NF-κB Promotes Breast Cancer Cell Migration and Metastasis by Inducing the Expression of the Chemokine Receptor CXCR4*

Received for publication, January 20, 2003, and in revised form, March 17, 2003
Published, JBC Papers in Press, April 9, 2003, DOI 10.1074/jbc.M300609200

Gregory Helbig‡‡, Kent W. Christopherson II§§,**‡‡, Poornima Bhat-Nakshatri**, Suresh Kumar†, Hiromitsu Kishimoto‡, Kathy D. Miller§§, Hal E. Broxmeyer**§§, and Harikrishna Nakshatri‡‡**‡‡

From the Departments of §Surgery, ¶¶Microbiology and Immunology, §§Medicine, and ¶¶Biochemistry and Molecular Biology and the ¶¶Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202 and the **Walther Cancer Institute, Indianapolis, Indiana 46208

Metastasis of cancer cells is a complex process involving multiple steps including invasion, angiogenesis, and trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth. While matrix metalloproteinases, urokinase-type plasminogen activator, and cytokines play a major role in invasion and angiogenesis, chemokines such as stromal derived factor-1α (SDF-1α) and their receptors such as CXCR4 are thought to play a critical role in motility, homing, and proliferation of cancer cells at specific metastatic sites. We and others have previously reported that the extracellular signal-activated transcription factor NF-κB up-regulates the expression of matrix metalloproteinases, urokinase-type plasminogen activator, and cytokines in highly metastatic breast cancer cell lines. In this report, we demonstrate that NF-κB regulates the motility of breast cancer cells by directly up-regulating the expression of CXCR4. Overexpression of the inhibitor of NF-κB (IκB) in breast cancer cells with constitutive NF-κB activity resulted in reduced expression of CXCR4 and a corresponding loss of SDF-1α-mediated migration in vitro. Introduction of CXCR4 cDNA into IκB-expressing cells restored SDF-1α-mediated migration. Electrophoretic mobility shift assays and transient transfection assays revealed that the NF-κB subunits p65 and p50 bind directly to sequences within the −66 to +7 region of the CXCR4 promoter and activate transcription. We also show that the cell surface expression of CXCR4 and the SDF-1α-mediated migration are enhanced in breast cancer cells isolated from mammary fat pad xenografts compared with parental cells grown in culture. A further increase in CXCR4 cell surface expression and SDF-1α-mediated migration was observed with cancer cells that metastasized to the lungs. Taken together, these results implicate NF-κB in the migration and the organ-specific homing of metastatic breast cancer cells.

* This work was supported by American Cancer Society Grant RPG-00-122-01-TBE, American Institute for Cancer Research Grant 00A047, National Institutes of Health Grant CA82208, and the Phi Beta Psi Sorority (all to H. E. B.) and by National Institutes of Health Grants DK53674 and HL67384 (to H. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. || To whom correspondence should be addressed: R4-202 Indiana Cancer Research Inst., 1044 W. Walnut St., Indianapolis, IN 46202. Tel.: 317-278-2238; Fax: 317-274-0396; E-mail: hnakshat@iuupui.edu.

Morbidity and mortality in cancer are mainly due to organ-specific metastasis and the failure of chemotherapeutic drugs to selectively kill cancer cells at the sites of metastasis. Metastasis is a non-random process, and each cancer type has its own preferred sites of metastasis (1). For example, breast cancer cells preferentially metastasize to the regional lymph nodes, lungs, liver, and bone (1, 2). Prostate cancers usually metastasize to bone. While there has been considerable progress in identifying genes that promote the metastasis of cancer cells, little is known about the genes that enable cancer cells to seed, survive, and proliferate at sites of metastasis. Three models of organ-specific metastasis are currently under consideration: 1) selective survival and proliferation of cancer cells in a particular organ due to local production of appropriate growth factors, 2) organ-specific endothelial cells trapping circulating tumor cells by expressing appropriate adhesion molecules on their surface, and 3) organ-specific attractant molecules helping in homing cancer cells to specific sites (3). While data supporting the first two models are still scanty, a recent study provided evidence supporting the third model. Muller et al. (4) demonstrated that metastatic breast cancer cells overexpress the chemokine receptor CXCR4. Additionally sites to which breast cancer cells metastasize express abundant amounts of stromal derived factor-1α (SDF-1α,* recently renamed CXCL12), the ligand for CXCR4. Moreover antibodies against CXCR4 significantly inhibited lymph node and lung metastasis in xenograft models of breast cancer. These results suggest that SDF-1α serves as a homing factor for cancer cells and that the signaling pathways activated upon interaction of CXCR4 with SDF-1α play a role in the survival and proliferation of cancer cells once they are localized in a specific organ.

The transcription factors that regulate CXCR4 expression in breast cancer cells are currently unknown. We considered the possibility that the extracellular signal-activated transcription factor NF-κB is involved in the expression of CXCR4 because NF-κB has been shown to up-regulate the expression of several prometastatic and proangiogenic genes including interleukin 6

* The abbreviations used are: SDF-1α, stromal derived factor-1α; CXCR, CXC chemokine receptor; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; IκBα, super-repressor; IL, interleukin; EMSA, electrophoretic mobility shift assay; uPA, urokinase-type plasminogen activator; MMP, matrix metalloproteinase; IKK, IκB kinase complex; NIK, NF-κB-inducing kinase; CAP, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; β-gal, β-galactosidase; XIAP, X-linked inhibitor of apoptosis protein; TRAF, tumor necrosis factor receptor-associated factor; cIAP, cellular inhibitor of apoptosis protein; TRPM-2, testosterone-repressed prostate message-2; SP-1, specificity protein-1; BLR, Burkitt’s lymphoma receptor; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoylphorbol-13-acetate.
mediated migration was observed in cancer cells that metastasize in xenograft models (9). NF-κB is a heterodimeric complex of Rel family proteins that is physically confined to the cytoplasm of normal cells through its interaction with inhibitor of κB (IκB) proteins (6). A heterodimer composed of p50 and p65 subunits is the predominant form of NF-κB, although several other cell type-specific heterodimers have been identified. Upon exposure of cells to growth factors and cytokines such as epidermal growth factor, IL-1, and tumor necrosis factor α, a series of signaling events target IκB for degradation, promoting the nuclear translocation of NF-κB. NF-κB binds to its response elements (5'-GGGGPuNNNNpPyCC-3' where Pu is a purine and Py is a pyrimidine) in the promoter region of target genes and activates transcription (5). CXCR4 may be one of the NF-κB target genes as a putative NF-κB binding site (5'-GAGGCAATTCC-3', −230 to −240) is present in the promoter region of CXCR4 (10).

Several laboratories, including ours, have demonstrated constitutive activation of NF-κB in a variety of cancers (11–17). We have shown that constitutively active NF-κB is responsible for overexpression of prometastatic and antiapoptotic genes in breast cancer cells (14, 18–20). In addition, we and others have shown that cancer cell-derived heregulin, IL-1α, and/or overexpression of epidermal growth factor receptor is involved in constitutive NF-κB activation in breast cancer (21–24). The present study was initiated to determine whether NF-κB promotes organ-specific metastasis by selectively up-regulating CXCR4. We demonstrate that NF-κB directly regulates the expression of CXCR4, which appears to be critical for the motility of cancer cells in response to SDF-1α in vitro. In addition, we also show that cells that express CXCR4 are clonally selected during their growth in the mammary fat pad of nude mice. A further increase in CXCR4 expression and SDF-1α-mediated migration was observed in cancer cells that metastasized to the lungs.

EXPERIMENTAL PROCEDURES

Breast Cancer Cell Lines—The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the ATCC and grown in minimum essential medium + 10% fetal calf serum. LxSN11, IκBαSR6, and IκBαSR10 cells, which are derived from MDA-MB-231 cells, have been described previously (19).

Plasmid Constructs and Transfection Assays—The CXCR4 promoter was cloned by PCR using a Genome Walker kit (Clontech). PCR-amplified DNA was cloned into the pBL-CAT3 vector and sequenced. Deletion mutants were also generated by PCR. The p65, p50, and Bcl-3 expression vectors were a generous gift from Dr. W. Greene (Gladstone Institute for Virology and Immunology, San Francisco, CA). The expression vectors for NIK, IKKα, IKKβ, and Bcl-3 expression vectors were a kind gift from Dr. G. Alkhatib (Indiana University School of Medicine). The CXCR4 expression vector was a kind gift from Dr. G. Alkhatib (Indiana University School of Medicine). MCF-7 and MDA-MB-231 cells were transfected with the CXCR4/CAT reporter and expression vectors along with a β-galactosidase expression vector (Bgal and pH110 for MCF-7 and MDA-MB-231, respectively) by the calcium phosphate method. The β-galactosidase activity and CAT activity in an equal number of β-galactosidase units were measured as described previously (14).

Electrophoretic Mobility Shift Assay (EMSA)—COS-1 cells were transfected with 10 μg of expression vectors, and whole cell extracts or nuclear extracts were prepared 48 h after transfection as described previously (14, 19). EMSA was performed with CXCR4, NF-κB, and SP-1 probes as described previously (14).

RNase Protection Assay—Total RNA was prepared using the RNeasy kit (Qiagen). The RNase protection assay with hCR6 or hAPO-5 probes (BD Pharmingen) was performed as described previously (19). The hCR6 probe set simultaneously measures the expression of CXCR1, CXCR2, CXCR3, CXCR4, BLR-1, BLR-2, and V28. The hAPO-5 probe measures XIAP, TRAF-1, TRAF-2, TRAF-3, TRAF-4, cIAP-1, cIAP-2, and TRPM-2.

Mouse Mammary Fat Pad Injection and Isolation of Cancer Cells—MDA-MB-231 cells (10⁶) were injected into the mammary fat pad of 7-week-old nude mice. Mammary tumors were resected after 6 weeks, minced, and digested for 1 h at 37 °C in digestion buffer (2 mg/ml collagenase type 3, 3 mg/ml hyaluronidase in 10 ml of PBS/g of tumor). Cell pellets were digested again for 20 min at 37 °C with protease (12.5 mg/ml in PBS). Cell pellets were washed in PBS and plated in growth medium. Metastatic cells from the lungs were similarly isolated with the exception that the lungs were collected 8 weeks after removal of the primary tumor. CXCR4 expression was measured periodically in cultured tumor- and metastasis-derived cancer cells. Data presented in the text were obtained from cells cultured for 1 month.

Cell Surface Expression of CXCR4—Cell surface expression of CXCR4 was measured by flow cytometry. MDA-MB-231 cells were stained with fluorochrome-conjugated monoclonal antibodies to either CXCR4 or an isotype control (BD Pharmingen) in accordance with the manufacturer’s specifications and then analyzed by flow cytometry (25). The staining protocol used was as follows. Cells were first washed in PBS, penicillin, streptomycin, 1% bovine serum albumin containing the appropriate antibody. Samples were mixed and incubated at 4 °C in the dark for 40 min. The cells were then washed twice in PBS, penicillin, streptomycin, 1% bovine serum albumin and fixed in PBS, 1% paraformaldehyde. One hundred thousand events were accumulated for each analysis. Samples were analyzed in triplicate, and the data were averaged for statistical analysis. Data are presented as means ± S.E., and comparisons were made using the two-tailed Student’s t test.

Chemosatosis Assay—Chemosatosis assays were performed using 96-well chemosatosis chambers (NeuroProbe) in accordance with the manufacturer’s instructions as described previously with minor variations (25). In brief, 0, 12.5, 25, 50, 100, 200, 400, or 800 ng/ml CXCL12/SDF-1a was added to 300 μl of phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum in the lower chamber. Twenty-five thousand fluorescent-tagged (4 μg/ml calcein AM, Molecular Probes, Eugene, OR) cells in 50 μl of medium were added to the upper chamber, separated from the lower chamber by a membrane...
NF-κB subunits. A, TPA and IL-1α induce CXCR4/CAT activity. MCF-7 breast cancer cells were transfected with the indicated CXCR4/CAT reporters (5 μg) and with RSVβ-gal (2 μg) as an internal control. TPA (125 nm) or IL-1α (5 ng/ml) was added 24 h after transfection. CAT activity in an equal number of β-galactosidase units was measured 36 h after transfection. B, IxBosSR reduces CXCR4/CAT activity in MDA-MB-231 cells. C, induction of CXCR4/CAT activity by p65, IKKα, and NIK. MCF-7 cells were transfected with the indicated CXCR4/CAT reporter constructs and expression vectors (0.5 μg) or the control vector pcDNA3. CAT activity was measured as described above. TNF, tumor necrosis factor.

for in vitro studies (26). Parthenolide reduced the expression of CXCR4 in MDA-MB-231 cells (Fig. 1B). To determine whether IL-1α, a potent inducer of NF-κB, alters CXCR4 expression, we performed an RNase protection assay with RNA from MCF-7 breast cancer cells containing the empty vector (pcDNA3) or cells that stably overproduce IL-1α. These cell lines have been described elsewhere. Consistent with the role of NF-κB in CXCR4 expression, IL-1α-overproducing cells displayed elevated CXCR4 compared with cells with the pcDNA3 vector (Fig. 1C).

To ensure that there is a correlation between CXCR4 mRNA and cell surface expression of the CXCR4 protein, we performed flow cytometric analysis with an antibody that specifically recognizes CXCR4 on the cell surface. Cell surface expression of CXCR4 was observed in MCF-7 cells that overexpress IL-1α but not in MCF-7-pcDNA3 cells (Fig. 2). Taken together, these results indicate that CXCR4 expression in breast cancer cells is regulated by NF-κB as well as by the cytokines that induce NF-κB.

NF-κB Directly Regulates the CXCR4 Promoter—To determine whether NF-κB directly regulates CXCR4 promoter activity, we performed a transient transfection assay in MCF-7 breast cancer cells with a CXCR4/CAT reporter. Two reporters, one with the −897 to +7 region (CXCR4(−897)/CAT) and the

---

2 S. Kumar, K. D. Miller, H. L. Chua, H. Kishimoto, R. M. Bigsby, and H. Nakshatri, submitted.
other with the −209 to +7 region (CXCR4(−209)/CAT) of the CXCR4 promoter, were used. Reporter activity was measured in the absence or presence of the NF-κB inducers tumor necrosis factor α, IL-1α, or TPA. IL-1α and TPA induced CXCR4/CAT reporter activity (Fig. 3A). Constitutive CXCR4 promoter activity in MDA-MB-231 cells was reduced by IxBoSR (Fig. 3B). Induction of CXCR4(−209)/CAT by IL-1α and TPA suggests that induction of CXCR4 by IL-1α and TPA is an indirect effect of NF-κB activation or that the NF-κB binding site in the CXCR4 promoter is distinct from the putative site predicted by the TFSEARCH computer program. To distinguish between these two possibilities, we generated additional deletion mutants (CXCR4(−121)/CAT and CXCR4(−66)/CAT) and tested them for activity in the presence of various subunits of NF-κB. The p65 but not the p50 subunit of NF-κB increased the activity of both reporters (Fig. 3C). Among the upstream kinases involved in NF-κB activation, NIK activated the CXCR4 promoter. IKKα but not IKKβ activated CXCR4(−897)/CAT. Although the basal activity of CXCR4(−66)/CAT was 5 times lower than that of CXCR4(−121)/CAT, the p65 subunit still activated this reporter. These results suggest that the NF-κB response element is located within the −66 to +7 sequence of the CXCR4 promoter.

We next determined direct binding of the NF-κB subunits to the −66 to +7 region of the CXCR4 promoter by EMSAs using extracts from COS-1 cells transfected with various subunits of NF-κB and the −66 to +7 region of CXCR4 as a probe. The p50 subunit, either alone or in combination with the p65 subunit, bound to the probe (Fig. 4, lanes 1–4). Similar results were obtained when nuclear extracts instead of whole cell extracts were used (Fig. 4, lanes 5–7). Neither c-Rel nor Bcl-3 bound to this region (data not shown). Unlabeled oligonucleotide with a classical NF-κB binding site from the immunoglobulin promoter but not the SP-1 binding site reduced the CXCR4 probe-protein complex formation (Fig. 4, lanes 8–10). The CXCR4 DNA probe-protein complex could be disrupted by an antibody against p65 and supershifted partially by an antibody against p50 (lanes 11–14). A nonspecific antibody (against the p110 subunit of phosphatidylinositol 3-kinase) had no effect on the CXCR4 probe-protein complex (lane 14). These results indicate that the p50 and p65 subunits of NF-κB directly bind to the CXCR4 promoter. Because this region of the promoter lacks a classical NF-κB response element, it appears that the p50 and p65 subunits bind to a non-classical response element. The exact p50 and p65 binding site sequence is yet to be determined because results of a DNase I footprinting assay were inconclusive (data not shown). Also we did not detect the binding of NF-κB subunits using EMSA when the probe contained only the −66 to −33 or the −32 to +1 regions of the CXCR4 promoter (data not shown). Thus, it appears that NF-κB DNA binding requires the entire −66 to +1 region.

NF-κB Regulates SDF-1α-mediated Migration of MDA-MB-231 Cells through CXCR4—To determine the consequence of NF-κB inhibition on SDF-1α-mediated migration of MDA-MB-231 cells, we performed a chemotaxis assay with LxSN11, IxBoSR6, and IxBoSR10 cells with increasing concentrations of SDF-1α. SDF-1α-induced motility was observed with LxSN11 cells but not with IxBoSR6 and IxBoSR10 cells (Fig. 5A). We confirmed the requirement of NF-κB for the SDF-1α-induced migration of MDA-MB-231 cells by performing a chemotaxis assay with cells pretreated with parthenolide. Parthenolide at 5 μM completely inhibits constitutive NF-κB DNA binding activity in these cells (19). Inhibition of SDF-1α-dependent migration was observed with cells pretreated with parthenolide (Fig. 5B).

Recent studies indicated that NF-κB regulates migration of MDA-MB-231 cells through up-regulation of uPA (27). In that case, the failure of IxBoSR cells and parthenolide-pretreated...
cells to migrate in response to SDF-1α could be due to reduced uPA instead of CXCR4 expression. To directly prove that reduced SDF-1α-dependent migration of IxBoSR cells is due to lower levels of CXCR4 in these cells, we reintroduced CXCR4 by transient transfection and performed a chemotaxis assay. SDF-1α-dependent migration was restored in IxBoSR6 and IxBo10 cells upon reintroduction of CXCR4 cDNA (Fig. 5C). These results confirm that NF-κB is directly involved in SDF-1α-mediated migration of breast cancer cells.

MDA-MB-231 Cells Selected after Growth in Nude Mice Express Higher Levels of Cell Surface CXCR4—Although a major function of SDF-1α is to transiently up-regulate the expression of integrins involved in the binding of CXCR4-expressing cells to the endothelium and egression from the circulation, SDF-1α is also known to activate other growth-promoting signaling pathways (28, 29). If that is the case, unlike in cell culture models, cancer cells that express CXCR4 should grow well in a xenograft model as these cells can take advantage of circulating SDF-1α. Because flow cytometry with an antibody against CXCR4 revealed that less than 10% of MDA-MB-231 cells grown in culture express CXCR4 on their surface, our hypothesis was that only those cells that express higher levels of CXCR4 should metastasize to organs such as the lungs. To test this possibility, we implanted MDA-MB-231 cells into the mammary fat pad of nude mice, resected the tumors after 6 weeks, and allowed the cancer cells isolated from the tumor to grow in culture. After an additional 8 weeks, cells from the lungs of these mice were cultured. MDA-MB-231 cells isolated after their growth in the mammary fat pad (named TMD231 hereafter) or those that metastasized to the lungs (hereafter called LMD231) expressed very high levels of CXCR4 compared with parental cells grown in culture (Fig. 6, A–C). In fact, the highest CXCR4 expression was seen in lung metastatic cells. Increased cell surface expression of CXCR4 in tumor- or lung-derived cells were maintained even after 3 months of growth in culture, which suggests that there is clonal selection of CXCR4-expressing cancer cells in the mammary fat pad. TMD231 and LMD231 cells are free of contaminating mouse cells as indicated by cell surface expression of the epithelial cell-specific antigen (Fig. 6, D–F, ESA). Both TMD231 and LMD231 cells showed enhanced SDF-1α-mediated migration compared with cells grown in culture (Fig. 6G). CXCR4 mRNA levels were increased in TMD231 and LMD231 cells compared with MDA-MB-231 cells as determined by an RNase protection assay using a human-specific CXCR4 probe (Fig. 7A). All three cell types expressed similar levels of XIAP, TRAF-3, and TRAF-4 transcripts, which suggests that there is no global increase in transcription in LMD231 and TMD231 cells compared with parental cells (Fig. 7B). Thus, it appears that cancer cells that express CXCR4 on their surface are selected during growth in the mammary fat pad, which could help cancer cells to respond to SDF-1α-mediated growth as well as migration signals. In addition, these CXCR4-expressing cells have a higher potential to metastasize to the lungs.

We next examined whether inhibitors of NF-κB can reduce CXCR4 expression in LMD231 cells. Cells were incubated with either parthenolide (5 μM) or MG132 (10 μM), a proteasomal inhibitor that inhibits NF-κB activation by reducing IκBα, for 4 or 8 h. Parthenolide inhibited CXCR4 expression by ~50%, whereas MG132 reduced CXCR4 expression by ~80% after 8 h of treatment (Fig. 7C). Thus, NF-κB inhibitors have the potential to reduce growth and survival of cancer cells at metastatic sites through inhibition of CXCR4 expression.

**Fig. 5.** IxBoSR reduces SDF-1α-dependent chemotaxis of MDA-MB-231 cells. A, cells transfected with IxBoSR, IxBoSR6 (squares), and IxBoSR10 (triangles) exhibit a loss of SDF-1α-induced migration when compared with empty vector LxSN11 (diamonds) (p ≤ 0.05 at 100, 200, 400, and 800 ng/ml, n = 6). B, loss of SDF-1α-induced migration was observed following 13 h of parthenolide treatment (p ≤ 0.05 at 100, 200, 400, and 800 ng/ml, n = 7). C, loss of migration induced by IxBoSR was partially rescued by transfection with CXCR4, IxBoSR6-CXCR4 (open squares), and IxBoSR10-CXCR4 (open triangles) compared with empty vector controls, IxBoSR6-pcDNA3 (filled squares), and IxBoSR10-pcDNA3 (filled triangles) (p ≤ 0.05 at 400 and 800 ng/ml, n = 6).

**DISCUSSION**

In this report, we show that the extracellular signal-activated transcription factor NF-κB regulates the expression of the chemokine receptor CXCR4, which has recently been implicated in organ-specific metastasis of breast cancer (4). NF-κB-dependent expression of CXCR4 appears to require a non-classical response element present within the −66 to +7 sequence of the CXCR4 promoter. This response element binds to either the p50 homodimer or p65-p50 heterodimer in vitro. Because the NF-κB-DNA complex generated with the CXCR4 promoter fragment and a classical response element displayed a similar mobility pattern in EMSA, it is less likely that interaction of NF-κB subunits to the CXCR4 promoter is facilitated by additional transcription factors. Moreover the reporter gene containing only the −66 to +7 region of the CXCR4 promoter was responsive to NF-κB, which implies that the transactivation by NF-κB subunits is direct. A number of known activators of NF-κB, including TPA and CD30, have previously been shown to induce CXCR4 (10, 30). Because of a lack of the
classical NF-κB-related binding sites in the promoter, it was suggested that transcription factors such as activator protein-1, SP-1, and nuclear respiratory factor-1 are responsible for constitutive and inducible expression of CXCR4. This study provides more compelling evidence for the direct involvement of NF-κB in the regulation of CXCR4 expression. Mapping of the precise NF-κB binding site within the −66 to +7 region may require additional studies such as chromatin immunoprecipitation assays.

Vascular endothelial growth factor has been shown to induce CXCR4 in breast cancer cells (31). Because vascular endothelial growth factor is a NF-κB-inducible gene (32), it is possible that the regulation of CXCR4 by NF-κB may be indirect in some cell types. However, vascular endothelial growth factor may not be responsible for the increased expression of CXCR4 in IL-1α-overexpressing MCF-7 cells as both parental and IL-1α-overexpressing cells contained similar levels of vascular endothelial growth factor transcripts (data not shown). Be-
cause both MCP-7 and MDA-MB-231 cells do not express IL-8 receptors CXCR1/2, IL-8 is less likely to be involved in CXCR4 expression (data not shown, also the riboprobe used in Fig. 7 measures CXCR1/2). Taken together, our results as well as published results by others (31, 32) suggest both direct and indirect regulation of CXCR4 expression by NF-κB in breast cancer cells.

Most of the current work on breast cancer metastasis focuses on the role of MMPs and the uPA/uPA receptor/plasminogen network (33, 34). It is believed that the uPA/uPA receptor/plasminogen network activates pro-MMP-1, -MMP-3, -MMP-9, and -MMP-13 produced by stromal cells. Activated MMPs break down the physical barriers of metastasis, thus promoting invasion, intravasation, and extravasation of cancer cells (33, 34). In addition, MMPs promote the growth of cancer cells at both primary and metastatic sites. We and others have shown that the expression of uPA and MMPs in cancer and stromal cells is regulated by NF-κB (8, 18). The present report adds CXCR4 to the list of prometastatic genes under the control of NF-κB. Thus, inhibitors of NF-κB should reduce breast cancer metastasis by reducing the expression of a number of prometastatic genes. NF-κB inhibitors should also reduce metastasis of other cancers as several cancers including melanoma, ovarian, prostate, brain, and pancreatic cancers are dependent on CXCR4 for migration, survival, and/or metastasis (3, 35–38). This also provides an explanation for the therapeutic benefits observed in patients treated with PS241, a proteasome inhibitor with anti-NF-κB properties (39).

Major observations in our present study are that cancer cells expressing CXCR4 are clonally selected during growth in the mammary fat pad of nude mice and that there is a further increase in CXCR4 expression in cancer cells that metastasize to the lungs. Because elevated CXCR4 expression was maintained in TMD231 and LMD231 cells even after 3 months in culture, it is less likely that the tumor microenvironment played any role in the transcriptional up-regulation of CXCR4. Thus, we believe that there is a clonal selection of CXCR4-expressing cells, which needs to be further verified by immunohistochemistry within the context of the primary tumor and the metastatic sites. However, we feel that that is beyond the scope of the current investigation because of the technical difficulties involved in such analysis. For example, the tumor microenvironment may contribute to the transient expression of CXCR4 in cells that are not clonally selected for metastasis.

Why CXCR4 expression is advantageous to cancer cells remains to be determined. The SDF-1α/CXCR4 activated signaling pathways may provide a growth advantage to cancer cells at both the primary and metastatic sites. SDF-1α has been shown to enhance tyrosine phosphorylation and association of components of the focal adhesion complex (28). In addition, it induces phosphatidylinositol 3-kinase and p44/42 mitogen-activated protein kinases but not stress-induced kinases such as p38 kinase and c-Jun amino-terminal kinase (29, 40). The AKT/PKB pathway, activated by phosphatidylinositol 3-kinase, protects a variety of cell types against cytokine-, stress-, and chemotheraphy-induced apoptosis (41). Thus, it is possible that cancer cells with cell surface CXCR4 are better equipped to protect themselves from host cytokine- as well as chemotheraphy-induced apoptosis. The mitogen-activated protein kinase pathway may provide proliferation signals for the cancer cells that express CXCR4 to grow out in the tumor microenvironment. SDF-1α/CXCR4 ligation-induced phosphatidylinositol 3-kinase along with uPA may promote the migration of these cells from the primary site. Taken together, our results reveal multiple functions of NF-κB in the growth, migration, and organ-specific metastasis of breast cancer cells, which in part appear to be mediated through the induction of CXCR4.

Acknowledgements—We thank Joan Dunn for technical assistance and Hui Lin Chua and Colin Crean for critical reading of the manuscript.

REFERENCES
1. Liotta, L. A. (2001) Nature 410, 24–25
2. Nicolson, G. L. (1993) Cancer Metastasis Rev. 12, 325–343
3. Moore, M. A. (2001) Bioessays 23, 674–676
4. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McNellan, W., Murphy, E. V., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. (2001) Nature 410, 50–56
5. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
6. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) Nat. Rev. Cancer 2, 301–310
7. Hansen, S. K., Nerlov, C., Zabel, U., Verde, P., Johnsen, M., Baeuerle, P. A., and Blasi, F. (1992) EMBO J. 11, 205–213
8. Sato, H., and Seki, M. (1993) Oncogene 8, 395–405
9. Andela, V. B., Schwarz, E. M., Puzas, J. E., O’Keefe, R. J., and Rosier, R. N. (2000) Cancer Res. 60, 6557–6562

![Fig. 7. CXCR4 mRNA is increased in TMD231 and LMD231 cells compared with parental cells. A, CXCR4 expression was measured by an RNase protection assay as described in Fig. 1. B, MD231, TMD231, and LMD231 cells express similar levels of XIAP, TRAF-3, and TRAF-4 transcripts. An RNase protection assay was performed with the hAPO-5 probe. C, parthenolide or MG132 inhibits CXCR4 expression in LMD231 cells. Cells were incubated with parthenolide (5 μM) or MG132 (10 μM) for the indicated time and subjected to an RNase protection assay as described in Fig. 1. Parth, parthenolide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](image-url)
10. Caruz, A., Samsoen, M., Alonso, J. M., Alcamí, J., Baleux, F., Virelizier, J. L., Parmentier, M., and Arenzana-Seisdedos, F. (1998) FEBS Lett. 426, 271–278
11. Bargou, R. C., Emmerich, F., Krupp mann, D., Bommert, K., Mapara, M. Y., Arnold, W., Royer, H. D., Grinstein, E., Greiner, A., Scheidereit, C., and Dorken, B. (1997) J. Clin. Investig. 100, 2961–2969
12. Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K., and Baldwin, A. S., Jr. (2000) Oncogene 19, 1123–1131
13. Dong, G., Chen, Z., Kato, T., and Van Waes, C. (1999) Cancer Res. 59, 3495–3504
14. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. (1997) Molecular Cell. Biol. 17, 3629–3639
15. Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M., and Baldwin, A. S., Jr. (1998) Genes Dev. 12, 968–981
16. Shattuck-Brandt, R. L., and Richmond, A. (1997) Cancer Res. 57, 3032–3039
17. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. (1997) J. Clin. Investig. 100, 2952–2960
18. Newton, T. R., Patel, N. M., Bhat-Nakshatri, P., Stauss, C. R., Goulet, R. J., Jr., and Nakshatri, H. (1999) J. Biol. Chem. 274, 18827–18835
19. Patel, N. M., Nozaki, S., Shortle, N. H., Bhat-Nakshatri, P., Newton, T. R., Rice, S., Gelfanov, V., Boswell, S. H., Goulet, R. J., Jr., Sledge, G. W., Jr., and Nakshatri, H. (2000) Oncogene 19, 4159–4169
20. Nakshatri, H., and Goulet, R. J., Jr. (2002) Curr. Probl. Cancer 26, 282–309
21. Biozzi, D. K., Cruz, A. P., Gansberger, E., and Pardee, A. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8542–8547
22. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6971–6976
23. Bhat-Nakshatri, P., Sweeney, C. J., and Nakshatri, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6971–6976
24. Bhat-Nakshatri, P., Sweeney, C. J., and Nakshatri, H. (2002) Oncogene 21, 2966–2978
25. Christopherson, K. W., II, Campbell, J. J., and Hromas, R. A. (2001) Blood 98, 3562–3568
26. Hembere, S. P., Hofmann, T. G., Droge, W., and Schmitz, M. L. (1999) J. Immunol. 163, 5617–5623
27. Silva, D., Rizzo, M. T., and English, D. (2002) J. Biol. Chem. 277, 3150–3157
28. Ganja, R. K., Brubaker, S. A., Meyer, J., Dut, P., Yang, Y., Qin, S., Newman, W., and Groopman, J. E. (1998) J. Biol. Chem. 273, 23169–23175
29. Kijowski, J., Baj-Krzyworzeka, M., Majka, M., Reca, R., Marcquez, L. A., Christofidou-Solomidou, M., Janowska-Wieczorek, A., and Ratjczak, M. Z. (2001) Stem Cells 19, 453–466
30. Vinante, F., Rigol, A., Scupoli, M. T., and Pizzolo, G. (2002) Blood 99, 62–60
31. Bachelder, R. E., Wendt, M. A., and Mercurio, A. M. (2002) Cancer Res. 62, 7203–7206
32. Shihata, A., Nagaya, T., Imai, T., Funahashi, H., Nakao, A., and Sano, H. (2002) Breast Cancer Res. Treat. 73, 237–243
33. Edwards, D. R., and Murphy, G. (1998) Nature 394, 527–528
34. Chambers, A. F., and Matrisian, L. M. (1997) J. Natl. Cancer Inst. 89, 1260–1270
35. Koshiba, T., Hiyama, R., Miya, T., Ida, J., Tsuji, S., Nakajima, S., Kagawuchi, H., Kobayashi, H., Doi, R., Hori, T., Fujii, N., and Imamura, M. (2000) Clin. Cancer Res. 6, 3530–3535
36. Sehgal, A., Skars, S., Boynton, A. L., Warrick, J., and Murphy, G. P. (1998) J. Surg. Oncol. 69, 239–248
37. Murakami, T., Mak, W., Cardone, A. R., Fang, H., Kiy, A. T., Nestle, F. O., and Hwang, S. T. (2002) Cancer Res. 62, 7328–7334
38. Scotton, C. J., Wilson, J. L., Scott, K., Stann, G., Wilbanks, G. D., Fricker, S., Bridger, G., and Balkawi, F. R. (2002) Cancer Res. 62, 5930–5938
39. Adams, J. (2002) Oncologist 7, 9–16
40. Lee, Y., Got, A., Kwon, H. J., You, M., Kohli, L., Mantel, C., Cooper, S., Hangoc, G., Miyazawa, K., Ohya, K., and Broxmeier, H. E. (2002) Blood 99, 4307–4317
41. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927