Award Number: DAMD17-98-1-8032

TITLE: Regulation of ErbB-2 and Src Signaling by CHK and Csk Tyrosine Kinases in Breast Cancer

PRINCIPAL INVESTIGATOR: Hava Avraham, Ph.D.
Arthur M. Mercurio, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Incorporated
Boston, Massachusetts 02215

REPORT DATE: May 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Regulation of ErbB-2 and Src Signaling by CHK and Csk Tyrosine Kinases in Breast Cancer

Hava Avraham, Ph.D.
Arthur M. Mercurio, Ph.D.

Beth Israel Deaconess Medical Center, Incorporated
Boston, Massachusetts 02215

havraham@caregroup.harvard.edu

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

The HER-2/neu/c-erbB2 and Src family members are implicated in the pathogenesis of breast cancer. In this study, we have examined the role of CHK kinase in suppressing the cell transformation mediated by ErbB-2 and src kinases. We generated stable transfected human breast carcinoma MCF-7 cells overexpressing CHK either active or inactive (kinase-dead). We showed that overexpression of active CHK (but not inactive CHK) inhibited in vitro MCF-7 cells growth, transformation (5-fold) and invasion (24% to 33%) induced upon HRG stimulation. In addition, in vitro Lyn tyrosine kinase activity was inhibited (5-fold) and entry into mitosis was delayed. Furthermore, in vivo tumor growth of MCF-7 cells transfected with active CHK (but not inactive CHK) and grafted in nude mice was significantly inhibited (97% to 100%) compared to untransfected MCF-7 cells (P < 0.05 and P < 0.03 for the two clones of MCF-7 cells transfected with active CHK that were tested). In conclusion, our data strongly support the role of CHK as a novel tumor suppressor gene for human breast cancer, acting through downregulation of Lyn kinase activity and regulation of the S-phase of the cell cycle.
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[Signature]

Date: 5/23/2000
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRONT COVER</td>
<td>1</td>
</tr>
<tr>
<td>STANDARD FORM (SF) 298</td>
<td>2</td>
</tr>
<tr>
<td>FOREWORD</td>
<td>3</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>4</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>5</td>
</tr>
<tr>
<td>BODY</td>
<td>5-12</td>
</tr>
<tr>
<td>KEY RESEARCH ACCOMPLISHMENTS</td>
<td>12</td>
</tr>
<tr>
<td>REPORTABLE OUTCOMES</td>
<td>12</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>12</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>n/a</td>
</tr>
</tbody>
</table>
FINAL REPORT FOR AWARD #: DAMD17-98-1-8032
Year 2 of 3

Title: Regulation of ErbB-2 and Src signaling by CHK and Csk tyrosine kinases in breast cancer

P.I.: Hava Avraham, Ph.D.

INTRODUCTION

A major means by which Src kinases are downregulated is through C-terminal tyrosine phosphorylation. The Csk family of protein tyrosine kinases comprises two members termed Csk and CHK. These enzymes CHK (originally termed MATK) shares ~50% homology with the Csk tyrosine kinase. CHK is expressed in malignant breast tissue but not in normal breast tissue. In addition, CHK, in contrast to Csk, has the unique ability to bind via its SH2 domain to a particular diphosphorylated sequence (Tyr1248) on the C-terminus of the ErbB-2 receptor when activated by hereregulin. Interestingly, this is the same sequence which confers oncogenicity to ErbB-2, suggesting a unique role for CHK in the regulation of ErbB-2 activation. Moreover, our preliminary results indicate that overexpression of CHK in MCF-7 breast cancer cells markedly diminished cell growth and inhibited tumor development of xenografts in nude mice. These results lead us to hypothesize that: (1) CHK may function as a negative regulator of both pp60src and ErbB-2, while Csk may function as a negative regulator of pp60src; (2) ErbB-2 activation results in the activation of Src kinases by their binding to an autophosphorylation site of ErbB-2, and that subsequent to this CHK binds to the Tyr1248 of the ErbB-2, resulting in phosphorylation and downregulation of the ErbB-2 and Src kinases; and (3) C-terminal phosphorylation of Src by Csk and CHK may be critical for downregulation of Src kinase activity and the inhibition of breast cancer growth. In order to test these hypotheses, we propose to focus on two basic aims: (a) To analyze the ability of CHK to downregulate ErbB-2 activated Src kinases; (b) To further characterize the effects of either CHK, Csk or both Csk and CHK, in preventing tumor development in tumor-bearing mice. New information gained from these studies in the role of CHK and Csk as putative negative growth regulators in breast cancer will advance current understating of oncogenic signal transduction mechanisms and may provide a basis for utilizing these tyrosine kinases to oppose the malignant process.

BODY

Stable transfection of the human breast cancer cell line MCF-7 with CHK. To evaluate the effect of CHK on breast cancer cells, we generated stable transfected cells overexpressing CHK either wild-type and active (wt), or mutated and inactive (mt) as a result of a point-mutation in the lysine of the ATP-binding site of the kinase domain (K262A). We used MCF-7 cells as a model system for human breast carcinoma. We analyzed two clones expressing CHK (wt), clone #5 and clone #10; and two clones expressing CHK (mt), clone #7 and clone #9. Control cells are untransfected MCF-7 cells (wt) and MCF-7 cells transfected with the empty vector (neo). We confirmed by Western blot analysis that the level of CHK protein expression was comparable in the different stable transfecants (Fig. 1). We did not detect any CHK expression in untransfected MCF-7 cells as previously described (2, 3).

CHK overexpression inhibits in vitro Lyn tyrosine kinase activity. We performed in vitro kinases assays to evaluate the biological function of the exogenous CHK.

We first analyzed stable transfectants for CHK kinase activity (Fig. 2). Upon stimulation with HRG, we measured tyrosine kinase activity in MCF-7/CHK(mt) cells but not in untransfected MCF-7 cells and
MCF-7/CHK(mt) cells (Fig. 2A). No CHK kinase activity was detected in the absence of HRG (data not shown). On the contrary, Csk kinase activity was detected at the same level in all types of MCF-7 cells (Fig. 2B), suggesting that CHK overexpression does not affect Csk activity.

Next, we investigated the modulation of Src family members by CHK. It has been reported that in MCF-7 cells two Src family protein tyrosine kinases could be activated, c-Src kinase which leads to MAP-kinase activation (8, 9, 10), and Lyn kinase which leads to JUN-kinase activation (11). We did not detect any significant Src tyrosine kinase activity (Fig. 2C). On the contrary, Lyn tyrosine kinase activity was detected and significantly decreased (5-fold) in MCF-7/CHK(wt) cells compared to untransfected MCF-7 cells and MCF-7/CHK(mt) (Fig. 2D).

**CHK overexpression inhibits in vitro MCF-7 cells proliferation and transformation.** It has been reported that Src family kinases are involved in cell proliferation and transformation induced in response to growth factor stimulation (1).

We first examined the cell proliferation of CHK-transfected cells upon HRG stimulation (Fig. 3A). MCF-7/CHK(wt) cells proliferation was not induced, whereas untransfected MCF-7 cells and MCF-7/CHK(mt) cells responded to HRG stimulation.

Next, we assessed the anchorage-independent growth of CHK-transfected cells in soft agar (Fig. 3B). The number of MCF-7/CHK(wt) colonies formed was significantly decreased (5-fold) compared to untransfected MCF-7 cells and MCF-7/CHK(mt) cells.

**CHK overexpression inhibits in vitro MCF-7 cells invasion.** It has been reported that Src family kinases are involved in cell invasion (12).

Therefore, we evaluated the ability of CHK-transfected cells to invade matrigel (Fig. 4). It has been reported that MCF-7 cells invasion could be induced after HRG stimulation (13). We observed that, in the presence of HRG, the matrigel invasion of MCF-7/CHK(wt) cells was significantly inhibited (33 %) compared to untransfected MCF-7 cells. No invasion was observed in the absence of HRG (data not shown). Interestingly, the matrigel invasion of MCF-7/CHK(mt) cells was also significantly reduced (24 %). These results suggest that the kinase activity of CHK is required but not sufficient for the invasion process.

**CHK overexpression delays in vitro entry into mitosis.** It has been previously demonstrated that Src family kinases are required for cell division to occur (14), and are specifically required at the transition from the G2 phase to mitosis in the cell cycle (15, 16). Furthermore, it has been recently shown that Lyn tyrosine kinase is involved in the G1/S transition through direct binding to and activation of the cyclin-dependent kinase 2 (Cdk2) (17). Since we demonstrated that CHK can downregulate Lyn kinase activity, we investigated whether the level of CHK expression might modulate cell cycle kinetics. A significant delay in the entry to S-phase (12 h) and an increase in G2/M phase (2-fold) was observed with MCF-7/CHK(wt) cells (clone #10) compared to the untransfected MCF-7 cells (Table I). Similar data were obtained with MCF-7/CHK(wt) clone #5 (data not shown).

**CHK overexpression inhibits in vivo MCF-7 tumor growth.** In vivo tumor development of MCF-7 cells grafted in nude mice was then studied. MCF-7 cells were inoculated subcutaneously and tumor size was followed for 60 days. The tumor growth of MCF-7/CHK(wt) cells was significantly inhibited compared to untransfected MCF-7 cells (97 % inhibition and P = 0.047 for clone #5; and 100 % inhibition and P = 0.028 for clone #10). No significant tumor reduction was observed for MCF-7/CHK(mt) cells (Fig. 5A). CHK expression was confirmed by Western blot analyses of the tumors taken when the experiment was terminated. The level of CHK protein expression was comparable in tumors obtained from CHK-transfected cells (Fig. 5B).
FIGURE 1 Stable transfection of the human breast cancer cell line MCF-7 with CHK.

<table>
<thead>
<tr>
<th></th>
<th>CHK (wt)</th>
<th>CHK (mt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt neo</td>
<td>#5 #10</td>
<td>#7 #9</td>
</tr>
</tbody>
</table>

![Western blot images](image)

MCF-7 cells were stably transfected with CHK either wild-type (clone #5, and clone #10) or mutated (clone #7, and clone #9). Control cells are non-transfected cells (wt) and cells transfected with the empty vector (neo). Total protein extracts from MCF-7 cells were prepared and analyzed for protein expression by Western blot using antibodies against CHK (upper panel), Csk (middle panel), and actin (lower panel).
FIGURE 2 Tyrosine kinase activity in MCF-7 cells overexpressing CHK.

Total protein extracts from MCF-7 cells induced with 10 nM HRG for 10 min were prepared then immunoprecipitated with antibodies against CHK (A), Csk (B), Src (C), and Lyn (D). The kinase activity of immunoprecipitates was determined using poly(Glu/Tyr) as a substrate. The data shown are the mean values ± SD of duplicate.
FIGURE 3 CHK overexpression inhibits in vitro MCF-7 cells proliferation and transformation.

A, MCF-7 cells were grown in the absence (-) or presence (+) of 10 nM HRG. Number of viable cells was quantitated by crystal violet staining. The data shown are the mean values ± SD of 4 wells.

B, MCF-7 cells were seeded in soft agar and allowed to grow for 2 weeks before counting viable colonies (3 cells or more per colony). The data shown are the mean values ± SD of 4 wells.
FIGURE 4 CHK overexpression inhibits in vitro MCF-7 cells invasion.

MCF-7 cells were tested for their ability to invade Matrigel in the presence of 10 nM HRG for 18 h. The data shown are the mean values ± SD of three experiments done in triplicate. Results are expressed as a percentage of the control (untransfected MCF-7 cells).
FIGURE 5 CHK overexpression inhibits in vivo MCF-7 tumor growth.

MCF-7 cells (10^7) were implanted subcutaneously into the mammary fat pad of female athymic nude mice (n = 6).

A, mice were followed for tumor growth. Data represent median tumor volumes as a function of time.
TABLE I CHK overexpression delays in vitro entry into mitosis of MCF-7 cells.
MCF-7 cells were starved in serum-free media for 72 h, then stimulated with 10% FBS and harvested at
the indicated time points. Cells were labeled with propidium iodide and subjected to flow cytometry
analysis. The percentage of cell in each phase of the cell cycle (G1, S, and G2/M) was calculated.

<table>
<thead>
<tr>
<th>time-points</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>77.1</td>
<td>16.4</td>
<td>6.5</td>
<td>78.0</td>
<td>8.7</td>
<td>13.3</td>
</tr>
<tr>
<td>6 h</td>
<td>80.2</td>
<td>10.9</td>
<td>8.9</td>
<td>74.0</td>
<td>8.5</td>
<td>17.6</td>
</tr>
<tr>
<td>12 h</td>
<td>70.3</td>
<td><strong>22.1</strong></td>
<td>7.6</td>
<td>74.8</td>
<td>9.2</td>
<td>16.1</td>
</tr>
<tr>
<td>18 h</td>
<td>66.3</td>
<td><strong>23.4</strong></td>
<td>10.3</td>
<td>76.1</td>
<td>11.3</td>
<td>12.6</td>
</tr>
<tr>
<td>24 h</td>
<td>63.7</td>
<td><strong>28.5</strong></td>
<td>7.8</td>
<td>65.2</td>
<td><strong>23.7</strong></td>
<td>11.1</td>
</tr>
</tbody>
</table>

KEY RESEARCH ACCOMPLISHMENTS:
Our studies demonstrate that CHK downregulated Src kinases activated by heregulin.
• CHK expression is upregulated in breast cancers and overexpression of CHK inhibits tumor
  formation in breast cancers grafted in nude mice.

REPORTABLE OUTCOMES:
We have submitted an abstract that was presented at the AACR meeting in April ’2000.

CONCLUSIONS:
A. Overexpression of CHK correlates with the known markers of breast malignancy.
B. Overexpression of CHK can negatively regulate the growth of MCF-7 breast cancer cells in nude
  mice.
C. These results suggest that CHK overexpression is associated with anti-proliferative activity and can
  reduce the transformation ability of breast cancer cells.

BIBLIOGRAPHY: n/a