CD44-Src signalling promotes invadopodia formation in prostate cancer (PC3) cells

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Abstract

Introduction
Src kinase activation has been reported in unrelated human cancers and considered a possible target of anti-invasive therapies. Our aim here is to identify the relationship of Src kinase with CD44-signalling. Although CD44 is implicated in the invasion of cancer cells, its role in invadopodia formation needs further elucidation.

Materials and Methods
To study the role of Src, PC3 cells were transfected with Src constructs by adenoviral-mediated delivery. Gelatin degradation assay was performed to determine the invasive property of PC3 cells. Immunoblotting and immunostaining analyses were used to determine complex interaction of proteins with CD44. PC3 cells knockdown of CD44 were used to characterize the function of CD44 in invadopodia formation and invasion.

Results
In this study, we show that expression of constitutively-active Src in prostate cancer 3 (PC3) cells significantly increased the invasiveness of PC3 cells via increasing the number of invadopodia as a result of CD44-associated complex (Src-Cortactin-Wiskott-Aldrich Syndrome Protein WASP) formation. The inhibition of invadopodial structures in CD44 knockdown PC3 cells resulted in reduced invasion.

Conclusion
Based on these results, we conclude that CD44-Src-cortactin-WASP is an invasion promoting complex. Proteins in this complex are relevant targets for intervening invadopodia formation and migration/invasion processes in prostate cancer cells.

Introduction
Prostate cancer is a disease of extensive metastases, with secondary lesions in lymph nodes, bones and sometimes in visceral organs, including liver, lungs and even the brain. Actin dynamics is a critical process that modulates cellular activity via regulation of signalling pathways through receptors and cell adhesion to the extracellular matrix (ECM). CD44 is a cell surface receptor and regulator of cell migration and tumour metastasis. CD44-Matrix metalloproteinase 9 (MMP9) complex generates motility enhancing signals through the degradation of ECM proteins. These cells use invadopodia-like structures for invasion into ECM. Localisation of MMP9 in the invadopodia assists in the focal degradation of matrix during invasion.

Signalling and actin-binding proteins (e.g. Src, cortactin, WASP and Arp2/3) have been shown to contribute to the formation of invadopodia. CD44 receptor has the potential to integrate adhesive and signalling activities to modulate migration/invasion processes during cancer progression. CD44 shares some properties with the integrin receptor. Similar to integrin, the cytoplasmic domain of CD44 associates with several proteins involved in signalling and actin dynamics. However, the role of CD44 in the formation of invadopodia is not clear.

PC3 cells knockdown of MMP9 (PC3/MMP9−/−) are highly adhesive which is due to the expression of highly glycosylated CD44 variant isoform v6. These cells are less invasive due to a deficiency in the expression of standard CD44 (CD44s) and failure in the formation of invadopodia. Pertinent to proteins that CD44 interacts with, it is highly possible that CD44 might regulate the formation of invadopodia. Therefore, we hypothesise that similar to integrins, CD44 associates with proteins involved in signalling, and these, in turn, are associated with actin and actin-binding proteins.

We show here that Src has a direct role in the formation of invadopodia through its interaction with CD44. CD44-associated signalling complex (Src, WASP, cortactin and Arp2/3) regulates the process of actin polymerisation involved in invadopodia formation.

Materials and Methods
The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Antibodies towards CD44, MMP9, Src, WASP, cortactin and cortactin (Y421) were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An antibody towards phospho-Src Y418 was obtained from Cell Signalling (Beverly, MA). Rhodamine phalloidin and other chemicals were purchased from Sigma. CY2 and CY3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

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Cell lines used for studies and culture conditions

PC3 cells were obtained from American Type Culture Collection (Manassas, VA). Stable PC3 cells expressing control scrambled RNA interference (RNAi) and short hairpin (shRNA) constructs to knock down CD44 (PC3/CD44-/-) were generated as described previously. These cells were cultured in RPMI-1640 media containing 10% FBS at 37°C as described earlier.

Preparation of cell lysates, cell surface labelling by biotinylation, immunoprecipitation and immunoblotting

PC3 cells were treated with an Src inhibitor (PP2; 100 nM) or transfected with Src constructs (constitutively active (CA) and kinase-defective (KD) Src) by adenoviral mediated delivery for 48–72 h as described previously. Surface labelling with NHS-biotin, preparation of cell lysates with RIPA lysis buffer, immunoblotting (25–50 µg), as well as immunoprecipitation/immuno blotting (100–200 µg) analyses were done essentially as described in earlier study.

Immunohistochemistry and actin staining

Cells were cultured onto cover slips in a 6- or 12-well dish for 14–16 h at 37°C. Cells were immunostained with antibodies of interest or stained for actin with rhodamine phalloidin as described previously. Cells were scanned in a Bio-Rad 6000 (Hercules, CA) confocal microscope and images were processed by the Adobe Photoshop program (Adobe Systems, Inc., Mountain View, CA).

Measurement of F-actin content using rhodamine phalloidin binding to PC3 cells

PC3 cells cultured in 24 culture plates were subjected to various treatments. For each treatment, six wells were used. Cells were labelled with rhodamine phalloidin (1:200) and processed as described previously.

Gelatin degradation and wound healing assays

Gelatin degradation was performed essentially as described previously.

Statistical analysis

All values presented as mean ± SEM. A value of p < 0.05 was considered significant. Statistical significance was determined by analysis of variance (ANOVA) with the Bonferroni corrections (Instat for IBM; Graph pad software).

Results

Src regulates the formation of invadopodia in PC3 cells

To determine the functional significance of c-Src in the formation of invadopodia, PC3 cells were infected with adenovirus containing CA- and KD-Src as shown previously. An increase in the phosphorylation of Src at Y418 corresponds with the increased levels of Src in PC3 cells transfected with CA-Src (Figure 1A and B, lane 1) as compared with control cells (Figure 1A and B, lane 3). Src phosphorylation in PC3 cells expressing KD-Src is significantly lower (Figure 1B, lane 2) than the control cells (Figure 1B, lane 3), although the expression levels of KD-Src and CA-Src are equal (Figure 1A, lanes 1 and 2).

Actin staining in these cells displayed an increase in the number of invadopodia in CA-Src expressing cells (Figure 1C) as compared with untransfected (-) cells (Figure 1E). An increase in the number of invadopodia corresponds with an increase in...
F-actin content in PC3 cells expressing CA-Src (Figure 1F). The number of invadopodia and F-actin content are significantly reduced below the control levels in KD-Src expressing cells (Figure 1D and F). Taken together, these results suggest that Src has a role in actin dynamics involved in the formation of invadopodia.

**Src regulates surface expression of CD44**

We subsequently determined the possible biochemical mechanisms by which Src may play a role in the regulation of invadopodia formation via CD44. PC3 cells surface labelled with NHS-biotin after various treatments were immunoprecipitated with a CD44 antibody or a non-immune serum (NI). An increase in the surface expression of CD44 (Figure 2B, lane 3) corresponds with an increase in the phosphorylation of CD44-associated Src at Y418 (Figure 2C, lane 3) and serine phosphorylation of CD44 (lane 3 in Figure 2D) in PC3 cells expressing CA-Src. A significant decrease below the control level (lane 2 in Figure 2B–D) was observed in the studied parameters in PC3 cells expressing KD-Src (lane 4; Figure 2B–D). Equal level of CD44 protein expression was observed regardless of the treatment (Figure 2A, lanes 2–4). The mechanisms by which Src regulates CD44 surface expression needs further study.

**Src regulates CD44-associated complex formation**

We proceeded to determine the role of Src in CD44-associated complex formation. We show here that Src regulates the phosphorylation state of WASP and cortactin (Figure 3A and B, lane 1). A significant decrease in the phosphorylation below the control level (lane 3) was observed in cells expressing KD-Src (Figure 3A and B; lane 2). Similar result was observed in cells treated with an Src inhibitor (PP2; lane 4 in Figure 3A).

To evaluate the role of Src in CD44-associated signalling complex formation, lysates (400 µg protein) made from PC3 cells expressing CA- and KD-Src were immunoprecipitated with an antibody to CD44 (lanes 2–4) and non-immune serum (NI; lane 1). CD44 immunoprecipitates were immunoblotted with an antibody to CD44 (A), streptavidin HRP (B), Y418 Src (C) and p-serine (D) antibody after stripping sequentially. Immunoprecipitation of equal amount of CD44 in panel (A) serves as a loading control. Results shown are representative of three different experiments.

**Knockdown of CD44 and expression of KD-Src in PC3 cells reduces the invasive property of PC3 cells**

We have shown earlier that invadopodia forms a focal degradation on the gelatin matrix. The effect of the expression of CA-Src and KD-Src as well as the knockdown of CD44 in the regulation of matrix degradation was determined (Figure 4). An increase in the number of invadopodia corresponds with the invasive nature of PC3 cells expressing CA-Src (Figures 1C and 4C). The depth and surface area of degradation was found to be more in CA-Src (Figure 4C) expressing cells than control cells (Figure 4A; scramble). As shown previously, neither focal adhesions nor invadopodia were observed in PC3 cells stably knockdown of CD44 (Figure 4B). Knockdown of CD44 and expression of KD-Src (Figure 4D) has the potential to decrease the invasive property of PC3 cells (B and D).

**Discussion**

Changes in the phenotype and architecture of cytoskeleton in PC3/MMP9/- suggest that CD44 may play a role in the formation of invadopodia and invasion in PC3 cells. Therefore, to best characterise the function of CD44 in invadopodia formation and invasion/migration processes, we used PC3 cells to stably knockdown of CD44. Neither invadopodia nor focal adhesions were observed in PC3 cells expressing KD-Src (Figure 4D) as compared to control cells (Figure 4A; scramble). As shown previously, neither focal adhesions nor invadopodia were observed in PC3 cells stably knockdown of CD44 (Figure 4B). Knockdown of CD44 and expression of KD-Src (Figure 4D) has the potential to decrease the invasive property of PC3 cells (B and D).

**Figure 2:** Src regulates CD44 phosphorylation on serine residues and surface expression. Equal amount of protein lysates were used for immunoprecipitation (IP) with an antibody to CD44 (lanes 2–4) and non-immune serum (NI; lane 1). CD44 immunoprecipitates were immunoblotted with an antibody to CD44 (A), streptavidin HRP (B), Y418 Src (C) and p-serine (D) antibody after stripping sequentially. Immunoprecipitation of equal amount of CD44 in panel (A) serves as a loading control. Results shown are representative of three different experiments.
observed in these cells. Knockdown of CD44 transforms highly invasive PC3 cells into a less adhesive/invasive cellular phenotype. Integrins play organisational roles in the adhesion and formation of invadopodia. Integrin αvβ3 is expressed in PC3 as well as PC3/MMP9-/- and PC3/CD44-/- cells (data not shown). However, integrin αvβ3 is not able to compensate for CD44 deficiency in the organisation of invadopodia in PC3/MMP9-/- and PC3/CD44-/- cells. Taken together, these observations suggest the importance of CD44 and the link between CD44 and actin dynamics in the organisation of invadopodia.

Highly invasive cancer cells and Src-transformed cells display actin-enriched invadopodia. Src kinase is the most ubiquitously expressed non-receptor tyrosine kinase. Src has been shown to localise in the invadopodia of mammary carcinoma cells where it specifically mediates the phosphorylation of cortactin. As shown by others in human cancer cells, the ability of Src to induce signalling involved invadopodia formation and is corroborated by the effects observed with a Src inhibitor (PP2). Cortactin phosphorylation is upregulated by oncogenes (e.g. Src) and growth factors and it was shown to play a role in bone metastasis of breast cancer cells. Cortactin phosphorylated on Y421 is identified in the invadopodia of PC3 cells. WASP-cortactin constitutes two distinct classes of Arp2/3 modulators. WASP is localised in the invadopodia of PC3 cells. Phosphorylation of Y291 in WASP increases the affinity of VCA-domain to Arp2/3 proteins and actin monomers, increasing actin polymerisation in osteoclasts. Weaver et al. have shown that when cortactin was added in combination with the active VCA-fragment of WASP, they synergistically enhanced Arp2/3-induced actin filament branching. Cortactin has been shown to stabilise WASP-Arp2/3 complexes. Inhibition of Src activity with PP2 or expression of KD-Src reduces the interaction of WASP and cortactin with CD44. Cortactin and WASP phosphorylation by Src seems a required mechanism for CD44 associated complex formation. CD44 interaction with Src promotes cytoskeletal reorganisation (Figure 5) as shown by others.

Conclusion
Our results suggest that CD44-Src-cortactin-WASP-Arp2/3 axis plays an important role in the formation of invadopodia. Src also has a role in the surface expression of CD44, which indicates that Src activation may occur independent of CD44 but dependent on integrin signalling. Additional experiments are needed to determine how Src increases the levels of CD44 on the cell surface. We conclude that reducing Src-mediated phosphorylation of cortactin and WASP or depletion of one of the components (e.g. CD44 or cortactin) of ternary complex

Figure 3: Src regulates CD44-associated complex formation. (A–B) Immunoblotting analyses with a phospho-specific cortactin (A) or phosphotyrosine antibody (B). Total cellular lysates (50 µg) made from indicated PC3 cells were used (A). WASP immunoprecipitates from total cellular lysates (100 µg) were used in (B). Blots were stripped and blotted with a cortactin and WASP antibody. Equal amount of cortactin and WASP serves as a loading control [lower panels in (A) and (B)]. (C–F) CD44 immunoprecipitates were immunoblotted with an antibody to CD44, WASP, cortactin and Src. Equal amount of CD44 in the upper panel serves as a loading control. (G) Confocal analysis of PC3 cells immunostained for indicated proteins is shown. Yellow aggregates represent invadopodia and colocalisation of CD44 with WASP, actin, p-cortactin and Src in invadopodia. Non-immune serum is used as a control for immunostaining. These results represent one of three experiments performed with the same results.
seems a possible strategy to reduce invadopodia formation or stability.

Abbreviations list
ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GADPH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitation; NI, non-immune.

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References
Research Study

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