

Mechanisms underlying differential expression of interleukin-8 in breast cancer cells

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ABSTRACT

We have recently reported that Interleukin-8 (IL-8) expression was inversely correlated to estrogen-receptor (ER)-status and was overexpressed in invasive breast cancer cells. In the present study, we show that IL-8 overexpression in breast cancer cells involves a higher transcriptional activity of IL-8 gene promoter. Cloning of IL-8 promoter from MDA-MB-231 and MCF-7 cells expressing high and low levels of IL-8, respectively, shows the integrity of the promoter in both cell lines. Deletion and site-directed mutagenesis of the promoter demonstrate that NF- κ B and AP-1 and to a lesser extent C/EBP binding sites play a crucial role in the control of IL-8 promoter activity in MDA-MB-231 cells. Knock-down of NF- κ B and AP-1 activities by adenovirus-mediated expression of a NF- κ B super-repressor and RNA interference, respectively, decreased IL-8 expression in MDA-MB-231 cells. On the contrary, restoration of Fra-1, Fra-2, c-Jun, p50, p65, C/EBP α and C/EBP β expression levels in MCF-7 cells led to a promoter activity comparable to that observed in MDA-MB-231 cells. Our data constitute the first extensive study of IL-8 gene overexpression in breast cancer cells and suggest that the high expression of IL-8 in invasive cancer cells requires a complex cooperation between NF- κ B, AP-1 and C/EBP transcription factors.

INTRODUCTION

The concept of autocrine and paracrine pathways in breast carcinogenesis has been proposed by several studies. Cytokines, in particular, are emerging as potential factors which could contribute to the progression of breast cancer. IL-8 is of particular interest as it is expressed by many types of tumors including prostate (Ferrer et al., 1998), colon (Brew et al., 1996), lung (Masuya et al., 2001), ovary (Xu & Fidler, 2000) and melanoma cancers (Nurnberg et al., 1999). IL-8 is a pleiotropic cytokine with a wide range of physiologic and pathophysiologic activities. In addition to its role as an immunomodulatory cytokine, IL-8 is thought to function as a growth and differentiation factor in human cancers, by modulating metastasis and angiogenesis (Xie, 2001)

Numerous studies have shown that estrogen receptor (ER)-positive breast cancers have a better prognosis (Osborne, 1998) than breast carcinomas which lack ER expression (Sheikh et al., 1994), as they exhibit a lower ability to metastasize. Interestingly, we have demonstrated recently an inverse correlation between expression of the α -chemokine IL-8 and ER levels in human breast cancer cell lines as well as in primary cancer cells (Freund et al., 2003). Moreover, IL-8 is able to increase the invasion capacity of ER-positive breast cancer cells (Freund et al., 2003).

Based on the association between the lack of ER expression and the aggressive phenotype of such breast cancers, it was of great interest to analyze the mechanisms underlying the differential regulation of IL-8 in ER-negative and ER-positive breast cancer cells. Our data show that IL-8 overexpression in ER-negative breast cancer cells occurs primarily at the transcriptional level by binding of NF- κ B members p50 and p65 to IL-8 promoter. AP-1 and to a minor extent C/EBP transcription factors, which are highly expressed in ER-negative breast cancer cells are also essential for full activation of IL-8 promoter activity.

RESULTS

Using cell lines and samples from patients, we previously showed (Freund et al., 2003) that IL-8 was overexpressed in ER-negative compared to ER-positive breast cancer cells. ER-negative breast cancers represent a subset of more aggressive cancers, notably by their higher invasiveness and metastatic potential. As, we have demonstrated that IL-8 is an enhancer of invasion, it was of particular interest to study whether IL-8 gene was functional in ER-positive breast cancer cells and what phenomenons could account for IL-8 overexpression in ER-negative breast cancer cells.

To analyze the functionality of IL-8 gene, we have chosen two widely used cell lines, MDA-MB-231 (ER-) and MCF-7 (ER+) cells, as prototypes of ER-negative and ER-positive breast cancer cells. Cells were treated with interleukin-1 α (IL1 α), interleukin-1 β (IL1 β) or tumor necrosis- α (TNF α), which are known activators of IL-8 production in other cell systems. As measured by ELISA (Fig. 1), in MDA-MB-231 cells, only TNF α was able to modestly increase the basal secretion of IL-8, certainly because of the high basal expression of IL-8 in this cell line. Lack of IL1 α and IL1 β responsiveness is due to the absence of IL-1 receptors in this particular cell line (data not shown). On the contrary, IL-8 secretion was strongly up-regulated in MCF-7 cells by treatment with IL1 α , IL1 β or TNF α , demonstrating that IL-8 gene is fully functional in MCF-7 cells.

In order to determine the reasons why ER-negative cells exhibit a higher basal expression of IL-8 gene (Freund et al., 2003), we first analyzed by southern blot the hypothesis that IL-8 gene could be amplified in MDA-MB-231 cells. We obtained the same signal of equivalent intensity in MCF-7 and MDA-MB-231 cells (data not shown), suggesting that IL-8 overexpression in MDA-MB-231 cells does not arise from gene amplification. We cloned IL-8 promoter from both cell lines to

ensure that the differential gene expression was not due to mutations. The sequence of IL-8 promoter was identical to the one reported by others (Mukaida et al., 1989)(data not shown), confirming that the promoter sequence in itself does not account for the differential expression of IL-8 between ER-negative and ER-positive breast cancer cells. Measure of IL-8 RNA levels in MDA-MB-231 and MCF-7 cells by quantitative PCR show that IL-8 RNA is about 80 fold more abundant in MDA-MB-231 compared to MCF-7 cells (Fig. 2A). We then performed run on experiments to assess IL-8 gene transcription rate (Fig. 2B). Transcription rate was much higher in MDA-MB-231 cells compared to MCF-7 cells, which could account for the differences in RNA and secreted IL-8 levels between these two cell lines. We analyzed IL-8 promoter activity by transient transfection in both cell lines (Fig. 2C). IL-8 promoter displayed a 10 fold higher activity in MDA-MB-231 cells compared to MCF-7 cells, whereas the anti-sense construct was not active.

We wished to determine whether the phenomenons observed in MDA-MB-231 and MCF-7 cells could be generalized to other cell lines. As shown previously (Freund et al., 2003), ER-negative cells (MDA-MB-231 and MDA-MB-436) secreted high levels of IL-8, whereas ER-positive cell lines secreted only very low levels of IL-8 (Fig. 2D left panel). The transcriptional ability of IL-8 promoter observed in these cell lines was in good agreement with the differences in IL-8 secretion (Fig. 2D right panel), suggesting that transcriptional regulation could be a general mechanism of IL-8 overexpression.

In order to delineate the sequences of IL-8 promoter involved in the differential regulation of IL-8 in MCF-7 and MDA-MB-231 cells, we first compared broad sequential deletions of IL-8 promoter (Fig. 3). Elimination of sequences located between -1481 and -272 bp of the promoter did not affect significantly the transcriptional activity of the construct. On the contrary, further deletion to -98 bp reduced by two thirds the activity of the construct compared to the wild-type reporter in MDA-MB-231 and MCF-7 cells. This deletion suppresses an AP-1 site which is crucial in the regulation of IL-8 promoter activity by TNF α in human gastric carcinoma cell lines (Yasumoto et al., 1992). The

50 bp construct which is deleted from the NF- κ B and C/EBP sites (Mukaida et al., 1994) showed no activity, suggesting that these sites are also important for IL-8 promoter regulation. To define more precisely the relevance of each site, we used point mutated constructs of AP-1, C/EBP and NF- κ B sites (Fig. 4A). Mutation of AP-1 and C/EBP sites decreased by about 70 and 50%, respectively, IL-8 promoter activity in MDA-MB-231 cells, whereas mutation of NF- κ B site shut down the promoter activity to a level comparable to that of MCF-7 cells, demonstrating that NF- κ B plays the main role in IL-8 gene regulation. On the contrary, combined mutation of both AP-1 and C/EBP sites reduced the activity of the promoter to the same level as NF- κ B mutation alone, suggesting that AP-1 and C/EBP sites are acting in coordination or are necessary for NF- κ B activity.

We then compared the ability of MCF-7 and MDA-MB-231 cell extracts to bind to these sites in gel shift assays (Fig. 4B). We observed a strong shifted complex with AP-1 and NF- κ B probes when using MDA-MB-231 cell nuclear extracts, whereas a barely detectable binding could be seen with MCF-7 extracts. Binding to C/EBP probe was much weaker than the one of AP-1 and NF- κ B in MDA-MB-231 cells (a longer exposure than the one of AP-1 and NF- κ B gels is shown here) and the difference in terms of binding was less pronounced between MDA-MB-231 and MCF-7 cell extracts. These data suggest that a stronger interaction of nuclear factors with AP-1, NF- κ B and in a lower extent C/EBP sites is responsible for the higher activity of IL-8 promoter in MDA-MB-231 cells as compared to MCF-7 cells. This led us to identify the factors interacting with the three sites in the ER-negative cell line.

To determine which isoforms of C/EBP present in MDA-MB-231 cells were able to bind to IL-8 promoter C/EBP site, we performed gel shift assays with MDA-MB-231 nuclear extract and C/EBP site from IL-8 promoter (Fig. 5A). C/EBP β and to a lesser extent C/EBP α interacted with C/EBP probe, as the shifted complex disappeared with the addition of the respective antibodies (Fig. 5A). AP-1 members bound to IL-8 promoter AP-1 site in MDA-MB-231 cells were identified as Fra-1

and Fra-2 and to a lesser extent as c-Jun (which generated a weak shifted complex) (Fig. 5B). Finally, p50 and p65 corresponded to the NF- κ B members of MDA-MB-231 cell extracts bound to the NF- κ B site of IL-8 promoter (Fig. 5C). To determine whether the differences observed in DNA binding were also reflecting variations in protein levels, we assessed the levels of AP-1, C/EBP and NF- κ B members involved in IL-8 gene regulation by western blot (Fig. 5D). We observed a strong overexpression of Fra-1, p50, p65, C/EBP α and C/EBP β in MDA-MB-231 cells compared to MCF-7 cells. On the other hand, Fra-2 and c-jun levels were slightly higher in MDA-MB-231 cells compared to MCF-7 cells.

We next focused on NF- κ B and AP-1 transcription factors, the main regulators of IL-8 promoter activity in MDA-MB-231 cells. We analyzed the global AP-1 activity in MDA-MB-231 and MCF-7 cells by using an AP-1 reporter in transient transfections (Fig. 6A). An 8-fold difference in AP-1 activity was found between these two cell lines, confirming our gel shift assays. We then analyzed the contribution of AP-1 pathway to IL-8 gene regulation by transfecting two inhibitors of AP-1 (JDP-1 and JDP-2) (Wardell et al., 2002) and a dominant-negative c-Jun mutant (Tam67) (Brown et al., 1993) (Fig. 6B). These three inhibitors reduced by about 60-70% IL-8 promoter activity. To further characterize the primary role played by NF- κ B to regulate IL-8 promoter, we evaluated NF- κ B activity in MDA-MB-231 and MCF-7 cells by transfection of a NF- κ B reporter (Fig. 7A). We observed that NF- κ B activity was about 5 fold higher in MDA-MB-231 cells compared to MCF-7 cells. When inhibiting NF- κ B activity with the adenovirus-mediated expression of a NF- κ B super-repressor, I κ B α I κ B(SA)2 (Haller et al., 2002), IL-8 promoter activity was reduced by about 80% (Fig. 7B), confirming the involvement of NF- κ B in IL-8 gene regulation.

One question which could be raised was whether ER was directly involved in the lower expression of IL-8 in ER-positive breast cancer cells. To test this hypothesis, we restored ER α expression in

MDA-MB-231 cells and evaluated IL-8 promoter activity (Fig. 7C). IL-8 promoter activity was down-regulated by about 40% by ER bound to E2 or not. We suspected that AP-1 and NF- κ B pathways could be affected by reintroduction of ER, which in turn could decrease IL-8 promoter activity. When transfecting a NF- κ B reporter, ER was able to inhibit by 40% the activity of the construct in a ligand-independent manner, whereas AP-1 activity was only inhibited by liganded-ER (Fig. 7C). The fact that ER was decreasing only modestly the activity of IL-8 promoter suggests that ER alone is certainly not the only factor involved in IL-8 expression.

We next wished to validate our *in vitro* results by analyzing the endogenous IL-8 gene regulation. To confirm the central involvement of NF- κ B in IL-8 gene regulation in MDA-MB-231 cells, we used the adenovirus encoding I κ B super-repressor. The blockage of NF- κ B pathway with I κ B super-repressor shut down by about 80% IL-8 secretion (Fig. 8A) and RNA levels (Fig. 8B) of MDA-MB-231 cells, underlining the key role of NF- κ B family in IL-8 regulation. Concerning AP-1 role in IL-8 regulation in MDA-MB-231 cells, we used RNA interference to suppress the expression of Fra-1, Fra-2 and c-Jun (Fig. 8C). Despite the efficiency of Fra-1, Fra-2, and c-Jun knock-down, IL-8 secretion by MDA-MB-231 cells was decreased by only 25 to 40% by RNA (Fig. 8C), confirming that although important, AP-1 members are not the critical regulators of IL-8 gene activity in ER-negative breast cancer cells.

Based on all the experiments presented above, NF- κ B seems to play the most important role in IL-8 gene regulation in ER-negative breast cancer cells, though AP-1 and C/EBP factors are also involved in IL-8 gene regulation in MDA-MB-231 cells. We thus tested whether the overexpression in MCF-7 cells of the combination of AP-1, C/EBP and NF- κ B members which bind to IL-8 promoter in MDA-MB-231 cells, could induce an IL-8 gene transcription comparable to that observed in ER-negative cells (Fig. 8D). We observed a very potent synergy between AP-1 and NF- κ B members. In these conditions, IL-8 promoter activity in MCF-7 cells reached levels very similar

to that of MDA-MB-231 cells, confirming the involvement of NF- κ B and AP-1 transcription factors in IL-8 gene regulation. NF- κ B and C/EBP transcription factors were able to regulate additively IL-8 promoter, whereas the combination of C/EBP and AP-1 members was not efficient. Notably, when combining AP-1, C/EBP and NF- κ B transcription factors, IL-8 promoter activity was lower than that in the presence of transfected NF- κ B and AP-1 cDNAs, underlining the central synergy between NF- κ B and AP-1.

DISCUSSION

Although progress has been made in breast cancer management over the last few years, the mechanisms underlying the more aggressive phenotype of estrogen-independent breast cancers compared to estrogen-dependent breast cancers remain elusive. We have shown that IL-8 is more expressed in ER-negative than in ER-positive breast cancers cells (Freund et al., 2003), and this has been confirmed more recently by another group (Lin et al., 2004). We have also shown that IL-8 could enhance the invasiveness of ER-positive breast cancer cells (Freund et al., 2003). In this study, we have now analyzed the factors responsible for the higher expression of IL-8 in ER-negative breast cancer cells.

We report that, despite a low basal level of IL-8 expression, IL-8 gene which can be induced by treatment with known activators, is functional in MCF-7. In fact, no mutation of the promoter region was found in MCF-7 cells as well as in MDA-MB-231 cells. Southern blot and run-on experiments, showed that constitutive IL-8 activation in MDA-MB-231 cells is directly correlated with IL-8 promoter activity rather than gene amplification. Detailed IL-8 promoter analysis enabled us to identify three regulatory elements, NF- κ B, C/EBP and AP-1, whose activity correlates with IL-8 expression. NF- κ B is the predominant site controlling IL-8 gene activity, as its mutation, completely abolished IL-8 promoter activity in MDA-MB-231 cells. The strong inhibition observed on endogenous expression of IL-8 gene, using infection with an adenoviral vector encoding the cytoplasmic NF- κ B repressor protein I κ B α (Haller et al., 2002) confirmed that NF- κ B constitutes the main regulator of IL-8 activity in these cells. These data are in agreement with several studies showing that NF- κ B is involved in the control IL-8 gene transcription by of numerous signals, including for instance LPS, TNF α , oxidative stress (Kunsch et al., 1994; Kunsch & Rosen, 1993; Roebuck, 1999). The effects of individual mutations of AP-1 or C/EBP were not as drastic as NF- κ B mutation, certainly because high levels of NF- κ B are present in MDA-MB-231 cells. Moreover, the use of siRNA against c-Jun, Fra-1 or Fra-2 only modestly diminished endogenous levels of IL-

8, confirming this hypothesis. Interestingly, mutation of both C/EBP and AP-1 sites led to an activity similar to that of the construct bearing the NF- κ B mutation. This suggests, that NF- κ B alone is not efficient and needs either C/EBP or AP-1 transcription factors to fully activate IL-8 gene transcription. This is in agreement with our transient transfection experiments in MCF-7 cells, showing that NF- κ B, AP-1 or C/EBP alone only modestly up-regulate IL-8 promoter activity. On the contrary, the restoration of NF- κ B, either with AP-1 or to a lesser extent with C/EBP transcription factors leads to a synergistic activation of IL-8 promoter. On the other hand, overexpression of both AP-1 and C/EBP is inefficient, demonstrating the key role played by NF- κ B. The coexpression of NF- κ B in combination either with AP-1 or C/EBP in MCF-7 cells was sufficient to retrieve an IL-8 promoter activity similar to that of MDA-MB-231 cells and simultaneous expression of the three factors was not more efficient. It is worth mentioning that introduction of C/EBP α and C/EBP β interfere with the synergy between AP-1 and NF- κ B. C/EBP might partially interfere with them, by steric hindrance. C/EBP and NF- κ B sites are partially overlapping and thus C/EBP might prevent proper contacts between AP-1 and NF- κ B. On the other hand, when AP-1 is not present in the cell, C/EBP would be able to partially palliate to this deficiency by synergizing with NF- κ B.

We have also evaluated to which extent ER by itself could contribute to the lower expression of IL-8 in ER-positive breast cancer cells. We previously showed that introduction of ER α or ER β in ER-negative cancer cells leads to a 40% ligand-independent decreased expression of IL-8 (Freund et al., 2003), suggesting that it could be the case. However, it is worth mentioning that the inhibition of ER activity with anti-estrogens in ER-positive breast cancer cells, does not increase IL-8 expression (data not shown). We now show that exogenous expression of ER α leads to a 40% ligand-independent decreased activity of IL-8 promoter, which is in agreement with a recent study (Lin et al., 2004). Moreover, our data suggest that this inhibition is likely to occur through a decreased NF- κ B activity as shown by transfection assay. AP-1 repression is only observed in the presence of

estradiol and thus would not account for the repression of IL-8 expression observed in the absence of estradiol. NF- κ B and AP-1 repression by ER have already been reported (Nakshatri et al., 1997; Philips et al., 1998). The fact that ER only decreases by 40 to 50% both IL-8 expression (Freund et al., 2003) and IL-8 promoter activity suggests that ER alone is not the only parameter accounting for IL-8 lower expression in ER-positive breast cancer cells. Indeed ER-negative and ER-positive breast cancer cells differ by many aspects in addition to the sole ER factor and we believe that NF- κ B, C/EBP and AP-1 are certainly more direct regulators of IL-8 gene.

Gel shift assay and western blot analysis show that NF- κ B, AP-1 and to a lesser extent C/EBP transcription factors are much more abundant in MDA-MB-231 cells than in MCF-7 cells. This is in agreement with previous studies showing that members of AP-1 family such as Fra-1 are overexpressed in ER-negative breast cancer cells (Philips et al., 1998). A recent study has highlighted also the possible role of C/EBP proteins in breast tumor progression. C/EBP β might be linked to tumor progression, indicative of an unfavorable prognosis and linked to ER-negative status, whereas C/EBP α , did not seem involved in breast cancer etiology (Milde-Langosch et al., 2003).

NF- κ B, the crucial factor controlling IL-8 expression in MDA-MB-231 cells regulates numerous genes, including those encoding a number of cytokines, cytokine receptors and growth factors. Constitutive NF- κ B activity is associated with aggressive forms of different types of cancers (Le et al., 2000; Nakshatri et al., 1997; Newton et al., 1999; Zerbini et al., 2003). Additionally, several studies suggest that NF- κ B transcription factors are involved in cancer growth and development (Bharti & Aggarwal, 2002; Gilmore et al., 2002). It has been demonstrated that in mammary cancer cells, NF- κ B promotes cell survival through inhibition of apoptosis (Sovak et al., 1997). Similarly, it has been reported recently that NF- κ B is constitutively activated in human melanoma and human pancreatic cancer cells, and that inhibition of NF- κ B activity results in down-regulation of IL-8 expression levels and inhibition of *in vitro* cell proliferation and metastasis, respectively (Fujioka et

al., 2003; Patel et al., 2002). Interestingly, constitutive transcriptional activity of NF- κ B has been associated to ER-negative MDA-MB-231 breast cancer cells and was shown to be suppressed by cotransfection of ER (Nakshatri et al., 1997).

In conclusion, we provide evidence that although NF- κ B is critical for IL-8 gene expression, cooperation with either AP-1 or C/EBP is required for optimal IL-8 gene activation in breast cancer cells. Overexpression of these three families of transcription factors accounts for the higher expression of IL-8 in invasive breast cancer cells and certainly for the aggressiveness of ER-negative breast cancers.

MATERIALS AND METHODS

Antibodies

Antibodies against AP-1 and C/EBP members were from Santa Cruz: c-fos (sc-52x), c-Jun (sc-45x), Fra-1 (sc-602x), Fra-2 (sc-604x), C/EBP α (sc-61x), C/EBP β (sc-150X), C/EBP δ (sc-151X), HDAC-1 (sc-7872). Antibodies against NF- κ B family members were from Trans-AM NF- κ B Family kit (Active Motif)

Cell Culture. Cells were maintained in media recommended by ATCC supplemented with 10% fetal calf serum (FCS) and gentamycin.

Plasmids. CMV5, CMV-ER α and (AP-1)₄-TK-CAT reporter plasmid have been previously described (Philips et al., 1998) (Lazennec et al., 2001). pNF κ B luciferase reporter was from Clontech. IL-8 promoter corresponding to -1481/+44 bp was cloned from MCF-7 and MDA-MB-231 cells using 5'-CGGATCCGAATTCGAGTAACCCAGGCATTATT-3' and 5'-CGGATCCAGCTTGTGTGCTCTGCTGTCTCTGAAA-3' primers. The corresponding plasmid was used for site-directed mutagenesis to mutate AP-1, C/EBP and NF- κ B sites. Primers used for mutagenesis were 5'-CAAATAGGAAGTGTGATactTggGGTTTGCCCTGAGGGGATGGGC-3' for AP-1, 5'-GAGGGGATGGGCCATCAGcTaCgAgTCGTGGAATTCCTCTGACA-3' for C/EBP and 5'-GGATGGGCCATCAGTTGCAAATCGTtaAcTTTCCTCTGACATAATG-3' for NF- κ B. Deleted IL-8 promoter constructs were constructed by PCR. CMV-GAL corresponds to the β -galactosidase gene under the control of the cytomegalovirus (CMV) promoter.

Transient Transfection. 3.10^5 cells were plated in 6-well plates in phenol red-free DMEM-F12 supplemented with 10% CDFCS 24 h before transfection. Transfections were performed with

lipofectamine according to the manufacturer's recommendations using 4 µg of luciferase reporter along with 100 ng of each expression vector and 0.8 µg of the internal reference reporter plasmid (CMV-Gal) per well. 24h after lipofection, cells were harvested and assayed for luciferase activity on Centro LB960 Berthold luminometer. CAT and β-galactosidase activities were determined as previously described (Lazennec et al., 2001).

Recombinant adenovirus construction, propagation and infection. The adenoviruses Ad5 and IκB(SA)2 (S32A and S36A mutations in IκBα) used in this study and their propagation have been described previously (Haller et al., 2002; He et al., 1998; Lazennec et al., 2001). MCF-7 and MDA-MB-231 cells were infected for 18h at a multiplicity of infection (MOI) of 100 with the different adenoviruses in DMEM/F12 10% CDFCS. The next day, the medium was changed and the cells were let to express IL-8 for 48h before collecting the medium.

RNA Extraction and Reverse Transcriptase, quantitative PCR. Total RNA was isolated with TRIzol reagent (Invitrogen) as described by the manufacturer. Reverse transcription was performed using 5 µg total RNA, random primers and Superscript II enzyme (Invitrogen). Quantitative PCR was performed with FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) on a Light Cycler instrument (Roche) as specified by the manufacturer. Ribosomal protein S9 (rS9) was used as an internal control. Primers (s: sense, as: anti-sense) used were:

IL8s: CACCGGAAGGAACCATCTCACT

IL8as: TCAGCCCTCTTCAAAAACCTTCTCC

rS9s: AAGGCCGCCCCGGGAAGTCTGAC

rS9as: ACCACCTGCTTGCGGACCCTGATA

Run-on Assay

Nuclear run-ons were performed as described previously (Milligan et al., 2000) with some modifications. Equal amounts (10^7 cpm/ml) of labeled nuclear RNA were hybridized at 65 °C for 24 h to Zeta-probe membranes (Biorad) previously bound with 5 µg of linearized plasmid DNAs. The immobilized plasmids used were pCR2-IL8 and pCR2-rS9. After washing, the filters were subjected to autoradiography. Radioactive transcripts were quantified on a Fuji Bas Reader. Data were normalized to transcription of the rS9 gene.

Nuclear-cell extract preparation, gel shift and western blot

Cells extracts and gel shift assays were performed as previously (Lazennec et al., 2001). Briefly, 30,000 cpm of the [32 P]-labeled double-strand oligonucleotides were combined with 1 µg poly (dI-dC) and 2 µg of nuclear cell extract. When indicated, 1 µg of antibodies were added. Oligonucleotides used were:

AP-1: AGTGTGATGACTCAGGTTTGCCC

AP-1 cons: CGCTTGATGAGTCAGCCGGAA

NF-κB: CGTGGAATTCCTCTG

NF-κB cons: AGTTGAGGGGACTTTCCCAGGC

C/EBP: CATCAGTTGCAAATCGTGG

C/EBP cons: TGCAGATTGCGGAATCTGCA

For western blot experiments, 30 µg of protein extracts were subjected to SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane. The blot was probed with the different antibodies and then incubated with horseradish peroxidase conjugated secondary antibody. An ECL kit (Amersham Pharmacia Biotech, Arlington, IL) was used for detection.

IL-8 ELISA. IL-8 concentration in culture supernatants was determined by ELISA as recommended previously described (Freund et al., 2003).

RNAi-mediated Knockdown

RNAi-mediated knockdown was performed with the 19-nucleotide targets for short, interfering RNAs (siRNAs) as follows: siRNA for Fra-1, 5'- CUGACUGCCACUCAUGGUG –3'; Fra-2, 5'- CUUUGACACCUCGUCCCGG-3'; control non-silencing GFP siRNA, 5'- CUUUGACACCUCGUCCCGG -3'; c-Jun, 5'- GAUCCUGAAACAGAGCAUG –3'. siRNA were transfected using 4 µl of Lipofectamine and 2.5 µg of each siRNA. 48h after transfection, cells and medium were harvested and processed for Western blot or ELISA, respectively.

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LEGENDS TO FIGURES

Fig. 1. IL-8 gene is functional in MDA-MB-231 and MCF-7 cells. Cells were treated for 48h with mock (NT), IL-1 α (50 ng/ml), IL-1 β (250 pg/ml), TNF α (50 ng/ml). Media were then collected to evaluate IL-8 levels by ELISA. Results are expressed as ng IL-8/ml/48h/million cells and represent the mean \pm SD of three independent experiments.

Fig. 2. The differential expression of IL-8 gene in MDA-MB-231 and MCF-7 cells takes place at the transcriptional level. **A.** IL8 RNA levels were quantified in MDA-MB-231 and MCF-7 cells by quantitative PCR. Results are expressed as arbitrary units corresponding to the ratio of IL-8 levels normalized by rS9 levels (n=3). **B.** The transcription rates of IL-8 and rS9 genes in MDA-MB-231 and MCF-7 cells were determined by run on assay. A representative experiment is shown here and the transcription rate, expressed in arbitrary units as the ratio of IL-8 signal over rS9 signal, represents the mean \pm SD of three independent experiments. **C.** IL-8 promoter was cloned in sense (xp2-IL8) or anti-sense orientation (xp2-IL8 AS) in xp2 reporter vector. MDA-MB-231 and MCF-7 cells were transfected with the different constructs along with CMV-GAL internal control. Results show relative luciferase activities (n=3) after normalization for β -galactosidase activity. **D.** Secreted IL-8 levels from MDA-MB-231, MDA-MB-436, CAMA-1 and MCF-7 cells were determined by ELISA (left panel). Results represent the mean \pm SD of three independent experiments. The cell lines mentioned above were transfected with xp2 or xp2-IL8 constructs along with CMV-GAL reporter (right panel). Results show fold activities (ratio of xp2-IL8 activity over xp2) after normalization for β -galactosidase activity (n=3).

Fig. 3. Determination of IL-8 promoter sequences involved in differential regulation of IL-8 gene activity. MDA-MB-231 and MCF-7 cells were transfected with reporter constructs

corresponding to the first 1481, 272, 98 or 50 bp of IL-8 promoter. Results show relative luciferase activities (n=3) after normalization for β -galactosidase activity.

Fig. 4. NF- κ B site is crucial for the high activity of IL-8 promoter. **A.** IL-8 promoter constructs harboring single, double or triple mutations of AP-1, C/EBP or NF- κ B sites were transfected in MDA-MB-231 and MCF-7 cells. Results show relative luciferase activities (n=3). **B.** Binding of nuclear factors to AP-1, C/EBP and NF- κ B sites was examined by gel shift assays using 2 μ g of nuclear extracts of MDA-MB-231 and MCF-7 cells. The right panel is a control of nuclear extract loading which corresponds to a western blotting of HDAC-1 antibody against MCF-7 and MDA-MB-231 nuclear extracts.

Fig. 5. Identification of the factors of MDA-MB-231 cells bound to IL-8 promoter.

A. Factors bound to C/EBP site of IL-8 promoter in MDA-MB-231 cells were identified by gel shift assay using C/EBP α , C/EBP δ and C/EBP β antibodies. Competition with a 10 fold excess of cold consensus C/EBP or NF- κ B oligonucleotides confirms the specificity of the complexes. **B.** The identification of AP-1 members of MDA-MB-231 cell extracts bound to AP-1 site was performed by gel shift assay using c-fos, c-Jun, Fra-1 and Fra-2 antibodies. Competition with a 10 fold excess of cold C/EBP or consensus AP-1 oligonucleotides confirms the specificity of the complexes. **C.** NF- κ B proteins present in MDA-MB-231 cells which bound to the NF- κ B site were identified by gel shift assay using p50, p65, c-Rel, p52 and RelB antibodies. Oligonucleotides competition were done with a 10 fold excess of cold AP-1 or consensus NF- κ B probes. **D.** Protein levels of Fra-1, Fra-2, c-jun, p50, p65, C/EBP α and C/EBP β were determined by western blot using 30 μ g of MDA-MB-231 and MCF-7 whole cell extracts. The upper band observed on Fra-1 blot corresponds to the phosphorylated form of the protein.

Fig. 6. AP-1 pathway is important for IL-8 gene activity.

A. AP-1 relative activity was assessed in MDA-MB-231 and MCF-7 cells by transfection of an AP-1 reporter. Results show relative CAT activities (n=3) after normalization for β -galactosidase activity. **B.** The importance of AP-1 pathway in IL-8 promoter activity regulation was evaluated by transfection of 100 ng of empty vector (C) or expression vectors of JDP1, JDP2 and Tam67 AP-1 inhibitors in MDA-MB-231 and MCF-7 cells, along with xp2-IL8 construct. Results show relative luciferase activities (n=3).

Fig. 7. NF- κ B factors are the primary regulators of in IL-8 promoter activity. **A.** NF- κ B relative activity was evaluated in MDA-MB-231 and MCF-7 cells by transfection of a NF- κ B reporter. Results show relative luciferase activities (n=3). **B.** The importance of NF- κ B in IL-8 promoter activity regulation was evaluated by infection of MDA-MB-231 and MCF-7 cells with Ad5 and Ad-I κ B viruses (MOI 100) and transfection of xp2-IL8 construct. Results show relative luciferase activities (n=3). **C.** xp2-IL-8, NF- κ B and AP-1 reporters were transfected in MDA-MB-231 cells along with CMV-GAL and control CMV5 or CMV-hER α expression vectors. Cells were treated or not with estradiol (10⁻⁸M) for 24h. Results show relative luciferase and CAT activities (n=3).

Fig. 8. NF- κ B, AP-1 and C/EBP transcription factors are acting together to control IL-8 gene expression.

A. MDA-MB-231 cells were infected at MOI 100 with backbone Ad5 or Ad-I κ B viruses. 24h after infection, the medium was collected and assayed for IL-8 levels by ELISA. Results represent the mean \pm SD of three independent experiments. **B.** RNA were collected from the same cells and used to measure IL-8 RNA levels by quantitative PCR. Results are expressed as arbitrary units corresponding to the ratio of IL-8 levels normalized by rS9 levels (n=3). **C.** Reduction of IL-8

expression using RNA interference against Fra-1, Fra-2 or c-Jun. MDA-MB-231 cells were transfected with siFra-1, siFra-2, sic-Jun or siGFP (as control). The extent of silencing of was determined by western blot using antibodies against Fra-1, Fra-2, c-Jun or β -actin (left panel). Secreted IL-8 levels were measured by ELISA 48h after transfection. Results represent the mean \pm SD of three independent experiments. **D.** xp2-IL8 reporter was cotransfected along with 100 ng of expression vectors of Fra-1, Fra-2, c-Jun, p50, p65, C/EBP α or C/EBP β in MCF-7 cells. Results show relative luciferase activities (n=3).