Anti-proliferative signalling of protein kinases

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ABSTRACT

We analysed various negative regulation mechanisms for signalling, which allow the maintenance of a non-proliferative state of epithelial cells. One of these mechanisms involves the multidomain junction protein AF-6 (1). It is a tumour suppressor-type protein, which has been described as fusion protein in ALL-1. Another tumour suppressor is Bcr, a quiescence-specific kinase, which contributes to CML as Bcr-Abl fusion protein. AF-6 and Bcr are colocalized at membranes of epithelial cells and downregulate proliferation by cross-talk with Ras/Raf-1 signalling (2). Bcr also negatively regulates Wnt signalling (3). The molecular mechanisms involved are distinct. Recently, we demonstrated that AF-6 binds via its PDZ domain to the C-terminus of c-Src and restricts its functions in epithelial cells (4). The number of substrates is reduced as well as its mobility and directionality of migration, as shown by optical analyses and time lapse microscopy (5,6). Lack of the c-Src PDZ ligand sequence allows wound healing, increases extracellular proteolytic activity and disrupts morphology of acinar structures. Src also interacts with the E3 ligase LNX1 and thereby is ubiquitinated (7). Thus, PDZ domain proteins are safeguards in epithelial cells to restrict c-Src or other kinases e.g. from inducing invasion or metastases. Other family members of c-Src such as Lck, Hck and Lyn, which mainly occur in blood cells, lack this C-terminal PDZ ligand sequence.

(1) Schneider S, et al, Moelling K and Hovens CM. *Nat Biotechnol*, **17**, 170-175 (1999); (2) Radziwill G, Erdmann RA, Margelisch U and Moelling K. *Mol Cell Biol*, **23**, 4663-72 (2003); (3) Ress A and Moelling K. *EMBO Rep*, **6**, 1095-1100 (2005); (4) Radziwill G, Weiss A, Heinrich J, Baumgartner M, et al and Moelling K. *EMBO J*, **26**, 2633–2644 (2007); (5) Lorger M and Moelling K. *J Cell Sci*, **119**, 3385-3398 (2006); (6) Baumgartner M, Lorger M, Radziwill G and Moelling K, under modification; (7) Weiss A, Baumgartner M, Radziwill G, Dennler J and Moelling K, under modification.

PDZ domain proteins and their ligands

Figure 1: Domain structure of proteins. AF-6: RBD, Ras-binding domain; FHA, forkhead associated domain; DIL, class V myosin homology region; PDZ, PDZ-domain; P, proline-rich region. Bcr: O, oligomerisation domain; GEF: guanine nucleotide exchange function; GAP: GTPase activating protein function; STEV: PDZ-binding motif. Abl: Cap, autoinhibitory domain; P, proline rich region; Nim nuclear import signal; DB, DNA binding region; FAB, F-actin binding site; Nex, nuclear export signal. β-catenin: TA,transactivation domain. Src: myr, myristylaton;SH Src homologie region; U, unique region; GENL, PDZ-binding motif; LNX1: RING, E3 ligase domain; NPAY, phosphotyrosine binding motif.

Bcr downregulates Ras/Raf signalling by interaction with PDZ protein AF6 (2)

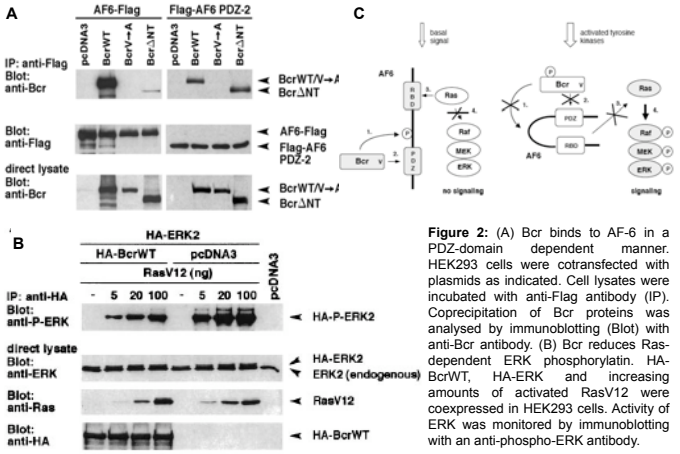


Figure 2: (A) Bcr binds to AF-6 in a PDZ-domain dependent manner. HEK293 cells were cotransfected with plasmids as indicated. Cell lysates were incubated with anti-Flag antibody (IP). Coprecipitation of Bcr proteins was analysed by immunoblotting (Blot) with anti-Bcr antibody. (B) Bcr reduces Ras-dependent ERK phosphorylatin. HA-BcrWT, HA-ERK and increasing amounts of activated RasV12 were coexpressed in HEK293 cells. Activity of ERK was monitored by immunoblotting with an anti-phospho-ERK antibody.

(C) Model depicting the effect of Bcr on Ras-dependent stimulation of ERK via AF-6. (Left) In quiescent cells the constitutively active Bcr phosphorylates AF-6 (step 1) which leads to the interaction of the PDZ domain of AF-6 with the PDZ binding motif of Bcr (step 2). This increases the affinity of AF-6 for Ras (step 3) and prevents binding of Raf to Ras (step 4). Under these conditions ERK is not activated. (Right) Phosphorylation of Bcr on tyrosine residues inactivates its protein kinase activity. Therefore, Bcr cannot phosphorylate (step 1) and cannot bind to AF-6 (step 2). Thus, AF-6 does not compete with Raf for Ras (step 3) and does not interfere with the Ras-dependent activation of the protein kinase cascade (step 4). P represents phosphorylation of proteins.

Bcr is a negative regulator of the Wnt pathway (3)

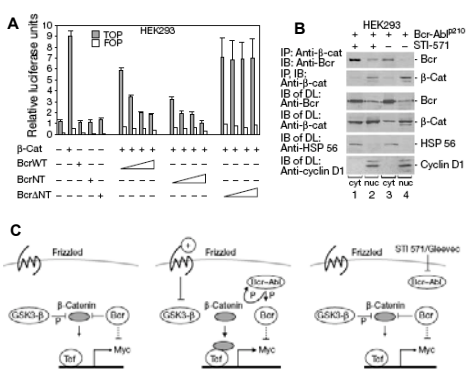


Figure 3: HEK 293 cells were transfected with TOPflash and its control FOPflash together with plasmid coding for b-catenin, BcrWT, BcrNT and BcrD]NT (1, 3, 4 and 7 mg each). Luciferase activity was determined by spectrophotometer. (B) Complex formation regulated by Bcr–Abl kinase. HEK 293 cells were treated with STI-571 (2μM, 1h), fractionated, IP and IB as indicated. (C) Model on Bcr effects on β-catenin. Negative regulation is indicated by bars and positive regulation by arrows. Dotted lines indicate downregulation of c-Myc protein levels by Bcr. The figure depicts an unstimulated cell (left), Bcr–Abl-expressing tumour cell (middle) and STI-571 effect (right). The thick arrows indicate strong activation of c-Myc.

c-Src is restricted in substrate phosphorylation by binding to the PDZ domain of AF-6 (4)

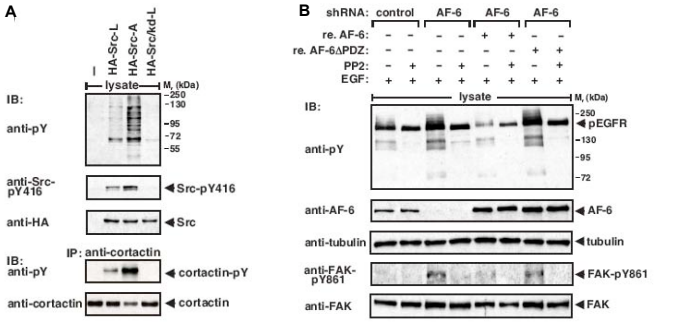


Figure 4: (A) Regulation of Src by its C-terminal sequence. HEK 293 cells were transiently transfected with constructs as indicated. Lysates of starved cells were analysed by blotting (IB) with anti-phospho-tyrosine (pY), anti-Src-pY416, anti-HA antibodies. For analysis of cortactin phosphorylation, lysates were precipitated (IP) with anti-cortactin antibody and blotted with anti-pY and anti-cortactin antibodies. (B) 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.

c-Src interacts with and is ubiquitinated by the PDZ protein LNX1 (7)

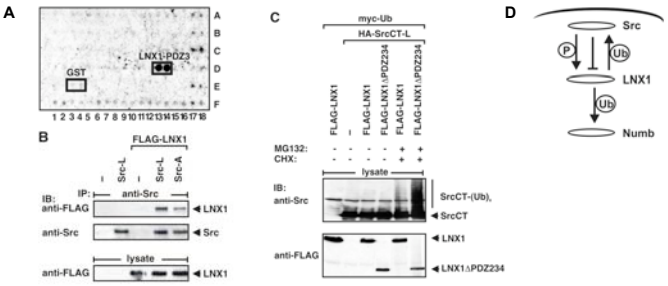


Figure 5: (A) PDZ arrays were incubated with HEK 293 cell lysates overexpressing c-Src. Bound c-Src was detected by blotting with anti-Src. (B) FLAG-LNX1, Src-L or Src-A were overexpressed in HEK 293 cells. Cell lysates were immunoprecipitated (IP) with anti-Src and immunoblotted (IB) with anti-FLAG. (C) HEK 293 cells were transfected as indicated and treated with or without 50 μM MG132 and 20 μg/ml cycloheximide (CHX) for 3 h prior to lysis. (F) Model of interactions between c-Src and LNX1.

Defect in PDZ ligand sequence increases invasive potential of c-Src (6)

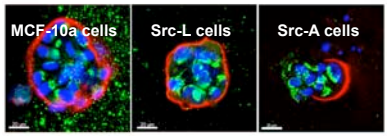
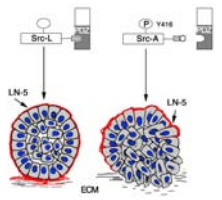


Figure 6: Expression of Src-A disrupts acinar structures. 11 days acinar cultures of MCF10A cells, or cells expressing Src-L or Src-A were analysed by confocal IF microscopy for Laminin-5 (red), Golgi (green) and nuclei (blue).



c-Src is negatively regulated by its novel "SH5" domain, a binder to PDZ domains

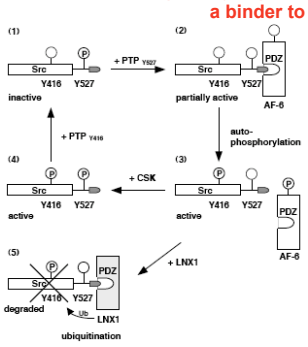


Figure 7: Model for the regulation of c-Src by PDZ proteins. For simplification only regulation of c-Src by phosphorylation of its C-terminal part is considered. (1) Inactive c-Src is phosphorylated on Tyr 527. (2) After dephosphorylation of Tyr 527 a PDZ protein restricts c-Src in a moderately active state. (3) Fully activated c-Src is auto-phosphorylated 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. Phosphorylation of c-Src by CSK followed by dephosphorylation of Y416 inactivates c-Src. (5) Activated Src is substrate for the E3 ligases LNX1 and c-Cbl, which ubiquitinates c-Src in a PDZ-dependent or PDZ-independent manner, respectively.