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Award Number: DAMD17-98-1-8310

TITLE: Regulation of ErbB Receptor Tyrosine Kinase Activities in Breast Cancer by the Kek Proteins

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REPORT DATE: October 2001

TYPE OF REPORT: Final

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

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 2001 | Final (1 Oct 98 - 30 Sep 01) |

**4. TITLE AND SUBTITLE**
Regulation of ErbB Receptor Tyrosine Kinase Activities in Breast Cancer by the KEK Proteins

**5. FUNDING NUMBERS**
DAMD17-98-1-8310

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 Words)*

We have found that the *Drosophila* transmembrane molecule *kekkon 1* (*kek1*) acts in a negative feed back loop to modulate the activity of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase. *kek1* is expressed in response to activation of the Gurken/EGFR signaling pathway during oogenesis. While loss of *kek1* activity is associated with a phenotype reminiscent of increased Grk/EGFR signaling, ectopic overexpression of *kek1* mimics the complete loss of EGFR activity. We found that the extracellular domain of Kek1 physically associates with the EGFR providing the basis for this inhibitory mechanism. Interestingly, we found that Kek1 is also a potent inhibitor of the EGFR in mammalian cells. First, Kek1 binds the EGFR and related proteins ErbB2, ErbB3 and ErbB4. Kek1 interferes with EGF mediated receptor tyrosine phosphorylation and activation of the downstream signaling molecules PI3-kinase and Erk/MAP kinases. Kek1 can also inhibit transformation in mouse mammary tumor cells with deregulated expression of receptors and ligands of the ErbB family.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer, IDEA | 19 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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## INTRODUCTION:

The aberrant activation of members of the EGF receptor (ErbB) family of receptor tyrosine kinases is thought to promote mammary tumor progression by stimulating tumor cell growth. We have found that Kekkon-1 (Kek1), a transmembrane leucine rich repeat (LRR) glycoprotein from the fruit fly *Drosophila melanogaster*, is capable of physically interacting with and suppressing the signaling activities of members of the mammalian EGF receptor family. Kek1 also potently suppresses the growth of mouse mammary tumor cells derived by ErbB receptor activation. These results indicate that LRR glycoproteins can act as direct modulators of growth factor signaling, and point to the possibility that these proteins could be products of tumor supressor genes.

## BODY:

### *Drosophila* Kek1 binds all known receptors of the ErbB/EGFR family

To explore the possibility that Kek1-like proteins might also suppress receptor signaling in mammalian tumors, we first assessed whether Kek1 is capable of interacting with each of the mammalian EGF receptor family members. To test the interaction of Kek1 with human EGF receptor and to examine the functional outcome of this interaction we established an inducible expression system for HA epitope-tagged Kek1 in 293 human embryonic kidney cells (HEK293-Ecr). With this system, HA-Kek1 is robustly expressed in an all-or-nothing manner with addition of the ecdysone analog ponasterone A (PonA). Fig. 1A shows that the endogenous EGF receptor may be co-immunoprecipitated with HA-Kek1 only when expression is turned on with PonA. Likewise, Kek1 may be co-immunoprecipitated with EGF receptor after PonA treatment. Similar results have been

have been obtained using Sf9 insect cells (see Fig. 3) and COS monkey cell expression systems (not shown), indicating that Kek1 has the ability to interact with mammalian EGF receptor.

To determine whether Kek1 is capable of interacting with the other three members of the ErbB receptor family, each of these proteins was expressed in Sf9 insect cells in the presence and absence of myc epitope-tagged Kek1. Immunoprecipitating with anti-myc antibodies, we observed that ErbB2, ErbB3 and ErbB4 are each co-precipitated with myc-Kek1 (Fig. 1B), while the *Drosophila* torso receptor tyrosine kinase only weakly co-precipitated with myc-Kek1 if at all. Taken together the results presented in Fig. 1 raise the possibility that Kek1 might suppress signaling through mammalian ErbB receptors.

**Kek1 inhibits EGF mediated activation of the EGF receptor and downstream signaling pathways**

To assess the functional consequence of Kek1 association with EGF receptor family members we examined two of the early biochemical events associated with EGF receptor activation, receptor autophosphorylation and the stimulation of the Erk1 and Erk2 mitogen-activated kinases (MAPKs). In these experiments we treated the HEK293-Ecr cells without or with Pon A to induce Kek1 expression (Fig. 2A, lower panel) and then starved for 4 hours without serum. We then treated cells without or with EGF for 5 minutes. In the experiment shown in Fig. 2A, upper panel, we immunoprecipitated cell lysates with anti-phosphotyrosine antibodies to isolate tyrosine-phosphorylated proteins, and then blotted precipitates with anti-EGF receptor. We observed that in the absence of Kek1 expression EGF potently stimulated the association of the EGF receptor with anti-phosphotyrosine. However the expression of Kek1 resulted in a loss of the receptor from anti-phosphotyrosine precipitates, indicative of an inhibition of receptor autophosphorylation.

In Fig. 2B we examined the stimulation of the Erk1 and Erk2 serine/threonine kinases by blotting lysates from treated cells with an antibody that recognizes the phosphorylated (activated) forms of these proteins. We observed that Kek1 inhibited the activation of Erks in response to EGF by ~75% in the HEK293-Ecr cells. These observations suggest that, consistent with its activity in flies, Kek1 interacts with the mammalian EGF receptor to suppress receptor activation and signaling through the MAPK cascade.

**Kek1 inhibits ligand binding and EGF receptor activation**

To examine the mechanistic details of Kek1 suppression of EGF receptor activity, we used the baculovirus/Sf9 insect cell expression system. This system was employed because the viral infection allows tight control of both the relative levels of proteins expressed in each cell and the number of cells expressing protein.

In the experiment depicted in Fig. 3A, myc-Kek1 or human EGF receptor were expressed alone, or the two proteins were co-expressed with EGF receptor in excess. Cells were treated without or with EGF, and lysates were immunoprecipitated with antibodies directed to either myc epitope (lanes 1-4) or EGF receptor (lanes 5-9). When precipitates were blotted with anti-phosphotyrosine, we observed a strong stimulation of receptor autophosphorylation by the growth factor in anti-receptor precipitates (upper panel, lanes 6-9), indicating that the total EGF receptor population responded strongly to ligand treatment. However, although the presence of EGF receptor was apparent in the anti-myc precipitates (middle panel, lanes 2 and 3), no stimulation of the tyrosine phosphorylation of this Kek-associated population of receptors was observed (upper panel, lanes 2 and 3). Moreover, EGF receptors in anti-myc precipitates were not capable of interacting with [$^{125}$I]EGF

(lower panel, compare lane 2 with lanes 6 and 8). These observations suggest that Kek1 directly interacts with the EGF receptor at the cell surface to inhibit ligand binding.

One possible explanation for the Kek1-mediated suppression of EGF binding and activation is that Kek1 becomes trapped in an intracellular compartment in Sf9 cells and retains a population of the EGF receptor. To examine this possibility we looked at the localization of myc-Kek1 and EGF receptor by immunofluorescence. In the experiment shown in Fig. 3B cells were infected at a low viral multiplicity of infection, so that only ~20% of the cells were infected with virus. Under these conditions many cells express either one or the other protein, and a few cells express both. Shown is a field exemplifying myc-Kek1 expression alone, EGF receptor expression alone, and the two proteins co-expressed. Individually, each protein exhibited a ring around the cell, the hallmark of cell surface expression, and merging of the images indicated that the two proteins co-localize in co-expressing cells. These observations indicate that Kek1 acts at the cell surface to suppress ligand binding and to interfere with EGF receptor signaling.

**Kek inhibits the growth of mammary cell lines with activated ErbB receptors**

Because of the known role of ErbB receptor activation in the genesis and progression of breast tumors, we sought to assess the functional outcome of Kek1/ErbB interactions by examining the impact of Kek1 expression on ErbB-mediated tumor cell growth properties. In this series of experiments we examined the effect of Kek1 expression on the anchorage-dependent and -independent growth of human and mouse cells, as well as the growth of cells as tumors in nude mice. Our studies included the HEK293-Ecr transfectants described above, and the human mammary tumor cell line MDA-MB-468. Of particular interest was a series of cell lines obtained from mouse mammary tumors generated by

expressing the EGF-like growth factor neuregulin-1 (IJ9921)[15], an activated form of ErbB2 (NF-639)[16], or activated ras (AC-816)[17] under the MMTV promoter in transgenic animals. Fig. 4A shows that the expression of Kek1 slowed the anchorage-dependent growth rate of every cell line examined except the AC-816 line. Likewise, Kek1 also potently suppressed the anchorage-independent growth and the growth of cells as tumors of the same cell lines (Fig. 4B). These observations suggest that Kek1 functions to disrupt tumorigenic and tumor growth processes initiated by ErbB receptor activation but not by the activation of downstream biochemical events.

## KEY RESEARCH ACCOMPLISHMENTS:

- ***Drosophila* Kek1 binds all known receptors of the ErbB/EGFR family**
- **Kek1 inhibits EGF mediated activation of the EGF receptor and downstream signaling pathways**
- **Kek1 inhibits ligand binding and EGF receptor activation**
- **Kek inhibits the growth of mammary cell lines with activated ErbB receptors**

## REPORTABLE OUTCOMES:

Amundadottir, L., Andresdottir, M., Ghiglione, C., Diamonti, A.J., Perrimon, N., Leder, P. and Carraway III, K.L. (2000) Kekkon-1 suppression of mammary tumor cell growth by direct interaction with ErbB growth factor receptors. PNAS. Submitted.

Ghiglione, C., Carraway III, K.L and Perrimon, N.(2001) Sturture/function analysis of the KEK proteins. In Preparation.

PATENTS: *Kekkon*genes and gene products, filed.

## CONCLUSIONS

*Drosophila* Kek1 was originally defined genetically as a feedback negative regulator of EGF receptor signaling in several developmental processes.[14] Epistasis studies placed the

action of Kek1 upstream of the fly EGF receptor. Since Kek1 is expressed in the same cell as the EGF receptor, these observations suggest that Kek1 interacts with either the receptor to suppress its signaling function or with the ligand to sequester its activity. Indeed, our previous observations indicate that Kek1 may be co-immunoprecipitated with *Drosophila* EGF receptor[14] but not the Gurken ligand (K. Carraway, unpublished) suggesting that Kek1 interacts directly with receptors to interfere with ligand binding activity.

In the present study we demonstrate that Kek1 can interact with all four mammalian ErbB receptor family members. When reconstituted in Sf9 insect cells, Kek1 blocked the binding of radiolabeled EGF to the population of EGF receptors associated with Kek1, but not the total receptor pool. Likewise, EGF-stimulated autophosphorylation of the Kek1-associated receptor population was blocked, but autophosphorylation of the total receptor pool was not. These observations suggest that Kek1 acts to suppress receptor signaling at least in part by physically interfering with ligand binding. However, other effects on receptor activation cannot be ruled out. We observed that Kek1 suppressed the growth properties of the NF-639 mouse mammary tumor cells, obtained from an activating point mutation in the transmembrane region of the ErbB2 receptor. Since this mutation is thought to generate constitutive receptor tyrosine kinase activity via a ligand-independent mechanism,[18] it is likely that Kek1 also acts to interfere with receptor dimerization or other events necessary for catalysis.

Our studies suggest that Kek1 is functionally similar to another Drosophila suppressor of EGF receptor signaling called Argos. Argos is also a transcriptional target of activated EGF receptor in developing tissues,[19] and it has been shown that Argos binds directly to *Drosophila* EGF receptor to inhibit the binding of the natural ligand Spitz.[20] However, the sequences of the two inhibitors are quite distinct. While Kek1 contains a series of leucine

rich repeats and an immunoglobulin-like domain in its extracellular region, Argos contains an imperfect EGF-like domain.[21] Given that at least two proteins in the *Drosophila* genome are dedicated to a similar purpose it seems quite likely that ErbB antagonists are also present in higher organisms.

**Kek1-related genes?**

Our previous studies indicated that the extracellular region of Kek1 was sufficient to mediate its biological activity as well as its interaction with *Drosophila* EGF receptor.[14] Our unpublished observations indicate that the LRR domain of the extracellular region is necessary for the suppression of EGF receptor-mediated developmental events in flies (C. Ghiglione, manuscript in preparation). These results suggest that Kek1/receptor interactions are mediated by the LRR domain, pointing to LRR-containing extracellular proteins as candidates for mammalian Kek1 homologues. Numerous mammalian LRR proteins have been described and several have arrangements of subdomains similar to Kek1, including the trk receptor tyrosine kinases,[22] LIG-1,[23] and a number of proteins of unknown function. The role of such proteins in ErbB-mediated developmental processes and tumor cell growth remain to be explored.

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## APPENDICES:

**Fig. 1** Kek1 association with mammalian ErbB receptor tyrosine kinases. ***a***, Association of Kek1 with human EGF receptor in two stably transfected HEK293-Ecr cell lines. Cells were treated without or with Ponasterone A (PonA) to induce HA-Kek1 expression, and lysates were immunoprecipitated (IP) with antibodies to either EGF receptor (left panel) or HA epitope (right panel). Precipitates were then immunoblotted with anti-HA (left panel) or anti-EGF receptor (right panel). Cell lysates (right lanes of each panel) were included as a positive control for blotting. ***b***, Association of Kek1 with ErbB receptors. Sf9 insect cells were infected with baculovirus encoding the indicated receptor tyrosine kinase (RTK) alone or together with virus encoding myc-Kek. Lysates (left two lanes) and anti-myc precipitates (right two lanes) were blotted with antibodies to RTKs.

**Fig. 2** Inhibition of EGF receptor signaling in HEK293 cells by Kek1. 293-Ecr stably transfected cells (clone 4) were treated without and with PonA for 24 hours, and then treated without and with EGF as indicated. ***a***, Inhibition of EGF receptor autophosphorylation.

Upper panel, lysates from treated cells were immunoprecipitated with anti-phosphotyrosine antibodies, and precipitates were blotted with anti-EGF receptor. Lower panel, lysates were blotted with anti-HA to detect Kek1 expression. ***b***, Inhibition of Erk1/2 activation. Lysates from treated cells were blotted with antibodies specific for phosphorylated Erk1 and Erk2 (upper panel) and re-probed with antibodies that recognize the total Erk2 population.

**Fig. 3** Mechanism of EGF receptor inhibition by Kek1. ***a***, Inhibition of EGF binding and EGF-stimulated receptor tyrosine phosphorylation by Kek1. Sf9 insect cells were infected with baculoviruses encoding either myc-Kek1 or EGF receptor, or co-infected with both viruses. Cells were treated without or with 30 nM EGF as indicated. For the [$^{125}$I]EGF crosslinking experiment (lower panel) trace levels (0.1 nM) of iodinated growth factor and 1 mM $BS^3$ crosslinker were added to all samples at the time of EGF addition. Lysates from cells were immunoprecipitated with antibodies to either myc epitope or to EGF receptor. Precipitates were exposed to autoradiography (lower panel), or were blotted with antibodies to phosphotyrosine (upper panel) or EGF receptor (middle panel). ***b***, Co-localization of Kek1 and EGF receptor at the cell surface. Sf9 insect cells were infected at a low multiplicity of infection with baculoviruses encoding myc-Kek1 and human EGF receptor. Cells were fixed and stained with both rabbit anti-EGF receptor (left panel) and mouse anti-myc epitope (middle panel). Images were merged to show co-localization (right panel).

**Fig. 4** Inhibition of cellular growth properties by Kek1. ***a***, Inhibition of anchorage-dependent cell growth. The growth rate of cells stably transfected with vector alone (v.o.) or cells transfected with epitope-tagged Kek1 were compared for MDA-MB-468 human mammary tumor cells, and NF-639, IJ9921 and AC-816 mouse mammary tumor cells. Growth rates of HEK293 cells treated without and with Kek1 induction by PonA were also compared. Experiments were carried out in triplicate and repeated at least three times.

***b***, Inhibition of tumorigenic growth properties by Kek1. The growth of cells in soft agar or as tumors in nude mice was compared. Plotted is the extent inhibition by Kek1 transfectants relative to vector alone transfectants. Error bars represent SE of 3-6 determinations. Experiments were repeated at least three times.

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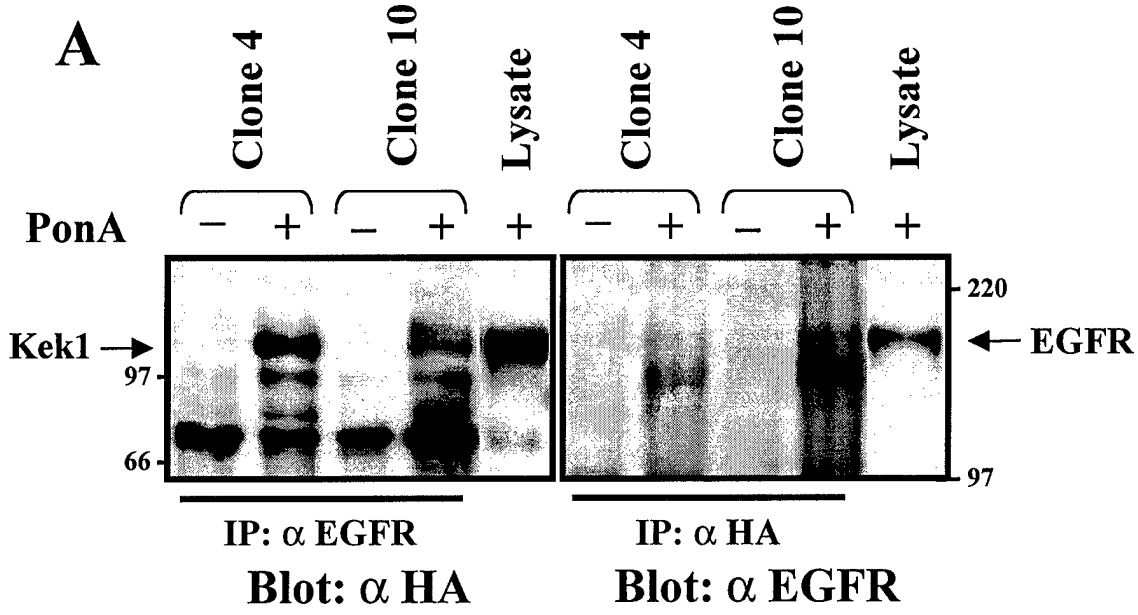
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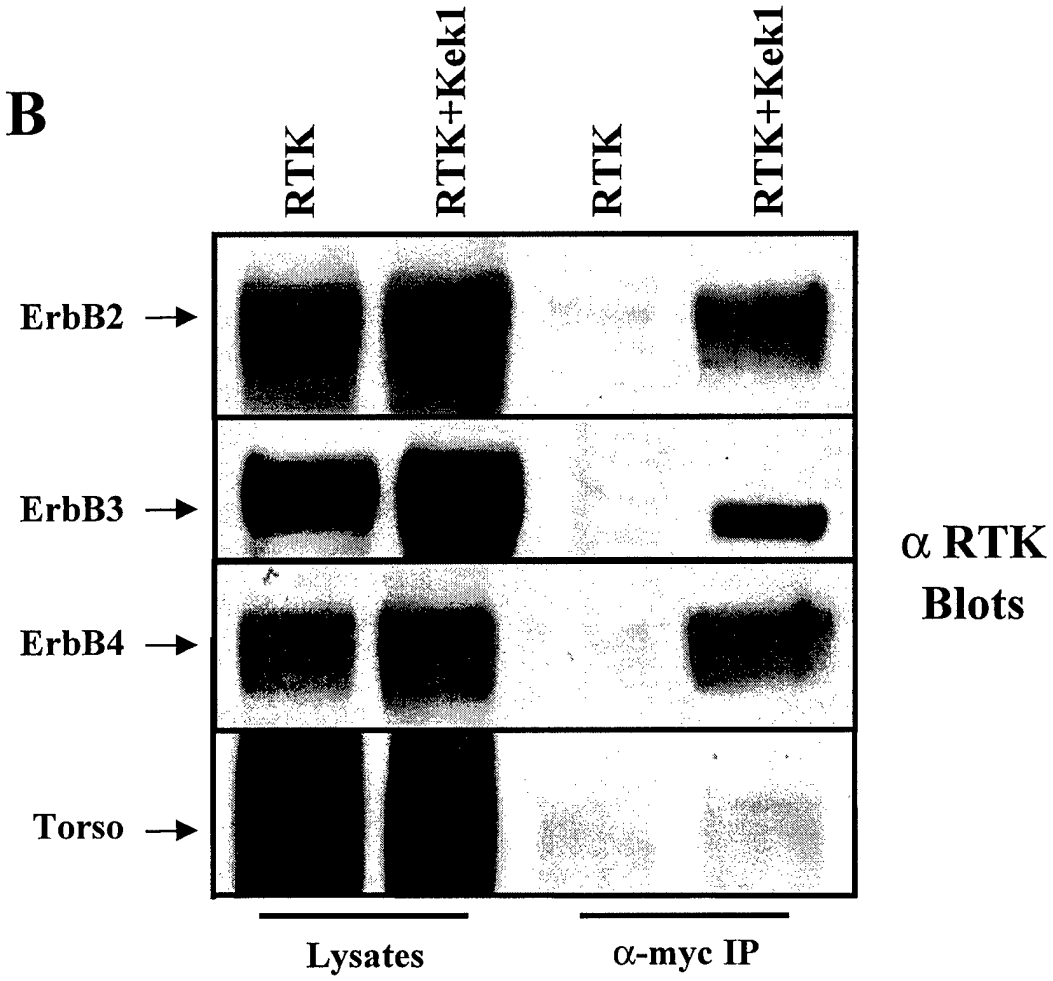
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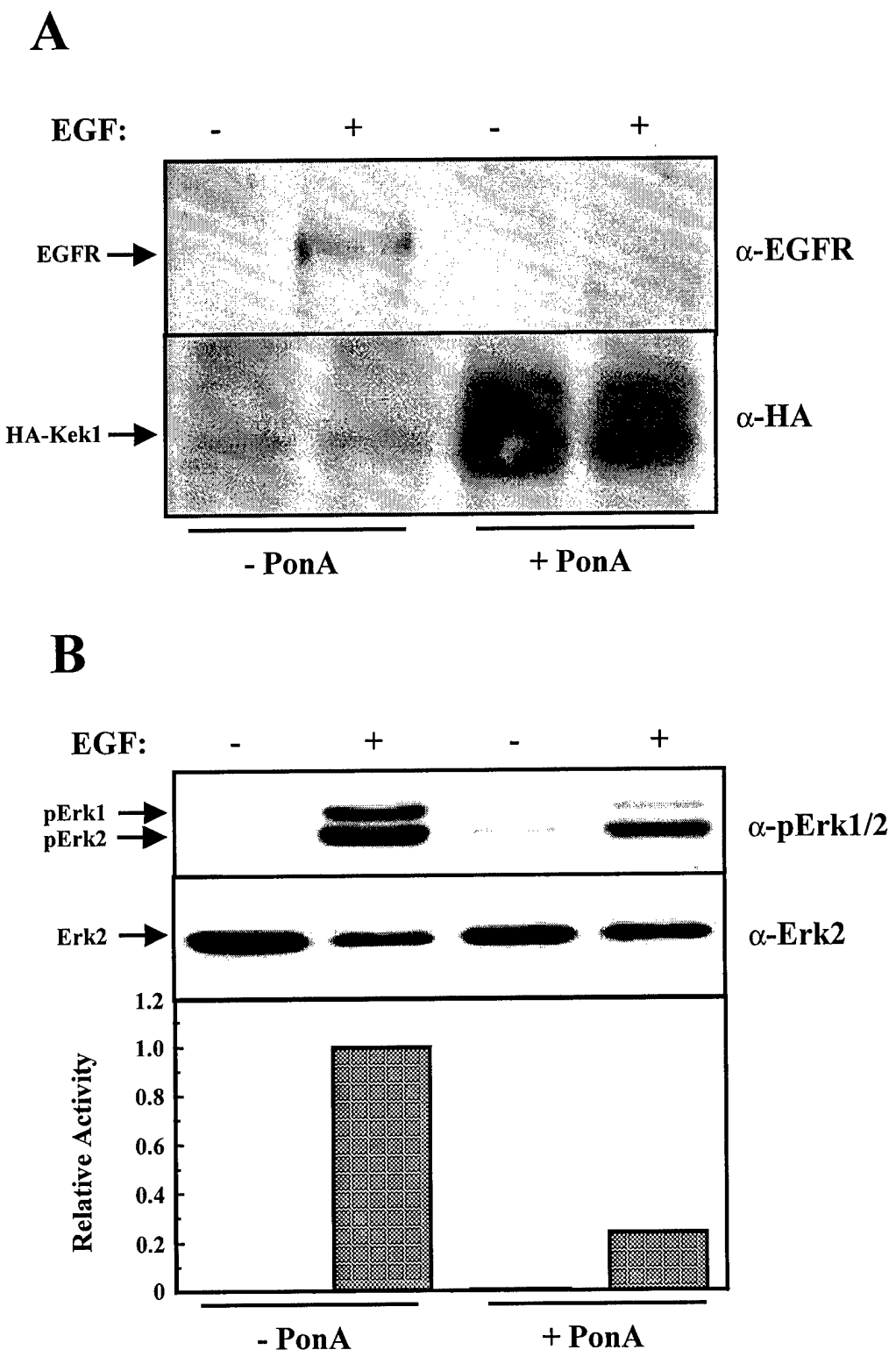
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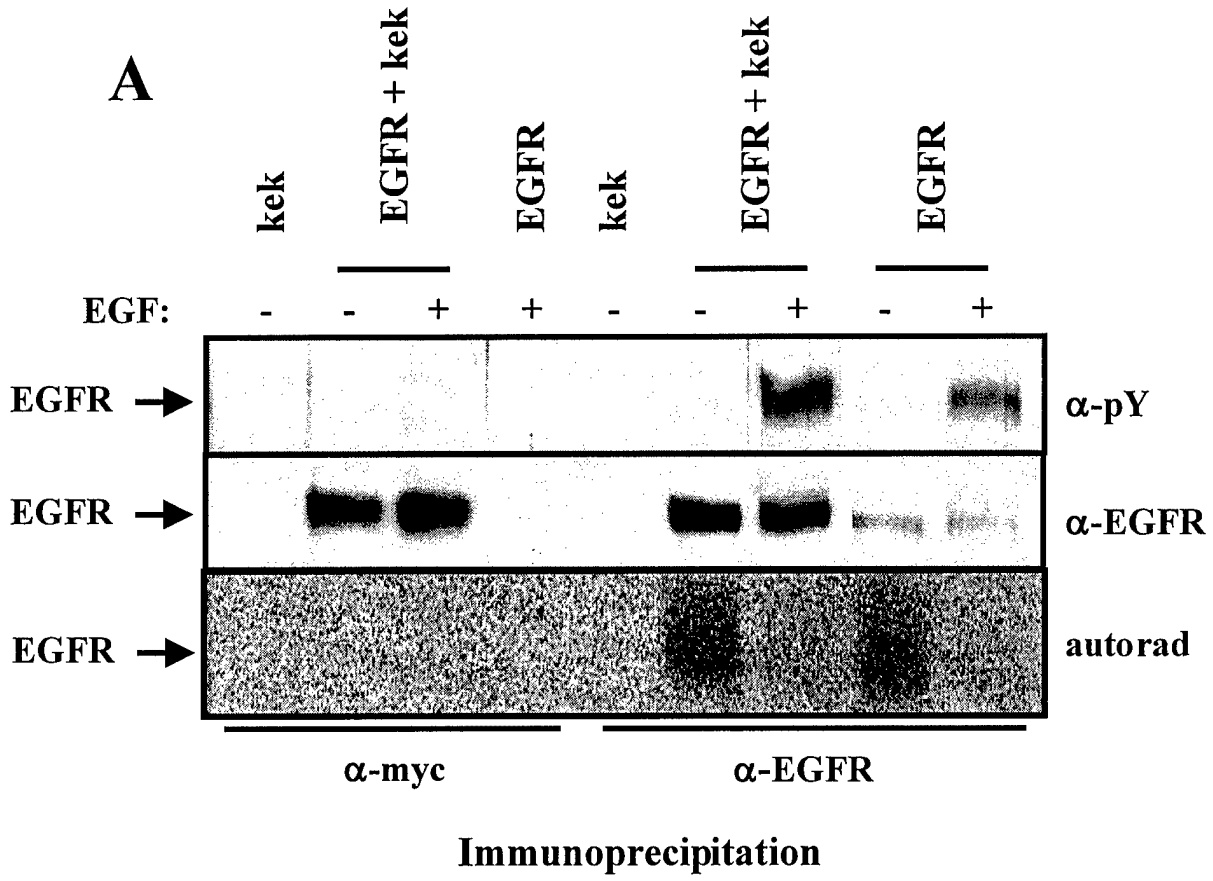
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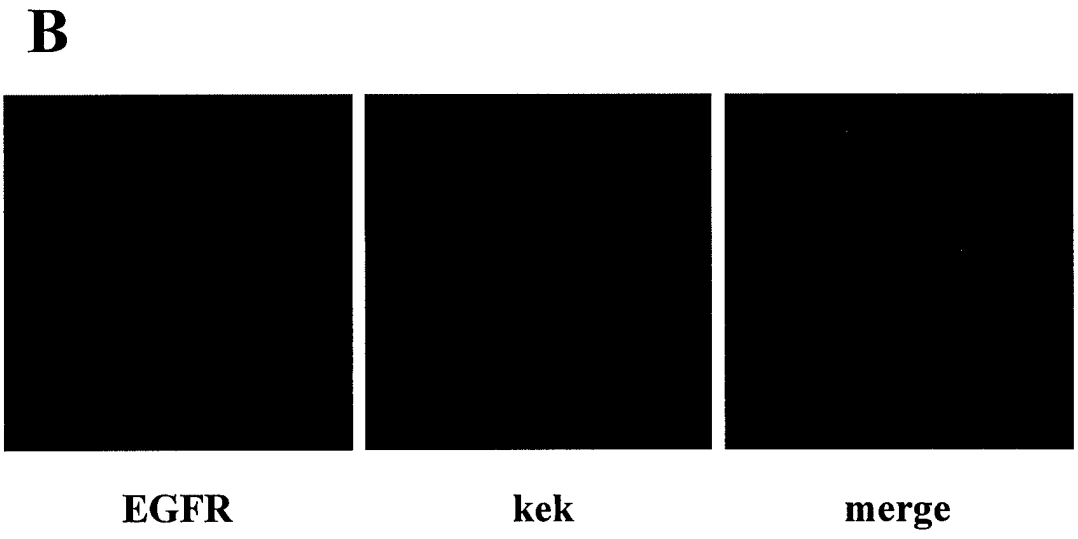
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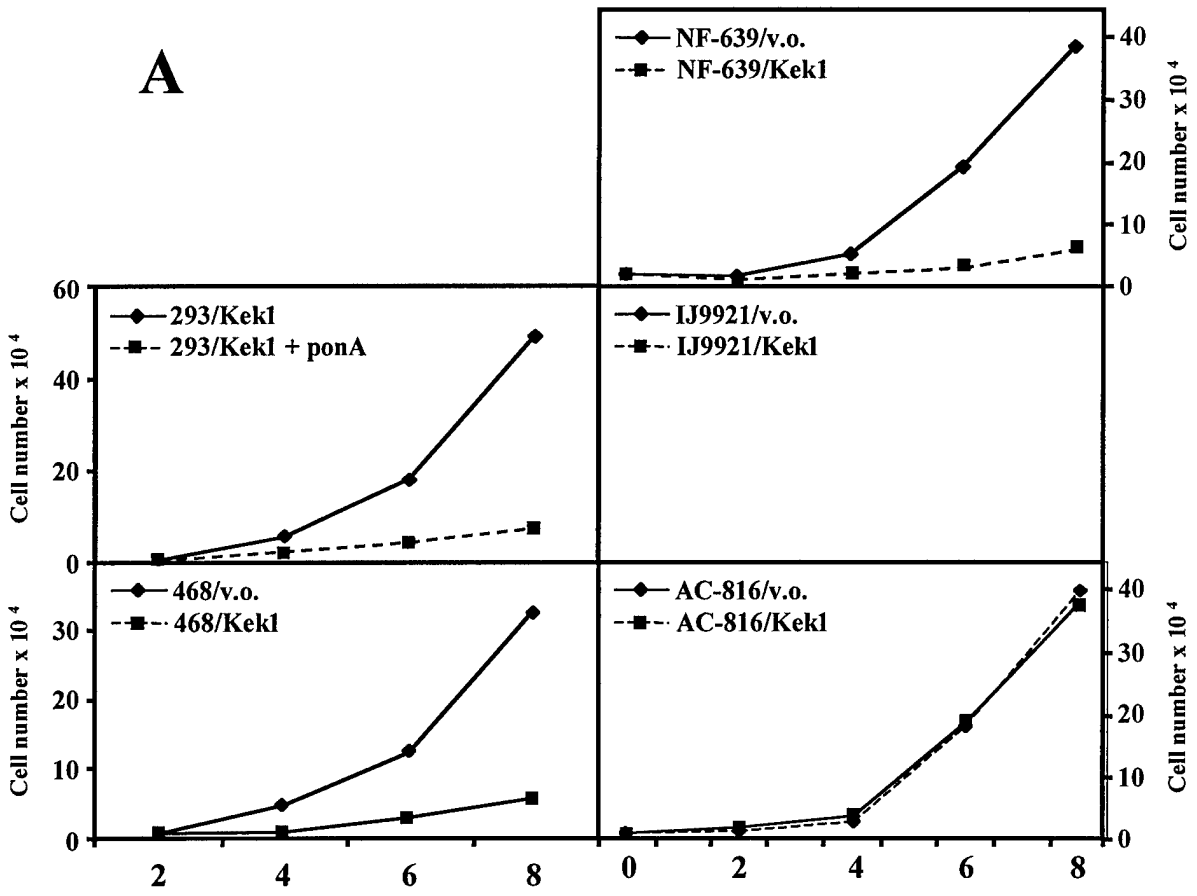
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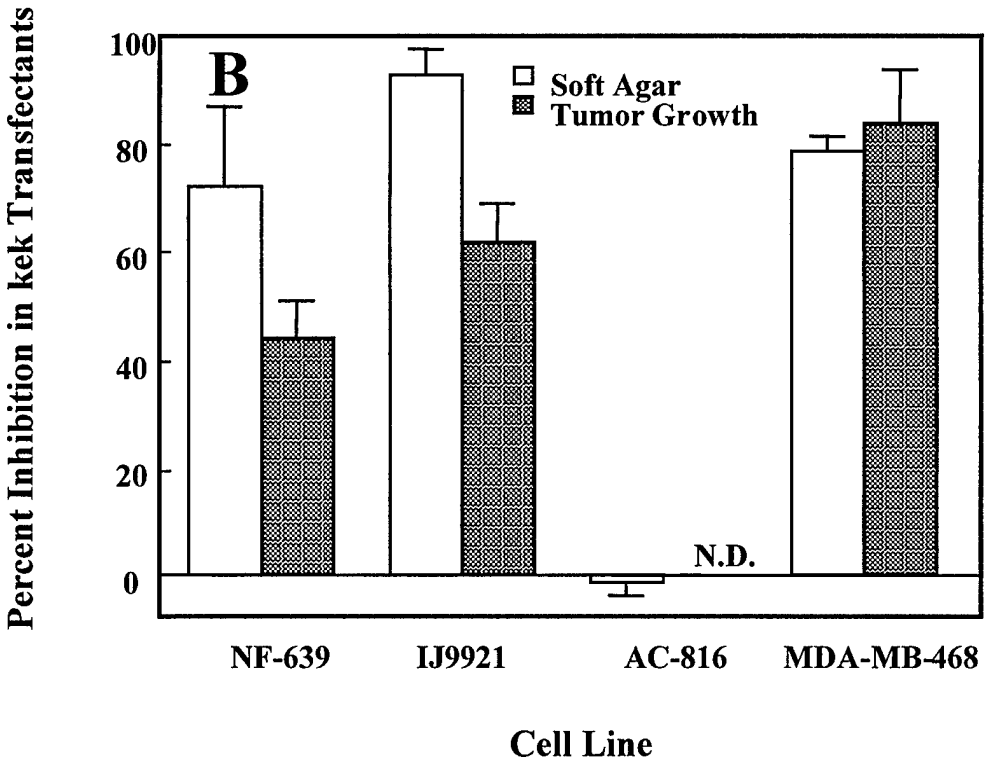
60


B
Soft Agar
Tumor Growth
Percent Inhibition in kek Transfectants
100
80
60
40
20
0
N.D.
NF-639
IJ9921
AC-816
MDA-MB-468
Cell Line