8-2000

The Role of NF-κb Activation in Breast Carcinoma and Glioblastoma Cell Lines

Kelly T. Murphy

Western Michigan University

Follow this and additional works at: https://scholarworks.wmich.edu/masters_theses

Part of the Biology Commons

Recommended Citation
https://scholarworks.wmich.edu/masters_theses/4621
THE ROLE OF NF-κB ACTIVATION IN BREAST CARCINOMA AND GLIOBLASTOMA CELL LINES

by

Kelly T. Murphy

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, MI, 49008
August 2000
ACKNOWLEDGEMENTS

I would like to acknowledge my mentor Dr. Bruce Bejcek for his patience and counsel. I also thank him for giving me the opportunity to contribute in his laboratory. I am indebted to him for my training and development of the analytical skills I acquired in his laboratory. I would like to thank the members of my committee, Drs. Karim Essani, John Geiser for taking the time to review this thesis and for their expertise and assistance throughout the duration of this work. I would also like to extend my sincere thanks to my colleagues Takeshi Shimamura and Santiago Navarro for their technical support and assistance in the laboratory. I would like to express my appreciation to Diane Stevenson, Lori Klingle, and Celene Jackson in the biological sciences department at Western Michigan University for helping to facilitate my successful completion.

I would like to lovingly acknowledge my wife, Mini Paulose-Murphy for her support and motivation throughout my graduate studies. I would like to wholeheartedly thank my sister Diane, my brothers Mike and Danny, for their encouragement and support. And lastly but never least, to my parents, words cannot express my gratitude for their unconditional love and guidance. This work is dedicated to my parents.

Kelly T. Murphy
THE ROLE OF NF-κB ACTIVATION IN BREAST CARCINOMA AND GLIOBLASTOMA CELL LINES

Kelly T. Murphy, M.S.

Western Michigan University, 2000

The nuclear factor-κB (NF-κB)/Rel family of inducible dimeric transcription factors regulates numerous genes associated with the immune, mitogenic, and apoptotic responses. Investigations were made into the role of NF-κB in breast carcinoma (MCF-7, T47D, MDA-MB-231) and glioblastoma (U87MG, U373MG, U118MG) cell lines. NF-κB binding to DNA was quantified using both gel mobility shift assays and transient transfections of a vector containing a luciferase gene under the control of 4 tandem copies of the NF-κB binding sequence. To determine the correlation between NF-κB activation, cellular proliferation, and foci formation, the tumor cells were cultured and analyzed in the presence of the NF-κB inhibitory compounds, prostaglandin A₁ (PGA₁) and acetylsalicylic acid (ASA). In addition, clones of dominant negative mutants which inhibit NF-κB activation were introduced into these tumor cell lines. One dominant negative mutant is a natural biological inhibitor of NF-κB, IκBα, modified so that it can no longer be phosphorylated and degraded. The other dominant negative mutant expressed a truncated form of p65, a monomer of the NF-κB complex. Breast carcinomas and glioblastomas showed elevated NF-κB activation when compared to normal breast epithelial cells. The tumor cells tested displayed reduced proliferation rates when subjected to NF-κB inhibition. Although, foci formation was reduced under the same inhibitory conditions this response was probably a direct result of decreased proliferation not cellular transformation. The investigations presented here demonstrate the involvement of NF-κB activation with proliferation of these cancer cell lines.
TABLE OF CONTENTS

ACKNOWLEDGMENTS...................................................................................... ii
LIST OF TABLES .................................................................................................. v
LIST OF FIGURES............................................................................................ vi
INTRODUCTION ............................................................................................... 1
  Epidemiology ............................................................................................... 1
  Breast Cancer ............................................................................................. 1
  Brain and Glial Cancer .............................................................................. 3
Platelet-derived Growth Factor Signaling in Breast Carcinomas and Glioblastomas ........................................................................ 4
NF-κB ............................................................................................................. 5
  Hypothesis .................................................................................................. 6
  Structure .................................................................................................... 7
  Inhibition by IκB ....................................................................................... 8
MATERIALS AND METHODS ........................................................................... 10
  Cell Lines and Culture .............................................................................. 10
  Chemical Inhibitors of NF-κB ................................................................... 11
  Quantification of NF-κB Transcriptional Activity ..................................... 11
    Preparation of of Plasmid DNA .......................................................... 11
    Transfection .......................................................................................... 12
    Luciferase Quantification .................................................................. 13
  Proliferation Assay ................................................................................. 13
Table of Contents—continued

Focus Formation Assay ................................................................. 14
Construction of IkBα, p65ΔC Dominant Negative Mutants .......... 14
  Preparation of IkBα, p65ΔC Clones ........................................... 14
  Stable Transfections ............................................................... 15
  Western Blot Analysis ............................................................. 16
Fluorescent Electrophoretic Mobility Shift Assay ......................... 17
  Preparation of Nuclear Extracts .............................................. 17
  Reactions With Molecular Beacon .......................................... 18
RESULTS .............................................................................................. 20
  Increased Proliferation and Focus Formation of Tumor Cell Lines .. 20
  Elevated NF-κB Transcriptional Activity in the Tumor Cell Lines ... 23
  Inhibition of NF-κB Transcriptional Activity by PGA₁ and ASA ... 24
  Inhibition of NF-κB Transcriptional Activity by Dominant Negative Mutants ................................................................. 31
    Characteristics of the Cells Transfected With pmelkBα ............ 31
    Characteristics of the Cells Transfected With p65ΔC ............... 37
DISCUSSION ......................................................................................... 42
APPENDICES
  A. Protocol Clearance From the Institutional Biosafety Committee 45
BIBLIOGRAPHY .................................................................................. 47
# LIST OF TABLES

1. Phenotypic Characteristics ............................................................................ 10

2. Net Proliferation Rates of the Tumor, pmelKBo, pRc/CMV Cells
   Quantified Using a Hemocytometer (A) and Cell Counting Kit (B)
   After 8 Days Growth .................................................................................. 34

3. Net Proliferation Rates of the Tumor, p65ΔC, p65 Cells Quantified
   Using a Hemocytometer (A) and Cell Counting Kit (B) After 8 Days
   Growth ........................................................................................................ 38
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Signal Transduction Pathway of NF-κB Activation</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Molecular Beacon and Oligonucleotides Used in Gel Shift Assays</td>
<td>19</td>
</tr>
<tr>
<td>3.</td>
<td>Elevated Proliferation Rates in Tumor Cell Lines Versus the Normal Breast Epithelial Cell Line</td>
<td>21</td>
</tr>
<tr>
<td>4.</td>
<td>Elevated Focus Formation in the Tumor Cell Lines Versus the Normal Epithelial Cell Line</td>
<td>22</td>
</tr>
<tr>
<td>5.</td>
<td>Elevated NF-κB Transcriptional Activation in Tumor Cell Lines Versus the Normal Breast Epithelial Cell Line</td>
<td>25</td>
</tr>
<tr>
<td>6.</td>
<td>Gel Mobility Shift Assays Demonstrating Increased Binding of NF-κB in Tumor Cell Lines Versus Normal Epithelial Cells</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>Chemical Inhibition of NF-κB Transcriptional Activity by PGA$_1$ and ASA</td>
<td>28</td>
</tr>
<tr>
<td>8.</td>
<td>Prostaglandin A$_1$ (PGA$_1$) and Acetylsalicylic Acid (ASA) Reduce Proliferation in Tumor Cell Lines</td>
<td>29</td>
</tr>
<tr>
<td>9.</td>
<td>Prostaglandin A$_1$ (PGA$_1$) and Acetylsalicylic Acid (ASA) Reduce Focus Formation in Tumor Cell Lines</td>
<td>30</td>
</tr>
<tr>
<td>10.</td>
<td>Reduced Levels of NF-κB Activation in Cells Transfected With pmelκBα</td>
<td>32</td>
</tr>
<tr>
<td>11.</td>
<td>Over-Expression of the IκBα Protein by Cells Transfected With pmelκBα</td>
<td>33</td>
</tr>
<tr>
<td>12.</td>
<td>Assays Demonstrating Reduced Proliferation in Cells Transfected With pmelκBα</td>
<td>35</td>
</tr>
<tr>
<td>13.</td>
<td>Assay Demonstrating Reduced Focus Formation in Cells Transfected With pmelκBα</td>
<td>36</td>
</tr>
<tr>
<td>14.</td>
<td>Reduced Levels of NF-κB Activation in Cells Transfected With p65ΔC</td>
<td>39</td>
</tr>
</tbody>
</table>
List of Figures—continued

15. Assays Demonstrating Reduced Proliferation in Cells Transfected With p65ΔC ................................................................. 40

16. Assay Demonstrating Reduced Focus Formation in Cells Transfected With p65ΔC ................................................................. 41
INTRODUCTION

Epidemiology

Over the last 150 years medical advances have reduced the mortality from infectious disease and greatly increased the life expectancy of individuals. As a result other diseases such as cancer have become a major cause of mortality in developed nations (Cairns, 1988) and cancer research has become the most funded issue in applied biology (Devesa et al., 1999). There are more than 40 specific categories of cancer based on the location and phenotype of the tumor cells with 90% of all cancers originating in epithelial tissue (Devesa et al., 1999). The majority of male mortality occurs from 4 primary sites of cancer including lung, prostate, colon, and pancreas (Cairns, 1988). Among females, 60 percent of the mortality occurs from lung, breast, colon, ovary, and pancreas (Cairns, 1988). In both genders, incidences of breast and brain cancer increase proportionally with additional genetic pressures from our environment.

Breast Cancer

In the United States, 200,000 women and 1,600 men are diagnosed each year with invasive breast cancer and nearly 2,000,000 deaths occurred during 1970-94 (Devesa et al., 1999). Recent advances in detection and screening have reduced the number of deaths in half to 43,000 annually. Women with a family history of
developing the disease are at greatest risk but only a small fraction of the breast cancer cases are attributed to inheritable genotypes (Davidson and Moses, 1998). Most cases are due to genetic alterations in epithelial tissue forming the ductile and tubular networks of the breast. Women, who were late in age at first birth and menopause, early in age for menarche, or go through hormone replacement therapy have significant increases in risk associated with developing breast cancer (Davidson and Moses, 1998). These facts helped establish that ovarian hormones such as estrogen, progesterone, and insulin-like growth factor play a central role in breast cancer development. Many of the new and effective agents entering the market such as Tamoxifen (Evista®) and Raloxifene (Nolvadex®) block estrogenic receptors (ER) from activating DNA transcription. The HER-2/neu proto-oncogene has also become an important target against breast cancer. The HER-2/neu gene encodes a tyrosine kinase receptor that closely resembles the epidermal growth factor receptor (Rubin and Farbel, 1997). The receptor functions primarily as a heterodimer involved with differentiation and cell division. Therapeutic antibodies, vaccines, and chemotherapeutic agents have been developed against both HER/neu and ER. Other receptors including transforming growth factor (TGF) α and β, platelet-derived growth factor (PDGF) α and β, fibroblast growth factor and retinoic acid (RAR) have been shown to be responsible for the development of breast carcinomas (Davidson and Moses, 1998, Ginsburg and Vonderhaar, 1991, Fan et al., 1999). Genetic mutations resulting in up regulation of proto-oncogenes (c-myc, Ha-ras, PTEN, BRCA-1, BRCA-2) and down regulation of tumor suppressor genes (p53, Rb) have
also been shown to initiate breast cancer (Davidson and Moses, 1998, Ginsburg and Vonderhaar, 1991, Fan et al., 1999, Rubin and Farber, 1997).

Brain and Glial Cancer

The cases of brain or nervous system cancer are fewer in occurrence than breast cancer with approximately 10,000 deaths annually. From 1970-94 there were 246,222 deaths reported from brain cancer (Devesa et al., 1999) with a bimodal distribution with respect to age. Young children with genetic predispositions and elderly adults have much higher incidences of occurrence than middle aged individuals (Devesa et al., 1999). Most cancers of the brain and nervous system originate from glial cells that function to support and protect the central nervous system (CNS) and its components (Xiang, 1995) and the collective terms used for glial cell tumors are glioblastomas or gliomas. Astrocytomas, the most common type of glioblastoma, are derived from astrocytes, the star-shaped glial cells, that attach neurons to their respective blood vessels and create the blood-brain barrier (Xiang, 1995). Glioblastomas are not as well understood as breast carcinomas. As a result, therapeutic targets remain to be identified and treatment options are limited to surgery or traditional high dose chemotherapy. Current research is focused on over-activation in the nuclear signaling pathways associated with nerve growth factor (NGF), epidermal growth factor (EGF), PDGF, pleiotrophin (PTN), and other neurotrophins (Couldwell et al., 1991, Hung et al., 2000, Nister et al., 1991). Several types of mutations within the EGF receptor gene were identified in multiple glioblastomas
Granulins, a relatively new class of growth regulators resembling EGF proteins, have been shown to be associated with glioblastoma proliferation in vitro (Liau et al., 2000). Recent investigations have also demonstrated that increased activity within the regulatory pathways associated with Akt and Bcl-2 plays a role in glioblastoma proliferation and progression (Frankel et al., 1999, Zundel et al., 2000).

Platelet-derived Growth Factor Signaling in Breast Carcinomas and Glioblastomas

Production of growth factors by breast carcinomas and glioblastomas is of central importance because the growth factors are implicated in mediating autocrine or paracrine stimulation of tumor growth (Rubin and Farber, 1997). Seymour and Bezwoda (1993) reported that breast cancer patients often have elevated PDGF levels in plasma. Many of the cell lines established from breast carcinomas and glioblastomas have been shown to express PDGF and their receptors (Romashkova and Makarov, 1999, Bronzert et al., 1987, Rozengurt et al., 1985). PDGF is one of the major mitogens found in the serum of higher mammals and has been shown to stimulate the growth and proliferation of connective tissue cells (Ross and Vogel, 1978). Over-expression of PDGF in several tissue types results in mitogenesis which leads to focus or tumor formation (Claesson-Welsh, 1996). PDGF and its secondary signaling molecules can act as agents for the malignant transformation in epithelial tissue (Claesson-Welsh, 1996). Other reports demonstrated that the T47D breast carcinoma cells had increased proliferation rates in response to exogenous PDGF
Structurally, PDGF is a dimer of some combination of chains A (17k) and B (14k) linked by disulfide bonds. Its α and β receptors are transmembrane tyrosine kinases that dimerize and autophosphorylate after PDGF binding (Claesson-Welsh, 1996). The α receptor binds all three isoforms of PDGF with high affinity whereas the β receptor binds AB and BB dimers. Recent investigations have led to the assumption that α and β receptors initiate different signals into the cell, however both receptors are potent activators of the PI₃ kinase, PKC, mitogen-activated protein kinase pathways (Claesson-Welsh, 1996). These signal transduction pathways are associated with many transcription factors including nuclear factor-κB (NF-κB) (Marumo et al., 1997, Yokote et al., 1996, Romashkova and Makarov, 1999). Nuclear signaling of PDGF has been shown to activate NF-κB (Romashkova and Makarov, 1999). As a result, NF-κB could potentially serve as an important component in transformation and proliferation of breast carcinomas and glioblastomas responsive to PDGF.

NF-κB

NF-κB activation occurs through a phosphorylation cascade from several cell-surface receptors that are mediated by cytokines and growth factors (Baeuerle, 1991). NF-κB is an important regulator of transcription for genes needed for cell growth and differentiation in many cell types. NF-κB has been identified as an inhibitor of apoptosis and a regulator of cellular transformation (Bauerle and Baltimore, 1996, Beg and Baltimore, 1996, Wang et al., 1996). Breast carcinoma and glioblastoma
formation occurs because the tumor cells have lost their ability to regulate their
growth and division making NF-κB an ideal target for research studies within these
tumor cells. Normal human cells will usually arrest development and then
differentiate in the first phase (G₁) of the cell cycle but cancerous cells rapidly
proceed through G₁ and advance into the DNA synthesis (S) phase of the cell cycle
(Baldwin, 1991). Inhibition of NF-κB has been shown to significantly reduce G₁-to-S
phase transitions in fibroblast cells through transcriptional regulation of cyclin D₁
(Guttridge et al., 1999).

Recent reports have linked NF-κB activation to carcinogenesis in a variety of
other types of cells (Abdel-Mageed et al., 1998, Bauerle and Baltimore, 1996,
Reuther et al., 1998). Studies have demonstrated that NF-κB activation is required to
promote tumor growth in Hodgkin’s lymphoma and induce cellular transformation in
myelogenous leukemias (Bargou et al., 1997, Daley and Baltimore, 1988). Transformation of normal epithelial and fibroblast cells by Bcr-Abl and oncogenic
Ras is also dependent on NF-κB activation (Chin et al., 1999, Finco et al., 1997,
Grimm and Baueerle, 1994, Reuther et al., 1998). Inhibition of NF-κB activity has
been shown to reduce the growth of MCF-7 breast carcinomas and friend
erythroleukemia tumor cells (Abel-Mageed et al., 1998, Marini et al., 1990).

Hypothesis

NF-κB is involved in a variety of cellular processes and it is present in a
majority of cell types as an inducible factor that is activated by multiple stimuli
including PDGF (Baeuerle, 1991, Olashaw, 1996). Once activated, NF-κB can control the transcription of numerous genes including inflammatory cytokines, chemokines, growth factors, and proteins associated with cell division, apoptosis, transformation, and viral replication (Baeuerle, 1991, Baeuerle and Baltimore, 1996, Guttridge et al., 1999, Verma et al., 1995). This study is designed to investigate the role that NF-κB inhibition could have on the reduction of focus formation and proliferation in breast carcinomas (T47D, MDA-MB-231, MCF-7) and glioblastomas (U87MG, U373MG, U118MG) that express forms of PDGF-like proteins and receptors. Representative cell lines established from breast carcinomas and glioblastomas were chosen specificity because they were shown previously to express PDGF receptors (Bronzert et al., 1987, Rozengurt et al., 1985). I hypothesize that inhibition of NF-κB will reduce proliferation and focus formation in these tumor cell lines.

Structure

The Rel/NF-κB family of transcription factors includes five distinct proteins that share an approximately 300 amino acid domain of high homology necessary for DNA binding (Baeuerle and Baltimore, 1996). All the proteins in this family can form homo- or heterodimeric DNA-binding complexes, each with differences in their DNA-binding specificity (Baeuerle, 1991). Three proteins, p65 (RelA), RelB and c-Rel, have both DNA-binding and transactivating domains. The other two proteins, p50 and p52, have only DNA-binding activity (Verma et al., 1995) although, the most
abundant complex of NF-κB is the heterodimer p65/p50. A consensus NF-κB recognition sequence has been developed based on the sequences bound by NF-κB from several genes (Baeuerle and Baltimore, 1996). This sequence contains 10 base pairs with guanines always the first three bases and the last two bases predominantly cytosines, 5'-GGGAATTTCC-3'.

Inhibition by IκB

A family (α, γ, β, δ) of inhibitory proteins commonly termed IκB, exerts post-translational control on NF-κB activity by their binding to cytoplasmic NF-κB dimers (Rice and Ernst, 1993). In vitro, IκBα binds to p65, RelB and c-Rel where it prevents NF-κB nuclear translocation (Verma et al., 1995). Stimulation of the IκB kinases (IKKα,β) results in phosphorylation of IκB causing it to be released from the NF-κB heterodimer and causing IκB degradation (Baeuerle, 1991, Rice and Ernst, 1993). Loss of IκB from the complex allows NF-κB to translocate from the cytoplasm into the nucleus where it binds to DNA and activates the transcription of genes needed for growth and proliferation (Figure 1).
Figure 1. The Signal Transduction Pathway of NF-κB Activation.
MATERIALS AND METHODS

Cell Lines and Culture

The primary tumor cells were isolated from human patients with malignant tumors. The breast carcinomas T47D, MDA-MB-231, MCF-7 and glioblastomas U87MG, U118MG were chosen for this study as previous reports have shown that they express PDGF-A, B and their receptors α, β (Bronzert et al., 1987, Rozengurt et al., 1985). The glioblastoma, U373MG, was also used in this study but mRNA and protein analysis revealed it did not express PDGF-B or the β receptor (Nister et al., 1991). Cell lines (Table 1) were purchased from American Type Culture Collection (ATCC) (Rockville, MD) and maintained in minimum essential medium (MEM), with 10% fetal bovine serum (FBS), 100U/ml of penicillin-G, 100µg/ml streptomycin, and 0.25µg/ml of amphotericin B. The normal breast epithelial cells, MCF-10A, were cultured in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Morphology</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87MG</td>
<td>brain glioma, astrocytomas</td>
<td>Epithelial</td>
</tr>
<tr>
<td>U373MG</td>
<td>brain, glioma, astrocytomas</td>
<td>Epithelial</td>
</tr>
<tr>
<td>U118MG</td>
<td>brain, glioma, astrocytomas</td>
<td>Epithelial</td>
</tr>
<tr>
<td>T47D</td>
<td>breast, ductal, carcinoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>MCF-7</td>
<td>breast, ductal, carcinoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>breast, adenocarcinoma</td>
<td>Epithelial</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>normal breast ductal tissue</td>
<td>Epithelial</td>
</tr>
</tbody>
</table>
modified Eagle’s medium (DMEM) with 2.5mM L-glutamine, 5% horse serum (HS), 100U/ml of penicillin-G and streptomycin, 0.25µg/ml of amphotericin B.

Chemical Inhibitors of NF-κB

Rossi et al. (1997) demonstrated that prostaglandin A₁ (PGA₁) at 24µM can inhibit the activation of NF-κB by inhibiting the phosphorylation of IκBα and as a result decreases the amount of NF-κB in the nucleus. Similar effects have been reported for acetylsalicylic acid (ASA) at 5mM (Castano et al., 1997, O’Neil, 1998, Yin et al., 1998). Knopp and Ghosh (1994) showed that ASA was capable of inhibiting the phosphorylation of IKK, a molecule responsible for phosphorylating IκB proteins. PGA₁ from Biomol Research Laboratories (Plymouth Meeting, PA) and ASA from Sigma (St. Louis, MO) were diluted in 95% ethanol to convenient working concentrations before addition to medium.

Quantification of NF-κB Transcriptional Activity

Preparation of Plasmid DNA

pNF-κB-Luc reporter vector was purchased from Clontech laboratories (Palo Alto, CA). Transfections with pNF-κB-Luc into cells allows the measurement of NF-κB binding through luciferase expression. The control vector without the NF-κB binding sites was produced through enzymatic digestion with the restriction enzymes Kpn I and Bgl II. pNFκB-Luc, control, and β-Gal plasmids were produced in shaking
cultures of *E. coli* (DH-10B) grown in LB medium with 100µg/ml ampicillin. Plasmids were purified using StrataPrep EF plasmid midiprep kit (Stratagene, LaJolla, CA), diluted in water or TE buffer and stored at -20°C. Plasmid DNA was quantified on the Beckman DU-7000 (Fullerton, CA) using spectrophotometric analysis at 260 and 280nm.

**Transfection**

Cells (10⁴ per well) were seeded onto 8 well culture plates and allowed to grow to approximately 80% confluence. In 1mL of medium free of serum, antibiotics, and antimycotics, 2µg of plasmid DNA was mixed with 10µl of transfection reagent (Gene Therapy Systems, San Diego, CA) for each well. After a 45min incubation, the DNA/reagent mixture was applied to the cells that were washed twice with phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄, pH 7.4). At 5 and 24 hours post transfection, fresh medium with 10% serum and the appropriate treatment was added. For chemical inhibition experiments, prostaglandin A₁ (PGA₁) and acetylsalicylic acid (ASA) were used at 24µM and 5mM respectively. Seventy-two hours after transfection, cells were washed with PBS buffer and harvested in 200µL of cell culture lysis reagent (25mM Tris-phosphate, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 10% glycerol, Triton® X-100, pH 7.8). The lysate was centrifuged at 12,000 x g for 1min to pellet cell debris. The supernatants were then transferred to new tubes and stored at -80°C.
Luciferase Quantification

Luciferase luminescence was produced using LucLite Plus™ substrate (Packard, Meriden, CT) and quantified on Packard TopCount™ Microplate scintillation counter. Samples containing 100µg of lysate protein, quantified by the bicinchoninic acid assay (BCA kit) (Pierce, Rockford, IL) were prepared in triplicate following manufacture’s protocol. Results were normalized to transfection efficiency determined by β-galactosidase expression in the tumor and normal cells transiently transfected with a β-Gal plasmid from Invitrogen (Carlsbad, CA) containing the lacZ gene construct. β-galactosidase expression was quantified using β-Gal assay kit and manufacturer’s recommendations (Invitrogen).

Proliferation Assay

Cell proliferation assays were performed in 96 well format using manufacturer’s recommendations and the Cell Counting kit-8 (Dojindo Molecular Technologies Inc., Gaithersburg, MD). The kit employs a novel tetrazolium salt that produces a highly soluble formazan dye upon biochemical reduction in the presence of an electron carrier. Four hours after cells were incubated with the tetrazolium salt, quantification of the dye was accomplished through the measurement of absorbance at 450nm. The cells were initially quantified using a hemocytometer and seeded approximately $10^4$ cells per well. Twenty-four hours after seeding, medium with 2% serum and the appropriate treatment was added. Medium with inhibitors, PGA1 at
24µM and ASA at 5mM, was replaced on the 1\textsuperscript{st}, 3\textsuperscript{rd}, and 5\textsuperscript{th} days of the experiment. Cell numbers were measured for 6 consecutive days. After generating a standard line for each cell line, absorbencies were then converted to cell number. Proliferation experiments involving mutants and MCF-10A were modified to the appropriate medium with 2% serum and assayed for 8 consecutive days with measurements taken every second day.

**Focus Formation Assay**

To evaluate cellular transformation, tumor and normal cell lines were seeded in 24 well culture plates with an initial cell density of ~20,000/cells per well and examined for contact inhibition or foci formation. The appropriate treatment was added 24 hours after seeding. Respective medium with 2% serum was replaced every second day. After ten-days, cells were stained with 0.25% crystal violet for quantification of foci formation.

**Construction of pmeIκBα and p65ΔC Dominant Negative Mutants**

**Preparation of pmeIκBα and p65ΔC Clones**

The pmeIκBα and p65ΔC, p65 gene constructs were the kind gifts of Drs. Nancy Rice and Nancy Colburn (National Cancer Institute, Bethesda, MD). All of these constructs contain the pRC/CMV vector (Invitrogen). pmeIκBα is a dominant negative mutant of IκBα while p65ΔC contains a C-terminal deletion of p65. NF-κB
inhibition by pmeIKBα and p65ΔC dominant negative mutants has been shown previously (Rice and Ernst, 1993, Guttridge, et al., 1999, Grimm and Baeuerle, 1994). The plasmids were grown in shaking cultures of E. coli (DH-10B) at 37°C with LB medium containing 100µg/ml ampicillin. Plasmids were purified using StrataPrep EF plasmid midiprep kit (Stratagene). The plasmid DNA diluted in water or TE buffer was quantified through spectrophotometric analysis at 260 and 280nm.

**Stable Transfections**

Stable cell lines expressing the dominant negative mutants and wild type genes were created for each tumor cell line using transfection recommendations and reagents from Gene Therapy Systems (San Diego, CA). Cells (10⁴ per well) were seeded in 8 well plates and transfected at ~80% confluence with 5µg per well of plasmid DNA and 10µl reagent (Gene Therapy Systems). After 24 hours of transfection, fresh medium with 10% serum was added and then replaced 48 hours post transfection with fresh medium containing 10% serum and G418 antibiotic at 400µg/ml or 800µg/ml for MDA-MB-231 cell line. The stably transfected cells were maintained permanently in medium containing the G418 antibiotic. Three individual clones were isolated from within each stable culture using cloning cylinders and 0.05% trypsin in PBS and 58mM EDTA for removal from the plates. The cells expressing the dominant negative mutants were assayed for NF-κB transcriptional activity, proliferation and foci formation as described previously. Clones stably
transfected with the empty pRc/CMV vector, p65 wild type and mock transfected cells were used as controls.

Western Blot Analysis

Western blotting was performed to ensure expression of genes introduced into the cells. Cells ($10^5$) were seeded in 100mm culture plates and grown until they were 80% confluent. The cells were washed twice with PBS and harvested with 500µL of cell lysis buffer (20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1mM β-Glycerophosphate, 1mM Na$_3$VO$_4$, 1µg/ml Leupeptin, 1% Triton X-100). Total protein concentrations of the lysate were determined using the BCA assay kit (Pierce, Rockford, IL). Samples with 10µg of lysate protein were prepared with reducing buffer (570mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins based on size (Laemmli, 1970). Samples were electrophoresed at 60V for 45min followed by 110V for 90min in 1X running buffer (250mM Tris-base, 192mM glycine, and 0.1% SDS). Proteins were transferred to a PVDF membrane through electroblotting for 2 hours at 4°C in buffer containing 250mM Tris and 192mM glycine. The membrane was first blocked overnight at 4°C with 5% I-block (Amersham Pharmacia Biotech, Piscataway, NJ) diluted in Tris-HCl (20mM), NaCl (137mM), and 0.1% Tween 20 (TBS-T). Then primary antibody anti-IκB (Upstate Technologies, Lake Placid, NY) was diluted to 0.5 µg/ml in I-block/TBS-T and also
incubated with the membrane overnight at 4°C. Anti-rabbit IgG (Amersham Pharmacia Biotech), conjugated with horseradish peroxidase, was diluted (1:2000) in fresh 1-block/TBS-T and incubated with the membrane at room temperature for 1 hour. After washing the membrane 3 times in TBS-T, the antibodies on the membrane were detected using the ECL Plus® Western Blotting Kit (Amersham Pharmacia Biotech) and exposed to Kodak X-omat film for 30min.

Fluorescent Electrophoretic Mobility Shift Assay

Preparation of Nuclear Extracts

The tumor and normal breast cells \(10^5\) were seeded in 100mm culture plates and harvested at approximately 80% confluence. Cells were washed quickly with 2ml of 1x PBS and then removed with 1.5ml of 0.05% trypsin/58mM EDTA left on for 3-5 minutes. Cells were collected and maintained on ice throughout the protocol recommended by Promega Inc., (Madison, WA). The harvested cells were centrifuged at 2,000 x g for 5min then resuspended and washed in 1ml buffer A (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.1% NP-40, and 0.5mM DTT). After recentrifugation, the cells were resuspended with gently pipetting in 80µl buffer A containing 0.1% Triton X-100. After incubation at 4°C for 10 minutes, the homogenate was centrifuged at 2,000 x g for 5min and the nuclear pellet washed in 60µl of 20mM HEPES, pH 7.9, 0.42M NaCl, 25% (v/v) glycerol, 1.5mM MgCl₂, and 0.2mM EDTA. This suspension was incubated on ice for 30 minutes and then
centrifuged at 16,000 x g for 20 minutes at 4°C. The resulting supernatant was stored at -80°C as nuclear extract.

Reactions With Molecular Beacon

Total protein concentration of the extracts was determined using the BCA assay (Pierce) and bovine serum albumin (BSA) as a standard. A molecular beacon containing a consensus NF-κB recognition sequence (Figure 2) and carboxyfluorescein (FAM) fluorochrome (Research Genetics Inc, Huntsville, AL) was used to probe for DNA binding proteins. The assays were performed in 40μL volumes containing 20μg of nuclear protein and 1pmol of molecular beacon and reverse compliment oligonucleotide (Life Technologies) in the binding buffer (10mM HEPES, 50mM KCl, 2.5mM DTT, 0.05% NP-40, 10% glycerol, pH 7.9) for 30 minutes at room temperature. Competition (unlabeled oligo) and specificity (mutated oligo) experiments were conducted using 50-fold molar excess of the competing unlabeled oligonucleotides. Super shift assays were conducted using 0.5pmol of antibody (Upstate Biotechnology) to the p65 subunit of NF-κB. Electrophoresis was performed on a 7% 1X Tris-glycine-polyacrylamide gel and run in 50mM Tris and 380mM glycine, pH 8.3 buffer. Following electrophoresis at 100V for 3hrs, the gel was analyzed by blue-excited fluorescence scanning with the 860 Storm FluorImager (Molecular Dynamics, Sunnyvale, CA).
Molecular Beacon: 5'-FAM-CATGAACCGGGAATTTCCAACATG-Dabcyl-3'
Reverse compliment: 3'-CCTTGGCCCTTTAAAGGTTAA-5'

Unlabeled oligonucleotide: 5'-CATGAACCGGGAATTTCCAACATG-3'
Mutated oligonucleotide: 5'-CATGAACCCGGAAATTTCCAACATG-3'
Reverse oligonucleotide: 3'-CCTTGGCCCTTTAAAGGTTAAGGTT-5'

Figure 2. Molecular Beacon and Oligonucleotides Used in Gel Shift Assays.

Underlined bases indicate point mutations in oligonucleotides used for specificity experiments.
RESULTS

Increased Proliferation and Focus Formation of Tumor Cell Lines

Proliferation assays were performed to compare the rate of tumor cells and the normal epithelial cells. The proliferation rates of the tumor cell lines were greater than the normal epithelial cells, MCF-10A (Figure 3). The intrinsic rate of increase for the 8 day experiment was greatest for the MCF-7 cell line with 9.2 fold increase per day followed by T47D (8.5), U118MG (7.1), MDA-MB-231 (6.8), U87MG (5.7), U373MG (4.8), and the rate for the normal cell line, MCF-10A, was 4.4. To further characterize the phenotypes of the tumor and normal cells, focus formation assays were also used to investigate if these cell lines could form foci when cultured over an extended period of time. Normal cell lines typically have a low occurrence of focus formation in culture and will arrest mitotic division when there is a single monolayer of cells covering the culture plate. In contrast, cancerous cells often form foci or multiple cell layers because they lack contact inhibition with other cells. The focus formation assay demonstrated that the tumor cells lacked contact inhibition and frequently form foci or multiple layers of cells when cultured (Figure 4). The normal MCF-10A cells behaved similar to other normal epithelial cell lines forming a single monolayer in adhesive cultures.
Cells were seeded $10^4$ per well in medium containing 2% serum that was replaced on the 2\textsuperscript{nd}, 4\textsuperscript{th}, and 6\textsuperscript{th} days of the experiment. Measurements were made every second day for 8 days. Glioblastomas and breast carcinomas are depicted by closed and open symbols respectively. Open circles illustrate the normal breast epithelial cell line, MCF-10A. The data represents the averages of two independent experiments.
Figure 4. Elevated Focus Formation in the Tumor Cell Lines Versus the Normal Epithelial Cell Line.

Cells were seeded $2 \times 10^4$ per well in medium containing 2% serum. Medium was replaced every second day. Cells were stained with 0.25% crystal violet after the tenth day of the assay. Bottom pictures taken at 40x magnification on the same cells demonstrate focus formation in representative cell types.
Elevated NF-κB Transcriptional Activity in the Tumor Cell Lines

Knowing that the tumor cells expressed PDGF like proteins and receptors, I was interested in quantifying the level of NF-κB transcriptional activity within these cells. Using transient transfections with either the pNF-κB-Luc plasmid or the control plasmid without the κB binding sequences, accurate determinations could be made into the amount of expression initiated by NF-κB. Seventy-two hours after transfection, the cells were washed and then harvested in lysis buffer. One hundred micrograms of lysate proteins from the individual samples were mixed with the Luclite Plus™ substrate (Packard) and luminescence was quantified on a Packard TopCount™ Microplate scintillation counter. The data from the transfection experiments suggests that luciferase expression was elevated in all the tumor cell lines assayed when compared to the normal breast epithelial cells (Figure 5). The glioblastoma lines, U87MG and U118MG, had the highest counts per minute (cpm) at 93193 and 98368 respectively. The glioblastoma, U118MG, showed the greatest level of expression with 14 times more luciferase activity as compared to the MCF-10A cells. In contrast to the other glioblastomas, the U373MG (16029cpm) did not have expression levels that were significantly different than the normal MCF-10A cell line (6967 cpm). The breast carcinomas MDA-MB-231, T47D, and MCF-7 had counts of 45524, 34733, 19759 cpm respectively. The MDA-MB-231 line had 5 times more expression than the normal cells followed by T47D (3) and MCF-7 (2). The overall expression in glioblastomas was doubled when compared to breast carcinoma
cell lines. The average NF-κB activation of luciferase expression in glioblastomas was 9 times higher than MCF-10A activation. The breast carcinoma cell lines on average had 5 times more expression than the normal breast epithelial cell line, MCF-10A.

Electrophoretic mobility assays also demonstrated that the tumor cells had a greater concentration of NF-κB in nuclear extracts (Figure 6). Samples that contained nuclear extracts from the tumor cells showed increased binding to the probe containing κB binding sequence as compared to the normal cell line. Competition experiments with 50 molar excess of unlabeled probe showed that binding could be reduced and was specific. Non-denaturing reactions with the antibody to the p65 subunit demonstrated NF-κB binding could be super shifted in the gel shift assays.

Inhibition NF-κB Transcriptional Activity by PGA₁ and ASA

To accurately assess the inhibition of NF-κB activity by PGA₁ (24µM) and ASA (5mM), the tumor cell lines were transfected with either the pNF-κB-Luc or control plasmids in the presence of the chemical inhibitors. Twenty-four hours after the initiation of transfection, fresh medium with the appropriate inhibitor was added. Cells were then harvested 72 hours after the initial transfection. The assays demonstrated that PGA₁ and ASA were able to reduce expression in all the cell lines tested (Figure 7). When compared to PGA₁, ASA was more effective in reducing expression by an average of 83% in breast carcinomas and 91% in glioblastomas. The average counts for U87MG, U373MG, U118MG were reduced by ASA from
Figure 5. Elevated NF-κB Transcriptional Activation in Tumor Cell Lines Versus the Normal Breast Epithelial Cell Line.

Activation quantified using transient transfections with pNF-κB-Luc vector (NF +) against control (NF -). The data represents averages (± SD) of samples assayed in triplicate following manufacture recommendations and protocol. Luciferase expression quantified through a Packard scinillation counter (Meriden, CT).
Figure 6. Gel Shift Assays Demonstrating Increased Binding of NF-κB in Tumor Cell Lines Versus Normal Epithelial Cells.

Nuclear proteins (20µg) were electrophoresed on 7% acrylamide gel for 3 hrs at 100V and supershifted with antibody against p65, a subunit of NF-κB complex. Unlabeled probe at 50pmol was used for competition experiments.
2125, 550, 2817 cpm to 327, 30, 1080 cpm respectively. ASA also decreased the activity in the breast carcinomas T47D, MCF-7, MDA-MB-231 from 224, 445, 451, to 25, 80, 172 cpm respectively. Expression in the presence of PGA_1 was reduced by an average of 31% for breast carcinomas and 56% for glioblastomas. PGA_1 inhibited the luciferase activity in U87MG, U373, U118MG, T47D, MCF-7, MDA-MB-231 from 2125, 550, 2817, 224, 445, 451 cpm to 941, 284, 918, 112, 331, 361 cpm respectively.

After demonstrating that PGA_1 and ASA were able to reduce NF-κB induced expression, the cell lines were cultured in the presence of these inhibitors to elucidate the effect on proliferation and foci formation. The rates of proliferation in the presence of the inhibitors were determined through the cell counting kit (Dojindo, Inc). Twenty-four hours after the cells were seeded, the appropriate treatment was introduced. ASA at 5mM and PGA_1 at 24µM were able to significantly decrease cellular proliferation in all tumor cell lines treated (Figure 8). PGA_1 produced an average reduction of 61% in proliferation of breast carcinomas and 60% in glioblastomas. ASA yielded reduced proliferation rates of 83% in breast carcinomas and 93% in glioblastomas. The proliferation assays with the inhibitors showed that division in these cells can be reduced proportional to NF-κB inhibition. To examine the effect on foci formation, PGA_1 at 6µM, 12µM, 24µM and ASA at 200µM, 1mM, and 5mM were introduced into the medium of tumor cells cultured in 24 well plates. Foci formation or loss of contact inhibition was reduced when the tumor cell lines were grown in the presence of PGA_1 or ASA (Figure 9). Tumor cells showed a
Figure 7. Chemical Inhibition of NF-κB Transcriptional Activity by PGA₁ and ASA.

NF-κB induced expression of luciferase quantified using pNF-κB-Luc vector (dark bars) against control (light bars). Untreated cells (U) were tested against cells that were cultured in the presence of PGA₁ (P) at 24µM and ASA at 5mM (A) or mock-transfected (M) cells to demonstrate the inhibition on NF-κB activation. The data represents averages (± SD) of samples assayed in triplicate.
Figure 8. Prostaglandin A₁ (PGA₁) and Acetylsalicylic Acid (ASA) Reduce Proliferation in Tumor Cell Lines.

Cell proliferation assays performed following the protocol as described. Cells (10⁴ per well) were cultured in MEM with 2% FBS and the appropriate inhibitor (PGA₁-24μM or ASA-5mM) for six days. Control cells were cultured in parallel concentrations of ethanol but without inhibitors. The results represent averages of two independent experiments.
Figure 9. Prostaglandin A1 (PGA₁) and Acetylsalicylic Acid (ASA) Reduce Focus Formation in Tumor Cell Lines.

The cells (2x10⁴ per well) were cultured in medium with 2% serum and the NF-κB inhibitors at increasing concentrations that was replaced every second day. Control cells were treated with parallel concentrations of ethanol. After a ten-day period, cells were stained with 0.25% crystal violet. The cells displayed a dose dependent response to the inhibitors.
dose dependent response to the inhibitors. The cells still had an increased tendency to form foci in the presence of the inhibitors relative to normal epithelial cells.

Inhibition of NF-κB Transcriptional Activity by Dominant Negative Mutants

**Characteristics of the Cells Transfected With pmelKBα**

To verify that NF-κB inhibition reduced proliferation and foci formation, stable cell lines expressing dominant negative mutants that inhibit NF-κB activity were established with pmelKBα and p65ΔC. Assays on the cells transfected with pmelKBα demonstrated that a 60% inhibition of expression occurred in these stably transfected cells created from each cell line as compared to the cells established with pRc/CMV (Figure 10). The three clones that were transfected with pmelKBα and isolated from the T47D cell line had the greatest inhibition that was 83% lower than the three clones transfected with pRc/CMV. The clones transfected with pmelKBα and established from the U87MG, U3737MG, U118MG, MCF-7, MDA-MB-231 cell lines had reduced expression by 78%, 47%, 33%, 62%, 45% respectively. Western blotting demonstrated that all the cells expressing pmelKBα for each tumor line were over expressing the IκBα insert relative to their wildtype and pRc/CMV cells (Figure 11). Their proliferation rates were determined through the cell counting kit (Donjindo, Inc) following the protocol as previously described. The cells were also counted using a hemocytometer on the 1st and 8th day to qualify the results obtained with the cell counting kit. Quantification by both methods produced similar rates.
Isolated clones expressing the dominant negative mutant pmeIKBα were compared to the clones transfected with the empty pRc/CMV vector. Transient transfections, with pNF-κB-luc vector (dark bars) and control (light bars), were used to examine the amount of NF-κB transcriptional activity in the cells. The data indicates the averages (± SD) of samples assayed in triplicate.
Figure 11. Over-Expression of the IκBα Protein by Cells Transfected With pmeIκBα.

Ten micrograms of protein were electrophoresied on a 10% Tris glycine polyacrylamide gel. Following electrophoresis proteins were transferred by electroblotting to a PVDF membrane, and IκB was detected using rabbit anti-IκB and ECLPlus® Western Blotting Kit (Amersham Pharmacia Biotech).
Table 2

Net Proliferation Rates of the Tumor, pmeIK.Ba, pRc/CMV Cells Quantified Using a Hemocytometer (A) and Cell Counting Kit (B) After 8 Days Growth

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast Carcinomas</strong></td>
<td></td>
<td></td>
<td><strong>Glioblastomas</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T47D wt</td>
<td>7.32</td>
<td>6.16</td>
<td>U87MG wt</td>
<td>5.49</td>
<td>5.71</td>
</tr>
<tr>
<td>pRc/CMV #1</td>
<td>6.47</td>
<td>5.86</td>
<td>pRc/CMV #1</td>
<td>5.83</td>
<td>3.46</td>
</tr>
<tr>
<td>pRc/CMV #2</td>
<td>8.30</td>
<td>6.21</td>
<td>pRc/CMV #2</td>
<td>4.32</td>
<td>4.26</td>
</tr>
<tr>
<td>pRc/CMV #3</td>
<td>5.36</td>
<td>5.65</td>
<td>pRc/CMV #3</td>
<td>3.88</td>
<td>2.84</td>
</tr>
<tr>
<td>pmeK #1</td>
<td>3.48</td>
<td>3.36</td>
<td>pmeK #1</td>
<td>3.24</td>
<td>3.33</td>
</tr>
<tr>
<td>pmeK #2</td>
<td>3.11</td>
<td>2.69</td>
<td>pmeK #2</td>
<td>2.65</td>
<td>2.12</td>
</tr>
<tr>
<td>pmeK #3</td>
<td>3.98</td>
<td>3.40</td>
<td>pmeK #3</td>
<td>2.23</td>
<td>2.54</td>
</tr>
<tr>
<td>MCF-7 wt</td>
<td>6.97</td>
<td>7.82</td>
<td>U373MG wt</td>
<td>5.96</td>
<td>3.85</td>
</tr>
<tr>
<td>pRc/CMV #1</td>
<td>6.45</td>
<td>6.29</td>
<td>pRc/CMV #1</td>
<td>3.43</td>
<td>3.24</td>
</tr>
<tr>
<td>pRc/CMV #2</td>
<td>7.03</td>
<td>8.93</td>
<td>pRc/CMV #2</td>
<td>4.92</td>
<td>3.36</td>
</tr>
<tr>
<td>pRc/CMV #3</td>
<td>7.21</td>
<td>7.64</td>
<td>pRc/CMV #3</td>
<td>3.22</td>
<td>2.19</td>
</tr>
<tr>
<td>pmeK #1</td>
<td>4.08</td>
<td>3.61</td>
<td>pmeK #1</td>
<td>2.79</td>
<td>2.36</td>
</tr>
<tr>
<td>pmeK #2</td>
<td>3.82</td>
<td>3.94</td>
<td>pmeK #2</td>
<td>3.48</td>
<td>2.44</td>
</tr>
<tr>
<td>pmeK #3</td>
<td>3.61</td>
<td>3.00</td>
<td>pmeK #3</td>
<td>2.34</td>
<td>2.02</td>
</tr>
<tr>
<td>MDA-MB-231 wt</td>
<td>6.78</td>
<td>4.31</td>
<td>U118MG wt</td>
<td>6.56</td>
<td>4.69</td>
</tr>
<tr>
<td>pRc/CMV #1</td>
<td>5.13</td>
<td>4.85</td>
<td>pRc/CMV #1</td>
<td>5.77</td>
<td>5.23</td>
</tr>
<tr>
<td>pRc/CMV #2</td>
<td>6.37</td>
<td>5.52</td>
<td>pRc/CMV #2</td>
<td>5.61</td>
<td>5.67</td>
</tr>
<tr>
<td>pRc/CMV #3</td>
<td>7.01</td>
<td>5.61</td>
<td>pRc/CMV #3</td>
<td>5.11</td>
<td>5.20</td>
</tr>
<tr>
<td>pmeK #1</td>
<td>2.89</td>
<td>3.04</td>
<td>pmeK #1</td>
<td>4.63</td>
<td>4.52</td>
</tr>
<tr>
<td>pmeK #2</td>
<td>4.53</td>
<td>2.53</td>
<td>pmeK #2</td>
<td>4.17</td>
<td>3.19</td>
</tr>
<tr>
<td>pmeK #3</td>
<td>3.45</td>
<td>2.46</td>
<td>pmeK #3</td>
<td>3.86</td>
<td>4.72</td>
</tr>
</tbody>
</table>

The assays involving cells transfected with pmeIK.Ba resulted in proliferation rates that were reduced ~50% from their wild type cells (Table 2). In the tumor cells measured with the kit, the average proliferation rate of the pmeIK.Ba cells was reduced 45% from their wild type and pRc/CMV transfected cells (Figure 12). Foci formation assays using the cells transfected with pmeIK.Ba and pRc/CMV gave insight into NF-κB's role on transformation. NF-κB inhibition in the pmeIK.Ba cells decreased the presence of foci but did not entirely prevent their formation (Figure 13).
Figure 12. Assays Demonstrating Reduced Proliferation in Cells Transfected With pmeIKBα.

Assays were performed following manufacturer’s recommendations (Dojindo, Inc). The cells (10^4 per well) were seeded in 96 well plates and cultured with the appropriate medium with 2% serum that was replaced every other day. The results represent (± SD) the averages of four independent experiments.
Figure 13. Assay Demonstrating Reduced Focus Formation in Cells Transfected With pmelκBα.

Cells were seeded $2 \times 10^4$ per well in 24 well plates and cultured with the appropriate medium containing 2% serum that was replaced every second day. After ten days the cells were stained with 0.25% crystal violet.
Characteristics of the Cells Transfected With p65ΔC

To further test their response to NF-κB inhibition, the tumor cell lines were stably transfected with p65ΔC and assayed for activation, proliferation, and focus formation as described previously. Comparisons between the cells expressing p65 wildtype and p65ΔC showed that the p65ΔC transfected cells inhibited NF-κB induced expression by 70% (Figure 14). The average counts per minute (cpm) for the three clones transfected with p65ΔC was reduced in U87MG (70%), U373MG (58%), U118MG (77%), T47D (65%), MCF-7 (57%), MDA-MB-231 (73%). All the p65ΔC transfected cells established, had expression levels lower than both the mock-transfected cells and cells transfected with p65 wildtype. The U87MG, T47D, MDA-MB-231 clones expressing p65 wildtype had higher expression levels than their mock-transfected cells similar to results described by Baldwin *et al.* (1995). The inhibition obtained with the cells transfected with p65ΔC was 15% greater than the results obtained with the cells transfected with pmeIkBα. p65ΔC transfected cells had decreased proliferation rates and focus formation relative to cells transfected with p65 wildtype and non-transfected tumor cells (Figure 15, 16). Proliferation in the p65ΔC transfected cells was decreased 50% from their mock-transfected cells and p65 transfected cells (Figure 15, Table 3). Comparing p65ΔC and p65 transfected cells, the greatest decrease in proliferation occurred within the U118MG (75%), MCF-7 (66%), T47D (50%), U87MG (48%) cell lines. In all the tumor cell lines, foci formation was significantly decreased in the p65ΔC transfected cells when
compared to the non-transfected cells and p65 transfected cells (Figure 16). The cellular transformation within the p65ΔC transfected cells was again incomplete and focus formation was inversely proportional to the proliferation rates. The non-transfected cells and p65 transfected cells reached full confluence faster and therefore had a greater opportunity for focus formation.

Table 3

Net Proliferation Rates of the Tumor, p65ΔC, p65 Cells Quantified Using a Hemocytometer (A) and Cell Counting Kit (B) After 8 Days Growth

<table>
<thead>
<tr>
<th>Breast Carcinomas</th>
<th>A</th>
<th>B</th>
<th>Glioblastomas</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D wt</td>
<td>6.53</td>
<td>8.48</td>
<td>U87MG wt</td>
<td>7.45</td>
<td>8.52</td>
</tr>
<tr>
<td>p65 #1</td>
<td>7.28</td>
<td>8.26</td>
<td>p65 #1</td>
<td>8.62</td>
<td>9.28</td>
</tr>
<tr>
<td>p65 #2</td>
<td>6.42</td>
<td>7.14</td>
<td>p65 #2</td>
<td>8.32</td>
<td>9.00</td>
</tr>
<tr>
<td>p65 #3</td>
<td>5.79</td>
<td>10.96</td>
<td>p65 #3</td>
<td>8.45</td>
<td>10.23</td>
</tr>
<tr>
<td>P65ΔC #1</td>
<td>4.65</td>
<td>6.41</td>
<td>P65ΔC #1</td>
<td>5.84</td>
<td>5.98</td>
</tr>
<tr>
<td>P65ΔC #2</td>
<td>5.12</td>
<td>2.77</td>
<td>P65ΔC #2</td>
<td>4.13</td>
<td>3.92</td>
</tr>
<tr>
<td>P65ΔC #3</td>
<td>4.08</td>
<td>3.41</td>
<td>P65ΔC #3</td>
<td>4.25</td>
<td>5.61</td>
</tr>
<tr>
<td>MCF-7 wt</td>
<td>6.42</td>
<td>6.29</td>
<td>U373MG wt</td>
<td>8.02</td>
<td>5.88</td>
</tr>
<tr>
<td>p65 #1</td>
<td>8.39</td>
<td>6.72</td>
<td>p65 #1</td>
<td>7.86</td>
<td>6.79</td>
</tr>
<tr>
<td>p65 #2</td>
<td>7.34</td>
<td>4.17</td>
<td>p65 #2</td>
<td>6.38</td>
<td>4.64</td>
</tr>
<tr>
<td>p65 #3</td>
<td>4.21</td>
<td>3.64</td>
<td>p65 #3</td>
<td>6.99</td>
<td>3.38</td>
</tr>
<tr>
<td>P65ΔC #1</td>
<td>4.47</td>
<td>3.11</td>
<td>P65ΔC #1</td>
<td>3.36</td>
<td>2.78</td>
</tr>
<tr>
<td>P65ΔC #2</td>
<td>2.89</td>
<td>2.85</td>
<td>P65ΔC #2</td>
<td>4.83</td>
<td>2.37</td>
</tr>
<tr>
<td>P65ΔC #3</td>
<td>3.48</td>
<td>2.79</td>
<td>P65ΔC #3</td>
<td>3.78</td>
<td>2.38</td>
</tr>
<tr>
<td>MDA-MB-231 wt</td>
<td>5.64</td>
<td>3.80</td>
<td>U118MG wt</td>
<td>6.42</td>
<td>6.13</td>
</tr>
<tr>
<td>p65 #1</td>
<td>6.33</td>
<td>4.32</td>
<td>p65 #1</td>
<td>8.04</td>
<td>7.36</td>
</tr>
<tr>
<td>p65 #2</td>
<td>6.82</td>
<td>3.03</td>
<td>p65 #2</td>
<td>7.20</td>
<td>5.25</td>
</tr>
<tr>
<td>p65 #3</td>
<td>5.93</td>
<td>5.49</td>
<td>p65 #3</td>
<td>7.81</td>
<td>6.25</td>
</tr>
<tr>
<td>P65ΔC #1</td>
<td>3.61</td>
<td>2.26</td>
<td>P65ΔC #1</td>
<td>3.17</td>
<td>3.99</td>
</tr>
<tr>
<td>P65ΔC #2</td>
<td>3.77</td>
<td>2.45</td>
<td>P65ΔC #2</td>
<td>3.65</td>
<td>3.10</td>
</tr>
<tr>
<td>P65ΔC #3</td>
<td>4.32</td>
<td>3.20</td>
<td>P65ΔC #3</td>
<td>4.53</td>
<td>2.91</td>
</tr>
</tbody>
</table>
Cells expressing the dominant negative mutant p65ΔC (p65DC) were compared to cells transfected with the normal p65 insert and the wildtype cells. Transient transfections, with pNF-κB-luc vector (dark bars) and control (light bars), were used to examine the NF-κB induced expression of luciferase. The data indicates averages of samples assayed in triplicate.
Figure 15. Assays Demonstrating Reduced Proliferation in Cells Transfected With p65ΔC.

Assay was performed following the protocol and recommendations from the Cell Counting kit (Dojindo, Inc). The cells were seeded $10^4$ per well in 96 well plates and cultured with the appropriate medium containing 2% serum that was replaced every second day. The cells were measured on the 2nd, 4th, 6th, and 8th day. Results are averages of four independent tests ($\pm$ SD).
Figure 16. Assay Demonstrating Reduced Focus Formation in Cells Transfected With p65ΔC.

Cells were seeded $2 \times 10^4$ per well in 8 well plates and cultured with the appropriate medium containing 2% serum that was placed every second day. After ten days the cells were stained with 0.25% crystal violet.
DISCUSSION

The transcription factor NF-κB plays an important role in a variety of cells through the initiation of transcription for genes involved in proliferation and mitogenesis for numerous cell types. The results presented here indicate that NF-κB also plays an integral part in the proliferation of glioblastomas, U87MG, U118MG, and breast carcinomas, T47D, MCF-7, MDA-MB-231 and to a lesser extent in U373MG. Through chemical and genetic inhibition of NF-κB, I was able to decrease the proliferation rates of these tumor cells. Unfortunately, inhibition of NF-κB induced expression did not completely restore the normal phenotypes of these tumor cells. Only at high concentrations of inhibitors was focus formation completely eliminated and the tumor cells were never sensitive to contact inhibition as was seen in the normal epithelial cells. The reduced focus formation in cells subjected to NF-κB inhibitory conditions was probably a direct result of reduced proliferation rates. Overall, the glioblastoma lines, U87MG, U118MG tended to be most responsive to NF-κB inhibition. The MDA-MB-231 breast carcinoma cell line was least effected by NF-κB inhibition but this observation could be influenced by low transfection efficiency within this line. The glioblastoma line, U373MG, consistently had lower expression levels compared to the other tumor cell lines. It is interesting to note that the U373MG line was the only tumor cell line not expressing the PDGF-B chain or β receptor. Future studies should include elucidation of the mechanisms responsible
for the increased NF-κB activity in these tumor cells. To definitively correlate PDGF receptor signaling to NF-κB initiation of transcription within the tumor cells, measurements of NF-κB activity need to be made in the presence of agonists and antagonists of PDGF. Proteins such as Akt, Ras, Rac, Raf, MEK, PI3K, and MAPK which are associated with PDGF and EGF nuclear signaling pathways could be ideal targets for follow up inhibitory studies. Inhibiting the EGF and PDGF-α,β receptors from phosphorylating cytoplasmic kinases within these cells could help establish the specific relationship between the receptor, proliferation, and cellular transformation. Differential expression studies between cells with NF-κB inhibition and without could provide essential information on the genes activated by NF-κB in these tumor lines. Expression studies with cell cycle proteins like cyclin D1, which are known to be dependent on NF-κB activation, could serve to give better evidence of cellular transformation. Because inhibition of NF-κB activity did not completely inhibit proliferation of the cells, future experiments should examine other transcription factors associated with growth and transformation of glioblastomas and breast carcinomas. In some research articles (Lim and Demo, 2000), the transcription factors, AP-1 and SP-1, have been shown to be activated along with NF-κB. Consideration has to be given to the AP-1 and SP-1 transcription factors because both have similar roles in cell cycle regulation as NF-κB (Lim and Demo, 2000). Therapeutic strategies associated with cancer treatment often involve prevention of G1-to-S phase transitions in actively dividing cells (Rubin and Farber, 1997). NF-κB
has been documented by many to be a major contributor of G₁-to-S phase transitions in cells (Baeuerle and Baltimore, 1996, Guttridge et al., 1999). The conclusions obtained in this investigation are similar to those gathered by other researchers on NF-κB inhibition in cancerous cells (Abel-Mageed et al., 1998, Bargou et al., 1997, Chin et al., 1999, Daley and Baltimore, 1988, Grimm and Baeurele, 1994, Marini et al., 1990). Therefore, it came to no surprise an important regulator like NF-κB was partly responsible for proliferation in these glioblastoma and breast carcinoma cell lines.
Appendix A

Protocol Clearance From the Institutional Biosafety Committee
Date: 8 May 1998

To: Bruce Bejcek, Department of Biological Sciences

From: Loreene L. Broker, Research Compliance Coordinator

Re: IBC Project Numbers 98-BBa, 98-BBb, and 98-BBc

This letter will confirm that your registration for the recombinant DNA projects listed below was reviewed by the Institutional Biosafety Committee (IBC) on 24 April 1998 and has been approved.

The approved projects are:

- 98-BBa: SSTR2 and SHP2
- 98-BBb: The Role of Syp and GRB2 in PDGF Transformation
- 98-BBc: gatf of HSVii, Its Role and Function in Morphological Transformation

Copies of the approved and signed registration documents are attached to this letter for your files.

If you have any questions, please call the research compliance coordinator at 387-8293. The Institutional Biosafety Committee wishes you success in the pursuit of your research goals.
BIBLIOGRAPHY


