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TITLE: The Role of p90rsk in Breast Cancer Cell Survival from
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13. ABSTRACT (Maximum 200 Words)
Evidence suggests that sensitivity to chemotherapy is largely due to a functional apoptotic pathway. Thus, a better understanding of the signal transduction pathways that lead to rescue from apoptosis might lead to improved modalities of treatment for unresponsive cancer types. The focus of this proposal was to elucidate the role of p90rsk in antagonizing apoptosis in breast cancer cells. P90rsk is a serine-threonine protein kinase in the Ras-Raf-ERK (extracellular signal-regulated kinase, also known as mitogen-activated protein kinase or MAP kinase) cascade that lies immediately downstream of ERK. Although the Ras pathway and ERKs have been the focus of much research in the cancer field, less is known about the role of p90rsk. We hypothesize that p90rsk may be particularly relevant to breast cancer cell survival because evidence suggests it can not only directly phosphorylate and activate the estrogen receptor but also has the potential to antagonize apoptosis through neutralizing BAD, a proapoptotic member of he Bcl family of proteins. In addition, new evidence suggests to its activation by PDK1 (3-phosphatidyl inositol 3, 4, 5 phosphate dependent protein kinase) in addition to ERK1/2 activation. We proposed to study p90rsk since it may provide a new target in breast cancer therapy.

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Introduction

Evidence suggests that sensitivity to chemotherapy is largely due to a functional apoptotic pathway. Thus, a better understanding of the signal transduction pathways that lead to rescue from apoptosis might lead to improved modalities of treatment for unresponsive cancer types. The focus of this proposal was to elucidate the role of p90^{rsk} in antagonizing apoptosis in breast cancer cells. P90^{rsk} is a serine-threonine protein kinase in the Ras-Raf-ERK (extracellular signal-regulated kinase, also known as mitogen-activated protein kinase or MAP kinase) cascade that lies immediately downstream of ERK1/2. Although the Ras pathway and ERKs have been the focus of much research in the cancer field, less is known about the role of p90^{rsk}. We hypothesize that p90^{rsk} may be particularly relevant to breast cancer cell survival because evidence suggests it can not only directly phosphorylate and activate the estrogen receptor but also has the potential to antagonize apoptosis through neutralizing BAD, a proapoptotic member of the Bcl family of proteins. In addition, new evidence suggests to its activation by PDK1 (3-phosphatidyl inositol 3,4,5 phosphate dependent protein kinase) in addition to ERK1/2 activation. We proposed to study p90^{rsk} since it may provide a new target in breast cancer therapy.

Specific Aims

1.   What is the contribution of the PDK1 pathway to activation of p90^{rsk1} in breast cancer cells?
        To date, p90^{rsk} activation has been assumed to be a result of phosphorylation by ERK. Recent evidence suggests otherwise—that in fact, PDK1 may in fact also play a major role in activating p90^{rsk}. These observations are important ones and need to be confirmed in breast cancer cells.

2.   Can p90^{rsk1} contribute to the antiapoptotic activity of growth factors such as IGF-1 in rescue of breast cancer cells from apoptosis following exposure to chemotherapeutic agents?
        A constitutively active p90^{rsk1} allele has been obtained enabling us to ask whether an active p90^{rsk1} alone in the absence of other activated kinases can contribute to escape from apoptosis. We will ask whether transfection of p90^{rsk1} (wild-type, WT; constitutively active, CA; or kinase-dead, DK) into breast cancer cells results in a change in the apoptotic response to chemotherapeutic agents.

3.   Characterization of phosphorylation of potential p90^{rsk1} targets, BAD, ER and IkBa.
        Kinases act by phosphorylating their substrates. In the first part of this Specific Aim, we propose to study the effect of transfection of p90^{rsk1} alleles on overexpression of the proapoptotic protein BAD. We will also determine whether immunoprecipitated p90^{rsk1} alleles can phosphorylate BAD in vitro. ER and IkBa have been reported to be substrates for p90^{rsk1}. We will ask whether constitutively active p90^{rsk1} results in hyperactivation of transcription mediated by ER and NF-kB (which is inhibited by IkBa).

Overall, the purpose of these experiments is to integrate new information on p90^{rsk1} that has recently become known to determine whether p90^{rsk1} is a major player in escape from apoptosis in breast cancer cell lines.

Research Accomplishments

The following is a synopsis of the research accomplishments achieved during the funding period of this grant. They are categorized by the tasks approved in the Statement of Work.

Task 1: Subcloning of p90rsk into mammalian expression vectors.
Task 2: Determine their expression upon transfection by Western analysis.

P90^{rsk} has been cloned into a retrovirus which was used to infect cells with extremely high efficiency. Retroviruses are enveloped and have RNA genomes that are typically approximately10 kb in size . Upon infection, the RNA genome is converted to double stranded DNA by the virally-encoded reverse transcriptase. The proviral cDNA integrates into the genome of the host through the activity of the virally-encoded integrase. Retroviral vectors that have all of the retrovirus genes removed and replaced by the gene of interest (in this case) are efficiently packaged either in cell lines that stably express the gag, pol, and env proteins or through transient transfection with plasmids that encode the gag, pol, and env proteins and the provirus of the vector. Env is a viral transmembrane protein that binds to the viral receptor on the host cell to initiate cellular uptake of the virus.
The genes were cloned into an HIV vector (Zufferey, et al., 1998) that encodes resistance to the antibiotic G418. A parallel construct with empty vector (*i.e.* p90rsk allele removed) was made as a control. Using transient transfection of 293T cells (Pear, et al., 1993), virus vectors can be generated. The vectors were pseudotyped using VSV-G and concentrated by centrifugation. The concentrated virus was titered using a colony forming assay in 293 cells and then used to transduce various an immortalized human mammary epithelial cell line and the MCF-7 breast carcinoma cell line. The cells were put under selection with G418 and cloned. Expression of p90rsk was examined and cellular growth characteristics were also examined.
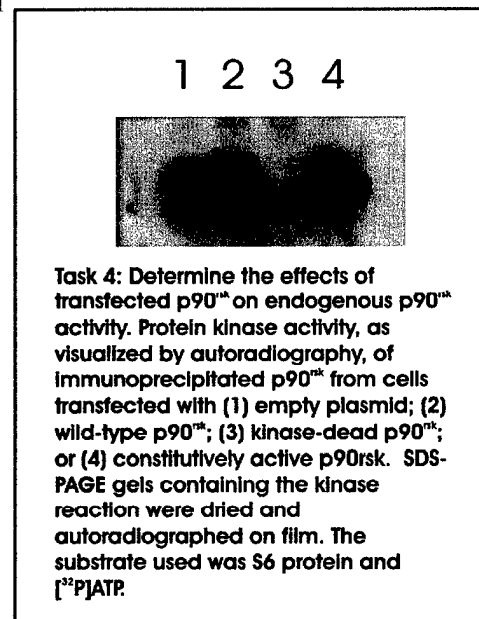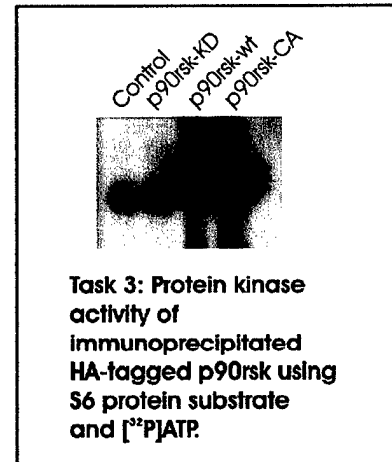

Task 3: Protein kinase activity of immunoprecipitated HA-tagged p90rsk using S6 protein substrate and [^{32}P]ATP.

Task 4: Determine the effects of transfected p90^{rsk} on endogenous p90^{rsk} activity. Protein kinase activity, as visualized by autoradiography, of immunoprecipitated p90^{rsk} from cells transfected with (1) empty plasmid; (2) wild-type p90^{rsk}; (3) kinase-dead p90^{rsk}; or (4) constitutively active p90rsk. SDS-PAGE gels containing the kinase reaction were dried and autoradiographed on film. The substrate used was S6 protein and [^{32}P]ATP.

     p90^{rsk} wt: wild-type allele
     p90^{rsk} CA: constitutively active allele
     p90^{rsk} DK: kinase inactivated allele

Task 3. Determine the kinase activity of the expressed p90^{rsk} alleles by immunoprecipitation/kinase assays using the hemagluttinin-tag (HA) epitope directed antibody. As shown in the accompanying figure, the behavior of the transduced kinases appears to be as expected with the kinase-dead allele having as much activity as the vector transduced and immunoprecipitated with HA-tag. The small amount of activity associated with lanes 1 and 2 can be attributed to background. By comparison, the two active p90^{rsk} alleles have approximately 1200-fold more activity as quantitated by scintillation counting.

Task 4. Determine the effects of transfected p90^{rsk1} on endogenous p90^{rsk1}.

We conclude from these experiments that the active transduced alleles (e.g. WT and CA) result in active protein kinase being immunoprecipitated by anti-p90^{rsk} antiserum. However, the KD allele results in significantly less kinase activity likely due to a dominant-negative effect. This is desirable as far as our experiments are concerned as it may be effective in silencing the endogenous p90^{rsk} activity.

Task 5. Determine the effect of heterologously expressed p90^{rsk} upon the activity of upstream kinases such as ERK and MEK as well as kinases of the PI3-kinase pathway. We performed experiments to test whether p42/44^{Erk} stimulation is abrogated or stimulated by p90^{rsk} alleles by stimulating cells transduced with the three p90^{rsk} alleles with EGF (100ng/ml) following a 2 hour serum starvation protocol. The cells were harvested and SDS-PAGE was performed on the extracts, followed by western analysis using anti-phospho-p42/44^{Erk}. The results, shown in the accompanying figure, suggest (1) that there is no 'retrograde' signaling since phosphorylation (and thereby activation) of p42/44^{Erk} does not appear to be occuring under basal conditions (compare lane 1 to lanes 2-4); and that (2) p90^{rsk} alleles including the kinase-dead allele do not interefere with p42/44^{Erk} signaling. Since MEK is upstream of p42/44^{Erk}, it would stand to reason that MEK is also not affected by p90^{rsk}.

Task 5: Determine the effects of p90rsk alleles on upstream kinases. Cells transduced with empty vector (lanes 1 and 5), kinase-dead p90rsk (2,6), wild-type p90rsk (3,7); or constitutively active p90rsk (4,8) unstimulated (lanes1-4) or one hour after being stimulated with EGF (lanes 5-8) were harvested and analyzed for phospho p42/p44 proteins by western blotting. Levels of unphosphorylated p42/p44 were unchanged (data not shown).

Task 6: Determine the contribution of the PI3-kinase pathway v.s. the Ras-Raf-ERK pathway on p90^{rsk1} activation.

Below, in Figure 1, I demonstrate the activities of cell lines generated by transduction of the p90^{rsk1} alleles, wild-type (WT), constitutively active (CA), and kinase-dead (KD).

In Figure 2 (next page), I show that the inhibition of PI3-kinase in MCF-7 cells is significantly inhibited by wortmannin, an inhibitor of PI3-kinase.
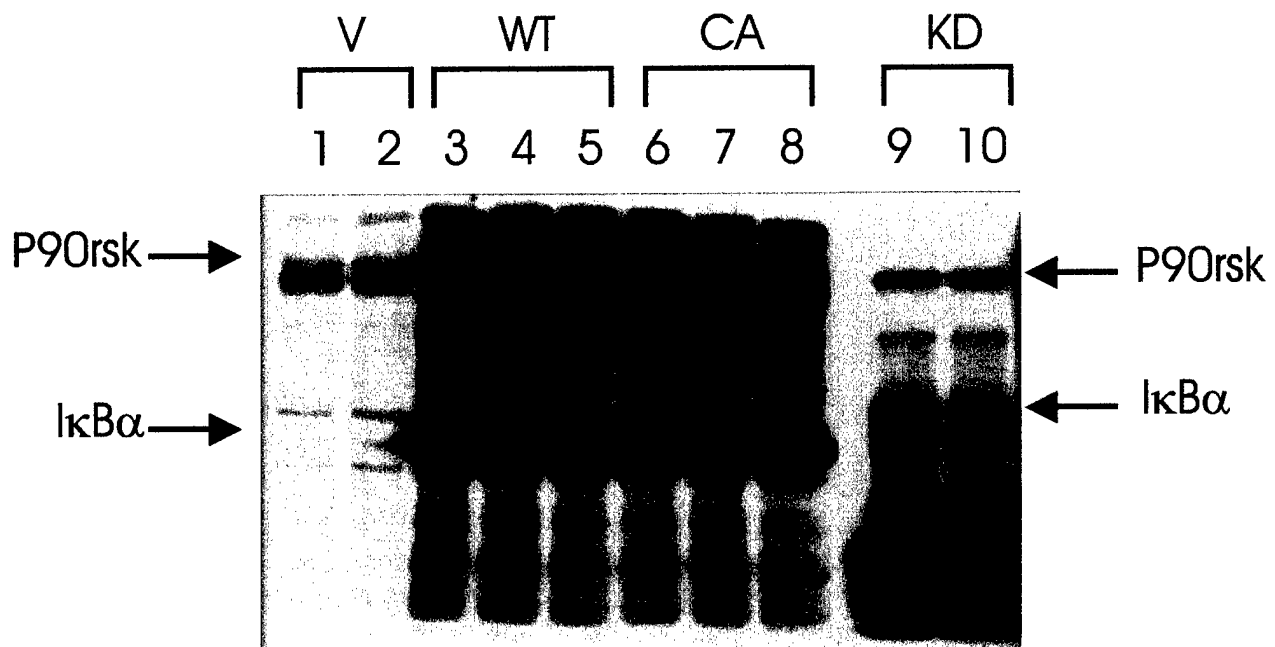

Figure 1: <u>Immunoprecipitation-Linked Protein Kinase Assays of Cell Lines Expressing Alleles of p90rsk.</u> Cells expressing vector (V), wild-type (WT), constitutively active (CA) and kinase-dead (KD) alleles of p90^{rsk} were immunoprecipitated with anti-HA antibody. The extracts analyzed were from: lane 1, HMV-4; 2, MCV-5; 3, HMWT-12; 4, MCWT-13; 5, MCWT-14; 6, HMCA-15; 7, HMCA-16; 8, MCCA-18; 9, HMKD-7; 10, MCKD-9. Lanes 1-8 are films of gels exposed for 1 hour. Lanes 9 & 10 were taken from a separate gel which was exposed for 18 hrs.
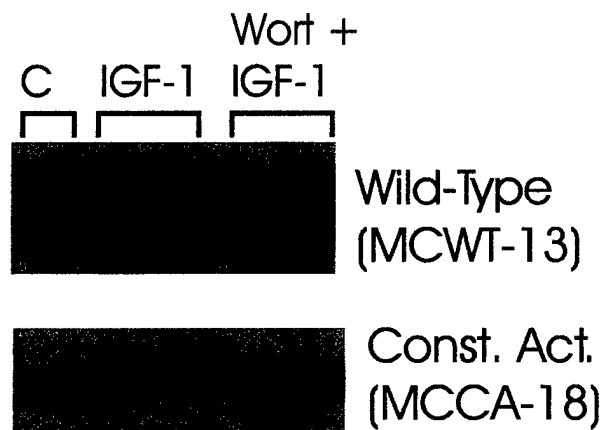
Wort +
C   IGF-1 IGF-1

Wild-Type
(MCWT-13)

Const. Act.
(MCCA-18)

Figure 2: Immunoprecipitation-linked protein
kinase activity of MCF-7 cell carrying WT and CA
alleles of p90rsk treated with IGF-1 or IGF-1 and
wortmannin. Control cells were starved overnight
but not treated with either IGF-1 or wortmannin.
Cells were either treated with 10 ng/ml of IGF-1 or
IGF-1 and 200 nM wortmannin.

Task 7 & 8: Generate a dose-response curve for adriamycin in breast
cancer cells and determination of apoptotic index.
Based on assays as represented below, we determined an optimal working
concentration for doxorubicin (adriamycin). The expression of constitutively
active or wild-type p90rsk significantly inhibited apoptosis.

Chart 1: Constitutively Active p90Rsk Protects MCF-7 Cells
From Adriamycin-Induced Apoptosis
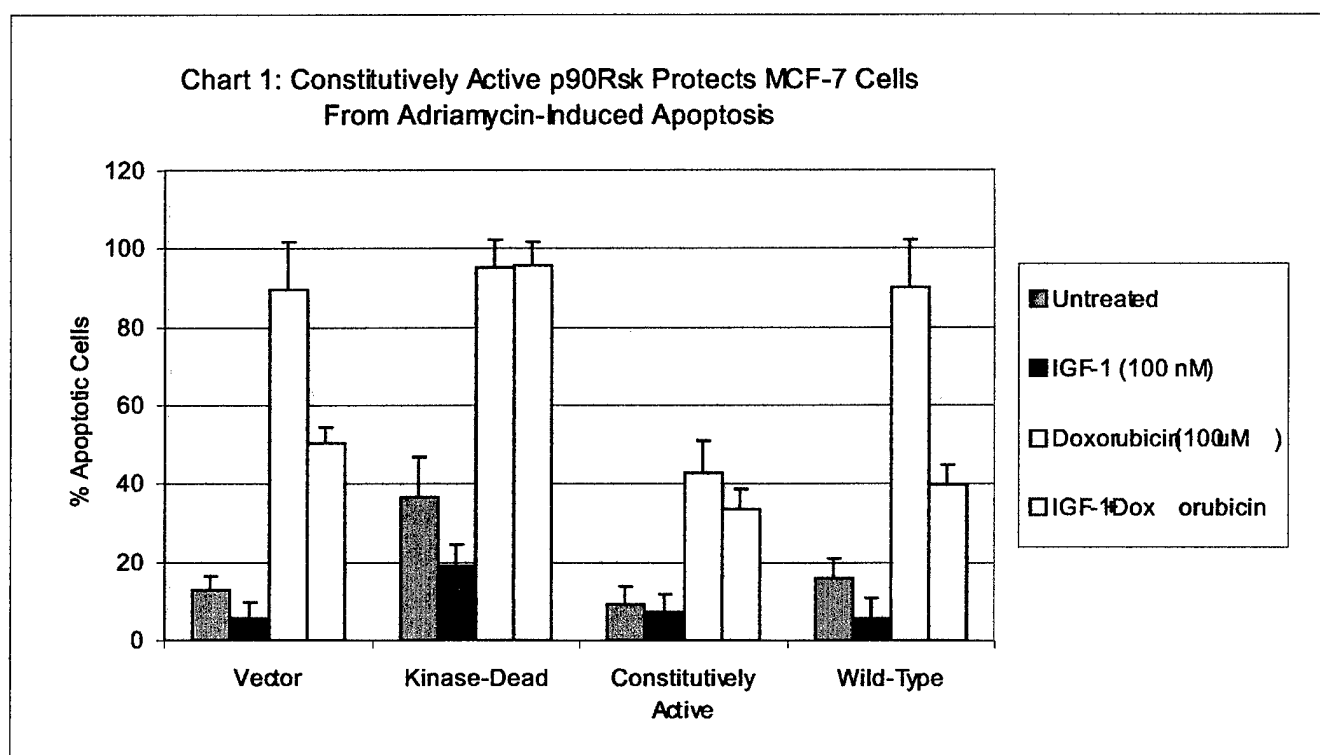
Task 9: Determine whether p90^{rsk1} phosphorylates ER and BAD.

In Figure 3, below, an *in vitro* immunoprecipitation-linked phosphorylation assay was performed using anti-p90rsk using GST-BAD and H1 as substrates. IGF-1(100ng/ml) was used to stimulate p90rsk activity (lanes 3,4,6). Wortmannin (10 uM) was used to inhibit PI3-kinase (lanes 2,4). Cells carrying the kinase-dead construct was assayed for BAD phosphorylating activity in lanes 5 and 6.

Figure 3: *In vitro* phosphorylation of GST-BAD by immunoprecipitated endogenous p90^{rsk}.

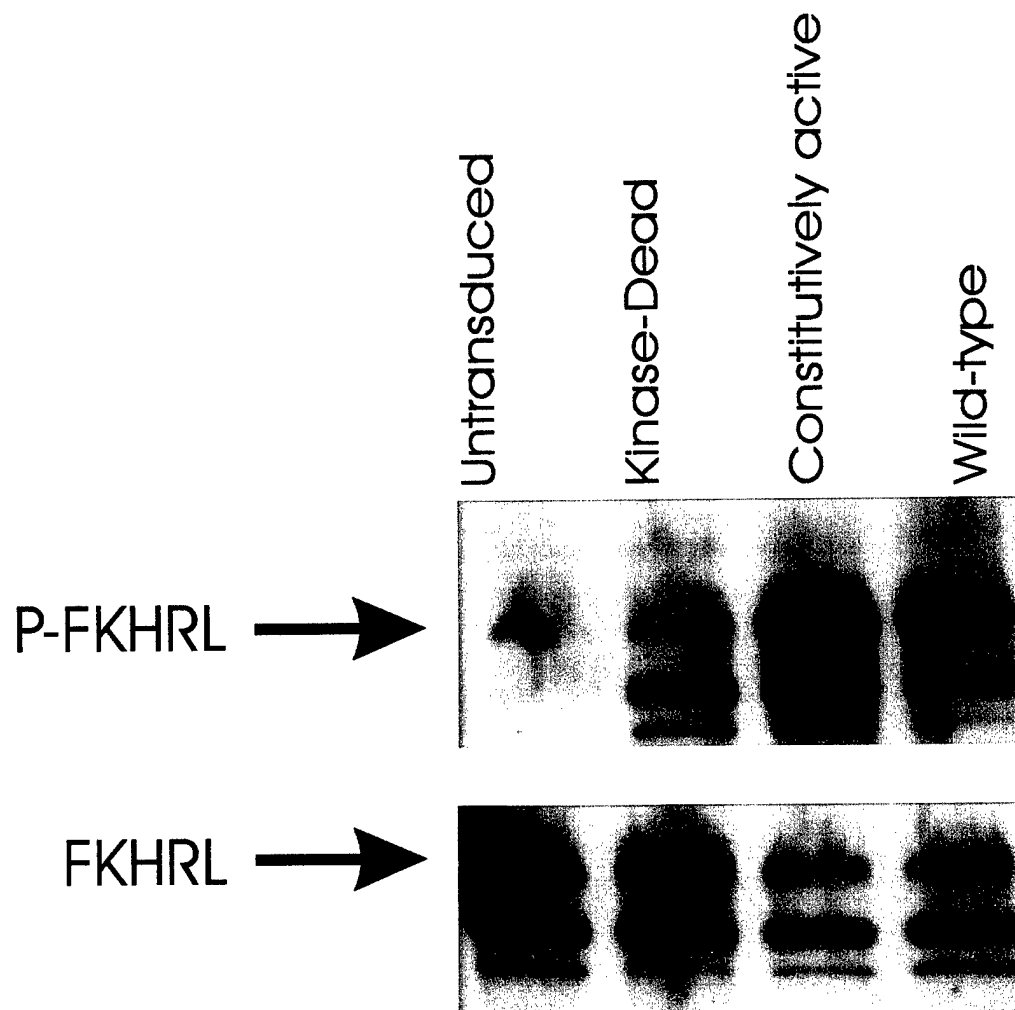|	1	2	3	4	5	6
IGF-1	−	−	+	+	−	+
Wortmannin	−	+	−	+	−	−
KD-p90rsk	−	−	−	−	+	+

GST-BAD →

H1 →
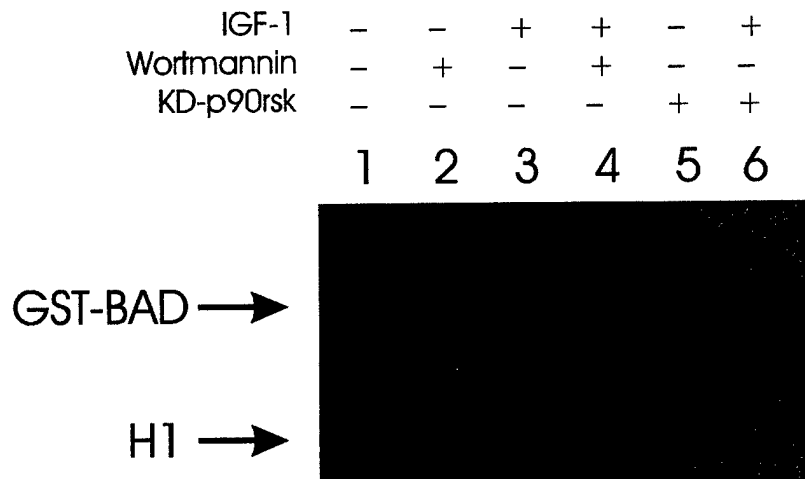
Figure 4: Forkhead phosphorylation can be regulated by p90rsk. MCF-7 cells were treated with 100 nM IGF-1 for 30 min and harvested for westerns. Top panel, anit-phospho-FKHRL1 antibody treatment of vector, kinase-dead, constitutively active or wild-type p90rsk -transduced cells. Bottom panel, the same extracts probed for total FKHRL.
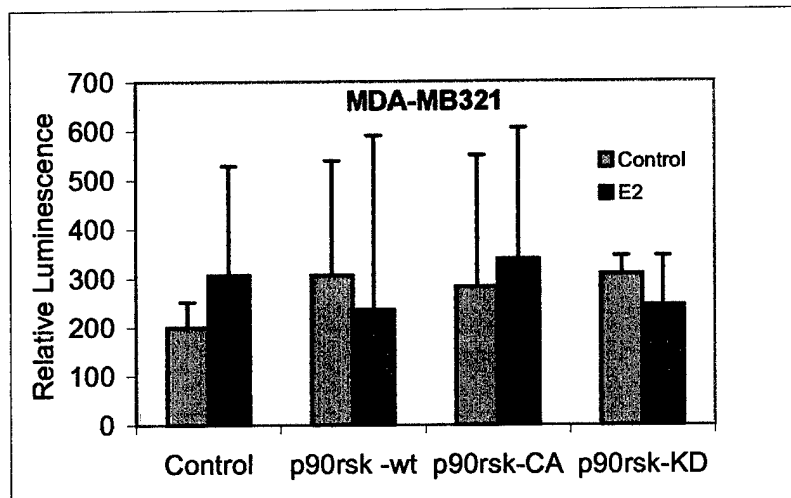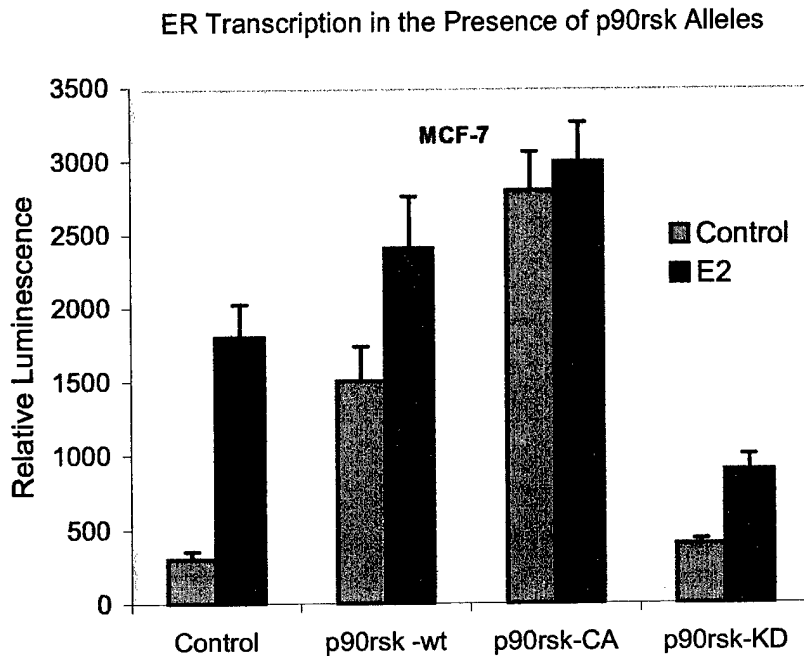
Chart 2: An estrogen response element (ERE) driven luciferase construct was supertransfected into MCF-7 cells (ER-positive) or MDA-MB231 cells (ER-negative) that were previously selected to express wild-type (wt), constitutively active(CA) or kinase-dead (KD) p90rsk. Cells were either treated (E2) or not (Control) and the resulting luminescence quantified.

Task 10. Determine whether cotransfection of p90^{rsk1} and BAD rescues apoptosis mediated by BAD.
The results obtained in this section were supportive of the hypothesis that BAD is phosphorylated and inactivated by p90^{rsk} but not conclusive. The experiments performed in this task were as follows:
Cells (MCF-7) were transduced with the three p90^{rsk} alleles, wild-type (wt), constitutively active (CA), and kinase-dead (KD) and a control containing no p90^{rsk} allele. Following selection and verification of expression of the above alleles both on an RNA and protein level (except for the control containing no p90^{rsk}), cells were transfected with wild-type BAD or the BAD allele lacking phosphorylatable sites at S112 and S136 (S112A/S136A). Transfectants for wild-type BAD were recovered only from the cells initially transduced with the constitutively active allele of p90^{rsk}. Under no circumstance were we able to recover any transfectants from the S112A/S136A BAD mutant. The transfections were repeated two more times (three times in all). In one case, a colony of sickly, slow-growing cells were recovered using wild-type BAD transfected into the wild-type p90^{rsk} expressing cells but were found to express little to no detectable levels of BAD protein. By RT-PCR, there was some detectable BAD mRNA however. These results are consistent with the known pro-apoptotic activity of BAD and is supportive of our hypothesis that p90^{rsk} can inactivate BAD but the inability to retrieve transformants was not the endpoint we were seeking as it prevents us from analyzing the results.

Task 11. Determine whether constitutively active p90^{rsk1} activates ER-mediated transcription.
We used a ERE-thymidine kinase/luciferase construct to transfect both ER-positive (MCF-7) and ER-negative (MDA-MB231) breast cancer cell lines selected for expression of the three p90^{rsk} alleles (and control). The results are shown in Chart 2.
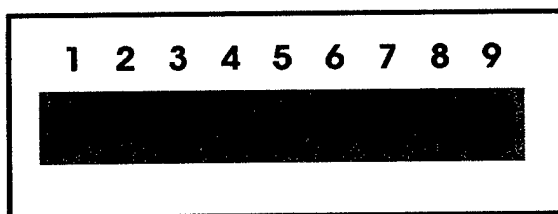
Figure 5: The effect of p90rsk alleles on NF-kB activity. EMSA showing binding of nuclear proteins to NF-kb consensus double-stranded oligonucleotide from extracts of cells that were treated with EGF (lanes 6-9) or untreated (lanes 1-5). Lane 1, untransduced; lanes 2 and 6, empty plasmid; 3 and 7, wild-type p90rsk; 4 and 8, constitutively active p90rsk; 5 and 9; kinase-dead p90rsk.

Task 12. Determine whether constitutively active p90^{rsk1} activates NF-κB mediated transcription. Experiments were performed as depicted in Figure 5 to ascertain the effect of the three Rsk alleles on NF-κB activity. Activity was measured by EMSA using double-stranded oligonucleotides encoding the NF-kB consensus binding sequence as previously publsihed (Sen, 1986) and nuclear extracts from cells transduced and treated as indicated. Treatment with EGF was included to activate the MAP kinase pathway (lanes 6 through 9). The results are strongly suggestive of no role for p90rsk in NF-κB-mediated transcription in these cells (MCF-7).

References Cited

Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46:705-16. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes Multiple nuclear factors interact with the immunoglobulin enhancer sequences. J Virol 72:9873-80.
Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, D. Trono, L. M. Staudt, H. Singh, R. Sen, T. Wirth, P. A. Sharp, and D. Baltimore. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery.

Reportable Outcomes

- p90^{rsk} protects cells against adriamycin-mediated apoptosis.
- p90^{rsk} phosphorylates BAD.
- p90^{rsk} phosphorylates FKHRL1.
- ER-mediated transcription is potentiated by active p90^{rsk}.
- p90^{rsk} does not activate NF-κB activity as measured by DNA-binding and EMSA analysis.

Conclusions

The investigations reported in this final report focuses on the role of p90^{rsk}, a serine-threonine protein kinase which lies immediately downstream of the classical growth factor activated MAP kinase (p42/44 ^{Erk}). While much research has focused on p42/44^{Erk}, somewhat less is known about the functional significance of p90^{rsk} activity. Clearly, p90^{rsk} must have substrates and functions distinct from that of p42/44^{Erk} – the phosphorylation site consensus sequence for p90^{rsk} is Arg-X-X-Ser/Thr which is different from p42/44^{Erk} (Ser-Pro and Thr-Pro) and the activation of p90^{rsk} requires PDK1 phosphorylation in addition to p42/44^{Erk}. PDK1 is also involved in activating Akt, an anitapoptotic protein kinase. It is interesting to note that p90^{rsk} consensus sites are in fact a subsequence of Akt and, based on this observation, we hypothesized a role for p90^{rsk} in the phosphorylation of and inactivation of two proapoptotic proteins substrates, BAD and FKHRL1. We also hypothesized that p90^{rsk} may potentiate the transcriptional activity of ER.

The results of our investigations point toward an anti-apoptotic role for p90^{rsk}, possibly through its phosphorylation of BAD and FKHRL. The evidence for bad is circumstantial as it was difficult to express BAD in our cell lines due, most likely, to its proapoptotic activity. To circumvent this, it will be necessary to make an inducible form of BAD whose expression is suppressed under basal conditions (*e.g.* tet-inducible system). Investigations into the role of p90^{rsk} in FKHRL1 are ongoing.

To summarize, p90^{rsk} could be a useful target for therapy as compounds that specifically inhibit protein kinases come to the forefront of cancer chemotherapy. Because of its involvement in antagonizing apoptosis and its ability to phosphorylate those substrates that are also substrates of Akt, it may be an important means of escaping apoptosis for some cells. It would be important to consider this aspect of p90^{rsk} in the development of agents that block Akt.

We are expanding on aspects of these investigations for publications purposes. Once they are accepted, copies will be sent to CDMRP.