Negative regulation of c-Src by binding to the PDZ domain of AF-6

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ABSTRACT

The protooncogene *c-src* encodes a tightly regulated non-receptor tyrosine kinase. c-Src plays a key role in cell adhesion, cell morphology and motility as well as in cell proliferation and survival. Elevated c-Src protein expression level and increased kinase activity have been found in several human cancers. Deletion of the C-terminal sequence results in a constitutively activated c-Src mutant detected in some human colon carcinoma. Replacement of the C-terminal sequence is characteristic for v-Src, the viral homologue of c-Src encoded by the avian Rous Sarcoma Virus (RSV), which causes tumours in chickens.

In this study we describe the very C-terminus of c-Src as ligand for a PDZ domain protein. PDZ domain proteins are multidomain proteins and function as scaffolds to organize cell adhesion complexes, to cluster transmembrane proteins, and to align proteins of a signalling cascade, e.g. shown for Src/Raf-1 signalling. In many cases PDZ proteins mediate anti-proliferative effects. Here, we demonstrate by mutational analysis that the C-terminal amino acid Leu of c-Src is essential for the binding to a PDZ domain. As candidate PDZ protein we identified AF-6, a junctional adhesion protein, involved in formation and maintenance of cell junctions and in negative regulation of signalling. We show that the PDZ domain of AF-6 strongly restricts the number of c-Src substrates, while knock-down of AF-6 has the opposite effect. The AF-6 PDZ domain interferes with phosphorylation of c-Src at Tyr 527 by the C-terminal Src kinase, CSK, and reduces autophosphorylation at Tyr 416 resulting in a moderately activated c-Src protein. The interaction of c-Src with a PDZ domain protein, e.g. AF-6, may spatially restrict c-Src to subcellular regions, e.g. cell-cell contacts. We identified a new type of negative regulation of c-Src through binding to a PDZ domain protein.

Regulation of c-Src by its very C-terminal sequence

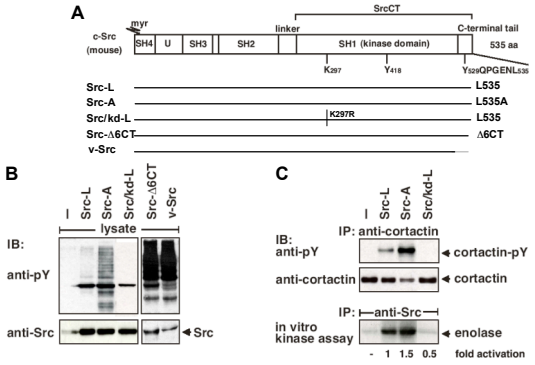

Figure 1: Regulation of c-Src by its C-terminal residue leucine. **(A)** Domain structure of murine c-Src. **(B)** HEK 293 cells were transiently transfected with empty vector (-) or constructs encoding Src-L, Src-A, Src/kd-L, Src-Δ6CT and v-Src as indicated. Transfected cells were lysed after starving for 18 h. Lysates were analysed by immunoblotting (IB) with anti-phospho-tyrosine (pY) antibody. Src expression was controlled by immunoblotting with anti-Src antibody. **(C)** For analysis of cortactin phosphorylation, lysates were immunoprecipitated (IP) with anti-cortactin antibody and immunoblotted with anti-pY and anti-cortactin antibodies. Immunopurified Src proteins were analysed by *in vitro* kinase assay with enolase as substrate. Numbers indicate fold activation.

Physical interaction between c-Src and AF-6 *in vitro* and *in vivo*

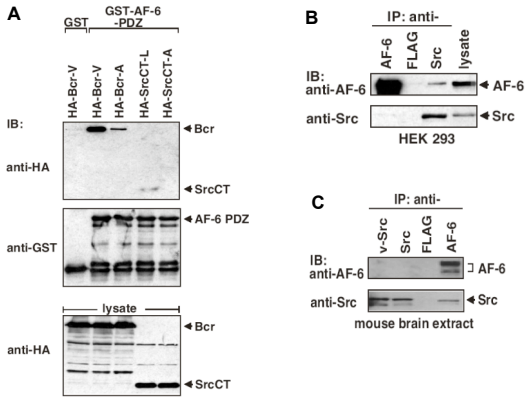

Figure 2: Interaction of c-Src with the AF-6 PDZ domain. **(A)** Pull-down assay: HEK 293 cells were transiently transfected with plasmids encoding HA-SrcCT-L, HA-SrcCT-A, HA-Bcr-V and HA-Bcr-A as indicated. Lysates were incubated with GST or GST-AF-6-PDZ, followed by immunoblotting with anti-HA or anti-GST antibodies. **(B)** Coprecipitation of endogenously expressed AF-6 and c-Src. Lysates of HEK 293 cells and mouse brain extracts **(C)** were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies.

The kinase activity of c-Src interferes with binding to AF-6

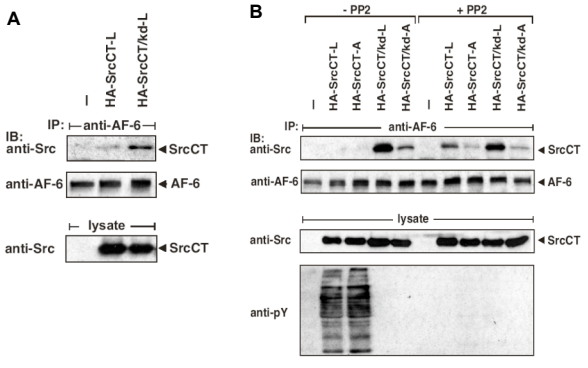

Figure 3: Effect of the kinase activity of c-Src on its binding to AF-6. **(A)** HEK 293 cells were transiently transfected as indicated. The interaction between endogenous AF-6 and SrcCT proteins was detected by immunoprecipitation with anti-AF-6 antibody and immunoblotting with anti-Src antibodies. **(B)** Cells were transfected as indicated and pretreated with or without the Src kinase inhibitor PP2 1 h prior to lysis. The interaction between AF-6 and SrcCT proteins was analysed as above. Immunoblotting with anti-pY antibody was used for control of Src activity.

Effect of AF-6 on phosphorylation of Src Tyr 416 and Tyr 527

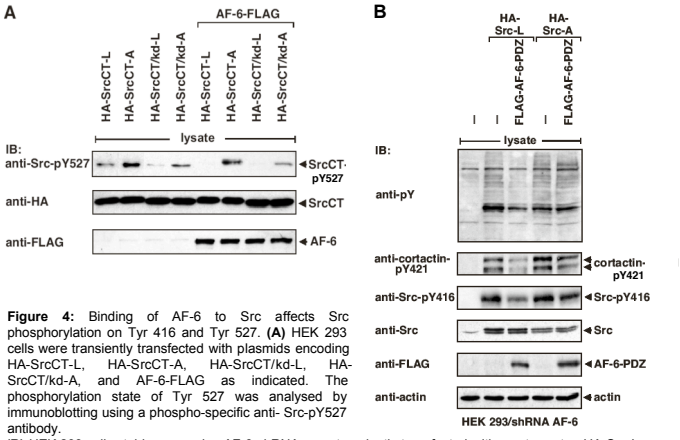

Figure 4: Binding of AF-6 to Src affects Src phosphorylation on Tyr 416 and Tyr 527. **(A)** HEK 293 cells were transiently transfected with plasmids encoding HA-SrcCT-L, HA-SrcCT-A, HA-SrcCT/kd-L, HA-SrcCT/kd-A, and AF-6-FLAG as indicated. The phosphorylation state of Tyr 527 was analysed by immunoblotting using a phospho-specific anti- Src-pY527 antibody. **(B)** HEK 293 cells stably expressing AF-6 shRNA were transiently transfected with empty vector, HA-Src-L or HA-Src-A with or without FLAG-AF-6-PDZ. Transfected cells were lysed after starving for 18 h. Lysates were analysed by immunoblotting with anti-pY antibody. For analysis of cortactin phosphorylation and Src autophosphorylation, lysates were immunoblotted with anti-cortactin-pY421 or anti-Src-pY416 antibodies, respectively.

AF-6 negatively regulates endogenous c-Src

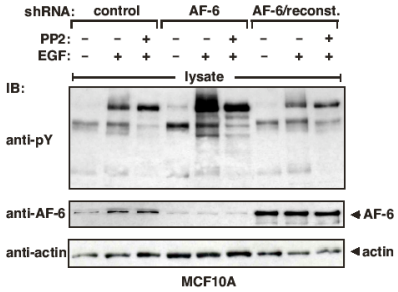

Figure 5: Increased Src-dependent phosphorylation by knockdown of AF-6. MCF10A cells stably expressing control shRNA or AF-6 shRNA or AF-6 knock down cells reconstituted for AF-6 (AF-6/reconst.) were starved for 18 h, pretreated with or without the Src kinase inhibitor PP2 1 h prior to lysis and stimulated with 20ng/µl EGF for 10 min as indicated. Tyrosine phosphorylation of proteins was analysed by immunoblotting using anti-pY antibody. Expression of AF-6 and equal loading was controlled by blotting with anti-AF-6 or anti-actin antibody, respectively.

Regulation of c-Src by PDZ proteins

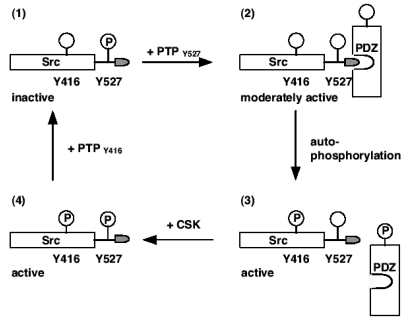

Figure 6: Model for the regulation of c-Src by PDZ proteins. For simplification only regulation of c-Src by phosphorylation of its C-terminal part is considered. (1) Inactive c-Src is phosphorylated on Tyr 527. (2) After dephosphorylation of Tyr 527 a PDZ protein restricts c-Src in a moderately active state. (3) Fully activated c-Src is autophosphorylated at Tyr 416 and unable to bind to AF-6 presumably due to phosphorylation of AF-6. (4) Release of c-Src from AF-6 may increase the accessibility of CSK to c-Src.

References:

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