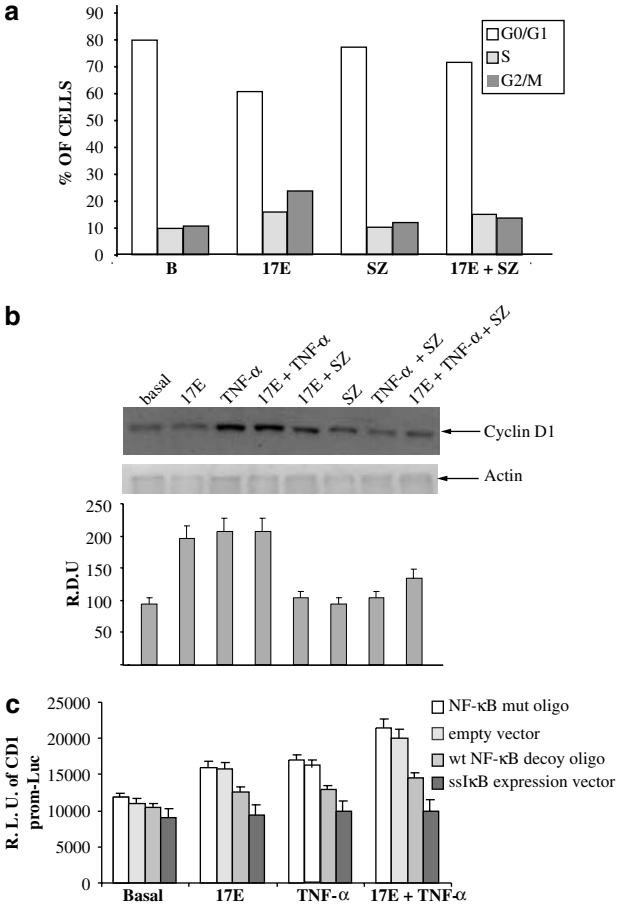

Figure 3 Effect of 17E (100 nM), TNF-α (10 ng/ml) and SZ (200 μM) on cell cycle and Cyclin D1 expression. (**a**) The diagram bars correspond to the cell cycle analysis of T47D cells after 24 h of stimulation. Data analysed with the CellQuest software. (**b**) Western blot of Cyclin D1 after 24 h of stimulation. Diagram bars correspond to the average of relative densitometric units with respect to the actin expression of five independent experiments. Similar results were obtained at 48 h. (**c**) Relative luminescence units (RLU) are the average of triplicate ± s.d., and were normalized with the corresponding β-galactosidase values. T47D cells were stimulated by 24 h as indicated, using 10 ng/ml of TNF-α and 100 nM of 17E. In the case of oligonucleotide decoy experiments, DNA was added 12 h before stimulation.

transfecting the T47D cells with a Luciferase expression vector under the control of a fragment of cyclin D1 promoter that we prepared, containing all the regulatory sequences described previously (Guttridge *et al*., 1999; Hinz *et al*., 1999; Castro-Rivera *et al*., 2001).

As shown in Figure 3c, 17E, TNF-α and the simultaneous treatment with both enhance the cyclin D1 promoter activity, similar to that observed on cyclin D1 protein levels. Moreover, we also performed experiments of NF-κB inhibition by using two specific and different experimental models. One of them was the addition of NF-κB decoy oligonucleotides, similar to that employed on proliferation assays, and the other was the transfection with an expression vector for the super-repressor IκB (ssIκB) (Werbajh *et al*., 2000). In both cases, the NF-κB inhibition significantly inhibits the promoter activity, confirming again the requirement of this transcription factor for cyclin D1 gene expression.

ER controls cyclin D1 gene expression through a complex containing NF-κB family members and the nuclear receptor coactivator RAC3

Previously, it has been demonstrated that estrogens inhibit NF-κB activation (Hsu *et al*., 2000; Evans *et al*., 2001). However, our results indicate that both 17E and TNF-α trigger a similar response, enhancing cyclin D1 and cell proliferation. Moreover, 17E-induced cyclin D1 expression requires active NF-κB. In order to demonstrate that estrogens do not antagonize NF-κB activation by TNF-α, we decided to perform electrophoretic mobility shift assay (EMSA) experiments. Our results show that, in the T47D cell line, TNF-α activates nuclear localization of several complexes containing members of the NF-κB family (Figure 4a, lane 3). The nature of each complex was revealed in super-shift experiments using the anti-p65- or -p50-specific antibodies (Figure 4a, lanes 4 and 5). Our data also show that these complexes were inhibited by SZ treatment (Figure 4b).

The simultaneous 17E plus TNF-α treatment did not induce NF-κB-binding inhibition to the specific oligonucleotide (Figure 4c, lane 3) as compared with TNF-α alone (Figure 4c, lane 2), indicating that the activated ER does not antagonize NF-κB activation. In addition, under these conditions, complexes similar to those induced by TNF-α single stimulation were identified, the homo-dimers Rel-A/Rel-A, p50/p50 and the hetero-dimers Rel-A/p50 being the most abundant (Figure 4c, lanes 3–5).

It has been previously reported that cyclin D1 gene promoter does not contain ER-responding elements (ERE) (Castro-Rivera *et al*., 2001). In order to determine whether the estrogen induction of cyclin D1 could be mediated by NF-κB binding, we analysed the possibility that both molecules could be part of the same protein complex. In this regard, we performed experiments of immunoprecipitation and Western blot analysis with proteins from T47D cells stimulated with TNF-α plus 17E.

Figure 5a shows that Rel-A was readily detected in the ER immunoprecipitate, and this protein was also detected in the Rel-A immunoprecipitate (Figure 5b). Therefore, this member of the NF-κB family and ER would both be part of the same protein complex. In addition, one of the ER nuclear receptor coactivators like RAC3, which is also an NF-κB-coactivator (as we have previously reported in Werbajh *et al*., 2000), was detected in the ER immunoprecipitate (Figure 5a), while Rel-A was also present in the RAC3 immunoprecipitate (Figure 5c). Interestingly, it has been reported that the nuclear receptor coactivator RAC3 and the IκB family member and coactivator Bcl-3 are overexpressed in breast tumors (Anzick *et al*., 1997; Cogswell *et al*., 2000; Planas-Silva *et al*., 2001; Westerheide *et al*., 2001).

In addition to the more ubiquitous Rel-A/p50 heterodimer, previous evidences have demonstrated that Bcl-3

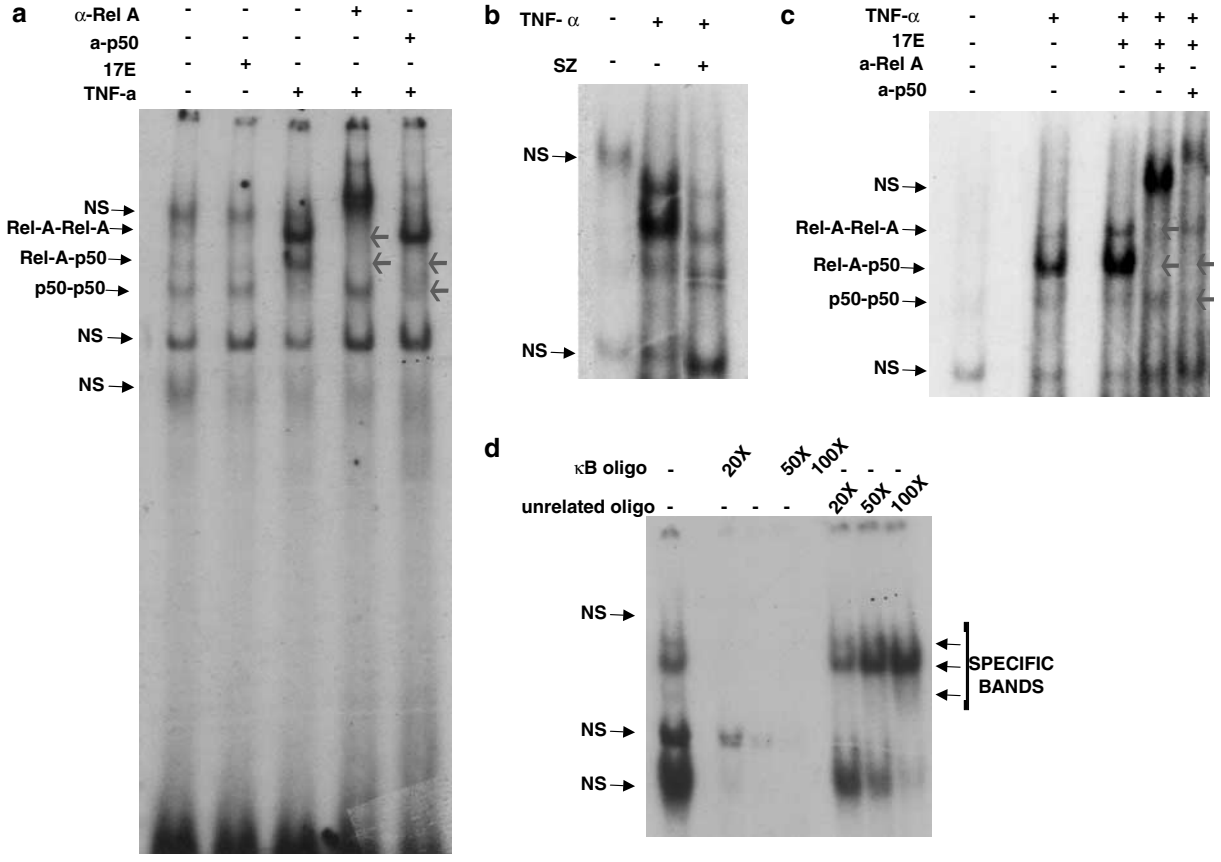

Figure 4 Effect of estrogens on NF-κB binding to their specific DNA elements. Nuclear extracts from T47D cells stimulated for 45 min with TNF-α (10 ng/ml) (**a**), TNF-α (10 ng/ml) plus SZ (200 μM) (**b**) or TNF-α (10 ng/ml) plus 17E (100 nM) (**c**) were incubated with double-stranded oligonucleotides containing an NF-κB-binding site and subjected to EMSA. The specificity of binding was determined by competition with the specific κB or the unrelated CCAAT oligonucleotides (**d**). The identities of the bound complexes were determined by super-shift with specific antibodies against RelA and p50, and are indicated by arrows at the left. Arrows at the right indicate the shift bands.

plus p52 could be forming an active transactivation compound (Westerheide *et al*., 2001). Moreover, it has been shown that this complex is involved in the control of cyclin D1 expression (Planas-Silva *et al*., 2001). Interestingly, we have found that there is a physical association between Bcl-3 and ER (Figure 5a). These results probably suggest that different types of ER/coactivator/NF-κB family member complexes could be controlling cyclin D1 expression levels and, therefore, proliferation in T47D cells.

Although cyclin D1 promoter does not show any ERE (Castro-Rivera *et al*., 2001), two D1-κB sites have been described in that region (Hinz *et al*., 1999). In order to determine if a protein complex containing the activated ER could be controlling cyclin D1 gene expression through κB-elements from its promoter, we performed chromatin immunoprecipitation experiments.

The DNA–protein complexes from cells simultaneously stimulated with 17E and TNF-α were immnoprecipitated with the specific antibody against ER-α and the bound DNA identified by polymerase chain reaction (PCR), by using the specific primers for the region containing the two distal κB sites of cyclin D1 promoter as shown in Figure 5d.

Figure 5e shows that ER coimmunoprecipitates with a fragment of DNA containing these specific κB elements, this binding being specifically inhibited by SZ treatment. These results show that TNF-α contributes to ER-dependent proliferation by NF-κB activation, and a complex containing the ER and this transcription factor may induce cyclin D1 expression.

Discussion

TNF-α was initially identified as a protein capable of inducing necrosis in mouse solid tumors (Larrick and Wright, 1990) and apoptosis in several different cell lines (Beg and Baltimore, 1996; Costas *et al*., 1996, 2000; Liu *et al*., 1996; Wang *et al*., 1998; Franco *et al*., 2002). Herein, we demonstrated that this inflammatory cytokine does not induce breast ductal tumor cell apoptosis, even in the presence of the NF-κB inhibitor SZ, in a dose that significantly inhibits this transcription factor binding to its specific DNA sequence. Moreover, our results show that TNF-α triggers a proliferative signal in ER-positive T47D and HC11 cells. To our knowledge, this is the first report indicating that this necrotic and

proapoptotic cytokine could be contributing to breast tumor development.

There is strong evidence that TNF-α, acting through its p55 receptor, simultaneously triggers divergent signaling pathways in the target cell. One of them involves caspase 8 activation, leading to apoptosis; on the other hand, NF-κB activation and the expression of antiapoptotic NF-κB target genes constitute part of a protective mechanism (Hsu *et al*., 1995; Beg and Baltimore, 1996; Franco *et al*., 2002). Therefore, the final target cell fate appears to be dependent on the balance between these two signaling pathways.

Although the proapoptotic and necrotic activity of TNF-α on several cell lines has been strongly investigated in the last two decades, its probable role as a proliferative signal through NF-κB activation in tumor cells has not received much attention. Nevertheless, it has been demonstrated that several cell lines are not sensitive to the proapoptotic signal unless *the novo* synthesis of antiapoptotic NF-κB target genes is blocked (Beg and Baltimore, 1996; Costas *et al*., 1996, 2000; Liu *et al*., 1996; Wang *et al*., 1998; Franco *et al*., 2002).

However, there are a few cell lines that are naturally sensitive to TNF-α-induced cell death, without require-

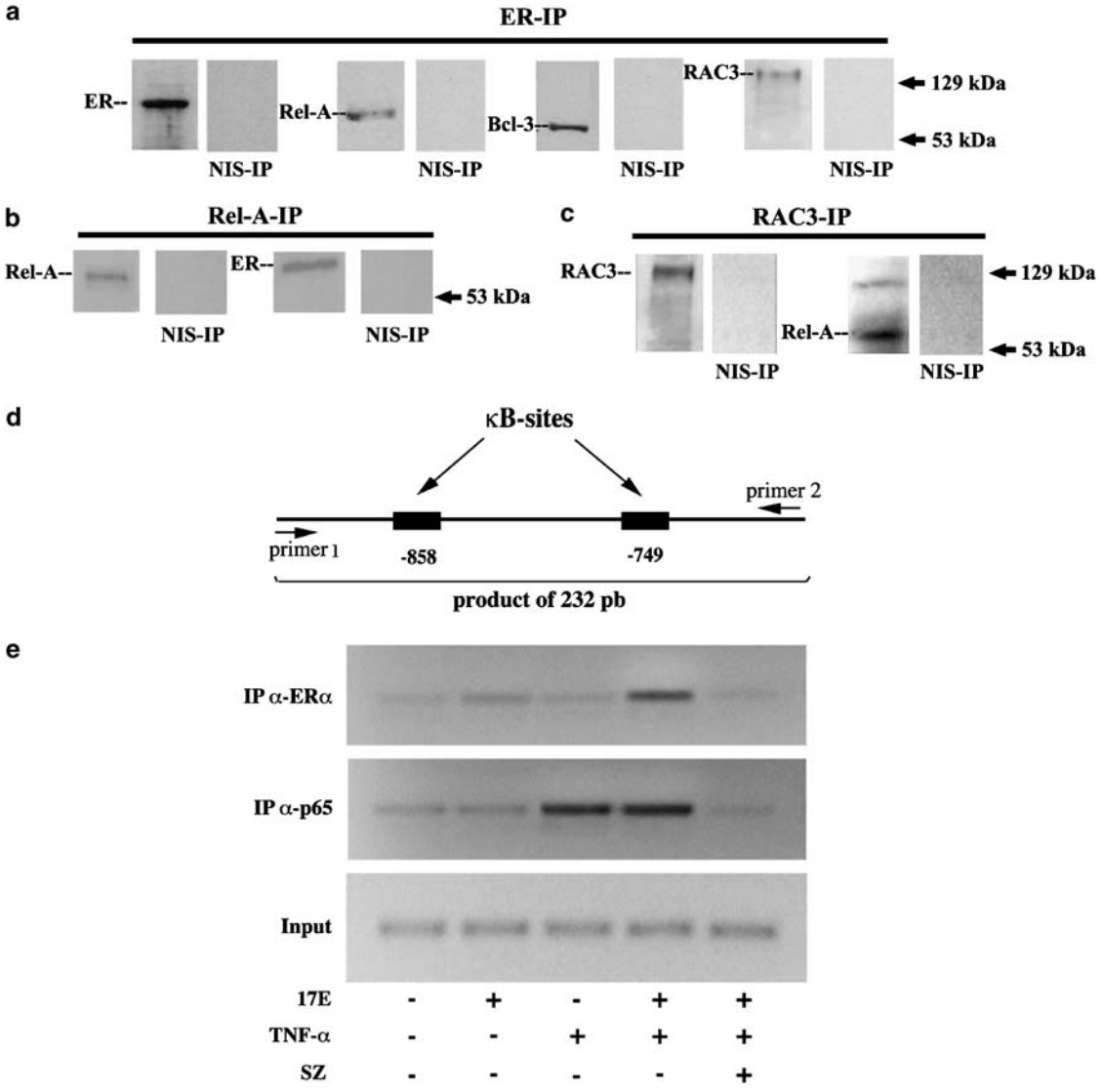

Figure 5 Activated ER interacts with κB elements of cyclin D1 promoter through a complex containing ER, NF-κB family members and the nuclear receptor coactivator RAC3. Western blot analysis of immunoprecipitates (IP) from T47D stimulated with TNF-α (10 ng/ml) plus 17E (100 nM) for 45 min. NIS-IP corresponds to the nonimmune serum immunoprecipitations. (**a**) Western blot of ERα, Rel-A, Bcl-3 and RAC3 from ER-α immunoprecipitates. (**b**) Western blot of Rel-A and ER from Rel-A immunoprecipitates. (**c**) Western blot of RAC3 and Rel-A from RAC3 immunoprecipitates. (**d**) Section of cyclin D1 promoter containing κB elements to be amplified by PCR. (**e**) The bands correspond to the fragment of cyclin D1 promoter containing the κB elements amplified by PCR from CHIP with α-ERα and α-Rel-A. No bands were detected in the absence of antibody or in the presence of the α-HA non-related antibody. (**f**) ER action model. One of the target genes involved in ER-induced proliferation is CD1. The CD1 promoter contains several specific sequences involved in the gene expression control that could be part of the ER response. ER could be found bound to CD1 promoter, because it can bind to distal κB elements through a complex with NF-κB (at least the Rel-a/p50 or Bcl-3/p52 heterodimers), and coactivators.

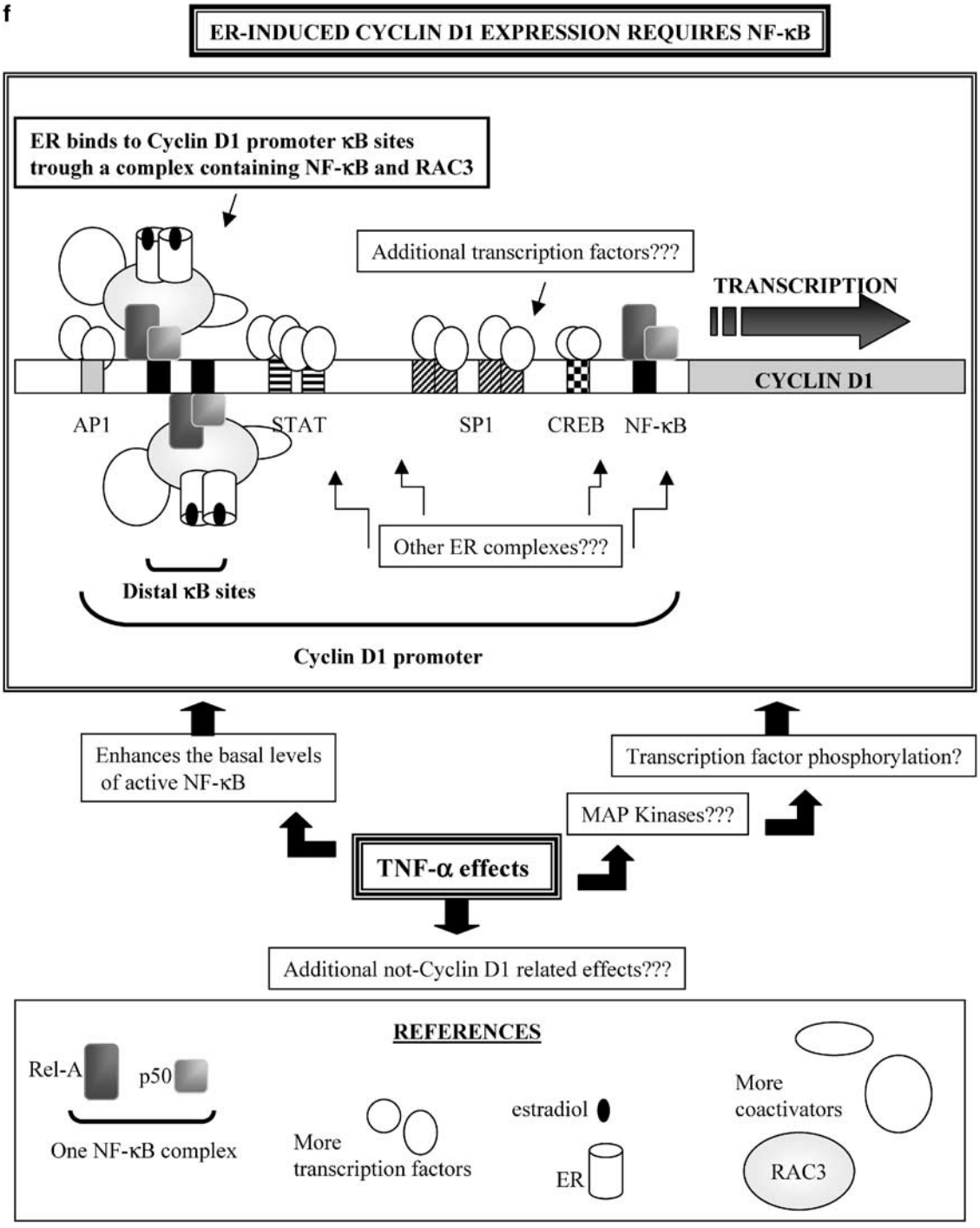

Figure 5 (*Continued*)

ment of the addition of any specific NF-κB inhibitor, such as the mouse fibroblasts L-929 (Costas *et al*., 1996, 2000) and the ER-positive breast cancer MCF-7 cell line (Zyad *et al*., 1994). In the latter line in which, differently from T47D and HC11 cells, TNF-α is not able to induce cell proliferation, our own unpublished results indicate that the ER-dependent proliferation is sensitive to SZ treatment (data not shown). Nevertheless, different options need to be analysed before assuming that NF-κB activation is also required for this cell line proliferation, for example, it cannot be excluded that the SZ-induced absence of an IKK-phosphorylated RAC3 could be affecting the ER proliferative response.

Supporting our hypothesis that NF-κB activation can induce mammary cell proliferation, other authors have shown that ER-negative breast cancer cells rely on this factor for aberrant cell proliferation, while avoiding apoptosis. Therefore, it can be assumed that blocking

NF-κB may have a potential therapeutic value in mammary tumors (Biswas *et al*., 2000, 2001).

Several specific sequences on cyclin D1 promoter have been described as involved in the estrogen-induced gene expression (Castro-Rivera *et al*., 2001). However, our results suggest that ER signaling requires NF-κB activation (Figures 2 and 5), even though other transcription factors could be activated and bound to cyclin D1 promoter (Figure 5f). Since NF-κB can be activated by multiple stimuli (Ghosh and Karin, 2002) and its constitutive activation has been found in several breast tumor cells during progression to hormone-independent growth (Nakshatri *et al*., 1997; Biswas *et al*., 2000; Pratt *et al*., 2003), it can be speculated that, in a physiological context, active NF-κB is probably available at compatible levels with ER requirement during the early stages of tumor progression when cells remain responsive to estrogens. This could be occurring in cells that are still sensitive to TNF-α-induced apoptosis and even in the absence of this cytokine. This idea is in agreement with our observations, which show that inhibition of NF-κB basal activation inhibits ER-induced response, including proliferation, cyclin D1 expression and binding to cyclin D1 promoter κB elements. In addition, observation carried out in wild-type mice as well as IKKα knockout mice clearly show that NF-κB plays an essential role during mammary gland development (Cao *et al*., 2001). In this study, the role of NF-κB in the proliferative response of ER-positive breast ductal cancer cells was examined. We find that activation of this transcription factor is crucial for cell proliferation not only in the absence of estrogen stimulation but also because the stimulated ER requires this activity.

There are several studies showing antagonism between ER and NF-κB (Hsu *et al*., 2000; Speir *et al*., 2000; Valentine *et al*., 2000; Evans *et al*., 2001). Some authors have reported that the molecular mechanism by which estradiol inhibits TNF-α-induced NF-κB is the induction of the κB member p105 that recruits the active NF-κB complex to the cytoplasm (Hsu *et al*., 2000). Others suggest a competition for nuclear receptor coactivators (Speir *et al*., 2000) between the activated ER and NF-κB, similar to that observed with the glucocorticoid receptor (GR) (Werbajh *et al*., 2000) However, other authors have identified residues into the ER ligand-binding domain that would be responsible for NF-κB transrepression and consider this competition as unlikely (Valentine *et al*., 2000).

On the other hand, our results show no antagonism between ER and NF-κB in T47D and HC11 cells at the TNF-α and 17E doses employed (Figures 1a and 2b). Moreover, we show that both molecules are part of the same protein complex, indicating that there is a physical association between them (Figure 5a–e). Nevertheless, because of our experimental design, we cannot determine if there is a direct association between ER and NF-κB.

It has been shown that, during cell cycle progression, the exit from G1 to S phase is NF-κB-dependent (Guttridge *et al*., 1999; Hinz *et al*., 1999). Interestingly, although the cyclin D1 promoter does not contain an ERE (Castro-Rivera *et al*., 2001), two D1-κB sites have been described in this region (Hinz *et al*., 1999). According to these evidences and additional previous works, the activated ER may only induce the cyclin D1 expression through indirect pathways that include activating extracellular signal-regulated kinase (ERK)-1 and 2 and protein kinase B (PKB), also called PKB/Akt (Castoria *et al*., 2001; Tsai *et al*., 2001; Hamelers and Steenbergh, 2003). In this regard, some authors have found that the estrogens induction of cell cycle progression would not be dependent on ER transcriptional activity since a transcriptionally inactive mutant of this receptor is able to induce the S-phase entry (Migliaccio *et al*., 1998). According to our results, ER would be able to control the cyclin D1 gene expression through at least the distal κB elements of the cyclin D1 promoter, by forming a complex with NF-κB family proteins (Figure 5f).

Several nuclear receptor coactivators have been described to be involved in ER transactivation, RAC3 being one of them (McKenna *et al*., 1999; Kato *et al*., 2000; List *et al*., 2001; Planas-Silva *et al*., 2001; Hamelers and Steenbergh, 2003). This is also a coactivator for NF-κB (Werbajh *et al*., 2000), and it has been shown to be amplified in several ovarian and mammary tumor cells such as T47D cell line (Anzick *et al*., 1997). In addition, the IκB family member and coactivator Bcl-3 is also overexpressed in breast tumors (Westerheide *et al*., 2001), and some authors have proposed that this oncoprotein induces cyclin D1 (Westerheide *et al*., 2001). Herein, we show that ER and NF-κB are part of the same complex and, as previously described in other cell lines, RAC3 coimmunoprecipitates with both ER and Rel-A (Webb *et al*., 1998; Klinge, 2000; Werbajh *et al*., 2000; Figure 5). Although NF-κB complexes, containing Bcl-3 or ER, were not detected in our EMSA experiments (data not shown), we have demonstrated that Bcl-3 can associate with the activated ER. The absence of DNA–NF-κB complexes containing ER or Bcl-3 could be due to the assay limitations, like the fact that the specific oligonucleotide used for these experiments does not display exactly the same DNA sequence that can be found at the cyclin D1 promoter region, or due to the lack of the surrounding sequences that could help stabilization of the transcriptional complex or the absence of chromatin.

As TNF-α triggers a complex signaling that involves several different kinases, we cannot exclude that it could be inducing a proliferative response through the regulation of additional NF-κB target genes or through NF-κB-independent pathways. However, our results suggest that TNF-α and the activated ER could be controlling tumor growth through, at least, a ternary complex containing the ER, RAC3 and members of the NF-κB family, whose direct action on the control of cyclin D1 expression has been clearly demonstrated (Guttridge *et al*., 1999; Hinz *et al*., 1999).

Taking into account that NF-κB shares some common coactivators with nuclear receptors (Sheppard *et al*., 1999; Werbajh *et al*., 2000), that it is overexpressed

in several tumors types and that it has a very important role in oncogenesis (Lin and Karin, 2003), our results could contribute to a better understanding of the mechanisms that underlie proliferation and tumorigenesis in cells that overexpress nuclear receptor coactivators.

Finally, in this work, we demonstrate for the first time that NF-κB, one of the TNF-α-triggered signals that could also be activated by multiple stimuli, is required for estrogen-mediated response, and that the mechanism of action involves, at least, the transcription factor recruitment of the activated ER to the κB target sequences into the cyclin D1 promoter (Figure 5f).

In conclusion, even though TNF-α effect on *in vivo* ductal tumor development remains to be elucidated, our results suggest that this cytokine could be contributing to mammary tumor development. Therefore, blocking TNF-α and/or its triggered signals could be a promissory tool, in combination with hormone therapies, for attacking ER+ breast cancer growth.

Materials and methods

Cells and reagents

The human breast tumor T47D and MCF7 cells and the mouse breast tumor HC11 cells were grown in Dulbecco's modified Eagle's F12 medium (DMEM-F12) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, Paisley, UK), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. At 48 h before starting the experiments, the medium was changed to DMEM without phenol red and supplemented with 10% charcoal-stripped steroid-free FCS. Unless stated, reagents were obtained from Sigma Chemical Co. (St Louis, MO), Pharmacia (Uppsala, Sweden) or Santa Cruz Biotechnology, USA.

Proliferation and viability assays

Cells were plated in 96-well flat bottom plates at a density of 8000 cells per well in 100 μl of medium. After 24 h, the cells were stimulated with hu-TNF-α (5–10 or 20 ng/ml) and/or 17E (100 nM) in the presence or not of the NF-κB inhibitor SZ 200–500 μM by 48 h. Cells were pulsed with 1 μCi/well of methyl[^{3}H]thymidine (^{3}H-Thy, New England Nuclear Life Science, Boston, MA, USA) during the last 8 h of cell culture, and harvested on glass fiber filters. Incorporated radioactivity was measured in a liquid scintillation β counter (Packard Instruments). Results are expressed as the mean c.p.m. of triplicate wells ± s.d.

In some experiments where the κB-consensus oligonucleotide was added, cell viability was determined by staining with crystal violet as described previously (Franco *et al*., 2002). Briefly, cells were plated at 1×10^4 cells/well in 24-well microtiter plates and transfected with 1 μM of the double-strand κB-consensus phosphorothioate modified oligonucleotides 5′-G*AGGGGACTTTCCC*T-3′ or the scrambled mutant 5′-C*TTTGAGGCCCTGG*A-3′. After 24 h of oligonucleotide addition, cells were stimulated with different doses of TNF-α, 17E and SZ. After 24, 48, 72 and 96 h, cells were fixed with 70% methanol and stained with 0.5% crystal violet. The absorbance corresponding to the stained surviving cells was determined at 570 nm. The percent of surviving cells was determined with respect to the basal conditions (without any treatment).

T47D transduction

Cell line was cultured to subconfluency and transduced with Ad5 IκB-α or Ad5 LacZ (Cafferata *et al*., 2001) in serum-free medium at a multiplicity of infection of 500 (1:500 T47D cells/viral particles) for 12 h with gentle agitation every hour. The adenovirus was washed off, fresh serum-containing medium was added, and the cells were incubated for a further 12 h. The cells were incubated in charcoal-stripped serum-containing medium for 48 h and then stimulated with 17E (100 nM), TNF-α (20 ng/ml) and SZ (200 μM) or the combined treatments.

T47D transfection

For assays of cyclin D1-promoter activity, T47D cells were transfected with the reporter plasmid CD1prom-Luc. In order to specifically inhibit NF-κB, two different experimental models were used. In one of them, cells were also transfected with ssIκB expression vector carrying mutated Ser 32 and Ser 36 to prevent phosphorylation and proteolysis of IκB, kindly provided by Dr G Cadwell and Dr M Karin (Werbajh *et al*., 2000) or the empty vector as control. In other experiments, NF-κB was inhibited by the addition of 1 μM of NF-κB decoy oligonucleotide or the scrambled mutant control 12 h before the stimulation. The first model is very useful for reporter assays, although it could be not used for proliferation assays, unless X-Gal-stained positive transfected cells were counted, because of the low efficiency of T47D transfection.

CD1prom-Luc construct was made as follows: a 915 bp fragment of human cyclin D1 promoter was prepared by PCR using T47D genomic DNA as template and oligonucleotides synthesized to the −890 to +25 published sequence. The PCR amplified product was subcloned in pGL3-Basic at the *Mlu*I and *Hin*dIII sites. The nucleotide sequence of the construct was confirmed by cycle sequencing.

Transfection was performed as described previously (Costas *et al*., 2000). Briefly, cells were cultured in 24-well plates at a density of 1×10^5 cells/well in DMEM without serum or antibiotics, and transiently transfected with a total of 0.8 μg of DNA (including 50 ng of RSV-β-Gal, using lipofectamine 2000 (GibcoBRL)). The medium was replaced by fresh and completed DMEM after 5 h. After 24 h, cells were stimulated with human TNF-α and 17E as indicated. The assays for luciferase and β-galactosidase activity were performed after 24 h of treatment using the appropriate substrates following the manufacturer's protocols (Promega corp.).

Cell cycle analysis

The number of cells at different phases of the cell cycle was monitored by determining the DNA content (Franco *et al*., 2002). After 24 h post-treatment with 17E, with or without SZ, cells were washed twice with PBS, trypsinized and resuspended in 70% ethanol. Subsequently, cells were precipitated, washed twice on PBS, and incubated for 3 h in 50 μg/ml propidium iodide solution (0.1% sodium citrate, 0.1% Triton X-100) at 4°C. Samples were measured in a BD FACSCalibur System. The data were analysed with the CellQuest software.

Immunoprecipitation and Western blot

Coimmunoprecipitations and Western blot were performed as described previously (Franco *et al*., 2002). Briefly, T47D cells were stimulated with 10 ng/ml of hu-TNF-α and 17E for 60 min and then lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40) containing the protease inhibitors 10 μg/ml leupeptin, 10 μg/ml aprotinin,

1 μg/ml pepstatin A, 1 mM phenylmethylsulfoyl fluoride and 1 mM dithiothreitol. Supernatants of lysates were incubated overnight at 4°C with the specific ER-α or Rel-A antibodies (Santa Cruz Biotechnology, USA) and immunoprecipitated for 2 h at 4°C with GammaBind G Sepharose (Pharmacia Biotech, USA). After six washes, Sepharose-bound immunocomplexes were separated on 6 or 10% SDS–PAGE and electro-transferred to PVDF membranes.

For experiments of cyclin D1 expression, cells were stimulated with hu-TNF-α or 17E, in the presence or not of SZ for 24 or 48 h. Then, cells were lysed in lysis buffer with protease inhibitors as described before. Samples were separated on 12% SDS–PAGE and electro-transferred to PVDF membranes.

Membranes were blocked for nonspecific binding with a solution containing 5% bovine serum albumin (IgG-free) and 0.05% Tween-20, and incubated for 60 min at room temperature in PBS and 0.5 μg/ml of Rel-A, RAC3, ER-α, Bcl-3 or Cyclin D1 antibodies (Santa Cruz Biotechnology, USA) in each experiment. Subsequently, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, and the proteins visualized by autoradiography using the chemiluminescence luminol reagent (Santa Cruz Biotechnology, USA).

Electrophoretic mobility shift assay

The EMSA was performed as described previously (Costas *et al*., 1996). Briefly, cells were stimulated by 45 min with 10 ng/ml of hu-TNF-α, 100 nM of 17E, or both simultaneously, in the presence or not of 0.5 mM of SZ. The nuclei were prepared by using the lysis buffer A (HEPES 10 mM, pH 7.9, $MgCl_2$ 1.5 mM, KCl_2 10 mM, Nonidet 40 0.5%). Nuclear proteins were prepared by washing the nuclear pellet with buffer B (HEPES 10 mM, pH 7.9, $MgCl_2$ 1.5 mM, KCl_2 0.42 M, EDTA 0.25 M, glycerol 20%) by continuous shaking for 15 min at 4°C. Binding to DNA reactions was performed in buffer C (HEPES 10 mM, pH 7.9, KCl_2 60 mM, EDTA 0.25 M, glycerol 20%). All the buffers contained the protease inhibitors 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The κB double-stranded oligonucleotide, containing the sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′, was end labeled using γ-^{32}P ATP and T_4 polynucleotide kinase. For each assay, 5 μg of nuclear extracts was incubated with 1 ng of κB oligonucleotide and 200 μg of poly dIdC for 15 min at room temperature. For supershift experiments, samples were preincubated for 1 h with the specific antibodies. In all, 5–20-fold excess of the unlabeled κB oligonucleotide or the unrelated oligonucleotide CCAAT (5′-GATCCCGGAGCCCGGGCCA ATCGGCGCA-3′) was utilized for competition assays. Complexes were separated in non-denaturing 5% PAGE. The gels were dried under vacuum and autoradiographed at −70°C.

Chromatin immunoprecipitation

Cells were stimulated for 45 min with 17E 100 nM and TNF-α 10 ng/ml in the presence or not of SZ 0.5 mM, and then fixed by adding formaldehyde to a final concentration of 1% by 10 min. Fixed cells were washed twice with cold PBS, scraped and centrifuged at 3000 r.p.m. for 5 min.

The cellular pellets were resuspended in chromatin immunoprecipitation (ChiP) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, containing the protease inhibitors 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol). Fragmentation of chromatin was performed by sonication on ice six times, 1 min at 40% and the size of DNA fragments (between 1 kb and 500 pb), checked by electrophoresis on 1.5% agarose gel. Immunoprecipitation was performed overnight with rotation at 4°C by using 25 μg of DNA in RIPA buffer with an anti-ER-α-specific antibody. Immunoprecipitates were incubated with GammaBind G Sepharose (Pharmacia Biotech, preabsorbed with sonicated salmon sperm DNA 50 μg/ml and BSA 200 μg/ml for 4 h at 4°C) for 2 h with rotation at 4°C. After three washes of pellet beads with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8), elution of DNA was performed with the elution buffer (1% SDS, 100 mM $NaHCO_3$) for 15 min at room temperature. After spin-down, proteins were removed by adding Proteinase K. Samples were heated with shaking at 65°C for 4 h to reverse the crosslink. After phenol/chloroform extraction, the DNA was precipitated with ethanol 70% and sodium acetate 0.3 M. Samples were washed once with ethanol 70% and resuspended in buffer TE. PCR was performed by using the specific primers: 5′-TCCATTCAGAGGTGTGTTT CTC-3′ and 5′-GGATTTCAGCTTAGCATGCG-3′ corresponding to the distal −749 and −858 κB elements of cyclin D1 promoter (Guttridge *et al*., 1999; Hinz *et al*., 1999) and performing 30 cycles.

Abbreviations

TRADD, TNF receptor-associated death domain; FADD, FAS-associated death domain; ER, estrogen receptor; SZ, sulfasalazine; 17E, 17-β-estradiol.

Acknowledgements

This work has been supported by grants from the Argentine National Research Council (CONICET), Agencia Nacional de Promoción Científica y Tecnológica and Fundación Antorchas, Argentina. We thank Dr Claudio Pereira, Dr Estela Medrano, Dr Christiane D Pasqualini and Dr Omar Coso for comments about the manuscript. We also thank Dr Nancy Hynes for kindly providing us the HC11 mouse mammary cell line, and Dr Fernando Pitossi, who has provided the adenoviral vector used in this work.

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