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Identification of Csk binding proteins as scaffolds for Src/Csk regulatory circuit

Src

Csk

A Doctoral Thesis

By

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Contents

Abbreviations

SFK: Src family tyrosine kinase

SH: Src homology

pY: phosphotyrosine

Csk: C-terminal Src kinase

Cbp: Csk binding protein

PAG: Phosphoprotein associated with GEMs

Dok: Downstream of tyrosine kinase

PH: preckstrin homology

PTB: Phosphotyrosine binding

IRS: Insulin receptor substrate

ZO: zona occludins

PDZ: PSD-95/Dlg/ZO-1

NLS: nuclear localization signal

Guk: guanylate kinase

PR: proline-rich

PTK: Protein tyrosine kinase

KD: kinase deficient

MEF: mouse embryonic fibroblast

EMT: epithelial-mesenchymal transition

EGF: Epidermal growth factor

DMEM: Dulbecco's modified Eagle's medium

FBS: fatal bovine serum

BSA: bovine serum albumin

HRP: horseradish peroxidase

Ig: immunoglobulin

ODG: *n*-octyl-β-D-glucoside

NP-40: Nonidet P40

MTT: 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide

TAP: tandem affinity purification

GFP: Green fluorescent protein

GST: glutathione-S-transferase

CBP: calmodulin binding peptide

TEV: tobacco etch virus

LC: liquid chromatography

MS: mass spectrometry

TOF: time of flight

Chapter 1

Analysis of Csk binding p62 phosphoprotein, Dok-1

Abstract

The Src family tyrosine kinases (SFKs) play pivotal roles in regulating proliferation, differentiation and adhesion of animal cells. SFK activity is controlled by Csk (C-terminal Src kinase), which phosphorylates a C-terminal regulatory tyrosine residue of SFKs to inhibit their activities. Csk is cytoplasmic, but is recruited to sites of SFK activity by tyrosine-phosphorylated Csk-binding proteins. To date, several Csk-binding proteins, i.e. paxillin, FAK, Cbp/PAG, have been identified, but it remains possible that there are additional Csk-binding proteins, because SFKs have more diverse functions which may independently require specific Csk-binding proteins for their regulations. Thus I attempted to comprehensively identify Csk-binding proteins using $Csk^{-/-}$ MEF, in which the activated status of SFKs can be fixed. Herein, I identified an adaptor protein Dok-1 as one of the major tyrosine phosphorylated Csk-binding proteins in these cells, and determined the mechanism of Csk/Dok-1-mediated regulation of SFK. Tyrosine phosphorylation of Dok-1 is stimulated by EGF, and enhances association with Csk. In SFK activated cells, phosphorylated Dok-1 localizes and recruits Csk to the membrane. Furthermore, I demonstrate that Dok-1 can associate with not only Csk but also c-Src, and that these associations are essential to Dok-1's ability to attenuate c-Src activity. These observations support the notion that Dok-1 function as negative feedback regulators of SFKs in EGF signaling.

Introduction

The Src family protein tyrosine kinases (SFKs) are proto-oncogene products and play pivotal roles in the initiation of variety of biological process including cell proliferation, differentiation and adhesion (Brown and Cooper, 1996; Thomas and Brugge, 1997). The structural features of SFKs and an oncogenic form of c-Src (v-Src) are shown in Fig.1. SFKs consist of an N-terminal fatty acylation sequence that is required for membrane anchoring, an SH3 domain that recognizes proline-rich motifs, an SH2 domain that specifically binds to motifs containing phosphorylated tyrosine residue, a catalytic domain that includes an autophosphorylation site (Y416) in the activation loop, and a C-terminal regulatory region containing a negative regulatory site (Y527). The activity of SFKs is strictly regulated by the phosphorylation of these critical tyrosine residues: the autophosphorylation of Y416 is required for full activity, and the phosphorylation of the C-terminal Y527 induces the intramolecular interaction with the SH2 domain to adopt an assembled inactive conformation (Xu et al., 1997). In the oncogenic v-Src, the lack of the C-terminal regulatory tyrosine makes the molecule constitutively active. In resting normal cells, most of SFKs are present as an inactive form. In response to extracellular signals, SFKs become activated either by the accumulation of specific adapter proteins that bind to the SH2 or SH3 domain of SFKs, or by dephosphorylation of pY527.

Phosphprylation of the C-terminal Y527 is catalyzed by another protein tyrosine kinase, C-terminal Src kinase (Csk) (Nada et al., 1991; Okada et al., 1991). Like SFKs, Csk consists of an SH3 domain, an SH2 domain, and a catalytic domain, but it lacks the N-terminal fatty acylation signal, autophosphorylation site, and the C-terminal regulatory site (Fig.1). Thus it belongs to an independent subfamily of tyrosine kinase. The critical role of Csk as a negative regulator of SFKs have been

demonstrated by the observation that Csk deficient mice, that die before birth, exhibit constitutive activation of SFKs (Nada et al., 1993). Structural analysis of Csk revealed that, unlike SFKs, the binding pockets of SH2 and SH3 domains of Csk are oriented outward, suggesting that function and/or subcellular localization can be regulated by the interaction with other molecules (Ogawa et al., 2002) (Fig.3). Since Csk is a cytoplasmic enzyme, it requires adaptor proteins to efficiently access to the membrane-anchored SFKs. To date, a variety of Csk-binding proteins including paxillin and FAK (Sabe et al., 1994), Dok-1 (Neet and Hunter, 1995), caveolin-1 (Cao et al., 2002), LIME (Hur et al., 2003), Cbp/PAG (Brdicka et al., 2000; Kawabuchi et al., 2000), have been identified. These proteins interact with the SH2 domain of Csk in a phosphorylation-dependent manner and can concentrate Csk onto the membrane structures where the function and regulation of SFKs take place. Furthermore, these Csk-binding proteins serve as good substrates of SFKs, thereby providing a platform for the negative feedback loop of SFK regulation. For example, focal adhesion proteins, paxillin and FAK, are phosphorylated by SFK upon cell adhesion, and the phosphorylated molecules then recruit Csk to the focal contacts, resulting in the termination of SFK-mediated adhesion signaling (Fig.4). In a detergent insoluble membrane fractions (lipid rafts), where many signaling molecules become accumulated upon cell stimulation, transmembrane adaptor proteins, Cbp/PAG and LIME, serve as Csk anchors to terminate SFK signaling (Kawabuchi et al., 2000) (Fig.2 and 4). Like these examples, each Csk-binding protein functions at a specific site to regulate the specific and localized function of SFKs. Since SFKs have quite diverse functions, it seems likely that there are more Csk-binding proteins that play roles in other specific sites of SFK activation, such as downstream of growth factor receptors and intracellular organelles and/or vesicles (Fig.4).

Dok-1, also known as p62^{dok}, was initially identified as a tyrosine-phosphorylated 62 kDa protein associated with p120^{RasGAP} in Philadelphia chromosome-positive chronic myeloid leukemia blasts and in v-Abl transformed B cells (Carpino et al., 1997; Yamanashi and Baltimore, 1997). It later turned out to be the prototype member of a new adaptor protein family, referred to as the Dok (downstream of tyrosine kinases) family. Members of this family become phosphorylated upon activation of many receptor tyrosine kinases and cytoplasmic kinases, including SFKs (Cong et al., 1999; Jones and Dumont, 1998; Jones and Dumont, 1999; Lock et al., 1999; Zhao et al., 2001). To date, based on amino acid sequence homology, seven members of the Dok family proteins have been identified; Dok-1, Dok-R (Dok-2), DokL (Dok-3), Dok-4, Dok-5, Dok-6, and Dok7 (Carpino et al., 1997; Cong et al., 1999; Di Cristofano et al., 1998; Grimm et al., 2001; Jones and Dumont, 1998; Lemay et al., 2000; Okada et al., 2006; Yamanashi and Baltimore, 1997). Structural characteristics of this family make them most similar to the insulin receptor substrate (IRS) family of proteins (Cong et al., 1999). The Dok family of proteins contains three distinct protein domains or regions, which include an amino-terminal pleckstrin homology (PH) domain, a central phosphotyrosine binding (PTB) domain, and a carboxy-terminal region containing multiple tyrosine residues. Dok-1, Dok-R, and DokL are preferentially expressed in hematopoietic cells and have been shown to primarily mitigate signals downstream of a wide array of receptor and nonreceptor tyrosine kinases (Jones and Dumont, 1999; Nelms et al., 1998; Yamanashi et al., 2000; Zhao et al., 2001), while Dok-4, Dok-5 and Dok-6, preferentially expressed in neural cells, have been shown to potentiate signals emanating from the c-Ret receptor (Grimm et al., 2001). On the other hand, Dok-7 have been shown to activate MuSK, a muscle-specific receptor PTK, in myotubes (Okada et al., 2006), indicating that

Dok-family proteins are not necessarily limited to playing an adaptor function downstream of PTKs. It has recently been proposed that Dok family members should be considered as a separate subgroup of the family based upon functional differences and different patterns of expression (Grimm et al., 2001; Okada et al., 2006).

In this study, to comprehensively explore Csk-binding proteins, I attempted to isolate Csk-binding proteins from Csk-deficient mouse embryonic fibroblasts (Csk$^{-/-}$ MEF), in which multiple SFKs are constitutively hyperactivated (Imamoto and Soriano, 1993; Nada et al., 1994; Nada et al., 1993). Since most of Csk-binding proteins so-far-identified serve as excellent SFK substrates, I thought that these cells would be a good source of the target proteins, and that the lack of Csk would also beneficial to pull-down the Csk-binding proteins using exogenously added bait proteins. In this chapter, I describe the identification of Dok-1 as a Csk-binding protein and the mechanism of Csk/Dok-1-mediated regulation of SFK.

Materials and Methods

Antibodies and reagents

Anti-v-Src (Ab-1) antibody was purchased from Oncogene Research. Anti-Csk (C-20), anti-Dok-1 (M-276), anti-Dok-1 mAb (A-3) and anti-RasGAP mAb (B4F8) antibodies were from Santa Cruz Biotechnology. Anti-Csk mAb was from Transduction Lab. Anti-Src[pY418] and anti-Src[pY529] antibodies were from Biosource. Anti-phosphotyrosine (anti-pY: 4G10) was from Upstate Biotechnology. Anti-myc (PL14) was from MBL. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG antibody were from Zymed Laboratories, Inc. EGF was from Peprotech. PP2, PP3 and SU6656 were from Calbiochem. Protein inhibitors cocktail was from nacarai tesque. Protein G Sepharose 4 Fast Flow and glutathione Sepharose 4 Fast Flow were from GE Healthcare.

Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were immortalized using the simian virus 40 large T antigen. Csk-deficient MEFs (Csk$^{-/-}$ MEFs) were prepared from Csk knockout mouse embryos lacking both Csk and the anti-oncogene product p53, and Csk$^{-/-}$ cells in which Csk was stably re-introduced (Csk$^{-/-}$ + Csk MEFs) were established as described previously (Nada et al., 1994). All cells used in this experiments (COS7, MEFs, Csk$^{-/-}$ and Csk$^{-/-}$ + Csk MEFs) were maintained in Dulbecco's modified Eagle' medium (DMEM; Nissui) supplemented with 10% fatal bovine serum (FBS; Equitech-Bio, Inc.) at 37 °C in a humidified atmosphere containing 5% CO2. For transient expression in COS7 cells, pcDNA3.1 (Invitrogen) was used for the expressions of c-Src, Csk and Dok1-myc. pEGFP-N1 (Clontech) was used for Dok-1. Transfections were carried out using Lipofectamine 2000 (Invitrogen) for according to the manufacture's instructions.

Immunoprecipitation

To prepare whole cell lysates, cells were washed twice ice-cold PBS, lysed ODG buffer (50 mM Tris-HCl, pH7.4, 0.15 M NaCl, 5% glycerol, 1% NP-40, 2% n-octyl-β-D-glucoside, 1 mM EDTA, 5mM β-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitors). After centrifugation for 30 min at 15,000 rpm, the supernatants were collected and measured by the Bradford method using bovine serum albumin (BSA) as the standard. Equal amounts of supernatants were incubated with the relevant antibody and Protein G Sepharose at 4 °C for 1 h. I washed the immunoprecipitates with ODG buffer for four times and subjected to immunoblot analysis.

Purification of Csk SH2 domain binding proteins

cDNA encoding SH2 domain of Csk (amino acid residue 73 to 181) and its deficient mutant (Csk SH2M; Arg-107 was changed to Lys) were subcloned into pGEX-6P-1 vector. A fusion protein of GST-Csk SH2 and GST-Csk SH2M were expressed in *Escherichia coli* BL21, and coupled with glutathione-Sepharose beads. The washed beads were incubated with whole cell lysate at 4 °C for 1 h, and then collected by centrifugation. After washing for four times, the proteins complexed with GST-Csk SH2 or GST-Csk SH2Msubjected to immunoblot analysis.

Immunoblot analysis

The whole cell lysates were mixed with equal volumes of 2 × SDS sample buffer (0.125 M Tris-HCl, pH6.8, 4% SDS, 10% β-mercaptoethanol, 10 % Sucrose, and 0.004% Bromophenol blue). Immunoprecipitates and the proteins purified by GST-Csk SH2 were mixed with 2 × SDS sample buffer equal to the volume of Sepharose resin. These

proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 1% BSA or 5% skim milk, probed with primary antibodies and further incubated with an HRP-conjugated secondary antibody. Antibody binding was visualized with a chemiluminescence system (Perkin Elmer Life Science).

Growth factor stimulation

To examine the effects of EGF stimulation, MEF cells were serum-starved overnight and then stimulated for the indicated time at 37 °C with 100 ng/ml EGF. Cells were incubated with 1 μM PP2 or DMSO for 1 h, prior to EGF stimulation.

Immunofluorescence staining and conforcal microscopy

COS7 cells co-trasfected with the pEGFP-N1 or pEGFP-Dok-1 with Csk KD (Kinase deficient; in which the Lys-222 ATP-binding site was changed to Arg) were grown on 24-well plates for two days before fixation. After 24 hour, these cells were re-plated on collagen-coated glass coverslips. I stained Csk by immunofluorescence using Alexa 594–conjugated anti-rabbit IgG antibody (Invitrogen). The specimens were observed using an OLYMPUS IX81 confocal microscope controlled by Fluoview FV1000 software.

Results

Identification of the Csk SH2 domain-associated 62 kDa protein

To comprehensively identify Csk-binding proteins, I used Csk$^{-/-}$ MEFs in which SFKs are constitutively activated (Nada et al., 1993). As a control, Csk$^{-/-}$ MEFs in which Csk was stably re-expressed (Csk$^{-/-}$ + Csk MEFs) were used. Whole cell lysates from adhered cells (A) and suspended cells (S) were subjected to immunoblot analysis with an anti-phosphotyrosine (pY) (Fig.5 A). In adhered states, various SFK substrates, especially ~120 and 65–75 kDa proteins that potentially correspond to FAK and paxillin, respectively, were tyrosine phosphorylated in either cell types, but with greater intensities in Csk$^{-/-}$ MEFs. When the cells were detached from the substrate, the levels of tyrosine phosphorylation were dramatically reduced in Csk$^{-/-}$ + Csk MEFs, consistent with the elimination of cell adhesion signaling mediated by integrin. In contrast, Csk$^{-/-}$ MEFs retained substantial levels of tyrosine phosphorylation even under suspended conditions. These observations represent that, in Csk$^{-/-}$ MEFs, SFKs are constitutively activated independent of extracellular stimuli, and suggest that the phosphorylation of potential SFK substrates that may be rapidly turned over in normal cells can be retained in these cells.

Many Csk-binding proteins so-far-identified create the binding sites for Csk SH2 domain. Upon cell stimulation, the phosphorylated Csk binding proteins, such as Cbp (Kawabuchi et al., 2000), bind to the SH2 domain of Csk through specific binding sites (pY314 in Cbp). Thus, I first attempted to concentrate Csk-binding proteins using Csk SH2 domain fused to GST (GST-Csk SH2) as an affinity ligand. Pull-down analysis of the GST-Csk SH2 binding proteins from adhered cells revealed that several major tyrosine phosphorylated proteins were bound to Csk SH2 domain (Fig. 5B). Some of them were turned out to be well-characterized Csk-binding proteins, such as paxillin,

FAK and Tensin. In addition to these, I recognized that a p62 kDa phosphoprotein was efficiently pulled down from Csk$^{-/-}$ MEFs independently of cell adhesion status, suggesting that the p62 kDa protein would be a potential SFK substrate that recruits Csk.

To identify the p62 protein, the lysates from Csk$^{-/-}$ MEFs cultured in adhesion were incubated with GST-Csk SH2, and the bound proteins were separated on a SDS-PAGE gel, followed research by a MALDI-TOF/MS analysis. However, I could not identify the 62kDa protein under these conditions (data not shown). Previous reports revealed that Csk binds to a p62 phosphoprotein in v-Src transformed MEFs (Neet and Hunter, 1995), and that the p62 protein is Dok-1 that has been originally identified as p120^{RasGAP} associated protein (Carpino et al., 1997; Yamanashi and Baltimore, 1997). To determine the identity between the p62 phosphoprotein found in my experiments and Dok-1, the GST-Csk SH2 bound proteins and the immunoprecipitates with anti-Dok-1 were immunoblotted with anti-phosphotyrosine (Fig.6, upper panel) and anti-Dok-1 (Fig.6, middle panel). These results revealed that Dok-1 is at least a major component of the p62Da phosphoproteins and one of the major Csk-binding proteins in Csk$^{-/-}$ MEFs.

Tyrosine phosphorylation of Dok-1 in EGF-stimulated mouse embryonic fibroblasts

Dok-1 has been shown as a adaptor protein involved in the negative regulation of growth factor signaling (Yamanashi et al., 2000). To confirm whether Dok-1 is indeed tyrosine phosphorylated in response to growth factor stimulation *in vivo*, I determined the changes in tyrosine phosphorylation of Dok-1 by EGF stimulation using wild type mouse embryonic fibroblasts (MEFs). After EGF stimulation, the whole lysates were subjected to immunoblot analyses with the indicated antibodies. Tyrosine phosphorylation of cellular proteins including the EGF receptor and Erk1/2 were

increased by EGF stimulation, indicating that these cells responded to EGF stimulation (Fig.7 A). The activation of SFKs was also observed in response to EGF stimulation, as indicated by an increase in the phosphorylation of autophosphorylation site, Tyr-418 (Fig.7 B). Dok-1 was then immunoprecipitated from the cell lysates, and the immunoprecipitates were subjected to immunoblotting with anti-Dok-1, anti-phosphotyrosine and anti-RasGAP (Fig.7 C). As expected, tyrosine phosphorylation of Dok-1 was transiently induced by EGF stimulation consistent with the activation of SFKs. These results suggest that Dok-1 is indeed tyrosine phosphorylated upon growth factor stimulation, and that there may be some functional link between Dok-1 phosphorylation and SFK activations.

To determine if SFKs contribute to the tyrosine phosphorylation of Dok-1 in EGF signaling, I have examined the effects of a SFK inhibitor, PP2, on the Dok-1 phosphorylation (Fig.8). The PP2 treatment did not inhibit activation of EGF receptor or ERKs, while it efficiently inhibited the phosphorylation of Dok-1. These results suggest that Dok-1 could serve as an *in vivo* substrate of SFKs.

Interaction of Dok-1 with Csk under the downstream of EGF signaling

To examine if endogenous Dok-1 could interact with endogenous Csk *in vivo*, I performed an immunoprecipitation assay from EGF treated MEFs. Whole cell lysates from MEFs unstimulated or stimulated with EGF were subjected to immunoprecipitation with anti-Dok-1 or anti-Csk, and the immunoprecipitates were probed with indicated antibodies (Fig.9). The results showed that Csk was co-immunoprecipitated with the phosphorylated Dok-1 from EGF-stimulated MEFs, suggesting that Csk could bind to phosphorylated Dok-1 under physiological conditions.

Intracellular association of Dok-1 with Csk

To further verify the *in vivo* interaction of Dok-1 with Csk, the Dok-1-GFP fusion protein was co-expressed with a kinase deficient form of Csk (Csk KD) in COS7 cells, and the location of Dok-1 and Csk were observed using confocal microscopy. Since Csk KD serves as a dominantly negative form, Csk KD expression induced activation of SFKs as well as elevations of phosphorylation of their substrates, including Dok-1 (data not shown). As shown in Fig.10, Csk-KD was distributed broadly throughout the cells in GFP expressing cells, while it became co-localized with Dok-1-GFP at the plasma membrane in Dok-1-GFP expressing cells. These observations suggest that Dok-1 was able to recruit Csk to the membrane upon phosphorylation by SFKs.

Csk binds to phosphorylated Tyr450 residue of Dok-1

The precise mechanism of Csk-Dok-1 interaction was then analyzed in *in vitro* expression system using COS7 cells. Myc-tagged wild type Dok-1 (W) or its mutant with a Tyr to Phe substitution at the potential Csk binding site Y450 (YF) (Neet & Hunter, 1995) were transiently expressed with or without c-Src in COS7 cells, and whole cell lysates were analyzed by immnunoblotting (Fig.11 A). Interestingly, it was found that the co-expression with Dok-1 greatly enhanced the tyrosine phosphorylation of cellular proteins, potentially reflecting that Dok-1 could activate c-Src. Immnuprecipitation assay with anti-myc showed that tyrosine phosphorylation of either type of Dok-1 was greatly enhanced by c-Src expression and that only wild type Dok-1 was co-immunoprecipitated with Csk (Fig.11 B). The inverse assay with anti-Csk also confirmed that wild type Dok-1, but not the YF mutant, was co-immunoprecipitated with Csk. These results demonstrate the phosphorylation of Y450 of Dok-1 is required for the interaction with Csk.

Contribution of Dok-1 to the regulation of SFKs activities

Finally, I examined the role of Dok-1 in the regulation of SFK activity. COS7 cells were co-transfected with c-Src and Mock vector (-), myc-tagged wild type Dok-1 (W) or its Y450F mutant (YF) (Fig.12 A), and the Dok-1 immunoprecipitates were then probed with indicated antibodies (Fig.12 B). It was shown that wild type Dok-1 could simultaneously interact with c-Src and Csk, and the phosphorylation of the C-terminal Y529 of c-Src was enhanced as a result of the close interaction with Csk on the same Dok-1 molecule. In contrast, the Y450F mutant did not interact with Csk, resulting in a reduced phosphorylation at Y529 of c-Src. These was no significant change in the binding to RasGAP between wild type and Y450F mutant, indicating the specific role of Y450 in the regulation of Csk. These data suggest that phosphorylated Dok-1 could simultaneously bind to c-Src and Csk to allow close interaction between enzyme and substrate, thereby efficiently terminating c-Src mediated cell signaling.

Discussion

In this chapter, I describe the identification and characterization of Dok-1 as a major Csk-binding protein that play roles in the regulation of SFK in EGF signaling. Previously, it was already shown in v-Src transformed MEFs that Csk binds to a p62 phosphoprotein (Neet and Hunter, 1995), which later turned out to be Dok-1 that was originally identified as p120^{RasGAP} associated protein (Carpino et al., 1997; Yamanashi and Baltimore, 1997). However, the role of Dok-1 in the regulation of SFK function has remained thoroughly unknown. In this study, I clearly showed that, once phosphorylated by SFK, Dok-1 binds to SFK and Csk to provide a platform where Csk-mediated inactivation of SFK can be efficiently performed (Fig.13). These observations are well consistent with the recent reports on Dok-1 and Dok-R from other groups. Dok-1 is shown to independently attenuates ERK pathway and Src/c-Myc pathway to inhibit PDGF signaling (Zhao et al., 2006), and Dok-R attenuates EGF-ERK pathway and Akt activation through processive recruitment of c-Src and Csk (Van Slyke et al., 2005). It is thus conceivable that Dok-1 is involved in the Csk-mediated SFK regulatory circuit.

The Dok family of proteins contains multiple domains and regions, which include a PH domain, a PTB domain, and a carboxy-terminal region containing multiple tyrosine residues. These domains and regions can mediate various combinations of protein-lipid and protein-protein interactions, indicating that Dok-1 could serve as a central hub of the cell signaling. Dok-1 subfamily (Dok-1, Dok-R and Dok-L) has been shown to primarily mitigate signals downstream of a wide array of receptor and nonreceptor tyrosine kinases (Jones and Dumont, 1999; Lemay et al., 2000; Mustelin and Tasken, 2003; Yamanashi et al., 2000). The interaction of membrane bound Dok-1 with RasGAP has been shown to play critical roles in suppressing growth factor singling. However, I observed that the Dok-1-RasGAP interaction was unchanged by EGF

stimulation, while Dok-1-Csk interaction was readily and transiently detected in early stages of cell stimulation consistent with the activation profile of SFKs. These suggest that Dok-1-Csk mediated SFK regulation system would largely contribute to the downregulation of EGF signaling at least in MEFs.

Upon cell stimulation, SFKs are transiently activated in the early phase of cell signaling. For example, SFK activity reaches maximal within 1 min after EGF stimulation, then it is rapidly downregulated within 5 min. From these features of SFKs, it has been difficult to capture the protein-protein interaction that is transiently formed during these short-term events. Therefore, for the study of molecular mechanism of function and regulation of SFKs, constitutively active forms of SFKs, such as v-Src and a mutated c-Src with a Tyr to Phe substitution at the C-terminal regulatory site Y527, has been widely used (Brown and Cooper, 1996; Frame et al., 2002). However, the overexpression of these active forms induces unusually high levels of tyrosine phosphorylation of various proteins, leading to strong cell transformation. Thus, to identify physiologically significant targets of SFKs that potentially bind to Csk, I here employed Csk$^{-/-}$ MEFs in which endogenous SFKs are constitutively activated by the reduced phosphorylation at the C-terminal regulatory site (Nada et al., 1994). These cells contain relatively high level of tyrosine phosphorylated proteins and some SFK signaling pathways were augmented, such as those involved in cell adhesion and cytoskeletal organization (Yagi et al., 2007). However, these cells do not show transformed phenotype. Based on these, I thought that the activated status of endogenous SFKs is fixed by the lack of Csk in Csk$^{-/-}$ MEFs, which would be beneficial conditions to capture the SFK substrates waiting for the recruitment of Csk. In this study, the pull-down assay using GST-Csk SH2 successfully identified not only previously characterized Csk-binding proteins but also Dok-1 that had not been detected in normal

cells. The further study using Csk$^{-/-}$ MEFs would give more information about the function and regulation of SFKs.

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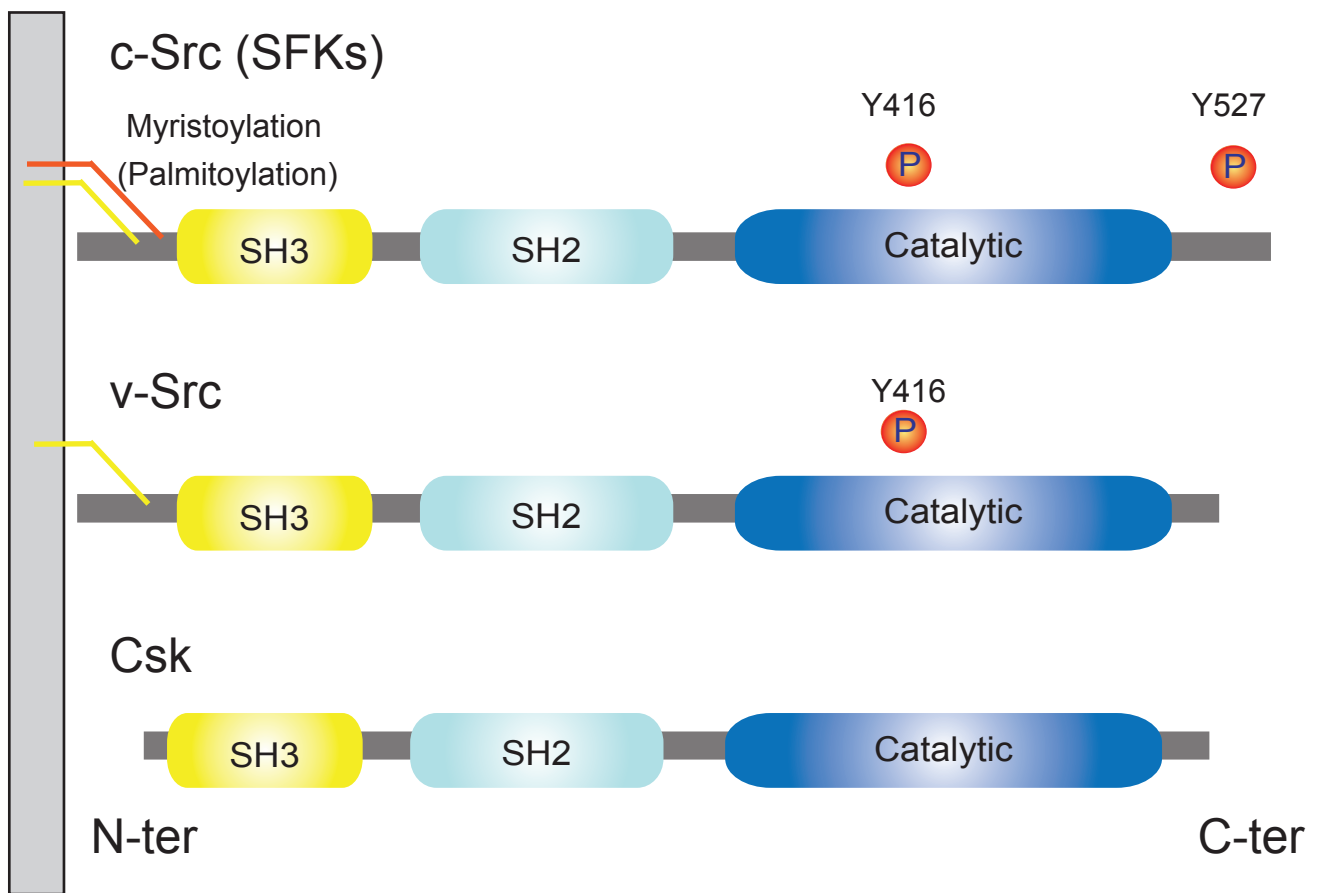
Figure 1. Domain structures of SFKs and Csk

Schematic models of domain structures of c-Src, v-Src and Csk are shown. All three proteins consist of a Src homology 3 (SH3), a Src homology 2 (SH2) domain and a catalytic domain. However, Csk lacks characteristic features conserved in SFKs, that include the N-terminal fatty acylation signal, the autophosphorylation site (Y416) in the catalytic domain, and the C-terminal negative regulatory site (Y527). v-Src lacks the C-terminal negative regulatory region, and instead contains unrelated 12 amino acids from virus gene and several point mutations throughout the molecule.
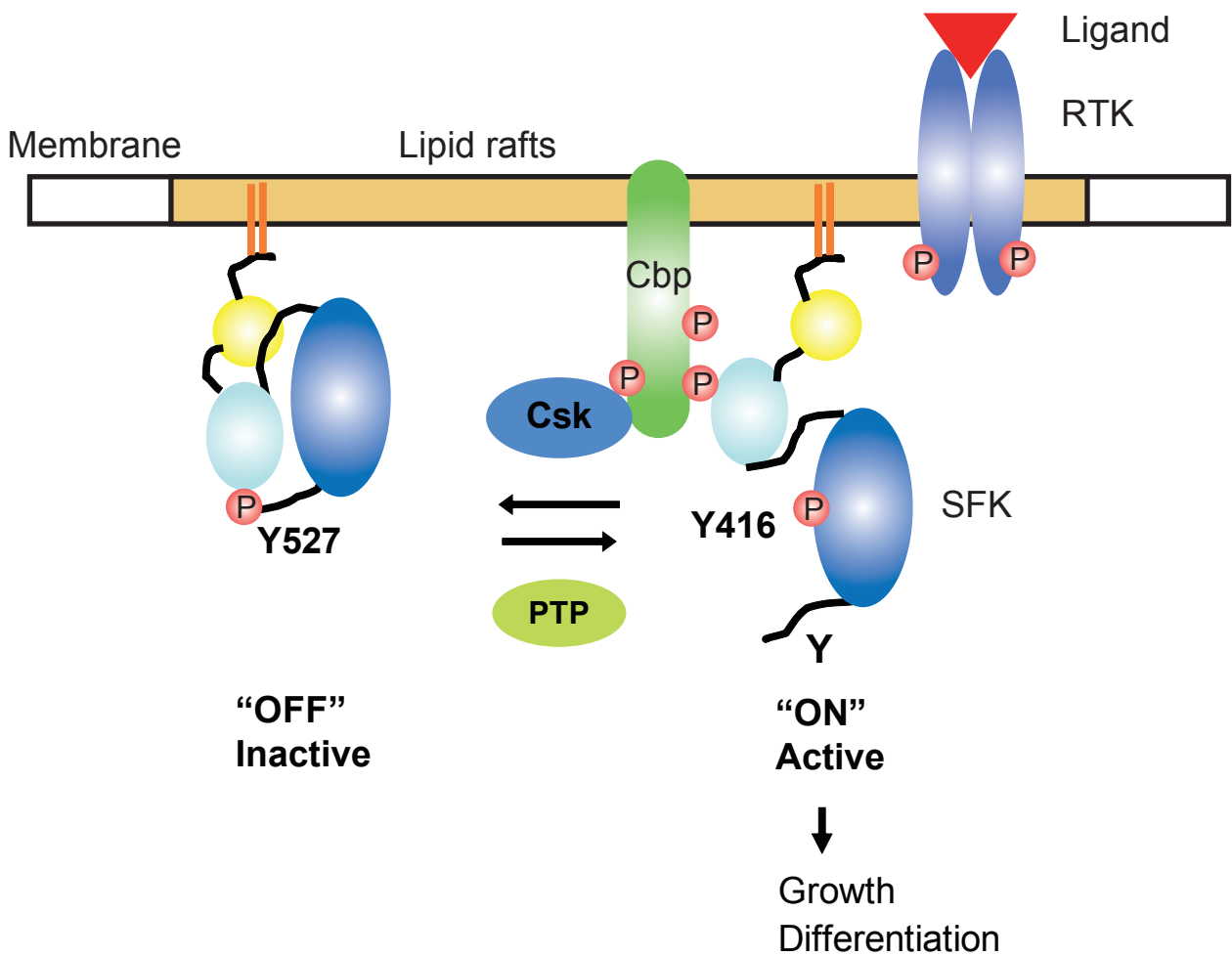
Figure 2. Regulation mechanism of Src family tyrosine kinases

Most of SFK members are preferentially localized in lipid rafts where a variety of signaling molecules, including Cbp, are concentrated. Under resting conditions, SFKs are present as an inactive form with a closed conformation. In response to extracelluar stimuli, SFKs become activated by displacement of inhibitory molecular interactions through binding to some adaptor proteins or by dephosphorylation of C-terminal tyrosine residue (Y527). Full activation involves autophosphorylation of tyrosine residue in the activation loop of SFKs (Y416). Activated SFKs phosphorylate several substrates, including Cbp, which induces recruitment of Csk to lipid rafts to allow the close interaction between SFK and Csk. The C-terminal tyrosine of SFKs is phosphorylated by Csk, which again induces the intramolecular domain interactions, resulting in a closed "inactive" conformation.
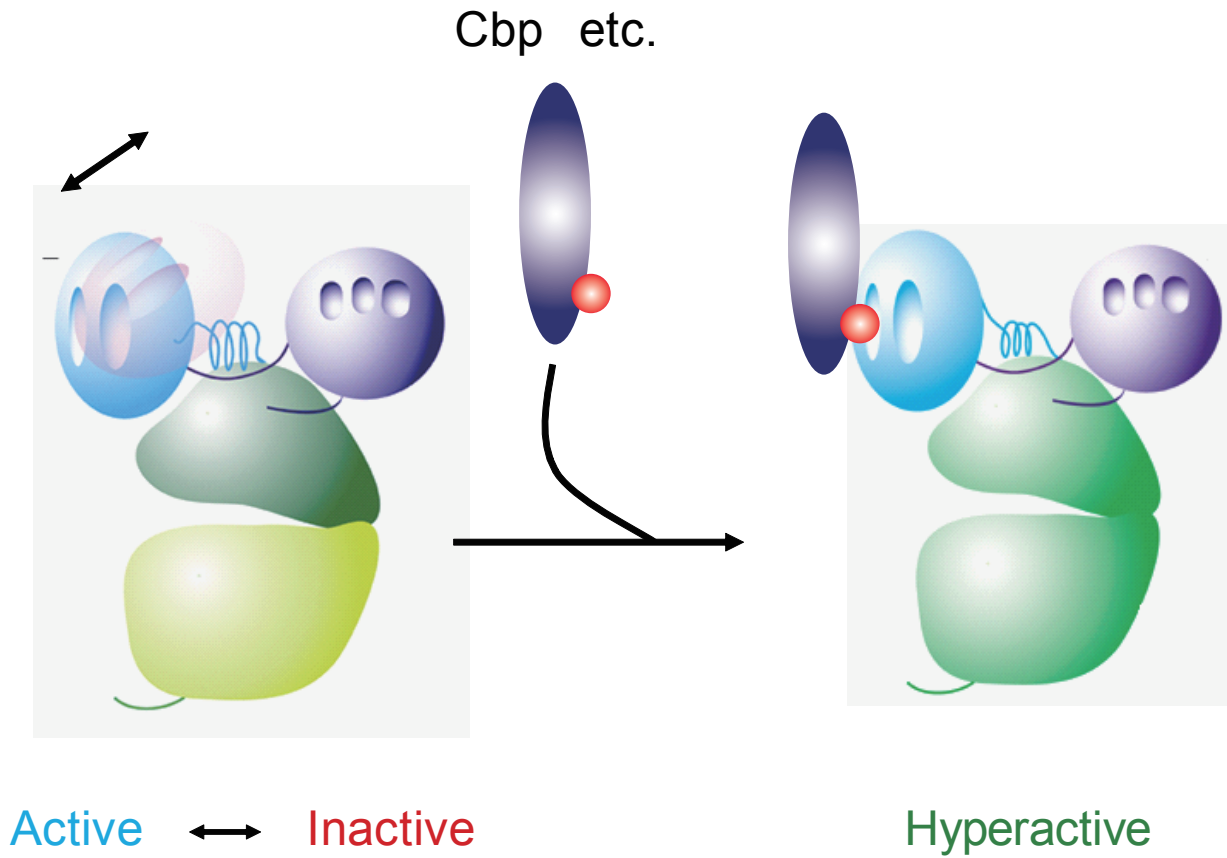
Figure 3. Regulation of Csk kinase activity
Schematic model of the structures of Csk. The crystal structure of Csk reveals that Csk has two different conformations, the active and the inactive forms. The SH2 domain of active and inactive molecules are shown in blue and red, respectively (left). Binding of a strong ligand, such as the phosphorylated Cbp/PAG, to the SH2 domain could elicit potentially full activity of Csk
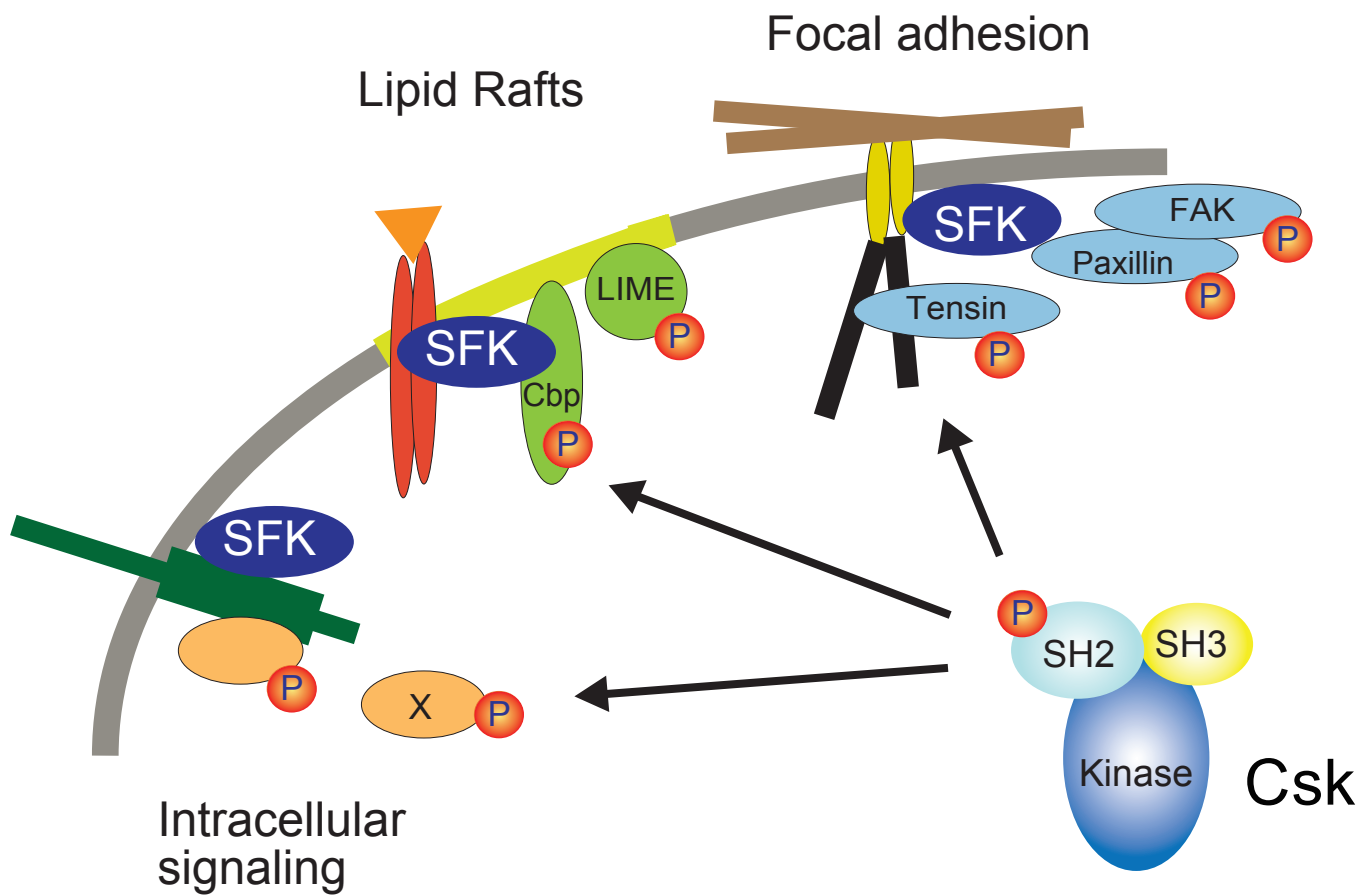
Figure 4. The diverse SFK functions are regulated by Csk *via* site specific adapter molecules

The kinase activity of SFKs is negatively regulated by the phosphorylation of a C-terminal regulatory Tyr by Csk. Since Csk is a cytoplasmic enzyme, it exploits adaptor proteins to efficiently access to the membrane-anchored SFKs. A variety of Csk-binding proteins, including paxillin, FAK and Tensin in focal adhesion, and Cbp and LIME in lipid rafts, have been identified. The SFK functions at specific sites are regulated *via* these site specific adapter molecules.
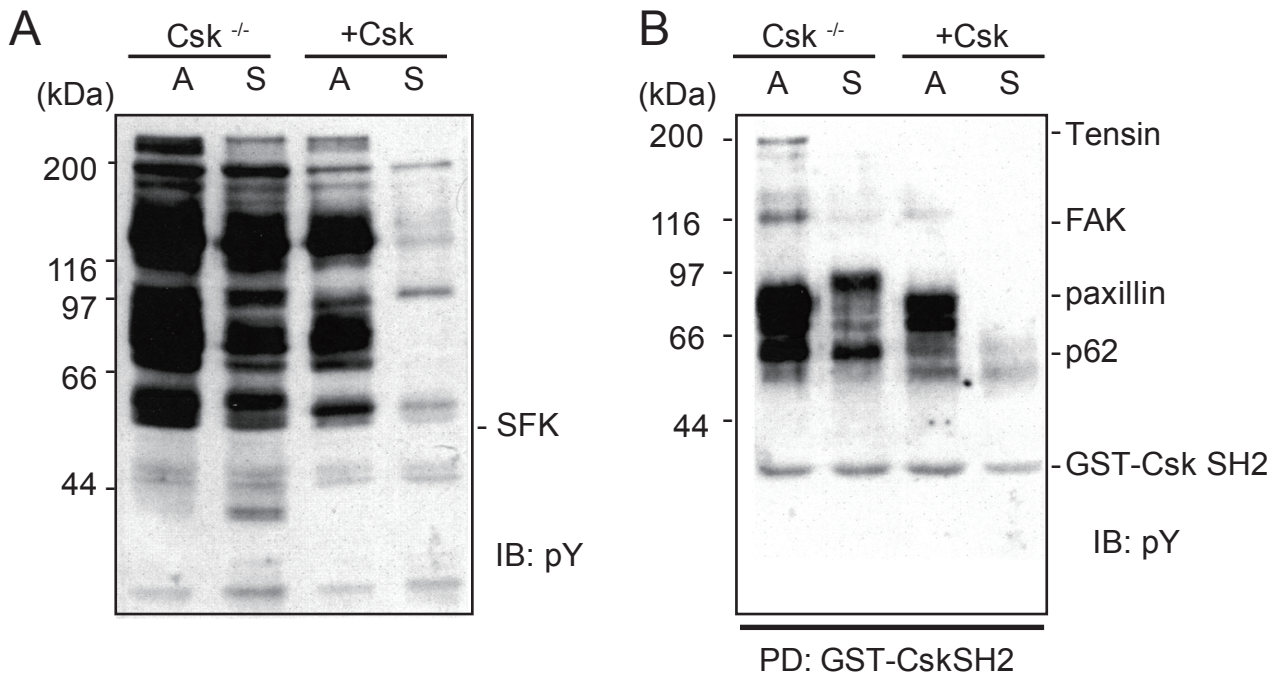
Figure 5. Interaction of Csk SH2 domain with phosphoproteins in Csk⁻/⁻ MEFs

A. Monolayer cultures of Csk⁻/⁻ MEFs and Csk⁻/⁻ + Csk MEFs were lysed with ODG buffer (A: adhered cells). The monolayer cultures were trypsinized, resuspended and held in suspension at 37 ° C for 1 h. These cells were collected and lysed with ODG buffer (S: suspended cells). The samples were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine (pY). **B.** Association of GST-Csk SH2 with 62kDa phosphoprotein in Csk⁻/⁻ MEFs. The samples pulled-down with GST-Csk SH2 were immunoblotted with anti-phosphotyrosine (pY).
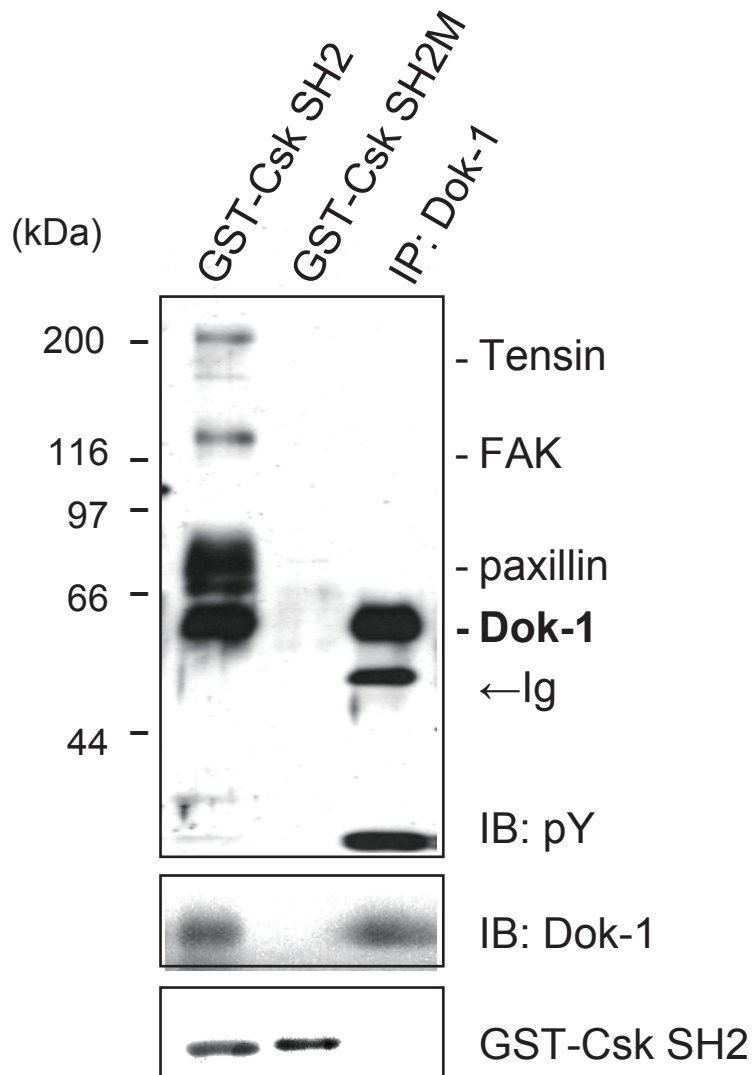
Figure 6. Dok-1 is a major component of p62 phosphoproteins
Whole cell lysates of Csk$^{-/-}$ MEFs were subjected to pull-down assay using GST fusion proteins fused to wild-type Csk SH2 or SH2 R107K mutant (SH2M). Dok-1 was immunoprecipitated from these cells. The precipitated samples were analyzed by immunoblotting with anti-phosphotyrosine and anti-Dok-1. Locations of identified proteins are shown on the right of the panel.
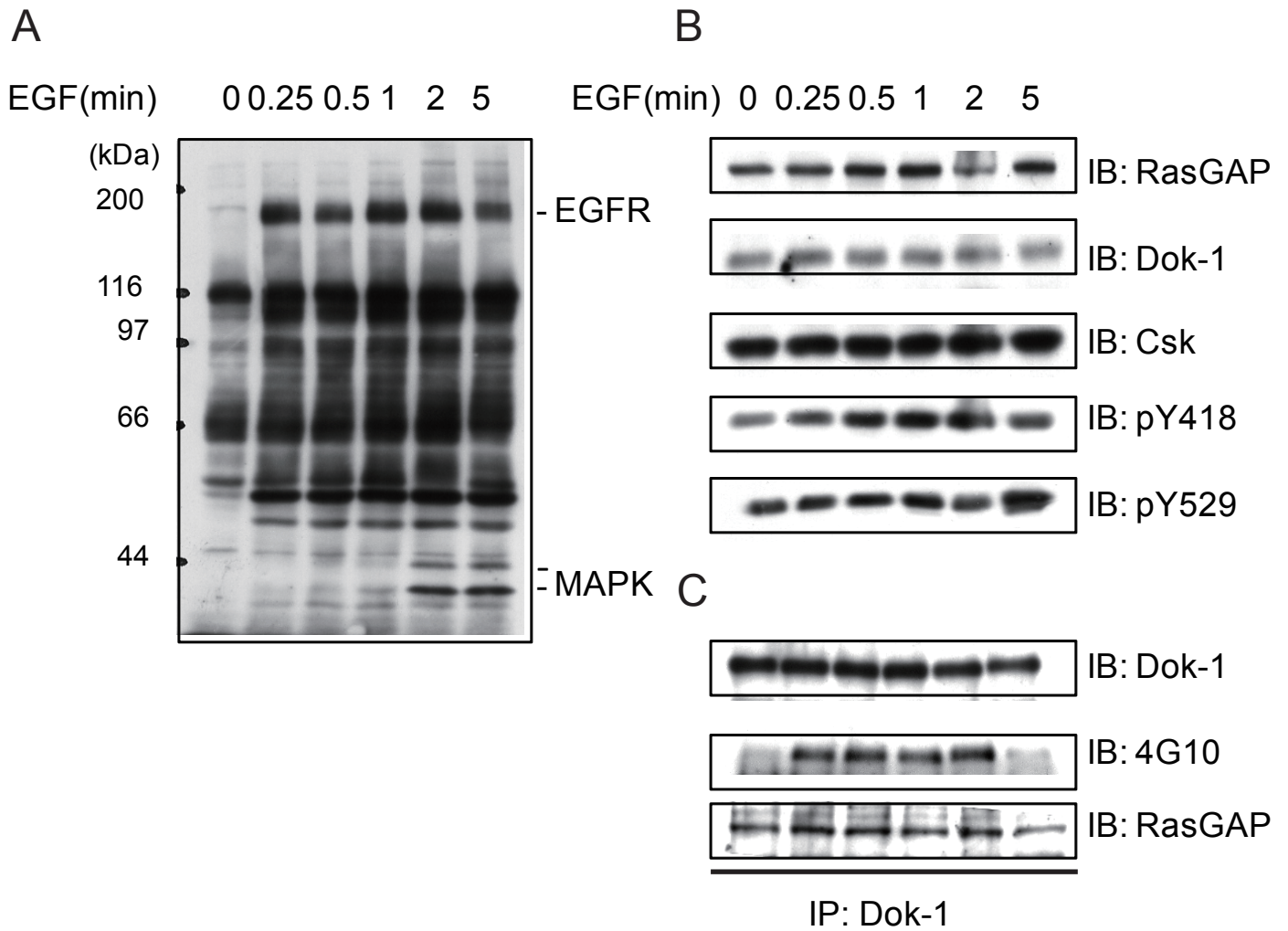
Figure 7. EGF stimulation of MEFs induces Dok-1 phosphorylation
A. and B. MEFs were starved overnight in serum-free DMEM, and then incubated with 100 ng/ml EGF for the indicated periods. The cells were lysed with ODG buffer, followed by immunoboloting with anti-phosphotyrosine (pY; A) and the indicated antibodies (B). C. Immunoprecipitated Dok-1 was subjected to imunoblotting with anti-pY, anti-Dok-1 anti-RasGAP.
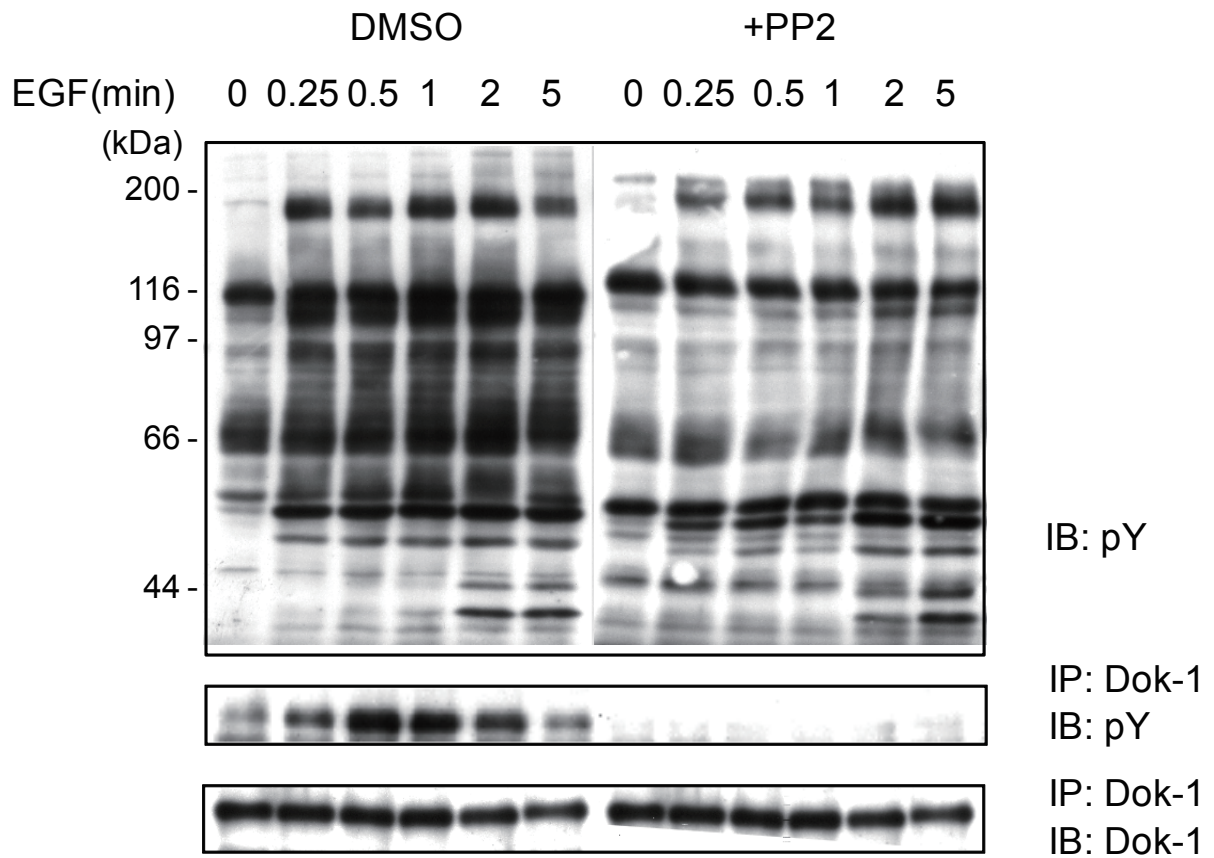
Figure 8. Inhibition of SFK activity reduces Dok-1 phosphorylation

Serum starved MEFs were pre-incubated with 5 µM PP2 or DMSO for 1 h and then stimulated with 100 ng/ml EGF for the indicated periods. Whole cell lysates (upper panel) and the immunoprecipitated Dok-1 (middle panel) were immunoblotted with the anti-pY. Dok-1 protein in the immunoprecipitate was confirmed by immunoblotting with anti-Dok-1 (lower panel).
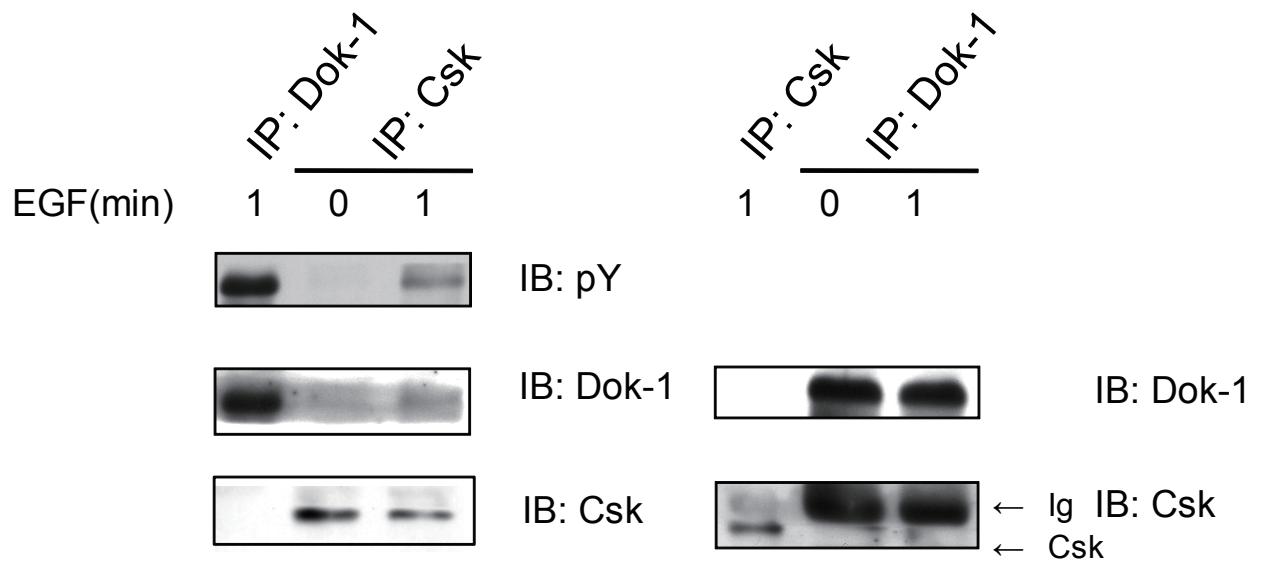
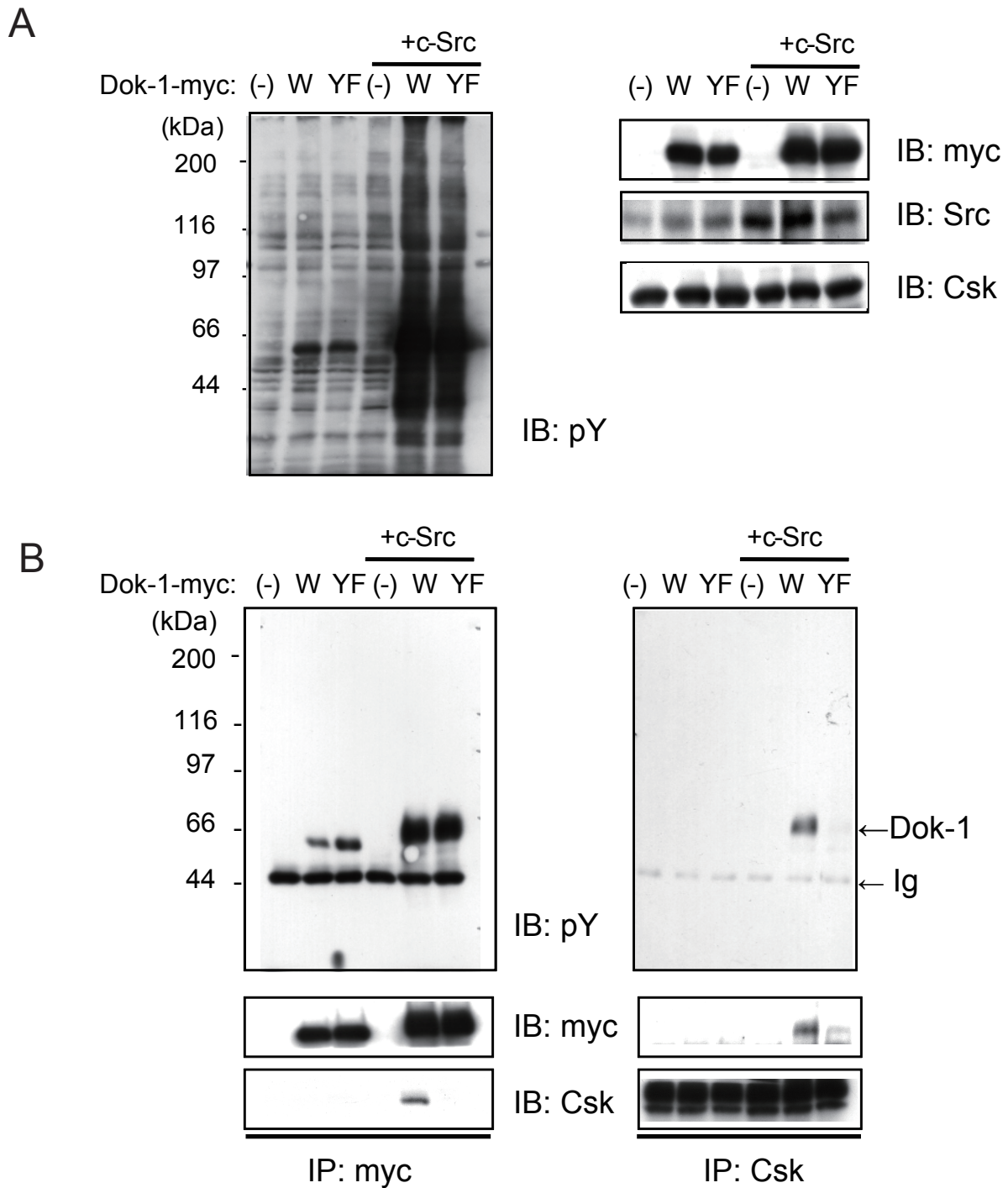**Figure 9. Interaction of Dok-1 with Csk in EGF stimulation**
Serum starved MEFs were pre-incubated with 5 µM PP2 or DMSO for 1 h and then stimulated with 100 ng/ml EGF for 1 min. Dok-1 and Csk were immunoprecipitated, and the complexes were immunoblotted with pY, anti-Dok-1 and anti-Csk.
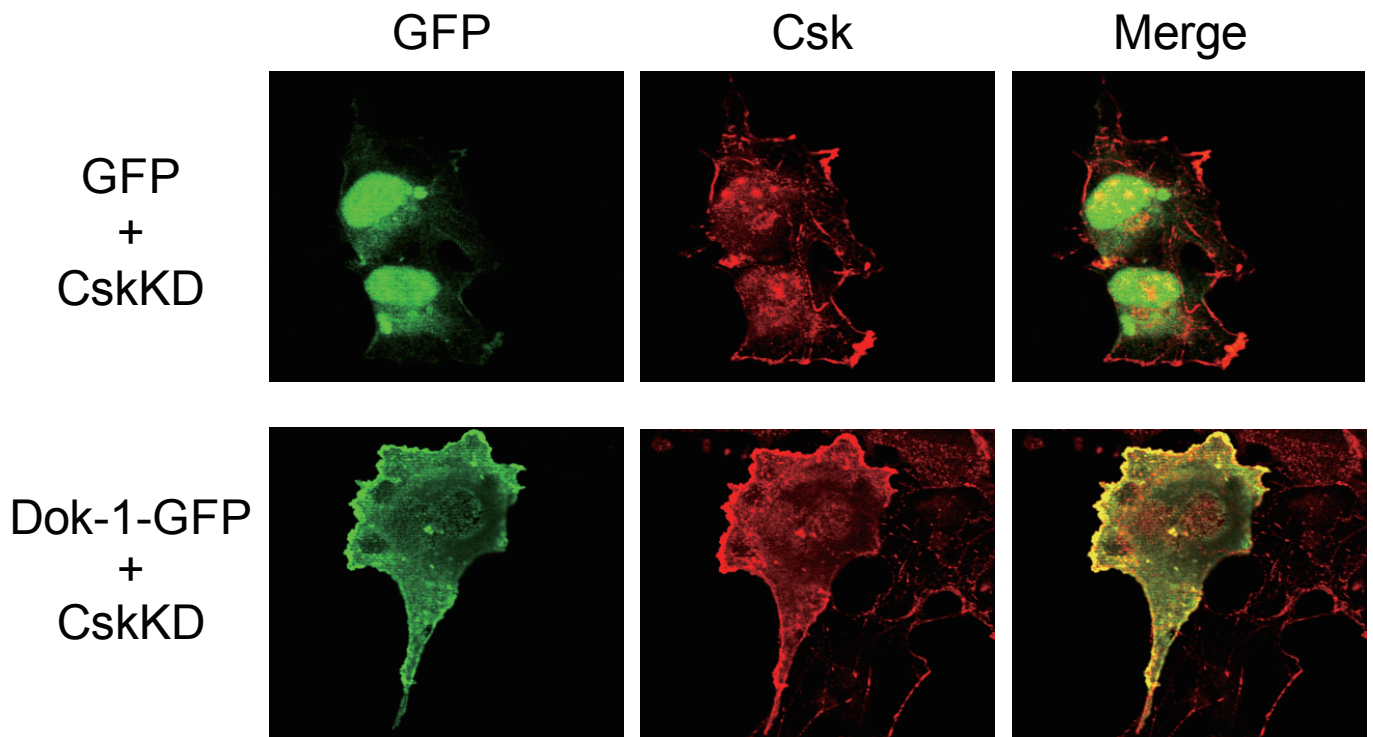
Figure 10. Co-localization of Dok-1 with Csk in COS7 cells

COS7 cells were transiently co-transfected with the vectors encoding Csk and GFP (upper panels) or Dok-1-GFP (lower panels). After 48 h, the cells were fixed and Csk was immunostained with anti-Csk antibody coupled with Alexa 594-conjugated goat anti-rabbit antibody (red). Dok-1 was located by GFP signals (green). Merged images are also shown (yellow). In COS7 cells, the level of endogenous Dok-1 in COS7 cells was negligible compared with that of exogenously expressed Dok-1-GFP.

Figure 11. Interaction of Dok-1 with Csk *via* pY450
A. COS7 cells were transfected with the indicated combinations of the expression vectors, and the whole cell lysates were immunoblotted with anti-pY, anti-myc and anti-Csk. (-): Mock, W: wild-type, YF: Y450F mutant. **B.** Dok-1-myc (left panels) and Csk (right panels) were immunoprecipitated, and the complexes were immunoblotted with anti-pY, anti-myc and anti-Csk.
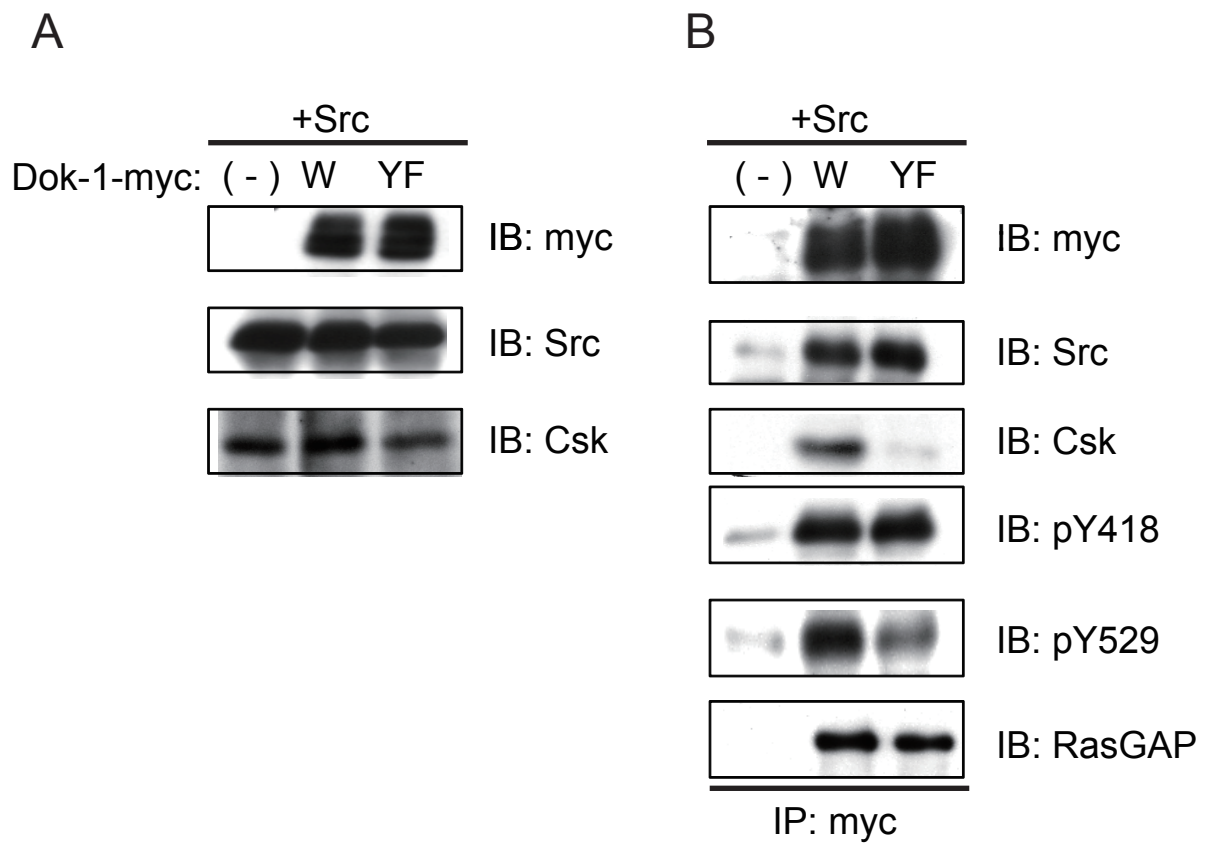
Figure 12. Interaction of Csk with Dok-1 regurates Src kinase activity
A. COS7 cells were transfected with the indicates combinations of the expression vectors, and the whole cell lysates were immunoblotted with anti-myc, anti-Src and anti-Csk. (-): Mock, W: wild-type, YF: Y450F mutant. **B.** Dok-1-myc was immunoprecipitated, and the complexes were immunoblotted with the indicated antibodies.
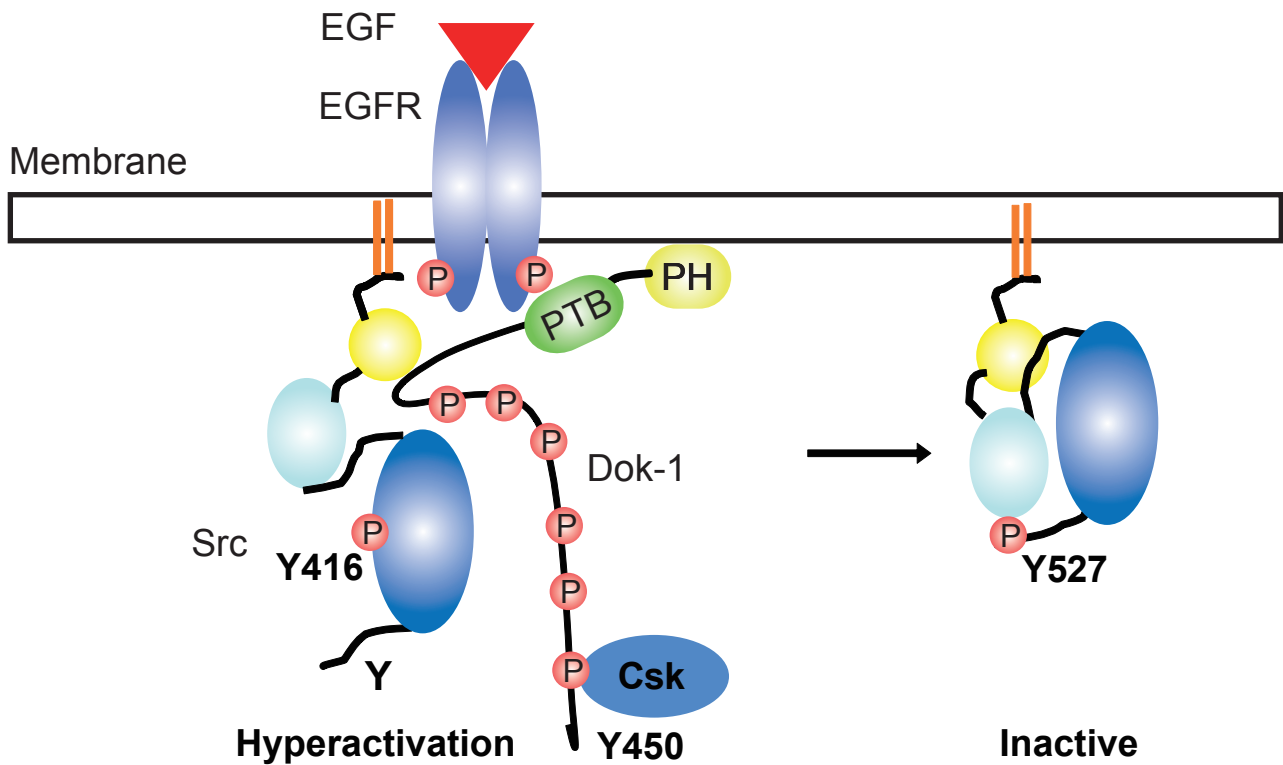
Figure 13. Dok-1 regulates Src activity through processive recruitment of Src and Csk

Src is tightly and dynamically maintained between active (pY416) and inactive (pY527) conformation. In the absence of growth factor stimulation (ex. EGF stimulation), a portion of Src constitutively interacts with Dok-1. On growth factor stimulation, Src is activated and phosphorylates Dok-1. Phosphorylated Dok-1 interacts with Src via SH3 or SH2 domain, where a transient Src hyperactivation occurs. Src dependent phosphorylation of Dok-1 Y450 facilitates the recruitment of Csk and subsequent inhibition of Src kinase activity.

Chapter 2

Proteomic analysis of novel Csk binding proteins involved in the regulation of c-Src transformation

Abstract

In Chapter 1, I identified Dok-1 as a scaffolding molecule for SFKs and Csk, and showed that Dok-1 regulates SFKs activity downstream of EGF receptor. In this chapter, to further elucidate the role of Csk-mediated SFK regulatory system, I explored more widely the scaffolding proteins for SFKs and Csk that are potentially involved in the regulation of cell transformation induced by c-Src. For this, I performed a proteomic analysis of tyrosine phosphorylated proteins that interact with c-Src and/or Csk. The c-Src interacting proteins were affinity-purified with Src SH2 domain from Src transformed cells. LC-MS/MS analysis identified several Src substrates, such as focal adhesion kinase, paxillin and ZO-1/2, as a transformation-dependent Src target. The Csk binding proteins were analyzed by a tandem affinity purification method (TAP). In addition to the previously identified Csk binding proteins, including Cbp/PAG, paxillin, and caveolin-1, I found that ZO-1/2 could also serve as a major Csk binding protein. ZO-2 was phosphorylated concurrently with Src transformation and specifically bound to Csk in a Csk SH2 dependent manner. These results suggest novel roles for ZO proteins as Src/Csk scaffolds potentially involved in the regulation of Src transformation.

Introduction

c-Src, a non-receptor tyrosine kinase, is the cellular prototype of v-Src, an oncogene product of Rous sarcoma virus (Takeya and Hanafusa, 1983). Since c-Src is frequently overexpressed and activated in a wide variety of advanced human cancers, such as colon and breast cancers, it has been implicated in malignant conversion in human cancers (Ishizawar and Parsons, 2004; Yeatman, 2004). Numerous studies have shown that the activation of c-Src induces the tyrosine phosphorylation of various cellular proteins including focal adhesion kinase (FAK) (Hanks and Polte, 1997), Cas and Crk (Sakai et al., 1994), Shc (Verderame et al., 1995), phosphatidyl inositol 3 kinase (PI3K) (Penuel and Martin, 1999), and signal transducers and activators of transcription 3 (STAT3) (Yu et al., 1995), resulting in the activation of signaling pathways associated with cancer progression. However, the complexity of these c-Src pathways have made it difficult to elucidate which pathways are critical for c-Src induced cell transformation and how these pathways are suppressed in normal cells.

The kinase activity of c-Src is negatively regulated by the phosphorylation of a C-terminal regulatory Tyr by C-terminal Src kinase (Csk) (Nada et al., 1991; Nada et al., 1993). Since Csk is a cytoplasmic enzyme, it exploits adaptor proteins to efficiently access to the membrane-anchored c-Src and its relatives (Src family kinases; SFKs). To date, a variety of Csk-binding proteins including paxillin and FAK (Sabe et al., 1994), Dok-1 (Neet and Hunter, 1995), LIME (Hur et al., 2003), caveolin-1 (Cao et al., 2002), and Cbp/PAG (Brdicka et al., 2000; Kawabuchi et al., 2000), have been identified. Furthermore, it has been shown that these Csk scaffold proteins could serve as substrates of SFKs by directly interacting with SFKs. These observations suggest that the diverse SFK functions are precisely regulated by Csk on a variety of Csk scaffold proteins whose expressions are temporally and spatially regulated within the cells.

However, it remains unclear which Csk scaffold proteins are involved in the regulation of the transforming ability of c-Src.

To address the regulatory mechanism of Src induced cell transformation, I here adopted a proteomic analysis of phosphoproteins that interact with c-Src and/or Csk in Src transformed cells. Recently, we have developed an experimental system using Csk-deficient mouse embryonic fibroblasts (Csk$^{-/-}$ MEF), in which wild-type c-Src can induce cell transformation (Oneyama et al., 2008). Using this system, I identified zona occludens-1/2 (ZO-1/2) as a new Src/Csk scaffolding protein that is potentially involved in the regulation of c-Src transformation.

Materials and Methods

Cell culture and retroviral-mediated transfection

$Csk^{-/-}$ and sibling $Csk^{+/+}$ MEFs immortalized using the simian virus 40 large T antigen were kind gifts of from Dr. Akira Imamoto (Thomas et al., 1995). These cells were cultured in DMEM supplemented with 10% FBS. All of the gene transfer experiments were carried out by using retroviral vectors, pCX4, as described previously (Akagi et al., 2003; Oneyama et al., 2008). Retroviral vectors encoding wild-type chicken c-Src and its constitutively active form (c-SrcYF) were kindly provided by Dr. Tsuyoshi Akagi. Wild-type rat Csk was expressed using a retroviral vector pCX4bleo. For transient expression in HEK293T cells, pcDNA3.1 (Invitrogen) was used for the expressions of c-Src, Csk, and Csk-HA. pCMV-Tag-1 (STRATAGENE) and pFLAG-CMV-2.1 (SIGMA) were used for ZO-2-myc and FLAG-ZO-2, respectively. Transfections were carried out using Lipofectamine 2000 (Invitrogen).

Immunochemical analysis and F-actin staining

Immunoblotting and immunoprecipitation were performed as described previously (Shima et al., 2003). The following antibodies were used: anti-phosphotyrosine (anti-pY: 4G10, Upstate), anti-PELP1 (Bethyl), anti-ZO-2 (Zymed), anti-FAK (C-20, Santa Cruz), anti-Cas (BD Biosciences), anti-GIT1 (H-170, Santa Cruz), anti-cortactin (Upstate), anti-Sam68 (C-20, Santa Cruz), anti-paxillin (Transduction Lab.), anti-Csk (C-20, Santa Cruz), anti-Csk mAb (Transduction Lab.), anti-myc (PL-14, MBL), anti-HA (Zymed), and anti-FLAG (M2, SIGMA). For F-actin staining, the fixed cells were stained with Alexa 594-phalloidin as described previously (Oneyama et al., 2008).

Soft-agar colony-formation assays

Single-cell suspensions of 4×10^4 cells were plated per 60-mm culture dish in 3 ml of DMEM containing 10 % FBS and 0.36 % agar on a layer of 5 ml of the same medium containing 0.7 % agar. Two weeks after plating, colonies were stained with 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Purification of Src SH2 domain binding proteins

Detergent soluble (non-Raft) fractions form Src transformed cells were prepared as described previously (Shima et al., 2003). Briefly, cells were lysed in a buffer (0.25% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4 and protease inhibitors), and separated by ultracentrifugation on a discontinuous sucrose gradient (5%-35%-40%). The heavy fractions containing 40% sucrose were collected and used as non-Raft fractions. A fusion protein of Src SH2 domain with GST (GST-Src SH2) was expressed in *Escherichia coli* BL21 using pGEX-6P-1 vector, and coupled with glutathione-Sepharose beads (GE Healthcare). The washed beads were incubated with non-Raft fractions at 4 °C for 2 h, and then collected by centrifugation. After extensive washing, the proteins complexed with GST-Src SH2 were eluted with 20 mM glutathione.

Tandem affinity purification of Csk binding protein

For tandem affinity purification (TAP) (Fig.1) of Csk binding protein, a kinase-deficient Csk (K222R) which was tandemly tagged at its C-terminus with an immunoglobulin G (IgG)-binding units of protein A from *Staphylococcus aureus*, a cleavage site for the tobacco etch virus (TEV) protease and a calmodulin binding peptide (CBP) (Burckstummer et al., 2006), was stably expressed in Csk-deficient cells using

pCX4bleo vector. The cells were lysed in ODG buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10% glycerol, 1% NP-40, 2% *n*-octyl-β-D-glucoside, 1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitors), and the cleared lysates were incubated with rabbit-IgG Sepharose (GE Healthcare) at 4 °C for 2 h. The beads were washed with ODG buffer and then with TEV-protease cleavage buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40 and 1 mM DTT) and the bound proteins were eluted by addition of TEV protease (16 °C for 1 h). The cleaved product was then incubated with calmodulin Sepharose (GE Healthcare) in the presence of 2 mM $CaCl_2$, and the bound proteins were eluted with EGTA.

Sample preparation and LC-MS/MS analysis

The purified protein complexes were separated by SDS-PAGE on a 10%T gel. After visualization of the proteins by silver staining, specific bands and/or regions of interest were excised from the gel and digested *in situ* with Achromobacter protease I (Lys-C) (Wako, Osaka) or TrypsinGold (Promega) essentially as described (Shevchenko et al., 1996). The digested samples were analyzed by nanocapillary reversed-phase LC-MS/MS using a C18 column (ϕ 75 µm) on a nanoLC system (LC Packing) coupled to a quadrupole time-of-flight mass spectrometer (QTOF Ultima, Waters). Direct injection data-dependent acquisition was performed using one MS channel for every three MS/MS channels and a dynamic exclusion for selected ions. Proteins were identified by database searching (Mascot Daemon, Matrix Science). Search parameters were as follows: MS tolerance of 0.2 Da, MS/MS tolerance of 0.4 Da, protease specificity allowing for one missed cleavage site, fixed modification of carbamidomethylation of cysteine residues, variable modification of oxidation of methionine residues, and variable modification of phosphorylation of tyrosine residues.

Results

c-Src induced transformation of Csk-deficient fibroblasts

Recently, we have developed an experimental system using Csk-deficient mouse embryonic fibroblasts ($Csk^{-/-}$), in which wild type c-Src can induce cell transformation (Oneyama et al., 2008) (Fig.2). Using the $Csk^{-/-}$ cells, we established a series of cell lines with different transformed phenotypes (Fig.2). $Csk^{-/-}$ cells had elevated levels of tyrosine phosphorylation of cellular proteins compared with Csk expressing cells, but were not sufficient for cell transformation (Oneyama et al., 2008). Overexpression of c-Src induced further elevation of tyrosine phosphorylation and transformed the cell. Expression of constitutively active form of c-Src, that has a Tyr to Phe substitution at the regulatory site (c-SrcYF), induced dramatic elevation of tyrosine phosphorylation concurrent with cell transformation(Fig.2 and 3). By comparing these cell lines, I first attempted to identify the critical c-Src targets required for cell transformation.

It is known that substantial fractions of c-Src and other SFK members are localized in a membrane microdomain, lipid raft, via their N-terminal fatty acid moiety (Galbiati et al., 2001). However, the elevation of tyrosine phosphorylation of raft proteins was not so remarkable as those in non-raft fractions (Fig.3). Furthermore, our recent analysis suggested that the transforming ability of c-Src is rather suppressed when c-Src is localized in rafts (unpublished observation). In this study, I thus focused on tyrosine phosphorylated proteins present in non-Raft fractions.

Identification of Src SH2 interacting proteins

To identify the critical Src targets in transformed cells, I first attempted to identify the Src interacting proteins. To evaluate the role of c-Src in cell transformation, I have recently developed an experimental system using Csk-deficient mouse embryonic

fibroblasts (Csk$^{-/-}$), in which c-Src can induce cell transformation (Oneyama et al., 2008). In this study, I used Csk$^{-/-}$ cells overexpressing c-Src or an active form of c-Src with a Tyr to Phe substitution at the regulatory site (c-SrcYF) as Src transformed cells. It is known that substantial fractions of c-Src are localized in a membrane microdomain, lipid rafts (Galbiati et al., 2001). However, as our recent analysis suggested that the localization of c-Src in non-raft compartments is critical for cell transformation (unpublished observation), I used non-raft fractions as starting materials. The non-raft fractions prepared from Src transformed cells were incubated with GST-Src SH2, and the bound proteins were separated on a SDS–PAGE gel (Fig.4 A). The LC–MS/MS analysis of the visible bands resulted in the identification of whole 24 proteins (data not shown). The representative proteins are shown on the right of the panel (Fig.4 A), and the identities of some representative proteins were confirmed by immunoblotting (Fig.4 B). A representative MS/MS spectrum assigned to ZO-2 is shown in Fig.5.

The identified proteins included well-characterized major Src targets, such as FAK, Cas, cortactin, Sam68, paxillin, and GIT1, indicating that our experimental system was functional. In addition to these, I identified poorly characterized Src targets, some of which are potentially involved in the Src signaling. Of these candidates, I identified ZO-1/2 as a Src interacting protein which was specifically detected in transformed cells (Fig.4 A and B). The tyrosine phosphorylation of ZO-1/2 by v-Src has been previously observed (Kaihara et al., 2003; Takeda and Tsukita, 1995), but its precise role in cell transformation still remains unclear. ZO-1/2 is a membrane-associated guanylate kinase (MAGUK) protein composed of three PDZ domains, an SH3 domain, a GuK domain, and an actin binding proline-rich domain (Fig.10) (Gonzalez-Mariscal et al., 2000). The PDZ1 domain and the GuK domain bind to claudins and occludens, respectively, indicating its critical roles as tight junction

proteins in epithelial cells (Umeda et al., 2006). However, it is also known that SH3-GuK domains are responsible for the binding to α-catenin and the proline-rich domain binds to actin filaments; these implicate the role for ZO proteins in the regulation of cadherin-based cell adhesion as well as cytoskeletal organization (Itoh et al., 1997). These observations suggest that ZO-1/2 would play crucial roles in the regulation of cell transformation.

Identification of Csk binding proteins

On the other hand, it is also known that the critical Src targets can interact with the Src inhibitor Csk. Csk contains an SH2 and an SH3 domains (Fig.6 A), and the SH2 domain is shown to interact with tyrosine phosphorylated Src targets (Sabe et al., 1994). Recruitment of Csk to the Src targets is believed to be critical for the efficient termination of the Src signaling. In this context, I next analyzed Csk binding proteins to identify functional Src targets. To comprehensively explore the proteins that specifically bind to Csk in the cells, I adopted a tandem affinity purification (TAP) method (Fig.1 and 6 A). To maintain the active state of c-Src in the transformed cells, I introduced a kinase-deficient form of tagged Csk (CskKD-TAP) or its mutant (SH3/2mt-TAP) in either non-transformed or c-Src transformed cells (Fig.6 B).

The Csk complexes were purified by a sequential column chromatography, and were separated by an SDS–PAGE (Fig.6 B, left panel). Immunoblot analysis showed that the Csk complex from c-Src transformed cells gave some specific tyrosine phosphorylated proteins, suggesting that there are some Csk binding proteins involved in cell transformation (Fig.6 B, right panel). The LC–MS/MS analysis of the silver-stained bands resulted in the identification of listed proteins that include known Csk binding proteins, such as Tensin, paxillin, Cbp, and caveolin-1 (Fig.7 and

Supplementary Data). In addition, we also identified ZO-1/2 as a major Csk binding protein (Fig.7 and S1) that corresponds to the tyrosine phosphorylated protein detected specifically in c-Src transformed cells (Fig.6 B). These results together with the identification of ZO-1/2 as a Src interacting protein raised the possibility that ZO-1/2 would be a critical scaffolding protein for the Src/Csk regulatory circuit.

Interaction of ZO-2 with Csk

To address the role of ZO-1/2 in cell transformation, the tyrosine phosphorylation status of ZO-2 was compared between transformed and non-transformed cells (Fig.8 A). ZO-2 in the cells transformed by c-Src or c-SrcYF gave stronger tyrosine phosphorylation signals compared with non-transformed cells, indicating the potential correlation of ZO-2 phosphorylation with cell transformation. The phosphorylation of ZO-2 in Src transformed cells was further confirmed in $Csk^{-/-}$ cells expressing Csk and c-SrcYF (Fig.8 B). Concurrent with transformation by c-SrcYF, Csk bound to various Src targets and ZO-2 became efficiently tyrosine phosphorylated. The interaction between ZO-2 and Csk was examined by an expression system using HEK293 T cells. Ectopically expressed ZO-2 was efficiently tyrosine phosphorylated by the expression of c-SrcYF (Fig.9 A). Immunoprecipitation assay showed that wild-type Csk (W) or its SH3 mutant (3M) was co-precipitated with ZO-2, whereas its SH2 mutant (2M) was less effectively co-precipitated (Fig.9 B). These observations demonstrate that ZO-2 binds to Csk in a manner dependent on the Csk SH2 domain.

Csk binding regions in ZO-2

Next, the interaction between ZO-2 and Csk was further examined using a series of ZO-2 deletion mutants (Fig.10). Full-length ZO-2, the fragment **a** (a.a. 4–652), and the

fragment **e** (a.a. 487–1167) were tyrosine phosphorylated (Fig.11, right middle panel) and co-immunoprecipitated with Csk (right bottom panel). These observations suggest that Csk binds to ZO-2 *via* the region containing Tyr 554, 575, and 618 (Fig.10, gray boxed). However, point mutagenesis studies showed that a single Tyr to Phe mutation of these residues did not affect the tyrosine phosphorylation of ZO-2 or the interaction with Csk, suggesting the redundant role of these residues in the interaction with Csk (data not shown). Although the most critical binding sites are yet to be determined, these results demonstrate that Csk could directly and specifically interact with ZO-2 in the cells.

Discussion

In this study, I have identified ZO-1/2 as a new scaffold for Src/Csk in Src transformed cells. The correlation between ZO-2 phosphorylation and Src transformation raised the possibility that the recruitment of Csk to ZO proteins might be involved in the suppression of transforming activity of c-Src. Many other Src target proteins including paxillin, FAK, Tensin, caveolin-1, LIME, and Cbp, have been shown to have the similar system. These molecules are tyrosine phosphorylated in accordance with the activation of c-Src and subsequently create the binding site for Csk. The recruited Csk inactivates c-Src on the scaffold proteins to efficiently terminate the c-Src signaling. These Csk scaffolds have unique localizations in the cells. Paxillin, FAK, and Tensin are localized to focal adhesions, while caveolin-1, LIME, and Cbp are exclusively localized in cholesterol-enriched membrane microdomain, caveolae or lipid rafts (Galbiati et al., 2001). As these intracellular domains have been shown as the origins of the Src signaling, the Csk scaffold proteins present in these domains are critical regulators of Src-mediated cell responses. In this context, ZO proteins should also play roles as another regulator of c-Src acting at a specific domain such as cell–cell junction. The activity of c-Src has been implicated in the regulation of epithelial-mesenchymal transition (EMT), which is a critical process of cancer progression (Frame, 2004; Rengifo-Cam et al., 2004). Since ZO proteins have been shown to interact not only with tight junction but also with adherence junction and actin filaments (Itoh et al., 1997), it is likely that ZO proteins contribute to the regulation of the multiple steps of EMT, that include cell–cell adhesion in epithelial cells and cytoskeletal remodeling in mesenchymal cells. Indeed, although the effect was not so remarkable, tyrosine phosphorylation of ZO proteins by v-Src has been shown to weaken junctional sealing (Takeda and Tsukita, 1995). A unique role of ZO-1 in regulating gene expression

associated with tumor invasion has also been reported (Polette et al., 2007). The more detailed analyses of the functions of ZO proteins as Src/Csk scaffolds would provide new clues to understanding the regulatory mechanisms of cancer progression particularly mediated by c-Src activation.

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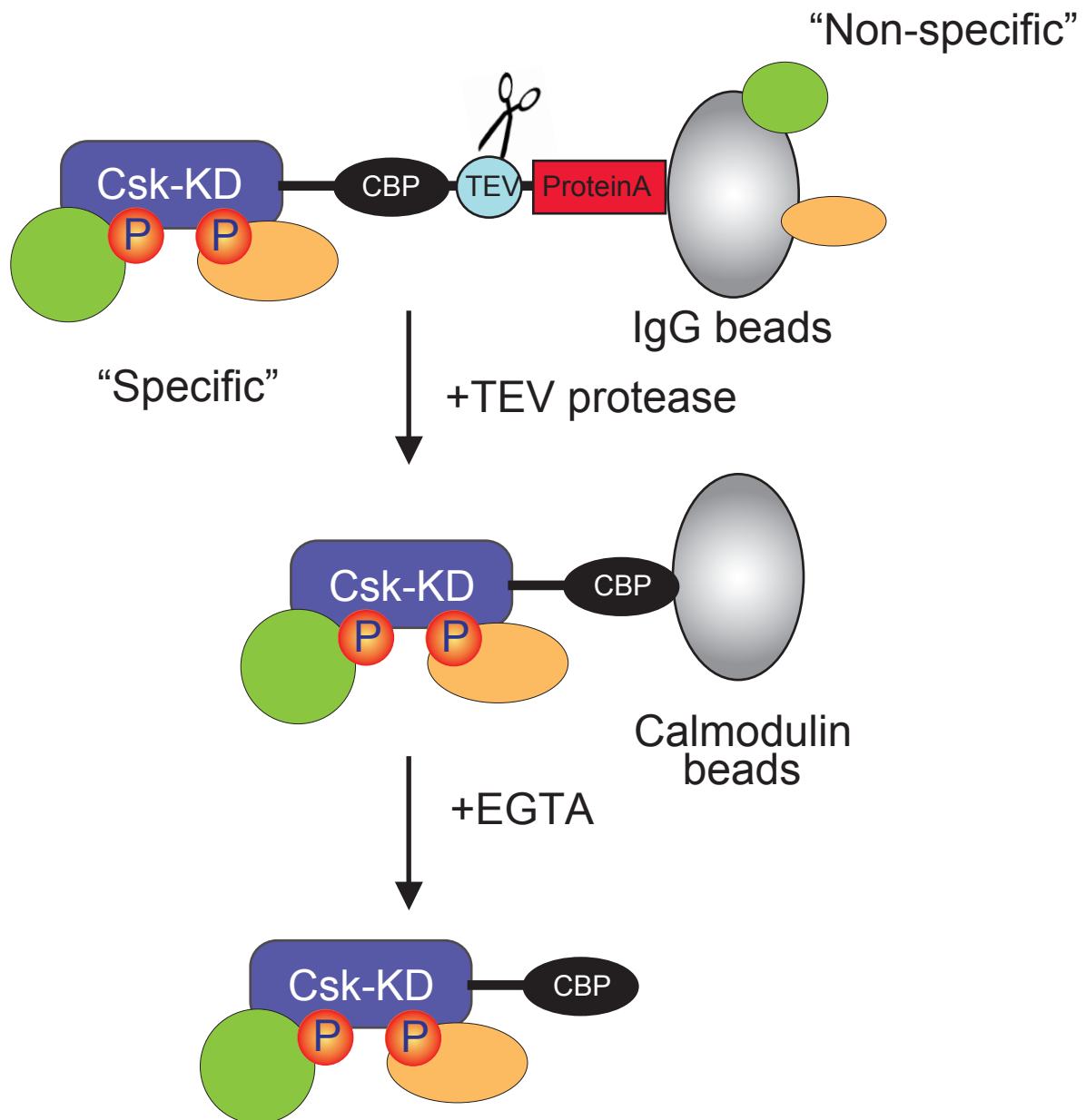
Figure 1. Principle of the TAP strategy

Tandem affinity purification (TAP) method is useful for the determination of protein partners quantitatively *in vivo*. The TAP tag used in this study consists of calmodulin binding peptide (CBP) from the N-terminal, followed by tobacco etch virus protease (TEV protease) cleavage site and Protein A, which binds tightly to IgG. The procedure is as follows; The TAP fused bait protein with the other constituents is collected by means of an IgG beads. After washing, TEV protease is introduced to release the bound material at the TEV protease cleavage site. This eluate is then incubated with calmodulin-coated beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as traces of contaminants remaining after the first affinity step. After washing, the bound material is then eluted with EGTA. The native elution consisting of the bait protein and its interacting protein partners is analyzed by SDS-PAGE, followed by the identification with mass spectrometry.
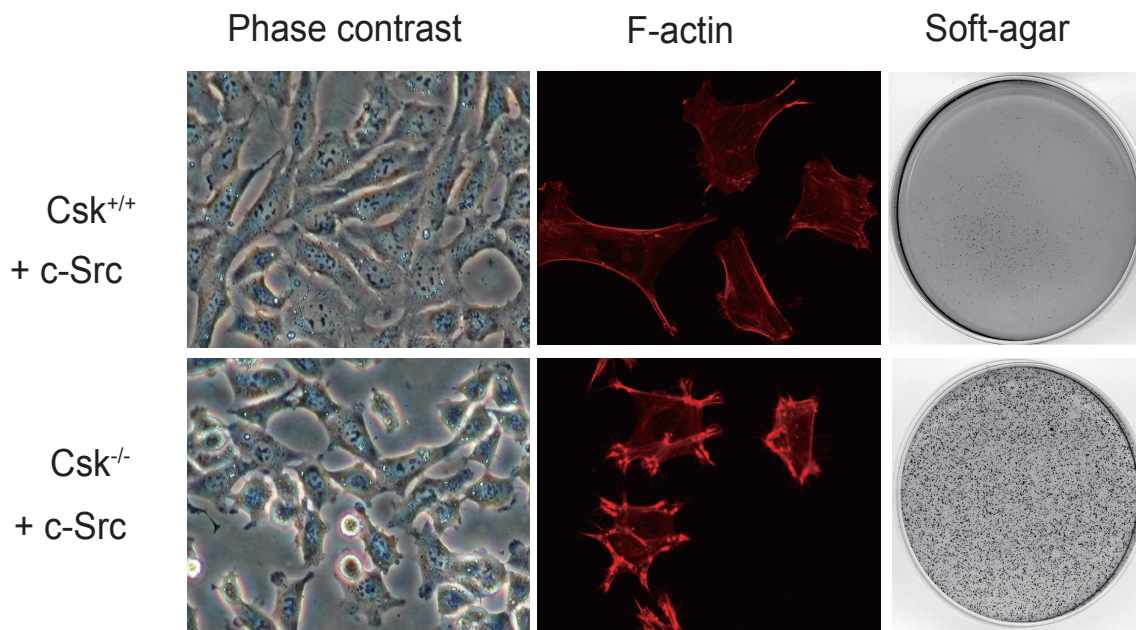
Figure 2. c-Src induced transformation of Csk-deficient fibroblasts

Csk$^{+/+}$ mouse embryonic fibloblasts (Csk$^{+/+}$ MEFs) and Csk$^{-/-}$ MEFs were infected with a retrovirus carrying c-Src. Stable cell lines were selected by treatment with puromycin. Cell morphologies were observed under phase contrast microscopy (left panels).The fixed cells were stained for F-actin with Alexa 594-phalloidin, and observed under confocal microscopy (middle panels). Transforming ability was examined by soft-agar colony formation assay (right panels). Cells (4×10^4) were plated in soft agar, and after 2 weeks, colonies were stained with MTT reagent and photographed.
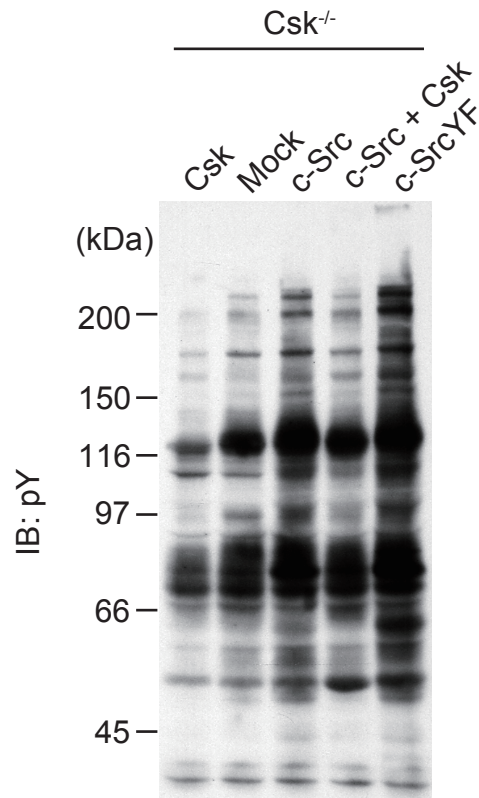
Figure 3. Tyrosine phosphorylation of cellular proteins in various cell types

Csk^{-/-} MEFs were infected with the indicated retrovirus vectors. Whole cell lysates of the stable transfectants were immunoblotted with anti-phosphotyrosine (pY).
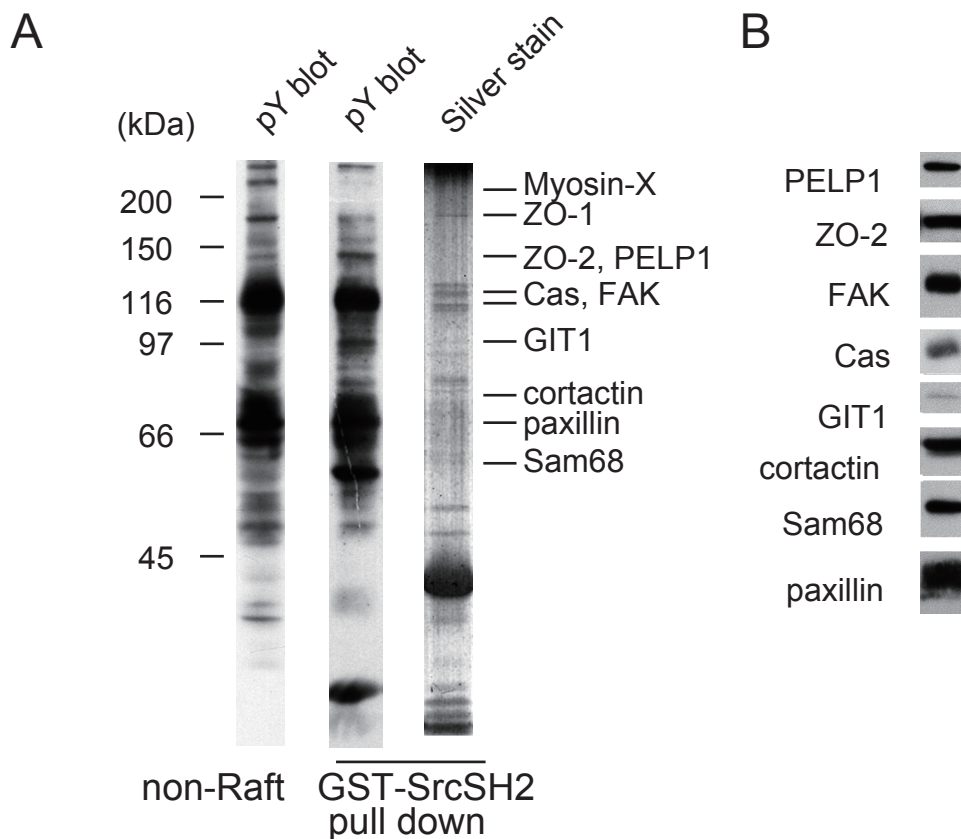
Figure 4. Identification of Src SH2 interacting proteins

A. The non-raft fractions were prepared form Src transformed Csk^{-/-} cells, and the tyrosine phosphorylated proteins were detected with anti-pY (left panel). The samples pulled down with GST-Src SH2 were immunoblotted with pY (middle panel) and stained with silver (right panel). The locations of some identified major proteins are indicated on the right of the panel. **B.** The pull-down samples were immunoblotted with the antibodies to the indicated proteins.

C

HPDIYAVPIK(616.82,2+)=mouse ZO-2 (a.a.1091-1100)
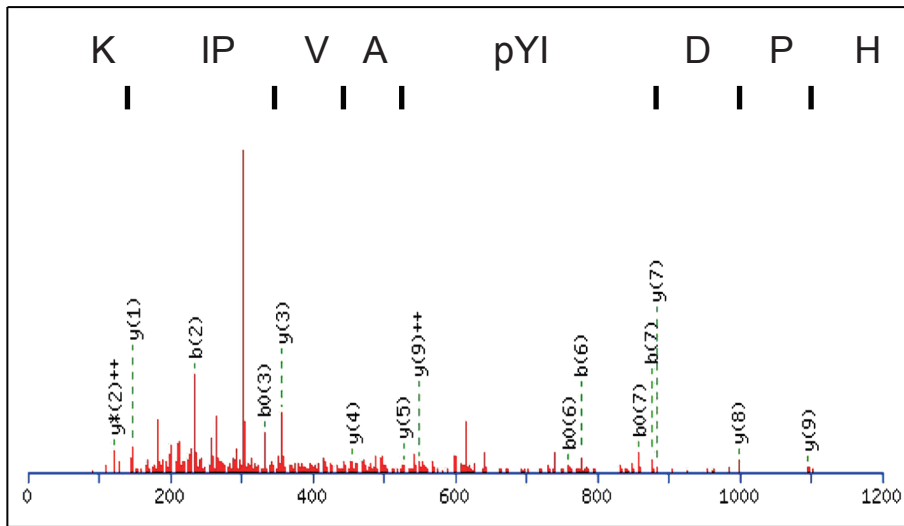
Figure 5. Identification of Src SH2 interacting proteins
A representative MS/MS spectrum assigned to ZO-2 is shown.
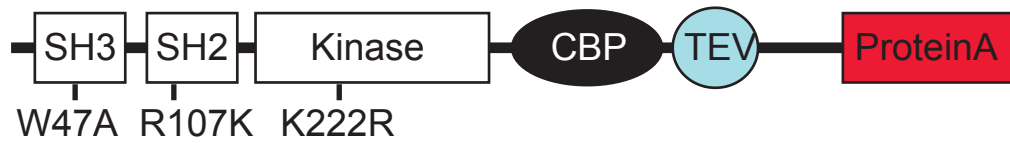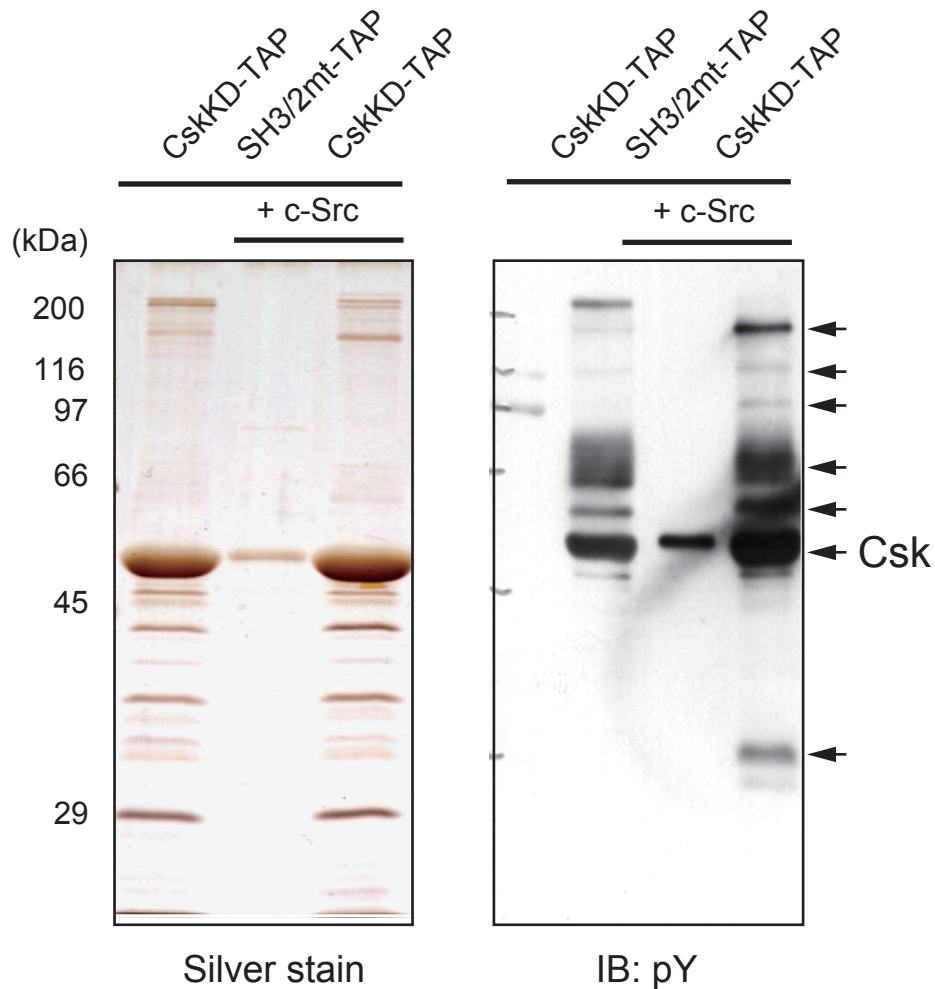
Figure 6. Identification of Csk binding proteins

A. A schematic representation of the kinase-deficient form of tagged Csk (CskKD-TAP). CBP, a calmodulin binding peptide; TEV, a cleavage site for the TEV protease. **B.** Csk binding proteins were purified by TAP method from Csk$^{-/-}$ cells transfected with CskKD-TAP (lane 1), c-Src plus Csk mutant (SH3/2mt-TAP) (lane 2), or c-Src plus CskKD-TAP (lane 3). The purified Csk complex was separated on a SDS–PAGE gel, followed by silver staining (left panel) and immunoblotting with anti-pY (right panel). Arrows indicate the locations of proteins that were specifically detected in c-Src transformed cells.

	CskKD-TAP + c-Src	(kDa)	Mascot Score
Tensin1		203	1155
ZO-1		194	57
ZO-2		131	511
Tensin3		157	181
paxillin		64	78
Cbp/PAG		48	55
(HSP70)		71	525
Csk		50	
(Csk)			
caveolin-1		21	34
14-3-3 δ / ζ		28	41
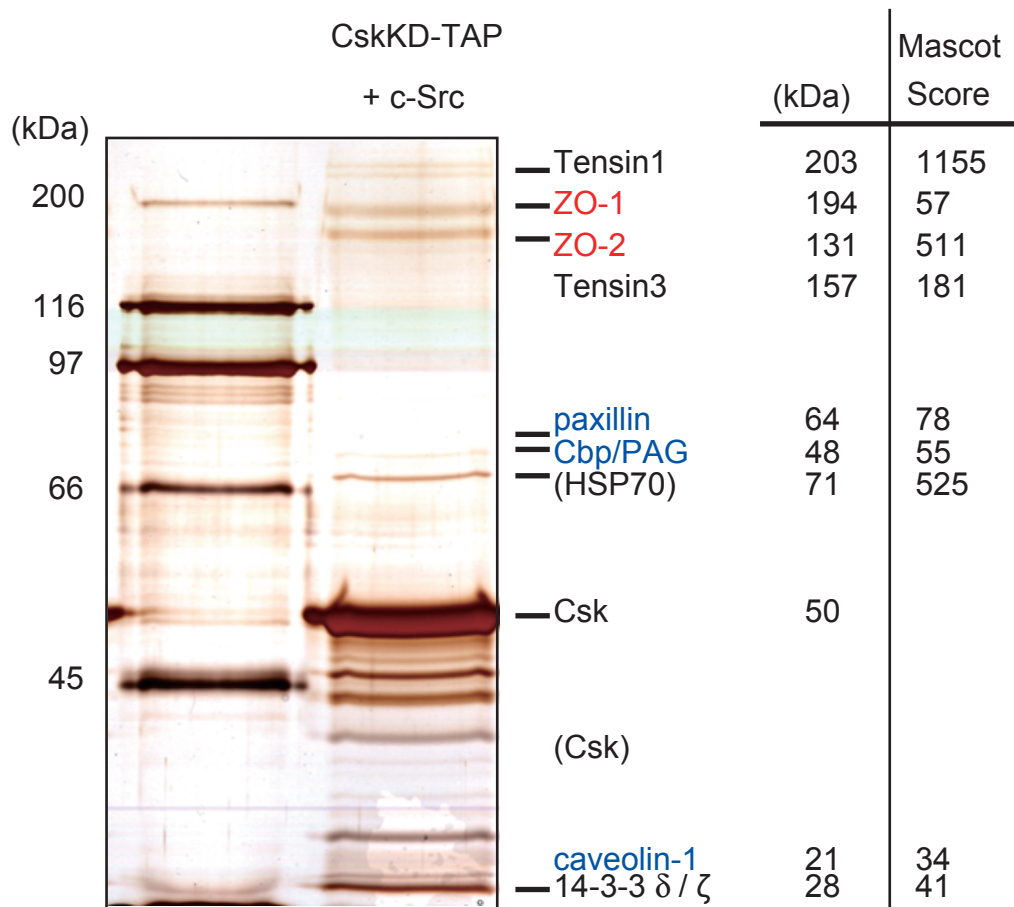
Figure 7. Identification of Csk binding proteins

Csk binding proteins purified by a larger scale of TAP method were detected by silver staining. Proteins identified by LC–MS/MS analyses are indicated.
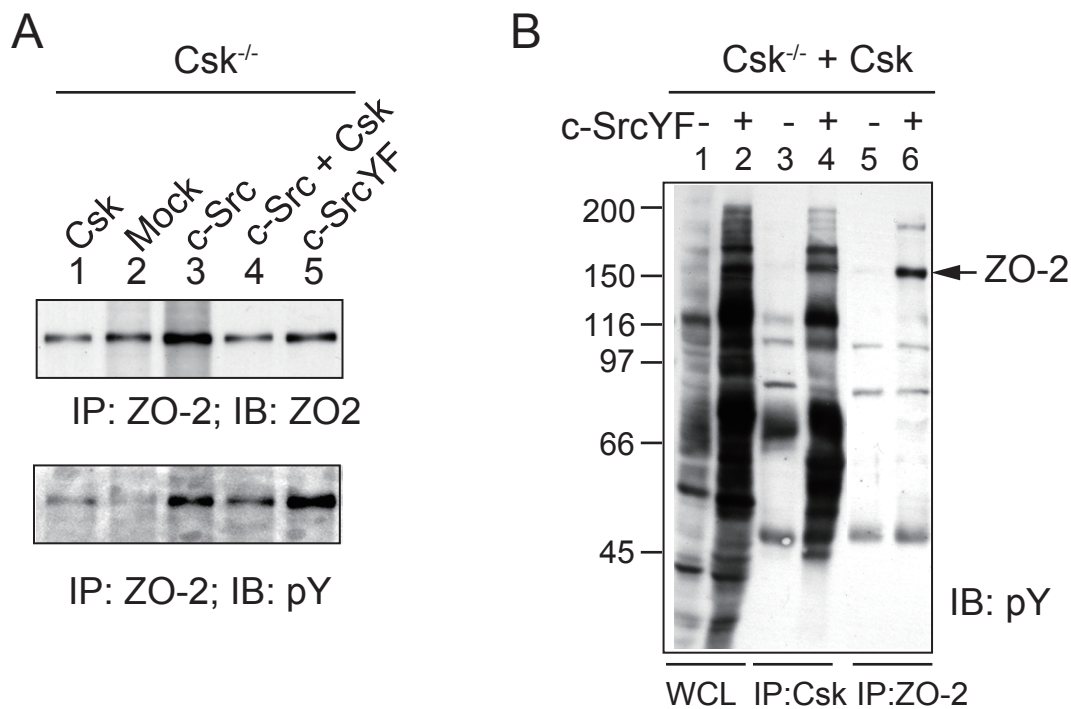
Figure 8. Phosphorylation of ZO-2 in transformed and non-transformed cells

A. Endogenous ZO-2 was immunoprecipitated from the indicated cell lines, followed by immunoblotting with anti-ZO-2 (upper panel) and anti-pY (lower panel). **B.** Whole cell lysates from Csk^{-/-} cells expressing Csk and those transformed by c-SrcYF were immunoblotted with anti-pY (lanes 1 and 2). Immunoprecipitates with anti-Csk (lanes 3 and 4) and anti-ZO-2 (lanes 5 and 6) were immunoblotted with anti-pY. An arrow indicates the location of ZO-2.
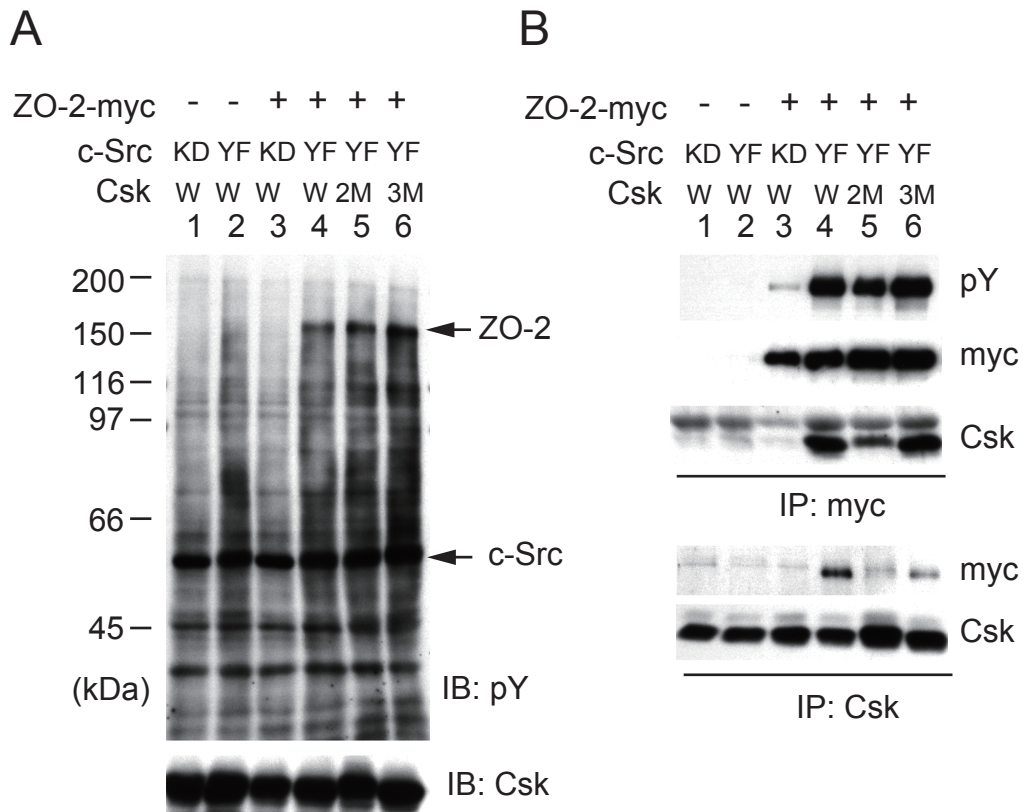
Figure 9. Interaction of ZO-2 with Csk SH2 domain
A. HEK293T cells were transfected with the indicated combinations of the expression vectors, and the whole cell lysates were immunoblotted with anti-pY and anti-Csk. KD: a kinase-deficient mutant, YF: a constitutively active c-SrcYF, W: wild-type, 2M: an SH2 mutant, 3M: an SH3 mutant. **B.** ZO-2-myc (upper panels) and Csk (lower panels) were immunoprecipitated, and the complexes were immunoblotted with anti-pY, anti-myc, and anti-Csk.
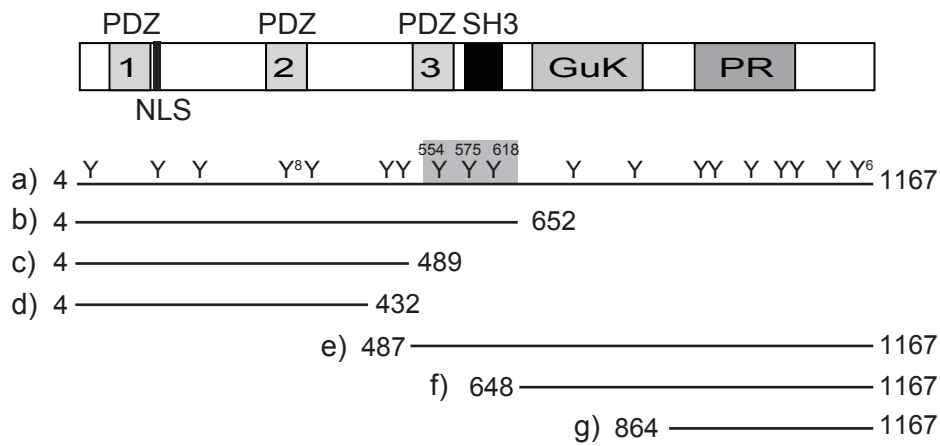
Figure 10. Csk binding regions in ZO-2

A schematic representation of ZO-2 structure and the deletion mutants (a–g) of ZO-2 used in this study. Locations of tyrosine residues are indicated on the full length ZO-2 (Y). The amino acid numbers at N- and C-terminus are shown. PDZ, PSD-95/Dlg/ZO-1; NLS, nuclear localization signal; GuK, guanylate kinase; PR, proline-rich domain.
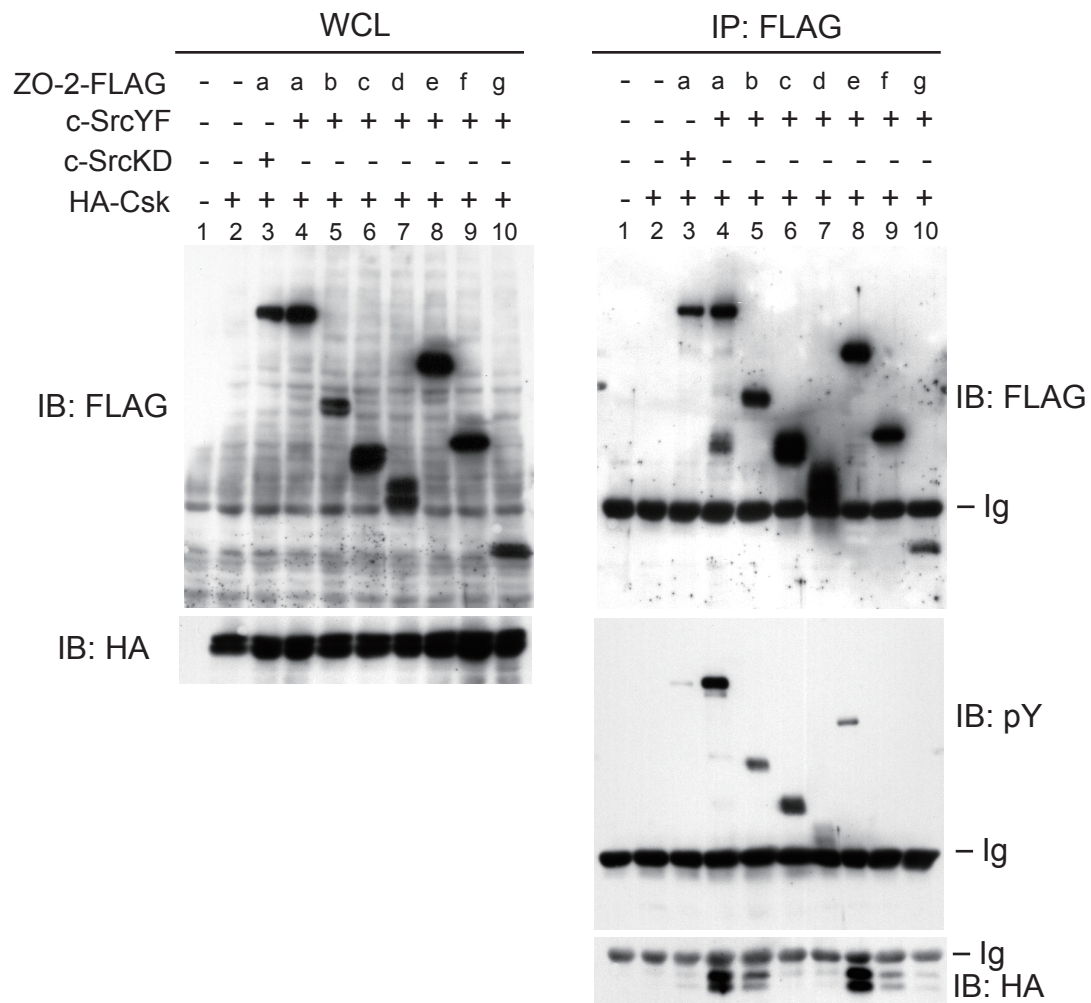
Figure 11. Csk binding regions in ZO-2

HEK293T cells were transfected with the indicated combinations of the expression vectors, and the whole cell lysates were immunoblotted with anti-FLAG and anti-HA (left panels). The lysates were then subjected to immunoprecipitation with anti-FLAG, and the precipitates were immunoblotted with anti-FLAG, anti-pY and anti-HA (right panels). The locations of immunoglobulin (Ig) are shown.
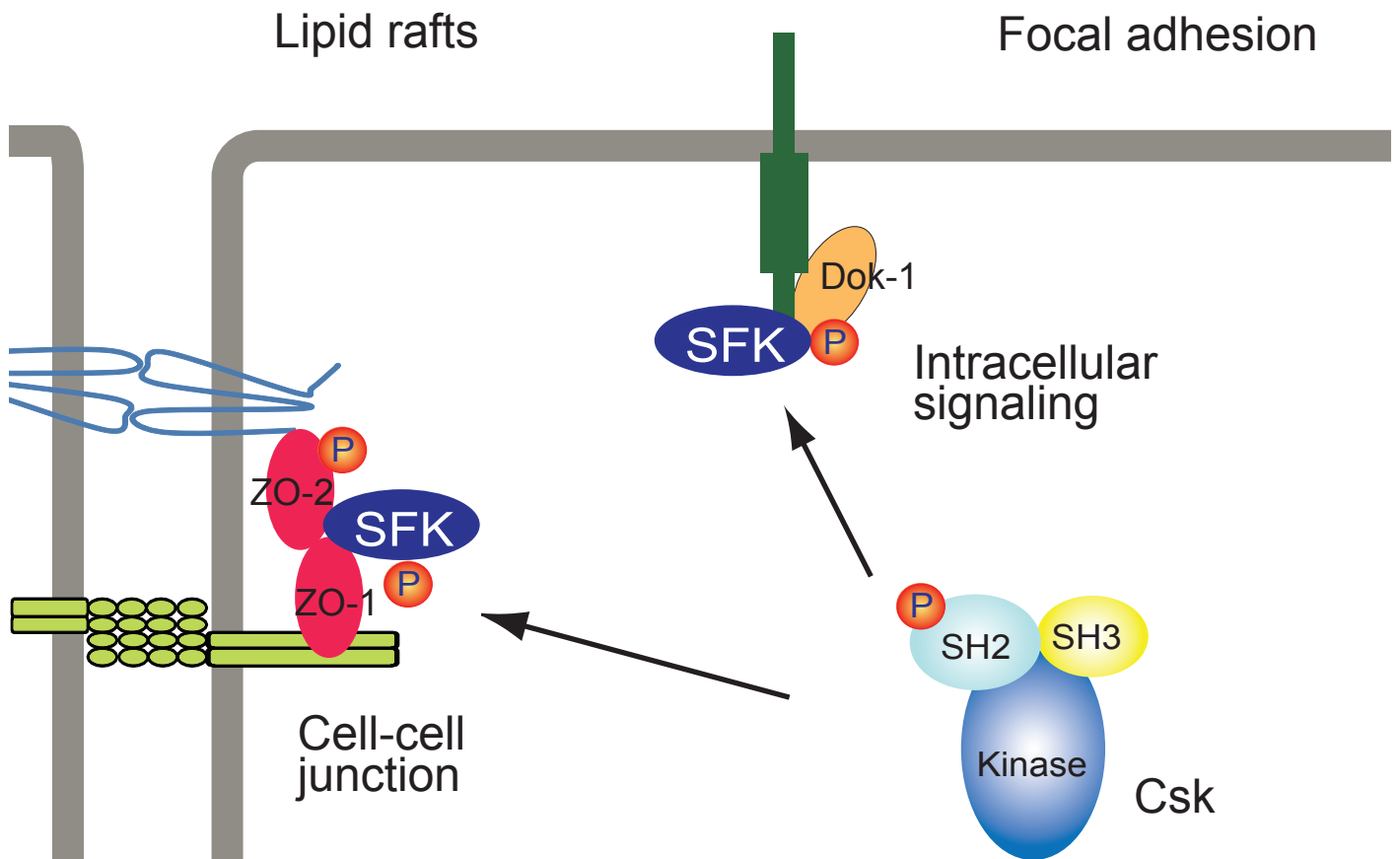
Figure 12. Roles of Dok-1 and ZO-1/2 in the regulation of SFKs by Csk

Dok-1 plays roles as a site specific Csk adaptor downstream of growth factor receptor.

ZO-1/2 is a site specific Csk anchor acting at the undercoat of cell-cell junctions.

Supplement Table 1. Analysis of Csk binding proteins by tandem mass spectrometry

PROTEIN NAME	ACESSION NUMBER	IDENTIFIED PEPTIDE SEQUENCE	MASS ERROR (ppm)	MASCOT SCORE	SEQUENCE COVERAGE
14-3-3 protein zeta/delta	gi|1526539	NLLSVAYK	95	29	8%
		DICNDVLSLLEK	9	12	
Caveolin-1	gi|6680854	HLNDDVVK	32	3	10%
		SFLIEIQCISR	13	31	
Cbp / PAG	gi|81868799	IPPENAVDEILTAR	46	55	3%
Heat shock 70 kDa protein 5	gi|31981722	VMEHFIK	855	4	15%
		TFAPEEISAMVLTK	46	30	
		ITPSYVAFTPEGER	54	45	
		TKPYIQVDIGGGQTK	44	27	
		NQLTSNPENTVFDAK	44	34	
		SQIFSTASDNQPTVTIK	78	21	
		IEIESFFEGEDFSETLTR	86	46	
Heat shock 70 kDa protein 8	gi|51702275	MVNHFIAEFK	22	48	23%
		FEELNADLFR	13	61	
		SQIHDIVLVGGSTR	26	52	
		TTPSYVAFTDTER	13	46	
		SFYPEEVSSMVLTK	27	31	
		NQVAMNPTNTVFDAK	15	70	
		IINEPTAAAIAYGLDK	60	73	
		TVTNAVVTVPAYFNDSQR	28	38	
		SINPDEAVAYGAAVQAAILSGDK	37	58	
		GPAVGIDLGTTYSCVGVFQHGK	11	48	
paxillin	gi|81902126	ELDELMASLSDFK	25	78	2%
Tensin-1 (Predicted)	gi|94363487	LFGFVAR	62	21	19%
		LLSGFGVDR	29	41	
		GDMTHELVR	145	40	
		HFLIETGPR	16	23	
		KGDMTHELVR	133	42	
		YWYKPEISR	11	24	
		SHPGGGPTVPSPGR	14	55	
		YSMPDNSPETR	91	14	
		VSSPPPTITQQGK	32	48	
		ERQPAEPPGPLR	22	26	
		TVGTNTPPSPGFGR	27	47	
		TPFQPMLDSSIR	17	44	
		VSAQGITLTDNQR	58	50	
		DSSGPANSTTDLLK	30	82	
		HAAYGGYSTPEDR	12	83	
		LVIPSRDPTDESK	11	29	
		YVHYFSGLLSGSIK	23	39	
		IIAVSFPSTANEENFR	43	66	

		TPEEEPLNLEGLVAHR	39	68
		VLEFGWPDLHTPALEK	29	48
		SQSFPDVEPQLPQAPTR	100	24
		FPDYGKVEFVFSYGPEK	122	7
		ATSETLAADPTPAATIVHFK	58	31
		HGGNYLLFNLSEQRPDITK	18	39
		SKPSPQLSAETPVAALPEFPR	36	35
		SQSVPGAWPGASPLSSQPLLGSSR	38	31
		SPVQCVSPELALTIALNPGGRPK	42	51
Tensin-3	gi|134152676	DDRFPDYGK	20	16	7%
		VSAQGITLTDNQR	22	57
		HVVPAQVHVNGDAALK	50	0
		YPPFSPPEPQLSSPASLHK	38	30
		IIAVSFPASCSEESYLHSLQEVTR	63	17
		LLIPERDPLEEIAENSPQTAANSAAELL	41	60
Tight junction protein 1, ZO-1	gi|15489342	GEEVTILAQK	222	2	1%
		EEAVLFLLDLPK	17	55
Tight junction protein 2, ZO-2	gi|25059002	LQLVVLR	55	25	15%
		LGHWLAVR	64	36
		HALLDVTPK	62	37
		YQEEGPVPQPR	75	28
		MQELQEAQNAR	58	52
		EDAVLYLLEIPK	89	61
		SSEPVQHEENIR	57	22
		STGDITAAGVTEASR	54	3
		LATELPDLFQTAK	46	35
		SNLPATAGSEIPGGSTK	63	49
		INGTVTENMSLTDAR	58	54
		RPVVLFGPIADIAMER	46	45
		VFLRPSPEDEAIYGPNTK	55	22
		VVMVNGTPMEDVLHSFAVQQLR	47	47

A EDAVLYLLEIPK(701.93,2+) = mouse ZO2 (a.a.549-560)
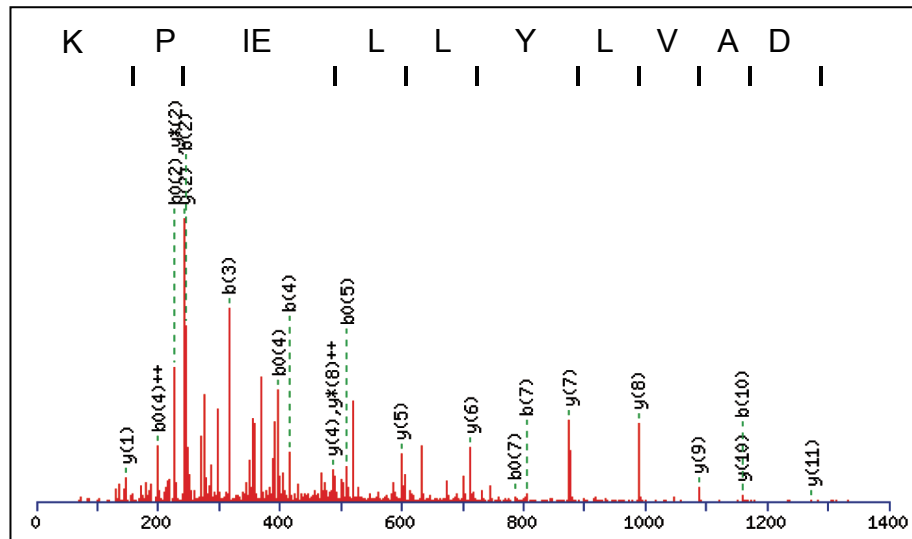
B

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   1 MEEVIWEQYT VTLQKDSKRG FGIAVSGGRD NPHFENGETS IVISDVLPGG
  51 PADGLLQEND RVVMVNGTPM EDVLHSFAVQ QLRKSGKIAA IVVKRPRKVQ
 101 VAPLQGSPPL SHDDRGFEVI EEFDGRSFRS GYSERSRHSS HDMLSHSWEG
 151 NRERGRPHQR TQSRERERSR GRSLERGLDQ EDYGRSRERS RGRSLERGLD
 201 RDFVSRDHSR GRSIDRDYDR DYERSYHEAY EPDYGGGYSP SYDRRAHPET
 251 RYERSRSREH LRSRSPSPES RSRHEHKGQH DPDRPIGVLL TKSKANEEYG
 301 LRLGSQIFIK EMTRTGLATK DGNLHEGDII LKINGTVTEN MSLTDARKLI
 351 EKSRGKLQLV VLRDSKQTLI NIPALNDSDS EVEDISEIES NRSFSPEERR
 401 QQYSDQDYHS STEKLKERPS SREETSGRLS RMGATPTPFK STGDITAAGV
 451 TEASREPRYQ EEGPVPQPRT APRVFLRPSP EDEAIYGPNT KMVRFKKGDS
 501 VGLRLAGGND VGIFVAGIQE GTSAEQEGLQ EGDQILKVNT QDFRGLVRED
 551 AVLYLLEIPK GETVTILAQS RADVYRDILA CGRGDSFFIR SHFECEKETP
 601 QSLAFTRGEV FRVVDTLYDG KLGHWLAVRI GNELEKGLIP NKSRAEQMAS
 651 VQNAQRENAG DRADFWRMRG QRSSGGVKKN LRKSREDLAA AVSVSTKFPA
 701 YEKVLLREAG FKRPVVLFGP IADIAMERLA TELPDLFQTA KTEPKDAGSE
 751 KSSGVVRLNT VRQIIEQDKH ALLDVTPKAV DLLNYTQWFP IVIFFNPDSR
 801 QGVKTIRQRL SPTSNKSSRK LFDQANKLKK TCSHLFTATI NVNSANDGWF
 851 GSLKDSIQQQ QNEAVWVSEG KMEGMDDDAE DRMSYLTAMG ADYLSCDSRL
 901 ISDFEDTDGE GGAYTDNELE EPAEEPLVSS ITRSSEPVQH EENIRKSSPE
 951 PRAQMRRAAS RDQLRDASPP PAFKPEPPKA RSQNREDSFD YSKSNLPATA
1001 GSEIPGGSTK GYPPPIAAKP AFGRPILKPS TPVPMPESEE VGESTEEQED
1051 APRSVLGRVK IFEKMDHKAK LQRMQELQEA QNARIEIAQK HPDIYAVPIK
1101 APKPDAGLPP HMSSRPPEPQ KAPSRLYQDT RGSYGSDPEE EEYRQQLAAH
1151 SKRGYYSQPS RYRDTEL
```

Supplementary figure S1. Identification of novel Csk binding proteins, ZO-2

(A) A representative MS/MS spectrum assigned to ZO-2 are shown. (B) Amino acid sequence of mouse ZO-2. Matched peptides shown in Red.
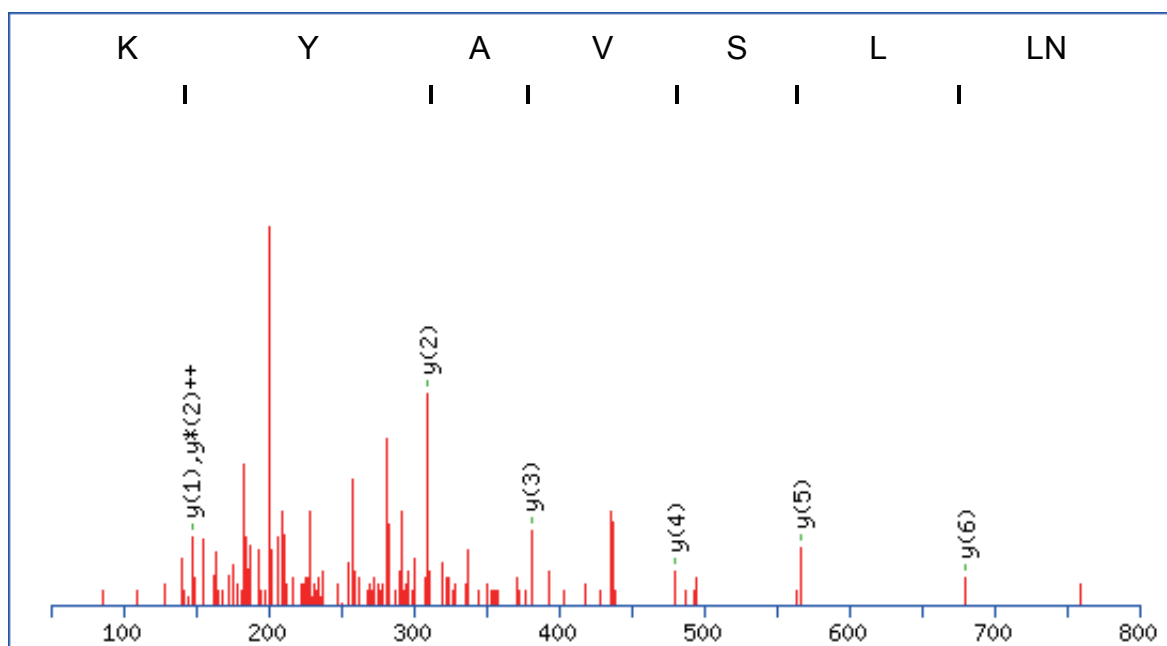
**A** EEAVLFLLDLPK(693.89,2+) = mouse ZO1 (a.a.481-492)
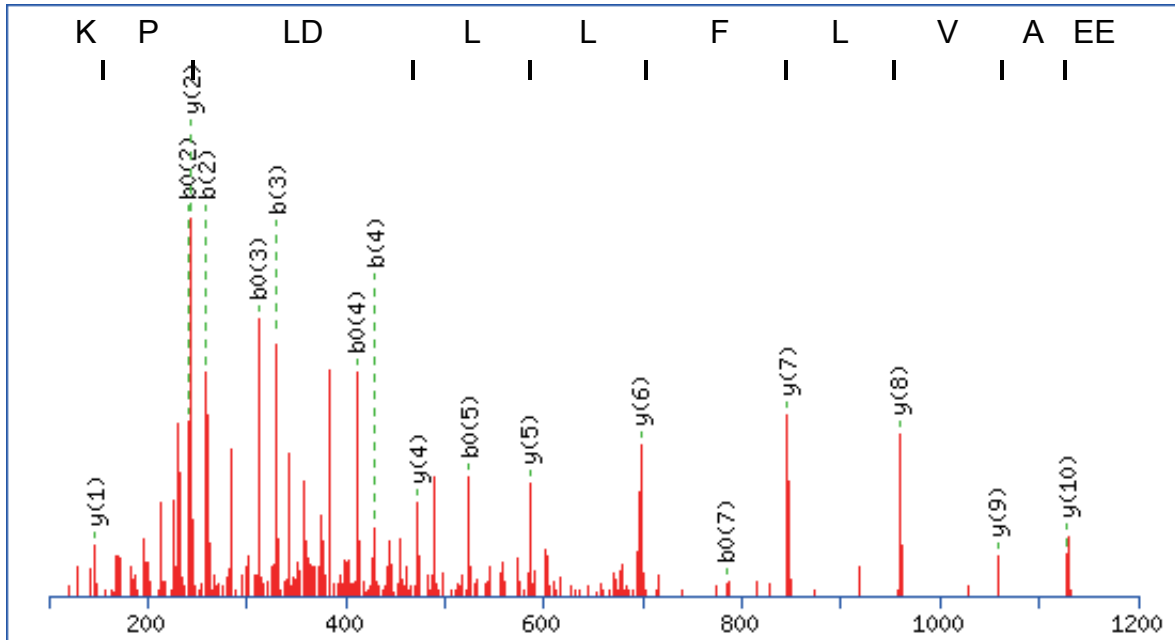
Sapplementary figure S2. MS/MS spectra of Single-peptide-based protein identification.

MS/MS spectura and amino acid sequence of ZO-1 (A), 14-3-3 ζ/δ (B), Caveolin-1 (C), Cbp/PAG (D) and paxillin (E). Each spectrum shows the predicted peptide sequence and the identified fragment.
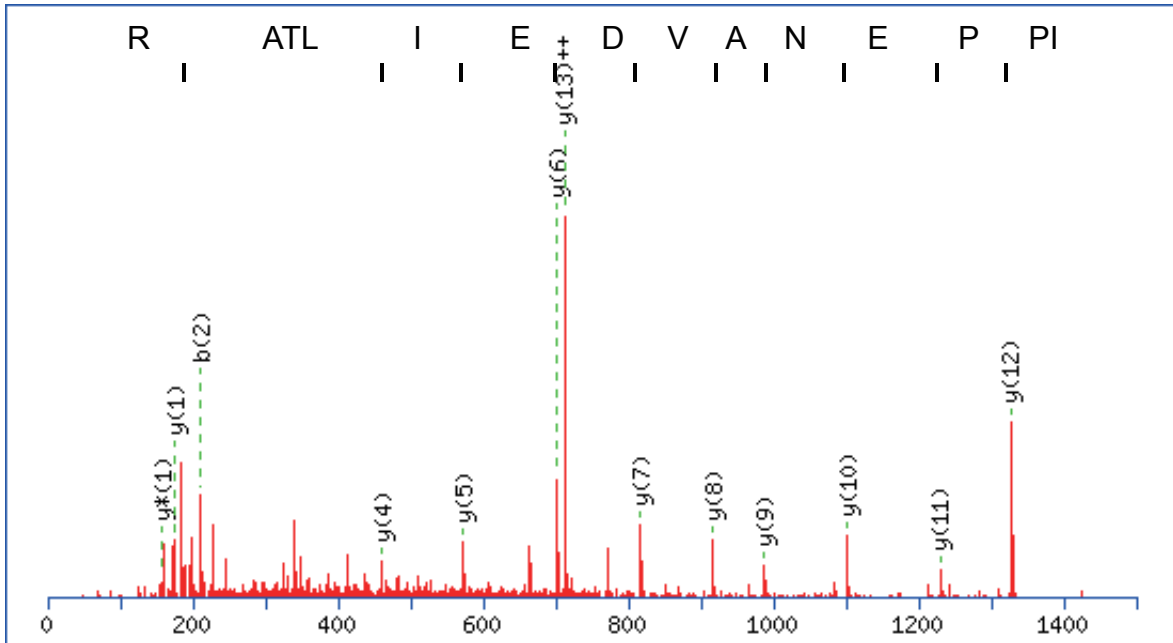
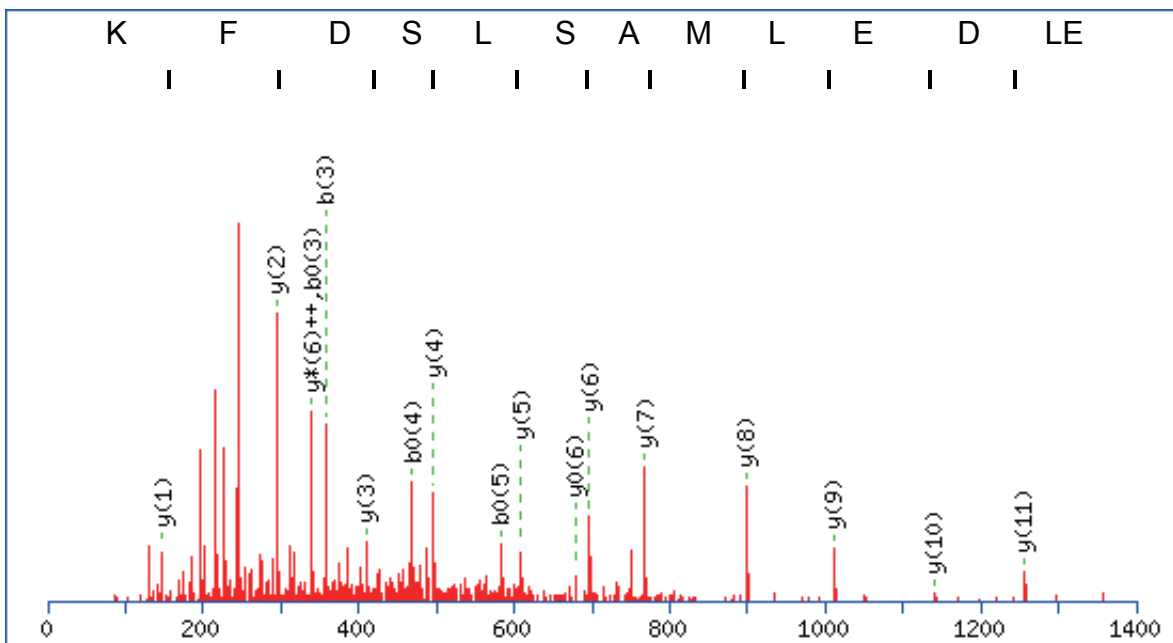B NLLSVAYK(454.76,2+) = mouse 14-3-3 protein zeta/delta (a.a.42-49)

C SFLIEIQCISR(683.36,2+) = mouse Caveolin-1 (a.a.136-146)
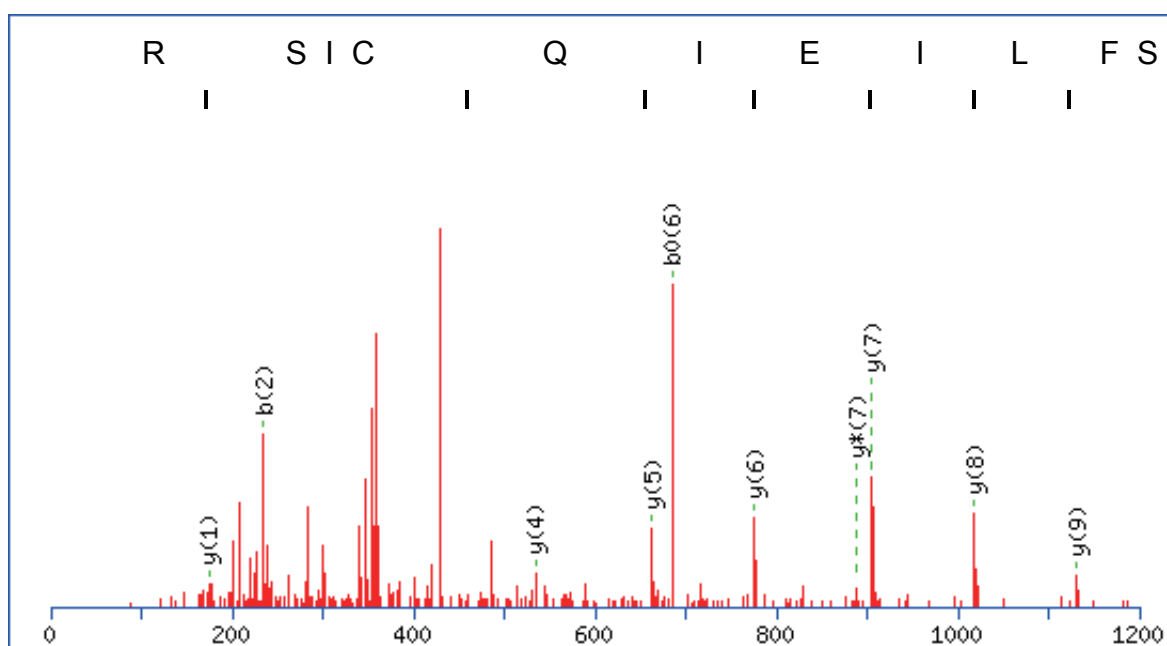
D IPPENAVDEILTAR(769.41,2+) = mouse Cbp/PAG (a.a.138-151)

E ELDELMASLSDFK(749.34,2+) = mouse Paxillin (a.a.265-277)

Acknowledgement

List of publications

1. Saito, K., Enya, K., Oneyama, C., Hikita, T. and Okada, M. (2008) Proteomic identification of ZO-1/2 as a novel scaffold for Src/Csk regulatory circuit. *Biochem Biophys Res Commun.* **66**, 969-975.

2. Nakanishi, A., Hirose, M., Yoshimura, M., Oneyama, C., Saito, K., Kuki, N., Matsuda, M., Honma, N., Ohnishi, H., Matozaki, T., Okada, M. and Nakagawa, A. (2007) Structural insight into the specific interaction between murine SHPS-1/SIRP alpha and its ligand CD47. *J Mol Biol.* **375**, 650-660.