The C-terminal PDZ ligand sequence GENL restricts the metastatic potential of c-Src in non-transformed human breast epithelial cells

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Background:

The c-Src kinase regulates cell behavior in response to adhesion to the substratum, to cell-cell contacts and to growth factors by integrating extracellular cues into intracellular signaling networks. Its state of activation is tightly regulated to prevent oncogenic transformation. The C-terminus of c-Src - GENL - is a ligand for PDZ proteins and might be involved in restricting c-Src activation and function.

Aims:

To explore the possible significance of the c-Src terminus GENL as a regulatory switch for c-Src activation, cell migration and metastatic transformation in non-transformed human breast epithelial cells (MCF-10A cells).

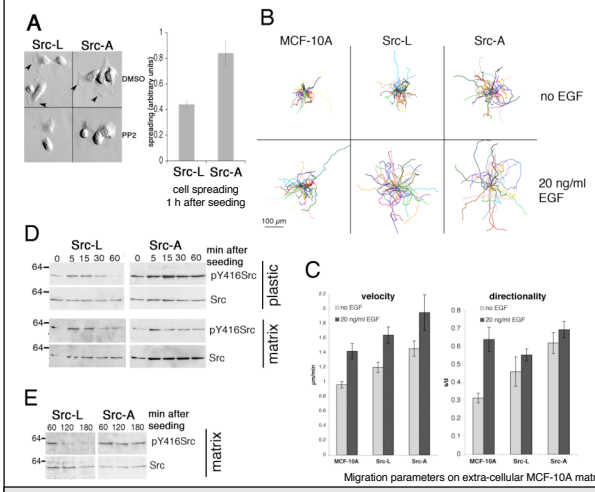
Results:

We found that c-Src with a point mutation in GENL (Src-A, L to A substitution) but not wild-type c-Src (Src-L) is activated in MCF-10A cells under conditions of growth factor and serum deprivation, while Src-A activity in cells in full growth medium is not increased. In the absence of EGF stimulation, c-Src phosphorylated on Tyrosine 416, indicative for Src activation, was markedly increased in membrane and cytoskeleton fractions of MCF-10A cells expressing Src-A **(Fig. 1)**. EGF-stimulated cell-sheet migration in wounding assays required intact PDZ ligand sequence and Src-A cells failed to polarize properly towards wound. Wound closure defects and impaired polarization correlated with a marked loss of E-cadherin and β-catenin from cell-cell contacts **(Fig. 2)**. Single cells expressing Src-A and migrating on reconstituted extra-cellular matrix of MCF-10A cells in the absence of growth factors, displayed velocity and directionality comparable to EGF-stimulated control cells **(Fig. 3)**. Concomitantly, Src-A increased phosphorylation of cortactin and focal adhesion kinase (FAK) on c-Src-specific sites and caused their accumulation at the leading edge **(Fig. 4)**. Comparable to control cells, Src-A expressing MCF-10A cells initiated the formation of spherical acinis. However, Src-A cells disrupted the laminin V layer on the basal side of the cells and displayed cohort-like migration into the surrounding extra-cellular matrix **(Fig. 5)**.

Conclusions:

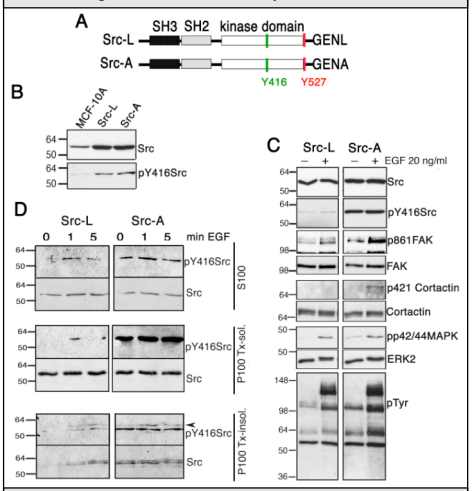
We propose that binding of PDZ domains to c-Src in epithelial cells is a safeguard mechanism to restrict c-Src functions, to maintain cell-sheet integrity, to decrease growth factor-independent adhesion turnover and to prevent morphological transformation towards an invasive phenotype.

Fig. 3 Src-A promotes EGF-independent migration of MCF-10A cells on extra-cellular matrix and increases velocity and directionality of migration



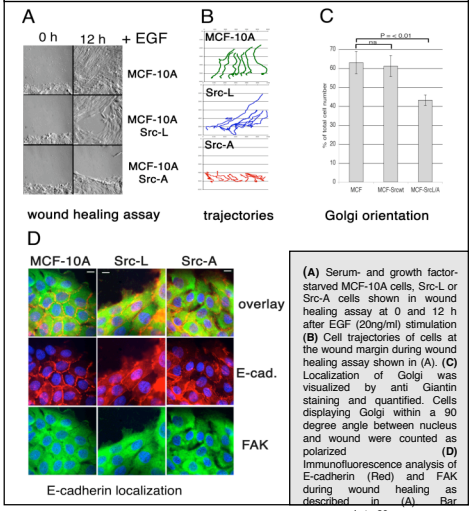
(A) Src-L or Src-A cells were seeded onto MCF-10A reconstituted extra-cellular matrix without or with the Src kinase inhibitor PP2 and visualized with live cell video microscopy. Src-L and Src-A cell spreading on MCF-10A matrix was quantified using NIH image software (right panel). Cell migration on MCF-10A matrix without or or with 20 ng/ml EGF was recorded by live cell video microscopy **(B)** and velocity and directionality of individual cells (n= 45-50 cells) was quantified **(C)**. Western blot analysis using c-Src- and activated Src family-specific (pY416Src) antibodies to determine activation status of Src-L and Src-A after adhesion for 1 h to plastic and MCF-10A matrix **(D)** or for 3 h to MCF-10A matrix **(E)**.

Fig. 1 PDZ ligand sequence restricts c-Src under conditions of growth factor and serum deprivation



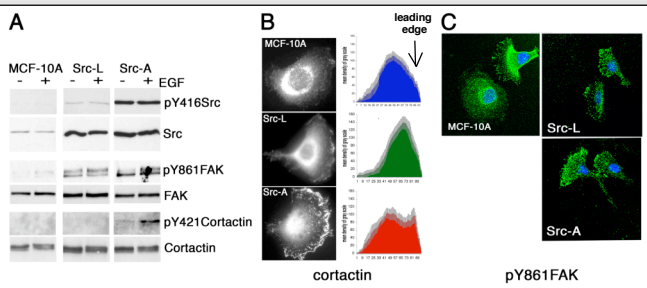
(A) Structure of c-Src (Src-L) and c-Src with leucine to alanine substitution in the C-terminal PDZ ligand sequence (Src-A). **(B)** Expression levels and activation status of c-Src, Src-L and Src-A in MCF-10A cells grown with serum and growth factors. **(C)** Phosphorylation and activation status of Src-L, Src-A and selected c-Src targets in MCF-10A cells expressing Src-L or Src-A without and with 20 ng/ml EGF stimulation for 5 min. **(D)** Activation status and expression levels of Src-L and Src-A in MCF-10A cells without and with 20 ng/ml EGF in cytosolic (S100) fractions, TritonX-100-soluble (P100 Tx-sol.) and -insoluble (P100 Tx-insol.) 100'000x g fractions. Arrowhead indicates position of Src-L and Src-A, respectively.

Fig. 2 Intact PDZ ligand sequence in c-Src is required for EGF-induced cell polarization and wound closure



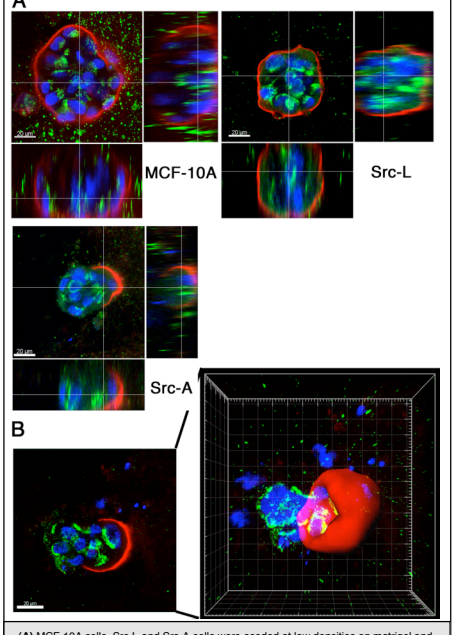
(A) Serum- and growth factor-starved MCF-10A cells, Src-L or Src-A cells shown in wound healing assay at 0 and 12 h after EGF (20ng/ml) stimulation **(B)** Cell trajectories of cells at the wound margin during wound healing assay shown in (A). **(C)** Localization of Golgi was visualized by anti Giantin staining and quantified. Cells displaying Golgi within a 90 degree angle between nucleus and wound were counted as polarized **(D)** Immunofluorescence analysis of E-cadherin (Red) and FAK during wound healing as described in (A). Bar corresponds to 20 µm.

Fig. 4 Src-A increases phosphorylation of focal adhesion kinase (FAK) and cortactin on c-Src-specific sites and causes their accumulation towards leading edge in migrating cells.



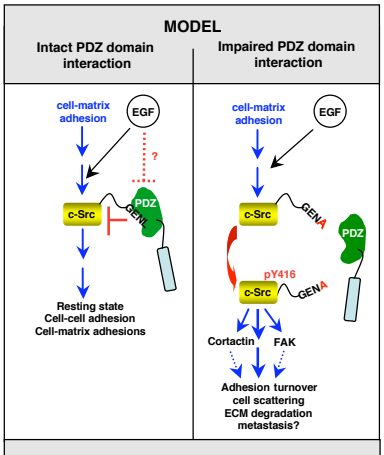
(A) Subconfluent MCF-10A, Src-L or Src-A cells were wounded multiple times to stimulate cell scattering. After 12 h without or with 20ng/ml EGF, cells were lysed and total lysates were probed by Western blotting with the indicated antibodies. **(B)** Cells were seeded onto MCF-10A extra-cellular matrix in the absence of serum and growth factors for 2 h and then fixed and stained for cortactin. Cortactin accumulation was quantified in polarized cells by densitometric analysis using NIH software. Plots in right panel show average relative intensities of cortactin staining throughout the cell body (tail towards leading edge) from at least 6 individual cells. **(C)** FAK phosphorylation on Tyr residue 861 on cells treated as in (B) was visualized by confocal microscopy using phospho 861FAK-specific antibodies.

Fig. 5 Src-A promotes degradation of basal laminin V and cell scattering in 3 dimensional cultures.



(A) MCF-10A cells, Src-L and Src-A cells were seeded at low densities on matrigel and allowed to form spherical cell aggregates (acini) in complete growth medium supplemented with 4% matrigel. After 11 days, acini were fixed and stained with anti Giantin (green) and anti laminin V (red) antibodies. Nuclei are in blue. Horizontal sections spaced by 2 µm were recorded by confocal imaging. Bottom up views and vertical cross sections are shown **(B)** 2 dimensional confocal image of MCF-10A Src-A cells escaping from acinus and corresponding 3 dimensional reconstruction (generated using Imaris).

MODEL



Under normal growth conditions, c-Src activity is maintained at a physiologically relevant level by adhesion and growth factor stimulation. This basal, tightly regulated c-Src activity is required for maintaining cell-cell and cell-matrix adhesions.

Our current model is that the C-terminal PDZ ligand sequence GENL restricts cell-matrix and cell-cell contact-induced c-Src activation trough interaction with PDZ domains. C-Src inhibition is relieved by growth factor stimulation, suggesting stimulation dependent regulation of c-Src–PDZ domain interaction.

Regulation of c-Src by PDZ proteins might be particularly important in epithelial cells where stimuli from cell-matrix (e.g. integrin) and cell-cell (e.g. E-cadherin) adhesion receptors need to be continuously integrated into intracellular signaling networks.

Mutation in GENL, which impairs PDZ interaction, or the elimination of a regulatory PDZ protein lead to a mode of aberrant activation of c-Src that is largely independent of growth factors. Consequences include increased substrate phosphorylation, growth factor-independent migration and the transformation of polarized epithelial cells within acini into cohorts of cells escaping into the surrounding extra cellular matrix.