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TITLE: Tumor Suppressor Activity of the EphB2 Receptor in Prostate Cancer

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14. ABSTRACT Mutations have been recently identified in the EphB2 receptor gene in prostate cancer suggesting that EphB2, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. Consistent with a tumor suppressor activity, we found that EphB2 is more highly expressed in non-transformed BPH-1 prostate epithelial cells than in several prostate cancer cell lines. Furthermore, EphB2 expression was rapidly lost in stably transfected DU145 prostate cancer cells, suggesting that EphB2 inhibits cell growth and/or survival. We plan to further examine the effects of EphB2 signaling on the behavior of cancer cells in tissue culture and elucidate the signaling pathways involved. We have also found that other Eph receptors that we detected in prostate cancer cells have tumor suppressor effects similar to EphB2. The information obtained from these studies will help guide the design of appropriate treatment strategies and determine if prostate cancers should be screened for Eph receptor and ephrin ligand expression for prognostic purposes.

15. SUBJECT TERMS Eph receptor, ephrin, tumor suppressor, signal transduction
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INTRODUCTION

Loss of function of tumor suppressor genes and increased function of tumor-promoting genes are critical steps in the development and progression of cancer. It is therefore important to identify these genes and understand how they affect cancer progression in order to develop new treatments. Previous work provided intriguing clues suggesting that the EphB2 receptor, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. In particular, inactivating mutations in the EphB2 gene were identified in clinical prostate cancer samples, and not in normal tissue, and forced expression of EphB2 was shown to suppress the growth of cultured prostate cancer cells that lack EphB2 expression (Huusko et al., 2004). Furthermore, a nonsense mutation in the EphB2 gene has been recently associated with prostate cancer risk in African American men with a positive family history (Kittles et al., 2006). Several mechanisms of EphB2 inactivation in colorectal tumors have also been recently reported, supporting the hypothesis that EphB2 functions as a tumor suppressor (Alazzouzi et al., 2005). However, the signaling mechanisms involved are completely unknown.

Our work with another Eph receptor in breast cancer supports that idea that activation of Eph receptor signaling pathways by their ligands, called ephrins, can inhibit the malignant properties of cancer cells (Noren et al., 2006). Our hypothesis is that signaling pathways activated by EphB2, and other Eph receptors, also negatively regulate the malignant properties of prostate cancer cells. Importantly, since submission of this application several reports have appeared in the literature highlighting the potentially critical role of different Eph receptors in the pathogenesis of prostate cancer (Lee et al., 2005; Xia et al., 2005; Fox and Kandpal, 2006). In addition, our recent work with EphB2 as part of this award has also supported the notion that reintroducing EphB2 in DU145 cells inhibits cell growth. We have now identified several potential Eph receptor-dependent tumor suppressor pathways in prostate cancer cells, which are described in this progress report.

BODY

Aim 1. Determine whether the growth-suppressing activity of EphB2 in prostate cancer cells depends on its activation by ephrin ligands

Task 1. Screen prostate cancer cell lines by immunoblotting and immunoprecipitation to determine expression and activation of selected Eph receptors and ephrins.

This was completed in the previous funding period. We have now compiled a table summarizing our expression data at the protein level as well as mRNA/protein data from the available literature (Table 1). We will continue to update this table as new information becomes available to guide the design of our experiments and help interpret the results obtained with regard to the activities of different Eph receptors in LNCaP, DU145 and PC3 prostate cancer cells.


Published data and results we obtained in the previous funding period indicate that forced expression of EphB2 in DU145 cells severely impairs their ability to grow, so that it is difficult to expand transfected cells for experimental analysis and to obtain stable clones (2006 Progress Report) (Huusko et al., 2004). Discussions with investigators working on methylation of tumor suppressor genes at the 2007 IMPaCT DOD Prostate Cancer Research Program Meeting in Atlanta reinforced the idea that it is very difficult to reintroduce tumor suppressor genes in cancer cells even when using inducible systems. This is because there is some basal expression of the transfected gene even when the cells are not induced. Our own recent work...
using a doxycycline-inducible system in breast cancer cells substantiates this. We found that the basal expression of ephrin-B2 in the absence of doxycycline was sufficient to cause the loss of the MDA-MB-435 breast cancer cells with high inducible ephrin-B2 expression (Fig. 1). In these cells, ephrin-B2 inhibits cell growth by activating the EphB4 receptor. Taken together, these results support the notion that transiently transfected EphB2 inhibits the growth of DU145 cells and that further information on the tumor suppressor role of EphB2 in prostate cancer can best be obtained using prostate cancer cells such as LNCaP and PC3, which endogenously express EphB2, as was proposed in Aim 2.

The other experiments proposed in this task, to examine how activation of other EphA and EphB receptors endogenously expressed in the DU145 cells (Table 1) affect cell growth, are in progress. We are using MTT assays to measure the effects of EphA receptor stimulation by ephrin-A1 Fc and EphB receptor stimulation by ephrin-B1 Fc on cell growth.

**Aim 2. Characterize the effects of EphB2 signaling pathways on prostate cancer cell survival, proliferation, migration and invasion**

**Task 7.** Confirm the involvement of downstream signaling pathways implicated by the inhibitor experiments of task 6. The focus in this task has shifted towards identification of possible tumor suppressor pathways regulated by Eph receptors in prostate cancer (see below).

**Task 8.** Examine the effects of EphB2 activation in prostate cancer cell lines where this receptor is present but not activated, if any are identified in the experiments in Aim 1.

**Task 9.** Examine the effects of ligand-mediated activation of other Eph receptors on DU145 cell survival, proliferation, migration, and/or invasion.

The effects of EphB2 forced expression in DU145 cells are consistent with tumor suppressor activities, and tumor suppressor activities in prostate cancer have been reported in the literature for other Eph receptors (Miao et al., 2001). Therefore, we proceeded to investigate possible tumor suppressor pathways downstream of EphB2 and other Eph receptors in LNCaP, DU145 and PC3 cells stimulated with ephrin ligands. We used ephrin-B1 to activate EphB2 and other EphB receptors expressed in the cells and ephrin-A1 to activate EphA receptors (Table 1). Future experiments will dissect the contribution of EphB2 to these pathways in LNCaP and PC3 cells, where EphB2 is endogenously expressed, by using an antagonistic peptide that we have identified and that selectively blocks activation of EphB2 by ephrin ligands (Koolpe et al., 2005). Analysis of the signaling pathways has produced very exciting and novel results. Therefore, we have focused our efforts on the characterization of the tumor suppressor pathways that we have identified downstream of Eph receptors in prostate cancer cells.

**Eph receptor-dependent inhibition of the Akt-TOR pathway**

The Akt-TOR pathway is of major importance for cell growth and transformation (Manning and Cantley, 2007). Typically, growth factor receptors activate this pathway through PI-3 kinase, which phosphorylates the phospholipid PI(4,5)P2 to produce PI(3,4,5)P3 (Fig. 2A). Binding to PI(3,4,5)P3 causes the relocalization of the serine/threonine kinase Akt to the plasma membrane, where Akt is activated by phosphorylation at two sites, including S473. Activated Akt phosphorylates and inactivates TSC2, which is a GTPase-activating protein for the Ras family protein Rheb. This leads to activation of Rheb and its downstream target TOR (target of rapamycin), which is also a serine/threonine kinase. TOR activation can be monitored by detecting phosphorylation of its substrate p70 S6 kinase at T389. The Akt-TOR pathway is often activated in cancer cells, including PC3 and LNCaP, due to the loss of the tumor suppressor Pten, which is a lipid phosphatase that dephosphorylates PI(3,4,5)P3 to PI(4,5)P2 (Sharrard and Maitland, 2007).
Surprisingly, we found that treatment of PC3 prostate cancer cells with either ephrin-A1 Fc or ephrin-B1 Fc results in substantial inhibition of the TOR pathway (as assessed by monitoring phosphorylation of S6 kinase; Fig. 2B). Inhibition of the TOR pathway downstream of Eph receptors is a completely novel finding and further substantiates the tumor suppressor activities of the Eph receptors in prostate cancer cells. Ephrin-A1 Fc treatment inhibits S6 kinase phosphorylation somewhat less than rapamycin, which is a drug that selectively inhibits TOR and is under evaluation in clinical trials for use as an anti-cancer agent (Fig. 2C). However, being a TOR inhibitor rapamycin only inhibits the more downstream steps in the pathway and does not inhibit TSC2 (Fig. 2C).

Given the importance and novelty of these findings, we have focused our efforts on investigating the mechanism by which Eph receptors inhibit the Akt-TOR pathway. We found that ephrin-A1 Fc stimulation of PC3 cells inhibits phosphorylation of TSC2 at the Akt target site T1462 (Fig. 2D). We therefore investigated whether Akt may itself be inhibited, and indeed we found decreased phosphorylation of S473, which is indicative of Akt activation (Fig. 2D). This is a particularly exciting finding because besides the TOR pathway, Akt also activates several other pathways that play an important role in oncogenesis (Manning and Cantley, 2007). Therefore, this suggests that ephrin-A1 Fc, unlike rapamycin, inhibits multiple oncogenic pathways (see also below).

We hypothesize that EphA2 activation is sufficient for downregulation of the TOR pathway downstream of ephrin-A1 Fc stimulation. EphA2 is highly expressed in PC3 cells and can be efficiently activated by ephrin-A1 Fc (as shown in our previous progress report) followed by downregulation, as expected (Fig. 2D). We are currently investigating the signaling connection between EphA2 and Akt. It has been recently reported that, following ephrin stimulation, EphA2 binds and activates the lipid phosphatase Ship2 through an interaction mediated by the SAM domains of both proteins (Zhuang et al., 2007). Like Pten, Ship2 dephosphorylates PI(3,4,5)P3, although to the different product PI(3,4)P2, and thus inactivates Akt and the TOR pathway. Interestingly, Ship2 is highly expressed in PC3 cells, providing support for this signaling mechanism (Sharrard and Maitland, 2007). We have prepared an expression construct encoding the SAM domain in the carboxy-terminal tail of EphA2 fused to EGFP. We will use this construct to investigate whether the EphA2-Ship2 interaction plays a role in downregulation of the TOR pathway (Fig. 2A). We are also collaborating with the laboratory of Dr. Maurizio Pellecchia at our institute, who has used NMR to characterize the structure of the EphA2 and Ship2 interacting SAM domains and the protein interface mediating the interaction. Dr. Pellecchia has also identified peptides that can be used to interfere with the EphA2-Ship2 association. It will also be important to determine if Ship2 also plays a role downstream of EphB receptors or if a different signaling mechanism plays a role when cells are stimulated with ephrin-B1 Fc. Furthermore, we are investigating regulation of the TOR pathway by ephrins in other prostate cancer cell lines. However, in DU145 cells the Ras-MAP kinase pathway appears to be a more prominently activated oncogenic pathway, as described in the next section.

Eph receptor-dependent inhibition of the Ras-MAP kinase pathway
The Ras-MAP kinase pathway is another major oncogenic pathway and activating mutations in this pathway are often found in cancer cells (Macrae et al., 2005). Interestingly, EphA2 expression is upregulated downstream of the Ras-MAP kinase pathway, which may explain the high level of EphA2 expression in many cancers (Macrae et al., 2005). It has been shown in a number of systems that EphA and EphB receptor activation by ephrins inhibits the activity of the Erk1 and Erk2 MAP kinases through activation of the GTPase-activating protein p120RasGAP, which inactivates H-Ras (Pasquale, 2005). In particular, ephrin-A1 Fc stimulation of PC3 cells has been shown to inhibit the Erk1 and Erk2 MAP kinases (Miao et al., 2001). We found that
ephrin stimulation can downregulate the MAP kinase pathway in PC3 cells and also in DU145 cells where this pathway is highly activated (Carey et al., 2007) (Fig. 3A). In PC3 cells, EphA2 activation is sufficient to mediate this effect because an EphA2-activating peptide that we identified (Koolpe et al., 2002) and an EphA2 activating antibody also inhibit Erk activation (Fig. 3C).

Eph receptor-dependent inhibition of integrin-mediated adhesion
Integrin mediated adhesion is critical for the establishment of tumors and for cancer invasion and metastasis (Slack-Davis and Parsons, 2004; Knudsen and Miranti, 2006). We found that ephrin-A1 Fc treatment of PC3 cells causes cell retraction and rounding (Fig. 4), as previously reported (Miao et al., 2000). We also demonstrated that these effects are due to EphA2 and not other EphA receptors expressed in PC3 cells (Table 1) because they can be blocked by treating the cells with a chemical compound that we have identified as part of a different project. This compound selectively inhibits ephrin-A1 binding to EphA2 (data not shown). The only other receptor inhibited by the compound is EphA4, which is not expressed in PC3 cells (Table 1). Based on our previous work with different cell types (Dail et al., 2006) and a previous report (Miao et al., 2000), this cell retraction phenotype suggests a decrease in β1 integrin mediated adhesion, which represents another possible Eph-dependent tumor suppressor mechanism (Slack-Davis and Parsons, 2004; Knudsen and Miranti, 2006). Experiments to determine if ephrin-B1 Fc-mediated activation of EphB2 and other EphB receptors also leads to PC3 cell retraction and rounding and similar experiments with LNCaP and DU145 cells are also in progress. We will also investigate whether a Eph receptor-mediated pathway leading to inactivation of the Ras family protein Rap1, which we have characterized in neurons (Richter et al., in press) and others have characterized in colorectal cancer cells (Riedl et al., 2005), is responsible for the rounding phenotype and decreased integrin-dependent adhesion in prostate cancer cells.

Cell growth and survival
MTT assays to measure cell growth and FACS analyses of propidium iodide-labeled cells to assess cell cycle progression and apoptosis following ephrin stimulation are also in progress.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that ephrin-B1 Fc treatment inhibits the Akt-TOR oncogenic pathway in PC3 prostate cancer cells. This is a novel effect of EphB receptors.
- Determined that ephrin-A1 Fc treatment inhibits the Akt-TOR oncogenic pathway in PC3 prostate cancer cells, including phosphorylation of Akt, TSC2 and S6 kinase. This is a novel effect of EphA receptors.
- Generated a construct encoding the EphA2 SAM domain fused to green fluorescent protein, which will be used to study the involvement of the EphA2-Ship2 association in inhibition of the TOR pathway.
- Determined that ephrin-A1 Fc and ephrin-B1 Fc treatment inhibits the Ras-MAP kinase oncogenic pathway in DU145 cells. This is interesting (and unexpected) because this pathway appears to be constitutively activated in DU145 cells.
- Determined that ephrin-A1 Fc and ephrin-B1 Fc treatment inhibits the Ras-MAP kinase oncogenic pathway in PC3 cells.
- Determined that EphA2 activation is sufficient to inhibit the Ras-MAP kinase oncogenic pathway in PC3 cells. Selective EphA2 activation was obtained using a peptide that we have identified and an EphA2 activating antibody.
• Determined that ephrin-A1 Fc treatment causes rounding of PC3 prostate cancer cells, suggesting inhibition of integrin-mediated attachment.

• Determined that EphA2 activation is sufficient to cause rounding of PC3 prostate cancer cells, suggesting inhibition of integrin-mediated attachment. The involvement of EphA2 was demonstrated by using a chemical compound that selectively inhibits ephrin-A1 binding to EphA2 and EphA4. Since the compound blocked the cell rounding effects of ephrin-A1 and since EphA4 is not present in PC3 cells, we conclude that EphA2 mediates the effects.

REPORTABLE OUTCOMES

Constructs:
pEGFP vector encoding the SAM domain of EphA2 as a fusion protein with enhanced green fluorescent protein (EGFP).

Abstracts:

Review Articles:

Training and Employment:
Dr. Severine Roselli, who was a Postdoctoral Associate trained with the support of this grant, received an Early Career Development award from the Cancer Institute NSW, Australia and obtained a Research Fellow position in the School of Biomedical Sciences at the University of Newcastle in Australia. Since she relocated to Australia, she has been replaced by another postdoctoral associate, Dr. Carlos Fernandez-Saez.

CONCLUSION

Our results indicate that activation of at least several Eph receptors by their ephrin ligands inhibits two major oncogenic pathways that have been implicated in prostate cancer progression, the Ras-MAP kinase pathway and the Akt-TOR pathway. Furthermore, Eph receptor activation inhibits integrin-mediated adhesion. These effects could explain the tumor suppressor activities of the Eph receptors. In addition to EphB2, we and others have gathered substantial evidence that the EphA2 receptor also has tumor suppressor activity in prostate cancer. Interestingly, EphA2 and EphB2 share the same chromosomal location at 1p36.1 (Pasquale, 2005). Therefore, the loss of heterozygosity reported for this locus in prostate cancer decreases not only EphB2 expression (Huusko et al., 2004) but also EphA2 expression.

Clearly, the tumor suppressor activities of the Eph receptors in prostate cancer represent an important area of investigation that will help understand the pathogenesis of this disease. The information obtained from these studies will help guide the design of novel treatment strategies and determine whether prostate cancers should be screened for Eph receptor and ephrin mutations for prognostic purposes.
REFERENCES


Table 1. Survey of Eph mRNA/protein expression in prostate cancer cells

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<tr>
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+++; high expression; ++, medium expression; +, low expression; nd, very low expression or not detectable.

1EphA10 and EphB6 are kinase-inactive receptors.

Fig. 1. Expression of ephrin-B2 inhibits the growth of EphB-expressing cancer cells. An MDA-MB-435 breast cancer clonal cell line stably transfected with an ephrin-B2 inducible construct was grown for 2 days either without doxycycline (− doxy) or with doxycycline (+ doxy) to induce expression of ephrin-B2. Cells were labeled with anti-ephrin-B antibodies (green) and DAPI to stain nuclei (blue). Despite the fact that the cells are clonal and were expanded in the absence of doxycycline, most cells have lost the ability to express high levels of ephrin-B2. This is presumably due to the fact that even the lower levels of ephrin-B2 expression under uninduced conditions can activate the EphB4 receptor expressed in these cells sufficiently to inhibit growth. Therefore, the cells with low ephrin-B2 expression can overcome the culture. The few cells that still express substantial levels of ephrin-B2 after induction are abnormally large and multinucleated (arrows), suggesting that they are unable to complete cytokinesis. Scale bar = 50 µM.
Fig. 2. Ephrin-A1 stimulation suppresses the Akt-TOR oncogenic pathway in PC3 cells. (A) Schematic representation illustrating regulation of the Akt-TOR pathway by growth factor receptors and the mechanism hypothesized to mediate suppression of the Akt-TOR pathway downstream of EphA2. (B) Stimulation of PC3 cells with 1 µg/ml ephrin-A1 Fc or 3 µg/ml ephrin-B1 Fc for 20 min inhibits phosphorylation of S6 kinase at threonine 389 (T389). Cell lysates were probed with antibodies to phosphoS6 kinase and reprobed for S6 kinase. (C) Ephrin-A1 stimulation inhibits not only S6 kinase phosphorylation but also phosphorylation of TSC2 at the Akt phosphorylation site (T1462). In contrast, rapamycin, a drug that targets TOR, inhibits S6 kinase phosphorylation but not TSC2 phosphorylation, as expected. Cell lysates were probed with antibodies to phosphoS6 kinase or phosphoTSC2 and reprobed for S6 kinase and TSC2. (D) Immunoblotting of cell lysates with anti-EphA2 antibody shows that stimulation of PC3 cells with ephrin-A1 Fc causes downregulation of the target receptor EphA2, as expected. Ephrin-A1 Fc also causes a decrease in phosphorylation of Akt at serine 473, indicating that ephrin-A1 suppresses Akt activation. Phosphorylation of TSC at the Akt target site (threonine 1462) is also reduced, together with phosphorylation of S6 kinase. Cell lysates were probed with the phosphospecific antibodies and reprobed with antibodies to Akt, TSC, and S6 kinase to show that total protein levels were not affected. (E) Ship2 is readily detected by immunoblotting in PC3 cell lysates.
Fig. 3. Eph receptors suppress the Ras MAP kinase oncogenic pathway in PC3 and DU145 cells. (A) Stimulation of PC3 and DU145 cells with 1 µg/ml ephrin-A1 Fc or 3 µg/ml ephrin-B1 Fc for 20 min inhibits phosphorylation (and therefore activation) of the Erk1 and Erk2 MAP kinases. Cell lysates were probed with antibodies to phosphoMAP kinases and reprobed for total MAP kinases. The relative levels of Erk1 and Erk2 are different in the two cell lines. A shorter film exposure was used for DU145 cells, consistent with their higher level of MAP kinase activation. (B) EphA2 selective activation using an antibody or the YSA peptide, which we identified (Koolpe et al., 2002), both inhibit MAP kinase phosphorylation in PC3 cells relative to Fc control treated cells. This indicates that EphA2 receptor activation is sufficient to inhibit MAP kinase activity.
**Fig. 4. Ephrin-A1 Fc treatment causes retraction and rounding of PC3 cells.** (A) Stimulation of PC3 cells with 0.5 µg/ml ephrin-A1 Fc for 15 min causes cell rounding. A small chemical compound that inhibits ephrin-A1 binding to EphA2 blocks the ephrin-A1-dependent cell rounding without affecting cell morphology on its own. Actin filaments are labeled with rhodamine-phalloidin (red) and nuclei are labeled with DAPI (blue). Scale bar = 50 µM. (B) The histogram shows the percentage of cells undergoing rounding (means ± SEM from triplicate measurements).