A c-erbB-2 Promoter-specific Nuclear Matrix Protein from Human Breast Tumor Tissues Mediates NF-κB DNA Binding Activity*

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The c-erbB-2 gene overexpression plays a major role in the pathogenesis of breast cancer. Binding studies detected a nuclear matrix protein (NMP) in human breast tumor tissues that recognizes a matrix attachment region (MAR) in the immediate vicinity of the c-erbB-2 gene promoter. This NMP is expressed in breast tumor tissues and cell lines along with c-erbB-2, but is not found in corresponding normal tissues. Furthermore, when NMP purified from the breast tumors by its affinity to the MAR sequence is added to nuclear extracts of breast cancer cells, it selectively stimulates the binding of the NF-κB transcription factor to DNA. A model is suggested in which the association of the MAR-like sequence with the nuclear matrix raises the local concentration of the specific NMP, which in turn interacts with the nuclear factor NF-κB to increase its local level. Such a complex could explain at a molecular level the “increase in NF-κB DNA binding activity” often observed in c-erbB-2- and BRCA1-positive human breast tumors. The increased NF-κB activity could thereby contribute to breast cancer progression.

Invasive breast cancer is the most common serious malignancy and a leading cause of death among women. It is generally believed that the overexpression of the c-erbB-2 gene (also known as HER-2 and Neu; Refs. 1–3) leads to abnormal growth, cellular transformation, and neoplasia (4–6); but the function of c-erbB-2 and the role of overexpression in tumor progression are still obscure.

Some functional hints have come from the detection of a c-erbB-2 promoter-specific DNA-binding nuclear protein that is present only in malignant human breast tissues (9) and induces mitogenesis and cell surface expression of the c-erbB-2 gene as well as BRCA1-positive human breast tumors. The increased NF-κB activity could thereby contribute to breast cancer progression.

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* The abbreviations used are: NM, nuclear matrix; NMP, nuclear matrix proteins; EMSA, electrophoretic mobility shift assay; MAR, matrix-attachment region.
Breast Cancer-specific NMP Mediates NF-κB DNA Binding Activity

FIG. 1. Breast tumor NM detects a MAR in c-erbB-2 gene promoter and a NMP present in the nuclear matrices of human breast tumor tissues and cell line only and not in benign breast tissues that bind to this MAR-DNA sequence. A, a schematic representation of the c-erbB-2 gene promoter's regulatory region (34). Distinct regions are shown, where +1 corresponds to the transcription start site. A+T-rich region (-450 to -390), GGA-rich region (-73 to -22), and B and SP1 binding positions are indicated. Underneath are the B recognition motifs from CTGAGAAACCCCA-3.

Preparation of Nuclear Extracts—Nuclear extracts from breast tissues and cell lines were prepared according to a method described earlier (9). Nuclear extracts were finally dialedysed against buffer (25 mM HEPES, pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride) and stored at -70 °C in small aliquots.

MAR Binding Assay—MAR binding assays were performed according to a standard procedure (33) with some modifications. A DNase I-treated, high-salt-extracted residual nuclear pellet was washed three times in RSB-0.25 M sucrose buffer and once in MAR binding buffer. The resulting pellet (nuclear matrix) was mixed with 5'-end-labeled oligonucleotide probes corresponding to regions: A+T (-450/-390), GGA-rich (-73/-22), -300/-280, and -22/+9 (34) of the c-erbB-2 gene in 100 μl of MAR binding buffer for 4 h at room temperature, with constant agitation. After three washings with MAR binding buffer the bound DNAs were further processed and gel electrophoresed as described in Ref. 33. In most of MAR binding assays, 5A293 of the NM and 50,000 cpm of 5'-DNA probes in the presence of 150 μg/ml Escherichia coli DNA were used, unless otherwise mentioned.

Southern Blot (DNA) Analysis—DNA isolated from 5A293 units of nuclear matrix were electrophoresed on agarose gel, transferred with GGA-specific (-79/-22) and non-specific (-22/+9) DNA probes. Lane 1, in both panels, DNA from normal breast nuclear matrix; lane 2, DNA from breast tumor nuclear matrix.

Electrophoretic Mobility Shift Assay (EMSA) and South Western Blot Assay—EMSA reactions were carried out with NMPs and nuclear extracts and with 32P-labeled double-standard oligonucleotide probes in the presence of 3 μg of poly[d(CT)] and KCl containing binding buffer, exactly as described earlier (8, 9). For competition studies, 50-fold molar excess of unlabeled double-stranded oligonucleotides were added. In South Western blot assay, 10 μg of NMPs were usually resolved on a 10% SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membrane and renatured as described (8, 9). The membrane was hybridized with 2.5 × 106 cpm of 32P-labeled oligonucleotide probes for 15 h at room temperature, with constant agitation. The rest of the steps were as described in the standard protocol.

Affinity Purification of Breast Tumor-specific Nuclear Matrix Protein—An affinity resin with -79/-22, GGA-rich, MAR-like sequences of c-erbB-2 was generated as described earlier (9). Complementary nucleotides corresponding to -79 to -22 sequences of c-erbB-2 promoter were annealed, phosphorylated, ligated, and coupled to CNBr-activated Sepharose 4B.

Large pools of NMPs from breast tumor tissues were used for the purification purposes. All the steps of the purification protocols were exactly as described earlier (9). Briefly, solubilized bulk NMP pools from breast tumor tissues were first mixed with total 200 μg of salmon sperm DNA for 15 min and then mixed with the nonspecific, -22/+9, DNA affinity resin for 6 h, all at 4 °C, with shaking. The low-salt flow-through from nonspecific affinity column was then mixed with the GGA-rich (-79/-22) DNA affinity column, and the rest of the purification protocol was followed as described (9).
polycrylamide gel and electrotransferred on to nitrocellulose membrane. Filters were incubated with NF-κB antibodies (anti-p65 and anti-p50 subunits of NF-κB) and anti-human nuclear matrix 45. The rest of the procedure followed was as suggested by the manufacturers (Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Upstate Biotechnology, Inc. (Lake Placid, NY).

RESULTS

To better understand the progression of breast tumorigenicity in c-erbB-2-expressing tumors, an attempt was made to study the possible involvement of protein interactions with DNA within the innermost nuclear structure, the nuclear matrix. To test for novel factor(s) in the nuclear matrix that bind to a specific segment of the c-erbB-2 gene regulatory sequences, MAR binding studies were conducted with many distinct segments of 5′-upstream regulatory regions of c-erbB-2 enhancer-promoter (Fig. 1A) and nuclear matrix preparations from human normal, c-erbB-2-3-expressing tumor breast biopsy tissues and breast cancer cell lines (33). Results (Fig. 1B) indicate that of the four regions tested, only one specific region, rich in GGA repeats (−79/−22) and in the immediate vicinity of the c-erbB-2 gene transcription start site, has the specific binding affinity observed only with the nuclear matrices of tumor tissues (Fig. 1B, lane 1) and not with the normal tissues (Fig. 1B, lane 2). This observation was further confirmed in the breast tumor cell line BT-20 (Fig. 1B, lane 3). The upstream region that contains a stretch of AT-rich sequences (conventionally a part of MAR) binds less effectively to the nuclear matrix than the GGA-rich region (Fig. 1B, lane 4). Breast tumor and BT-20 cell nuclear matrices binding with the GGA-rich DNA element were tested with more stringent conditions by using increasing amounts of a competitor E. coli DNA, where the GGA-rich DNA element still appears to be the dominant bound material (Fig. 1C, lane 3, breast tumor NM, lane 4, BT-20 NM with 200 μg/ml E. coli DNA versus lanes 1 and 2 of same NMs with 100 μg/ml E. coli DNA). A Southern blot analysis with DNAs from tissue nuclear matrices further demonstrates a strong binding with GGA-rich DNA probe (Fig. 1D, lane 2), not observed with the nonspecific (−22/+9) DNA probe. DNA from normal breast nuclear matrix failed to show any affinity with either probes (Fig. 1D, lane 1). These results (Fig. 1B–D) demonstrate the presence of a MAR-like DNA element within the immediate upstream of c-erbB-2 promoter. These sequences are rich in GGA and not in AT, which traditionally is part of, but not a prerequisite for, MAR. This GGA-rich region (−79/−22) has also been mentioned as an activator of gene promoter and an alternate transcription start site region of the c-erbB-2 gene (35, 36). Other groups have reported the presence of MAR in chicken oviduct, chicken lysozyme gene, avian-globin gene, and 5′-upstream regulatory region of human H4 histone gene promoter (25, 31, 32, 37, 38).

To reaffirm the results of Fig. 1, whether this DNA attachment onto the nuclear matrix of breast tumors is mediated by any specific protein factor(s), we performed DNA-protein binding gel shift assays with NMPs from breast biopsy tissues. Solubilized NMPs devoid of intermediate filaments (29) from several normal (benign), tumor breast biopsy tissues and breast cancer cell line, BT-20, were mixed with the radiolabeled GGA-rich DNA probe, in DNA binding assays. The DNA binding EMSA clearly demonstrates a strong DNA-protein complex formation only with the NMPs of breast tumors (Fig. 2A, panels a–c, lanes 2, 4, 6) and not with NMPs of their normal breast tissue counterparts (Fig. 2A, panels a–c, lanes 1, 3, 5). The tumor cell line BT-20 displays the same complex as the tumor tissue NMP (Fig. 2A, panel d, lane 8), while no such complex is observed with a normal breast cell line MCF-10A (Fig. 2A, panel d, lane 7). Different groups have reported the presence of MAR-binding proteins, such as: SATB1, OCT-1, nucleolin, and recently from human breast carcinomas (26, 28, 37–40).

To test whether this binding activity of NMPs from breast tumors is a specific phenomenon, we examined the status of NMP binding with other region of c-erbB-2 (−22/+9). Again the

![Fig. 2. Breast tumor NMPs contain a unique sequence-specific DNA-binding protein.](image-url)
gel shift assay from two more sets of breast tumor, but not breast normal NMPs clearly demonstrates the formation of a specific DNA-protein complex only with the GGA-rich, −79/−22, DNA probe (Fig. 2B, panel a, lanes 2 and 4 versus lanes 1 and 3; BT-20 NMP, lane 6 versus MCF10A NMP, lane 5). No binding is observed in these NMP extracts with other DNA (−22/+9) probe (Fig. 2B, panel b). These results were supported by a SouthWestern blot assay (Fig. 2C), probed with the same two DNA probes. The MAR-like GGA probe (−79/−22) identifies a dominant nuclear matrix protein of 68 kDa, seen only in the breast tumor NMPs and not in their normal counterparts (Fig. 2C, lanes 2 and 4 over lanes 1 and 3 as well as in BT-20 NMP, lane 5). The second DNA probe (−22/+9) fails to demonstrate any binding. Furthermore, we investigated whether this binding activity of GGA with breast tumor NMPs is localized in the nuclear matrix domain only or is a general phenomenon, found in other compartments of the cell. Results (Fig. 2D) of a DNA binding assay (EMSA) with GGA-rich probe and breast tumor extracts show an optimized binding activity with the nuclear matrix (lane 4) and solubilized nuclear matrix proteins (lane 5) in comparison with cytoplasmic extract (lane 1), nuclei (lane 2) and nuclear extract (lane 3). A slower migrating intense DNA-protein complex seen only in the nuclei (lane 2) and nuclear extract (lane 3) could probably be due to CAAT and TATA (as well as some other unknown)-binding proteins, since the specific probe (−79/−22) also contains CAAT and TATA binding sequences. This complex is totally absent from the nuclear matrix compartment (lanes 4 and 5), affirming the fact that the nuclear matrix is devoid of these high-salt-sensitive nuclear factors.

Together, the results of Figs. 1 and 2 clearly demonstrate the presence of a MAR-like non-conventional (rich in GGA and not in AT) element in the close vicinity of the c-erbB-2 gene transcription start site, whose attachment to the breast nuclear matrix is mediated by a sequence-specific DNA-binding NMP that is expressed only in the malignant tissues and breast cancer cell line BT-20 and not in the normal breast tissues.

To elucidate a functional role(s) of the identified NMP factor, we affinity-purified the protein from the breast tumor NMP pools on the GGA-rich DNA (−79/−22) affinity column. The purified NMP from breast tumors displays a dominant polypeptide of 68 kDa (Fig. 3A, panel a, lane 2). This purified NMP binds the GGA-rich (specific) probe in a sequence-specific manner, as demonstrated by SouthWestern (Fig. 3A, panel b) and EMSA (Fig. 3B) assays. GGA-rich (specific) probe observes a strong band with 50 ng of purified NMP and 10 μg of breast

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**Fig. 3.** DNA affinity purification of the MAR-specific NMP from the human breast tumor NMP pools. Large NMP pools from breast tumor tissues were purified on the GGA-rich DNA affinity column (see “Materials and Methods”). Purified NMP was tested by SDS-polyacrylamide gel electrophoresis and SouthWestern and EMSA assays. A: panel a, a silver stain of protein gel. Lane 1, 5 μg of breast tumor NMP pool; lane 2, 30–40 ng of purified NMP. Panel b, SouthWestern blot assay of the same samples with specific (GGA-rich, MAR, −79/−22) and nonspecific (−22/+9) oligonucleotide probes. The arrow indicates the position of the protein (68 kDa) in panels a and b. B, EMSA with the purified NMP. Lane 1, GGA, −79/−22, probe alone; lane 2, 10 μg of breast tumor NMP pool; lane 3, 50 ng of purified NMP; lane 4, 50 ng of purified NMP with 50-fold cold specific probe (−79/−22); and lane 5, 50 ng of purified NMP with 50-fold cold nonspecific (−22/+9) probe. The arrow indicates the DNA-protein complex formation with NMP pools (lane 2) and purified NMP (lane 3), which is abolished in the presence of specific (lane 4), but not with nonspecific (lane 5), probe.

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**Fig. 4.** Purified NMP from breast tumor tissues stimulates NF-κB DNA binding activity in breast cell nuclear extracts in a specific manner. EMSA was performed with three labeled oligonucleotide probes: −22/+9 of c-erbB-2 promoter (A), −78/−42 of exon 1 of BRCA1 (16) (B), and NF-κB sequences (see “Materials and Methods”) (C) with two breast cell lines, MDA-MB231 and BT-20, nuclear extracts. Increasing amounts (50, 100, and 150 ng) of purified NMP were added to 10 μg of nuclear extracts of MDA-MB231 and BT-20, together with 15,000 cpm of 5’-labeled probes, and the EMSA reactions were resolved on 6% native acrylamide gels in 1 × TBE (8, 9). A, with −22/+9, c-erbB-2 probe. Lanes 1 of MDA-MB231 and BT-20 without NMP; lanes 2–4, with increasing amounts (50, 100, and 150 ng, respectively) of purified NMP, B, same as in A but with −78/−42 of exon 1 of BRCA 1, C, same as in A but with NF-κB probe; lanes 1–4 and lanes 5–8 are exactly similar as in A and B; lane 9, with 10 μg of BT-20 nuclear extract and 50-fold cold NF-κB oligonucleotide; lane 10, same as in lane 9 except with 50-fold cold mutant NF-κB oligonucleotide; and lane 11, with anti-p65 antibody. The top band in C is the specific NF-κB complex. Unlike in A and B, a gradual increase in NF-κB DNA binding activity in C is observed due to the addition of the purified NMP.
tumor NMP pools (Fig. 3A, panel b, lanes 2 and 1, respectively), while the nonspecific (−22/+9) DNA probe fails to bind these NMPs. The EMSA result (Fig. 3B) with 50 ng of purified NMP displays the same DNA-protein complex (Fig. 3B, lane 3) as in breast tumor NMP pools (10 μg, lane 2), which is competed out with specific (GGA-rich, −79/−22) cold DNA (lane 4), but not with nonspecific (−22/+9) cold DNA (lane 5).

To ascertain a functional role that this specific NMP factor may be contributing, a functional test was performed. The purified NMP was added to the nuclear extracts from various tumor cell lines in an EMSA binding reaction, using defined DNA probes from c-erbB-2, BRCA1, exon 1 regulatory sequences, and NF-κB binding sequences (see “Materials and Methods,” “Oligonucleotide Probes”). Addition of increasing amounts of purified NMP into MDA-MB231 and BT-20 nuclear extracts and with DNA probe (−22/+9) of c-erbB-2 does not have any effect on the DNA-protein complex formation (Fig. 4A, lanes 2–4 versus lane 1). Similarly addition of NMP into these extracts and with DNA probe (−78/−42) of BRCA1 also does not influence the DNA-protein complex (Fig. 4B, lanes 2–4 versus lane 1). Interestingly, addition of purified NMP to these nuclear extracts and with NF-κB binding DNA probe appears to have a selective stimulatory effect on the DNA binding activity of nuclear factor NF-κB (Fig. 4C, lanes 2–4 versus lane 1 and lanes 6–8 versus lane 5). Cold competition with κB DNA (lane 9), with mutant κB DNA (lane 10), and with anti-p65 antibody (lane 11) confirm the specificity of the NF-κB complex. This increase in NF-κB activity appears to be highly selective, which is not observed with other two probes (of −22/+9 of c-erbB-2 and −78/−42 of BRCA1) tested (Fig. 4, A and B). Purified NMP factor alone does not bind to κB or any other probe except its specific GGA-rich recognition sequence (−79/−22).

To further evaluate whether NF-κB is present in the nuclear matrix of breast tissues, we performed a Western blot analysis of the nuclear matrix preparations from normal and tumor breast tissues, which were subsequently probed with anti-p65 and anti-p50 antibodies of NF-κB. As per our prediction, reasonable amounts of NF-κB (p65 as well as p50) subunits were found preferentially associated with the breast tumor nuclear matrix (Fig. 5A, panel a; lane 3 in both panels) and not with the normal breast nuclear matrix (Fig. 5A, panel a; lane 3 in both panels). Lane 1 in both panels are standard control p65 and p50 peptides. To demonstrate that equal amounts of proteins were used, we silver-stained a parallel gel with these samples and also performed a Western blot assay with a control anti-human nuclear matrix antibody. The results of the silver stain (Fig. 5A, panel b) and Western blot analysis (Fig. 5A, panel c) confirm that equivalent amounts of protein were used from the tumor (lane 2) and normal (lane 3) breast tissue nuclear matrices. Additionally, we also performed a NF-κB-specific Western blot analysis with the total nuclear content along with the nuclear matrix from the breast tumor tissue. The results of Fig. 5B clearly demonstrate that a much smaller yet a reasonable fraction of total nuclear NF-κB is associated with the nuclear matrix (Fig. 5B, panel a, lane 3 versus lane 2). A silver stain of the nuclear extract (lane 2) and nuclear matrix (lane 3) are shown underneath (Fig. 5B, panel b) to show that equivalent amounts of protein were used for the analysis.

**DISCUSSION**

The proteinaceous network of the NM is believed to be involved in DNA organization, DNA replication, gene transcription, and RNA splicing and processing (17–20). The protein components (NMPs) of the NM provide the structural framework for loop domains of DNA, attached at MARs (15, 21, 22).

Several groups (12–14, 25–28, 41) have demonstrated that sequence-specific DNA-binding proteins can be components of nuclear matrix attachment sites. In recent years, intensive studies have also suggested possibly related role(s) of the nuclear matrix in tumor progression (10–16). In cancer cells, some transforming proteins appear to be associated with the matrix (23, 24), and there are also indications of specific alterations in the protein composition of the matrix as cells undergo differentiation (25) and during the invasion and proliferation of tumors (14, 15, 24, 25, 42, 43). Could a “tumor-specific” NMP be involved in a transition associated with tumor progression? The results reported here may provide such an instance, based on a sequence-specific DNA-binding nuclear matrix protein exclusively present in human breast tumor tissues.

Concerning the association with DNA, the protein is unlikely to be part of one of the integrated structures that generates loop domains of chromatin. Such binding sequences, found in DNase I-sensitive sites near the bases of the loops, are generally rich in AT sequences. In contrast, the sequence-specific DNA-binding protein studied here mediates the attachment of a GGA-rich region of c-erbB-2 to the nuclear matrix.
This is the other recognized mode of attachment to the nuclear matrix: transient and based on enhancer regions upstream (5') of actively transcribed genes [31, 32].

Based on these findings, the juxtaposition of DNA binding factors and DNA regulatory elements at the matrix may increase the local concentration of various transcription factors (25, 29, 30). We suggest that the specific binding of the c-erbB-2 DNA element to the breast tumor nuclear matrix thereby concentrates nuclear factor NF-κB at those sites. It can thereby rationalize the increased NF-κB DNA binding activity observed in malignant breast and other solid tumors that express c-erbB-2 (and BRCA1) genes (44, 45). Increased transcription of this tumorigenic protein could then be a factor in tumor progression; but one must now approach the difficult question of how the specific NMP is expressed and regulated in breast tumors.

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