A new method for isolating tyrosine kinase substrates used to identify Fish, an SH3 and PX domain-containing protein, and Src substrate

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We describe a method for identifying tyrosine kinase substrates using anti-phosphotyrosine antibodies to screen tyrosine-phosphorylated cDNA expression libraries. Several potential Src substrates were identified including Fish, which has five SH3 domains and a recently discovered phox homology (PX) domain. Fish is tyrosine-phosphorylated in Src-transformed fibroblasts (suggesting that it is a target of Src *in vivo*) and in normal cells following treatment with several growth factors. Treatment of cells with cytochalasin D also resulted in rapid tyrosine phosphorylation of Fish, concomitant with activation of Src. These data suggest that Fish is involved in signalling by tyrosine kinases, and imply a specialized role in the actin cytoskeleton.
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Introduction

The viral Src protein (v-Src) was discovered more than 20 years ago. In the intervening years intense study of both v-Src and its cellular counterpart, c-Src, has focused on issues of regulation, transforming activity and physiological function.

The Src family of protein tyrosine kinases comprises eight members in mammals, some broadly expressed, some restricted to certain tissues (Cooper, 1990; Bolen *et al.*, 1992). All are tightly regulated *in vivo*; catalytic activity is repressed by interactions involving the SH3 domain, the SH2 domain, and the phosphorylated C-terminus (Superti-Furga and Courtneidge, 1995). Recent crystal structures of Src and Hck have revealed exactly how this repression takes place (Sicheri *et al.*, 1997; Williams *et al.*, 1997; Xu *et al.*, 1997). Morphological transformation is frequently the result of the expression of catalytically de-repressed forms of Src family kinases, especially in fibroblasts (Cooper, 1990). There is also a large body of circumstantial evidence supporting the view that hyperactive Src family kinases may play a role in several human epithelial malignancies, particularly of the colon and breast (Cartwright *et al.*, 1989, 1990; Ottenhoff-

Kalff *et al.*, 1992). In normal cells, Src family kinases act in signal transduction cascades. Typically they transduce signals from cell surface receptors, of both the kinase and non-kinase type (Erpel and Courtneidge, 1995), and mediate the activation of a variety of pathways, including those involving the mitogen-activated protein (MAP) kinases (Dikic *et al.*, 1996; Luttrell *et al.*, 1996; Sadoshima and Izumo, 1996), the phosphatidylinositol 3-kinase (PI 3-K) (Cantley *et al.*, 1991), and Myc (Barone and Courtneidge, 1995).

Over the years, many putative substrates of Src have been identified by a variety of methods, including direct analysis of candidate proteins such as tensin (Davis *et al.*, 1991) and paxillin (Turner and Miller, 1994), the use of phosphotyrosine antibodies for proteins such as actin filament associated protein (AFAP-110; Flynn *et al.*, 1993), cortactin (Wu *et al.*, 1991), p120^{ctn} (Reynolds *et al.*, 1992), p130^{Cas} (Sakai *et al.*, 1994) and p62^{dok} (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), and the analysis of Src-associated proteins such as Sam68 (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994) and Efs (Ishino *et al.*, 1995). Many substrates are cytoskeletal or membrane components (Davis *et al.*, 1991; Wu *et al.*, 1991; Rothberg *et al.*, 1992; Flynn *et al.*, 1993; Turner and Miller, 1994), others are enzymes (Cobb *et al.*, 1994), and others adaptor or docking proteins (McGlade *et al.*, 1992; Sakai *et al.*, 1994; Ishino *et al.*, 1995; Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997). Yet, despite the seeming plethora of substrates, it seems likely that physiologically relevant substrates remain undiscovered; for example, there are mutant forms of Src which seem able to phosphorylate all known substrates, yet fail to transform (Erpel *et al.*, 1995). Perhaps some relevant substrates are not stably associated with Src, or are not abundant in cells, and were therefore missed by the available strategies. We therefore sought a new way to identify tyrosine kinase substrates.

Results

A method to identify tyrosine kinase substrates

We wanted to develop a method for identifying substrates for tyrosine kinases that could be applied to a variety of kinases and cell types, and that was based on a cDNA expression system so that sequence information was immediately available. We therefore chose to adapt a method, described by Kavanaugh *et al.* (1995), that was used to identify binding partners for the Shc phosphotyrosine-binding (PTB) domain. The method in Figure 1A and described in detail in Materials and methods, consisted of phosphorylating a λ gt11 cDNA expression library, derived from mRNA of mouse Swiss 3T3 fibroblasts, with Src kinase, and then detecting phosphorylated proteins with an anti-phosphotyrosine antibody. Successive rounds of phosphorylation and antibody screening (Figure 1B) were

Fig. 1. Identification of potential substrates of Src. (A) Phosphorylation screening protocol. Petri dishes containing a Swiss 3T3 cell cDNA expression library in λ gt11 were overlaid with nitrocellulose filters containing IPTG and incubated for 16 h to induce expression of recombinant proteins. Replicate filters were incubated with extracts containing baculovirally expressed human Src kinase and ATP. Individual plaques containing tyrosine-phosphorylated protein were identified with an anti-phosphotyrosine (anti-pTyr) antibody using a standard immunoblotting protocol. Positive cDNA clones were purified by successive rounds of phosphorylation with Src kinase and immunodetection with anti-pTyr antibody. (B) Immunodetection of positive plaques. Immunoblot analysis of filters with an anti-pTyr antibody during (i) primary and (ii and iii) secondary rounds of phosphorylation screening showing various positive clones (arrow heads).

used to plaque-purify several cDNAs, and the inserts were then sequenced.

Several of these clones, which we have named Tks (tyrosine kinase substrates), are listed in Table I. Conceptual translation showed that each insert corresponded to a partial cDNA with an open reading frame containing at least one, and usually several, motifs which closely resemble the consensus sequence, EEEIYG/EEFD, for phosphorylation by v-Src kinase (Songyang and Cantley, 1995). One clone, Tks-7, was identical to part of p130^{Cas}, a known substrate of both Src and focal adhesion kinase, and a Crk binding protein (Sakai *et al.*, 1994; Polte and Hanks, 1995; Harte *et al.*, 1996). Tks-1, Tks-2 and Tks-9 correspond to partial cDNAs for recently described proteins with C-terminal SH3 domains. Tks-1 encodes a portion of a mouse cDNA product (designated s19) which was identified as a Src SH3-domain binding protein (Yamabhai and Kay, 1997). Tks-2 corresponds to part of SH3P7, a protein identified on the basis of its ability to interact with SH3-binding peptide ligands (Sparks *et al.*, 1996). Tks-2 also shares significant sequence similarity with a neuronal actin binding protein, drebrin E (Toda *et al.*, 1993). Tks-9 corresponds to the mouse homologue of human CIP4, a proposed target protein of the activated form of the Rho-family GTPase Cdc42 (Aspenstrom, 1997) and of a rat protein, STP, that was identified independently as a possible regulator of cation transport in yeast (Tsuji *et al.*, 1996). A portion of CIP4 was also described as Trip10, a thyroid hormone receptor interacting protein (Lee *et al.*, 1995). Tks-3 was identical to a portion of Mem3, a protein encoded by a mouse maternal embryonic mRNA, which is related to a yeast vacuolar sorting protein (Hwang *et al.*, 1996). The Tks-5 and Tks-14 clones encoded novel proteins. Inspection of Tks-5 showed that in addition to three possible Src phosphoryl-

ation sites, it contained part of an SH3 domain. We chose to analyse this clone further.

Identification of Fish, a novel multi-SH3 domain-containing protein

Several overlapping cDNA clones encompassing the entire coding region of Tks-5 were isolated from a mouse embryonal cDNA library with a probe corresponding to the original Tks-5 clone. These cDNAs represented two distinct transcripts (data not shown), encoding isoforms of 1124 and 1081 residues (Figure 2A). The larger isoform contained two insertions of 15 and 28 amino acids (underlined), which were absent in the smaller isoform. The predicted translational start site, which is shared by both transcripts, is preceded by stop codons in all three reading frames and conforms quite closely to the consensus for translation initiation (Kozak, 1991). It is noteworthy that the initiator methionine of p47^{phox}, the protein most closely related to that described here, is in the analogous position (see Figure 2C). These data strongly suggest that this residue is indeed the translational start site. Both predicted isoforms have five SH3 domains (boxed), a phox homology (PX) domain (overlined), and consensus sequences for SH3 domain binding (double underlined) and Src phosphorylation (ringed). The 15 amino acid insertion (underlined) occurs between the PX domain and the first SH3 domain, and the 28 amino acid insertion (underlined) is between the first and second SH3 domains. The original λ gt11 clone is encompassed by the arrows. Outside of the described domains, the amino acid sequence shows no significant homology with other proteins in any accessible databases (data not shown) and is therefore unlikely to contain any catalytic domain. We have named the protein encoded by these clones Fish (five SH3 domains).

The overall topography of Fish is shown schematically in Figure 2B, where it is compared with other proteins which also contain PX and/or SH3 domains. The PX domain, whose function is as yet unknown, was recently described as a conserved domain in many eukaryotic proteins (Ponting, 1996), including those shown in Figure 2B and C. The PX domain of Fish is most closely related to the PX domains of p47^{phox} and p40^{phox} (Volpp *et al.*, 1989; Wientjes *et al.*, 1993), Bem1 and Scd2 (Chenevert *et al.*, 1992; Chang *et al.*, 1994), and Cpk (MacDougall *et al.*, 1995; Molz *et al.*, 1996; Virbasius *et al.*, 1996; G.Plowman, K.Joho and S.A.Courtneidge, unpublished data). All of the SH3 domains are most similar to each

other and to the two SH3 domains of p47^{phox} (Volpp *et al.*, 1989), as shown in Figure 2D. Pairwise comparison of the Fish SH3 domains indicate that they share 27.5–48% identity with each other, 24–47% identity with the two SH3 domains of p47^{phox}, but only 13.7–23.5% identity with the SH3 domains of Src and the p85 subunit of the phosphatidylinositol 3-kinase (PI 3-K).

Expression and tyrosine phosphorylation of Fish

To assess the expression profile of Fish mRNA we analysed various adult mouse tissues by Northern blot hybridization using a probe representing the Tks-5 cDNA (Figure 3). We detected a prominent transcript of ~10.5 kb in most tissues examined, with the exception of spleen and testis which contained relatively low and undetectable levels of Fish mRNA, respectively. We also noted much weaker expression of a message of ~6 kb in those tissues containing the 10.5 kb mRNA. Whether the smaller transcript corresponds to a differentially spliced mRNA or perhaps a Fish-related transcript, remains to be established. Both the 10.5 and 6 kb transcripts are significantly larger than the Fish coding region (~3.5 kb) present in the two Fish cDNAs that we identified, suggesting that they may also contain extensive 5'- and/or 3'-untranslated sequences.

We raised polyclonal antibodies against a GST fusion protein containing the original Tks-5 cDNA product, and used these to examine the expression of Fish in cells (Figure 4A). Three forms of Fish, of ~130, ~140 and ~150 kDa, were detected in immunoprecipitates from NIH 3T3 cells. We think it unlikely that p130 and p140 represent proteolytic breakdown products of the p150 form of Fish, since they were also detected in lysates of cells generated under denaturing conditions (data not shown), and perhaps represent products of other RNA splice variants. Transient transfection of COS cells with an expression construct encoding the small form of Fish (see Figure 2A) gave rise to two bands, the largest of which was 150 kDa. The smaller product most likely corresponds to a truncated or proteolytically degraded product, since expression of a construct encoding Fish with a myc epitope tag at its N-terminus also generated two analogous products that were each recognized by the myc antibody (data not shown). To test whether Fish was indeed a substrate for Src, we co-transfected 293 cells

Table I. A summary of clones obtained from anti-phosphotyrosine screening of a phosphorylated mouse fibroblast expression library

Clone	Possible Src phosphorylation sites	Size (bp)	Identity (functional information)
Tks-1	QRLSYGAFT	838	s19 (Src SH3 binding protein)
Tks-2	REQRYQEQH PETSYGREH EGTYEVPP QDTLYEEP	533	SH3P7 (SH3 ligand binding protein)
Tks-3	EGPIYEGLI	1250	Mem3 (relative of yeast vacuolar sorting protein VPS35)
Tks-5	LKVKYEEPE EEPEYDVPA EETIYENEG	1020	novel (SH3 domain containing adaptor protein now named Fish)
Tks-7	PQDIYDVPP LPNQYGQEV LLDVYDVPP HHSVYDVPP	364	p130 ^{Cas} [substrate of Src and focal adhesion kinase (FAK), Crk binding protein]
Tks-9	EVQKYEAWL LGAGYGLLS EVQKYEAWG DTPITYTEFD	1000	homologue of human CIP4, Trip10 and rat STP (Cdc42 interacting protein, thyroid hormone receptor interacting protein, cation transport regulator)
Tks-14	EEPVIYEMV SEAIYEEMK EEMKYPLPE	789	novel (contains two YxxM motifs)

Fig. 2. Amino acid sequence of murine Fish. (A) Predicted amino acid sequence of two Fish isoforms. The sequence shown was compiled from five overlapping cDNAs that were isolated from a murine day 11.5 embryonal cDNA library using a probe corresponding to Tks-5, a partial cDNA for Fish that was identified in the initial screen for Src substrates using the Swiss 3T3 cDNA library. The boundaries of the Tks-5 cDNA product are marked by arrows and potential Src tyrosine phosphorylation sites therein are indicated with ovals. Insertions of 15 and 28 amino acids that were identified in one clone, and are likely to be derived by alternative mRNA splicing, are underlined. Sequence numbers are shown at the right. The phox homology (PX) domain is overlined and five SH3 domains are boxed. Three polyproline motifs that represent potential SH3 binding sites are doubly underlined. (B) Topology of selected proteins containing a PX domain. Domains are represented by boxes as indicated: PX domains are white; SH3 domains are black; the C2 domain is light grey; the phosphatidylinositol 3-kinase catalytic domain (PI 3-K) is dark grey; the p110 homology domain is dashed; the pleckstrin homology (PH) domain is striped and the Rho-GAP domain is checked. (C) Comparison of the phox homology (PX) domains of Fish and selected proteins. This alignment was created using a similar approach to that reported previously (Ponting, 1996). Briefly, iterative profile searches were conducted using SearchWise (Birney *et al.*, 1996) and multiple alignments using Clustal W (Thompson *et al.*, 1994). Definition of the domain boundaries and introduction of gaps (indicated by dashes) required inspection and some hand editing, and was helped by protein termini and neighbouring domain boundaries. Names of proteins and species are shown at the left of the alignment using the following abbreviations: h is human, m is mouse, dm is *Drosophila melanogaster*, sc is *S.cerevisiae* and sp is *S.pombe*. PLD1 refers to phospholipase D1. Sequence numbers are shown at the end of the PX domain. Numbers at the top of the alignment refer to the Fish sequence. Amino acids are shown using the single letter code. Colours are used to indicate conserved features. The basic residues histidine, lysine and arginine (H, K and R, respectively) are shown in green, the hydrophobic residues alanine, phenylalanine, isoleucine, leucine, methionine, proline, valine and tryptophan (A, F, I, L, M, P, V and W, respectively) are shown in purple, the aromatic residues phenylalanine, tryptophan and tyrosine (F, W and Y, respectively) are shown in yellow and the acidic residues aspartate and glutamate (D and E, respectively) are shown in pink. (D) Comparison of the SH3 domains of Fish with those of p47^{phox}. Multiple alignments of the SH3 domains from Fish, p47^{phox}, Src and PI 3-K were conducted using MegAlign (DNASTar). The % amino acid identity is shown.

A

MLAYCQDATVVDVEKRSPSKHYVYINVTWSDSTSTQTIYRRYSKFFDL 50
QNLQDKPFTIEGQKDKPQRIIPFLPGKILFRSHIRDAVAKRLKPIDEY 100
CRALVRLPHITSCDEVFRFFEARPELVNPKEDYGSSKRKS~~VWLS~~SWAE 150
SPKKDV~~GADTNAEPMT~~~~ECYVVVS~~NYKKQENSELSLQAGIEVDVTEKNE 200
SGWVFSTSEEGQWVPATYLEAQ~~Q~~TRDSDSDINTSKTGEVSKRRKAHLRR 250
LDRRWTLGGMVNRQHSR~~REKYVT~~VQPYTSQSKDEIGFEKGVTVIRKNTL 300
EGWNYIRYLKKEGWAPASYLKRA~~DD~~LP~~TT~~KKNLAGPVEIIGNIMEISNL 350
LNKKASGDKEAPAEEGSEAPITKKEISLPILCNASNGSALAIPERTTSK 400
LAQGSFAVARIAPQRAQISSPNLRTPPPPRESSLG~~FQ~~L~~PKPPEP~~SVH~~Q~~ 450
BYVNAEFQSCISD~~GIS~~FRGGQKA~~EV~~IDRNSGGWYVQIGKEGWAPASY 500
IDKRRKPNLSRRRTSTLTPKVPFPAPP~~SK~~KEAEENFVGACESQGSPLKV 550
K~~Q~~EP~~PD~~QVPAFGFDEPEMNEEPSGDRGSGDKHPAQPRRISPASSLQRA 600
HPKVGESSEDALEETI~~Q~~ENEGRPYTEDTLSARGSSGSDSPGSSLS 650
LAVKNSPKSDSPKSSSLKLKAEKNAQELKQNSISFSSSVTISTTCS 700
SSSSSSLSKNGNDLKRPSASDAGIRD~~TF~~KVGT~~KD~~PDV~~KAG~~LASCARAK 750
PSVRPKPVLNRAESQSQEKMDISSLRRLQ~~RT~~QGLRGGLKGRSSEDS 800
PQMAEGSRRGSADIILPLTATPPCVPKKEWEGQ~~Q~~TYVIC~~SA~~YQKVQDS 850
EISFPEGA~~EV~~HL~~FK~~AVSGWYVVRGELEGWAPSHYLVA~~EE~~NQOPDTASK 900
EGDTGKSSQNEGKSDSLEKIERVQALNTVNQSKRAT~~EP~~PSKPPGGFGK 950
TSGTVAVKMRNGVRQAVRPPQSVFPPPPKDNNSCALRRNESLTATDSL 1000
RGVRRNSFSTARSAAAEAKRLRAEASQGESPLPTQ~~R~~KGI~~P~~VPSPVR 1050
PKPIEKSQFIHNNL~~Q~~VYIS~~AD~~YEGDETAGFQEGVSMEVLEKNPN~~GW~~ 1100
YCQLDEVKFPKGVWPSNYLEKK~~Q~~ 1124

B

C

D

Fish SH3#1	Fish SH3#2	Fish SH3#3	Fish SH3#4	Fish SH3#5	p47 phox SH3#1	p47 phox SH3#2	Src	p85 PI3K	
100	41.2	33.3	48.0	27.5	47.1	40.0	17.6	23.5	Fish SH3#1
	100	49.0	46.0	29.4	37.3	44.0	19.6	15.7	Fish SH3#2
		100	44.0	27.5	33.3	30.0	13.7	13.7	Fish SH3#3
			100	30.0	36.0	42.0	16.0	16.0	Fish SH3#4
				100	31.4	24.0	19.2	16.7	Fish SH3#5
					100	36.0	17.6	25.5	p47 phox SH3#1
						100	22.0	16.0	p47 phox SH3#2
							100	28.8	Src
								100	p85 PI3K
(% identity)

Fig. 3. Tissue distribution of Fish mRNA. Northern blot analysis of poly(A)⁺ RNA from the indicated adult mouse tissues using a ³²P-labelled Fish cDNA probe (upper panel). Hybridization analysis of the same Northern blot using a β -actin cDNA probe (lower panel).

with cDNAs for Fish, with or without wild-type, activated or kinase-inactive Src. Anti-phosphotyrosine immunoblotting revealed that Fish was tyrosine-phosphorylated when co-expressed with active forms of Src (Figure 4B).

We next determined whether Fish was also tyrosine phosphorylated in Src-transformed fibroblasts. A comparison of NIH 3T3 cells, and counterparts transformed with an activated allele of Src in which the regulatory C-terminal tyrosine is replaced with phenylalanine, showed that Fish was tyrosine-phosphorylated in Src-transformed cells (Figure 5A). To determine whether Fish was likely to be a direct substrate of Src, we used Rat1 fibroblasts transformed with a temperature-sensitive version of v-Src. At the non-permissive temperature, a low basal level of tyrosine phosphorylation of Fish was detected. However, within 10 min of placing the cells at the permissive temperature, the tyrosine phosphorylation of Fish increased, and was maximal within 1 h of the shift (Figure 5B). These kinetics are similar to those seen for the reactivation of Src kinase activity (S.A.Courtneidge, unpublished observations).

Fish contains several motifs and domains that are known to be involved in protein-protein interactions; we therefore looked for Fish-associated proteins. Fish isolated from NIH 3T3 cells was not associated with tyrosine-phosphorylated proteins (Figure 5C). In contrast, in Src-transformed cells Fish immunoprecipitates contained several tyrosine phosphoproteins. Several of these proteins, most notably those of 125, 115 and 65 kDa, were recognized by two different antibodies raised against different regions of Fish, and are therefore likely to represent Fish-associated

Fig. 4. Expression and tyrosine phosphorylation of Fish by transient transfection. (A) The Fish cDNA (short form) encodes a 150 kDa protein. Fish was immunoprecipitated from extracts of NIH 3T3 cells (lane 1) or COS cells transfected with an expression vector containing the Fish cDNA in the anti-sense (-)(lane 2) or sense (+)(lane 3) orientation. Samples were analysed by SDS-PAGE and immunoblotting with anti-Fish.1 serum. The Fish cDNA product and a possible premature termination product are indicated by arrow heads. Three forms of endogenous Fish in NIH 3T3 cells, designated p150, p140 and p130, are also indicated. (B) Fish is tyrosine-phosphorylated in 293 cells which coexpress active Src. Lysates of 293 cells transfected with either NmycFish cDNA alone, encoding an N-terminal myc epitope-tagged form of Fish, or in combination with cDNAs for SrcK⁺, SrcK⁻ or SrcY527F, as indicated, were analysed by SDS-PAGE and immunoblotting with anti-Fish.2 (upper panel) and anti-Src (EC10; middle panel). Anti-Fish.2 immunoprecipitates from these lysates were analysed by SDS-PAGE and immunoblotting with anti-pTyr (lower panel).

proteins. We are currently characterizing these proteins further, although our preliminary analyses have ruled out rasGAP, Fak, AFAP-110 and p120^{Ctn}.

Many proteins phosphorylated on tyrosine in Src-transformed cells are also transiently phosphorylated in normal cells in response to growth factors or other stimuli. We therefore asked whether growth factor stimulation resulted in tyrosine phosphorylation of Fish. Our initial tests used NIH 3T3 cells, where we were unable to find convincing evidence for Fish phosphorylation in response to a variety of stimuli (data not shown). However treatment of Rat1

Fig. 5. Phosphorylation of Fish in Src-transformed NIH 3T3 cells and temperature-sensitive v-Src-transformed fibroblasts, and association with tyrosine-phosphorylated proteins. (A) Fish is tyrosine-phosphorylated in Src-transformed NIH 3T3 cells. SDS lysates prepared from NIH 3T3 cells (C17) and SrcY527F-transformed NIH 3T3 cells (527) were immunoprecipitated either with rabbit Ig or anti-Fish.2 antibody, and analysed by SDS-PAGE and immunoblotting with anti-pTyr (upper panel) or anti-Fish.2 (lower panel). The apparent molecular weight of the tyrosine-phosphorylated species is 150 kDa. (B) Fish is tyrosine-phosphorylated in temperature-sensitive v-Src transformed fibroblasts following a shift to the permissive temperature. Lysates were prepared from ts68.10 cells following a time course of shifting from the non-permissive temperature (34.5°C) to the permissive temperature (39.5°C) and immunoprecipitated with anti-Fish.2 antibody, followed by analysis by SDS-PAGE and immunoblotting with anti-pTyr (upper panel) or anti-Fish.2 (lower panel). The apparent molecular weights of the two forms of Fish detected in Rat1 cells are 140 and 150 kDa; only p150 is tyrosine-phosphorylated. (C) Fish is associated with several tyrosine-phosphorylated proteins. Lysates prepared from NIH 3T3 cells (C17) or SrcY527F-transformed NIH 3T3 cells (527) were immunoprecipitated with anti-Fish.1 or anti-Fish.2 antibodies and analysed by SDS-PAGE. Proteins were transferred to nylon and immunoblotted with anti-pTyr. Tyrosine-phosphorylated proteins that appear to co-associate with Fish in immunoprecipitates with both antibodies (bands at ~65, ~115 and ~125 kDa) are indicated by arrows.

fibroblasts with PDGF (Figure 6), LPA and bradykinin (data not shown) did lead to an increase in tyrosine phosphorylation of Fish. The kinetics of this phosphorylation was quite slow, with increases still being detected 2 h after stimulation. It will be interesting to determine whether tyrosine phosphorylation of Fish has a functional role in the cell's response to growth factors.

One response commonly elicited by growth factors, and frequently involving proteins with SH3 domains and PX domains, is reorganization of the cortical actin cytoskeleton. Indeed, as shown in Figure 7A, disruption of the actin cytoskeleton by treatment with cytochalasin D (cytD) resulted in a dramatic change in the profile of tyrosine-phosphorylated proteins, with both increases and decreases in phosphorylation being detected. One set of proteins which showed increased phosphorylation had molecular weights in the range of 150 kDa. Immunoprecipitation and anti-phosphotyrosine immunoblotting confirmed that these were the Fish proteins (Figure 7B, left panel). Identical results were obtained when latrunculin B (a marine toxin that also disrupts the actin cytoskeleton) was used (data not shown). Further analysis showed that Fish proteins became phosphorylated within 2 min of cytD treatment (Figure 7B, right panel), and at sub-micromolar concentrations of the drug (Figure 7C). These results strongly suggest that the tyrosine phosphorylation of Fish is connected to the integrity of the actin cytoskeleton. Src-transformed cells have a disorganized actin cytoskeleton, and many of the known Src substrates are cytoskeletal proteins. Yet to our knowledge, there have been no reports that disruption of the actin cytoskeleton can activate Src. To examine this, we measured Src activity, both by autophosphorylation and enolase phosphorylation, from untreated and cytD-treated cells. cytD had no effect

Fig. 6. Fish is tyrosine-phosphorylated in Rat1 cells in response to PDGF. Lysates prepared from Rat1 cells following treatment with 50 ng/ml PDGF for the times indicated were immunoprecipitated with anti-Fish.2 antibodies and analysed by SDS-PAGE. Proteins were transferred to nylon and immunoblotted with anti-pTyr (upper panel) and anti-Fish.2 (lower panel). The apparent molecular weights of the two forms of Fish detected in Rat1 cells are 140 and 150 kDa; only p150 is tyrosine-phosphorylated.

on the level of Src in the cell, but it did lead to a modest increase in its intrinsic kinase activity (Figure 8), suggesting that it is activated under the same conditions that we detected Fish phosphorylation. We have detected this activation using both a Src-specific monoclonal antibody and anti-cst.1, an antibody that recognizes Src, Fyn and Yes. The average activation that we detected in five separate experiments was 1.8-fold.

Discussion

Despite the large number of substrates for Src already described in the literature, several proteins phosphorylated on tyrosine in Src-transformed cells remain uncharacterized, and many important targets may have so far gone undetected because of their low abundance. We therefore sought a method to detect substrates for Src that would not rely on association with Src or relatively high abundance in the cell, and that would allow rapid cloning of the cDNAs encoding them. The method we devised requires

Fig. 7. Fish is tyrosine-phosphorylated in response to treatment with cytochalasin D. (A) Treatment of NIH 3T3 cells with cytD results in changes in total cellular phosphotyrosine. Lysates from NIH 3T3 cells treated with either DMSO alone or 10 μ M cytD for 10 min were analysed by SDS-PAGE. Proteins were transferred to nylon and immunoblotted with anti-pTyr. Black arrows indicate bands that have increased following cytD treatment and white arrow heads indicate bands that have decreased. The two panels derive from the same immunoblot, with the top panel being a lighter exposure than the bottom. (B) Fish is tyrosine-phosphorylated rapidly following cytochalasin D treatment. Lysates from NIH 3T3 cells treated with either DMSO alone or 10 μ M cytD for 10 min were immunoprecipitated with anti-Fish.2 antibodies, analysed by SDS-PAGE and immunoblotted with anti-pTyr (left panel). NIH 3T3 cells were treated with 10 μ M cytD for the times indicated, and lysates were prepared and immunoprecipitated with anti-Fish.2 antibodies. Proteins were analysed by SDS-PAGE and immunoblotted with anti-pTyr (right panel). (C) Fish is tyrosine-phosphorylated following treatment with low concentrations of cytochalasin D. Lysates were prepared from NIH 3T3 cells treated with the different concentrations of cytD as indicated for 15 min, and immunoprecipitated with anti-Fish.2 antibodies. Proteins were analysed by SDS-PAGE, and immunoblotted with anti-pTyr (upper panel) and anti-Fish.2 (lower panel).

a bacteriophage expression library, an enriched source of Src kinase (e.g. baculovirus-infected insect cell extracts) and phosphotyrosine antibodies. Using this approach we identified cDNAs for several candidate Src substrates. One of the cDNA clones, Tks-7, encoded a fragment of a known Src substrate, p130^{Cas} (Sakai *et al.*, 1994), implying that the method is a valid way to detect tyrosine kinase substrates. Intriguingly, several of the cDNAs identified by this method encode proteins with SH3 domains (p130^{Cas}, SH3P7, s19, CIP4/STP and Fish), although their SH3 domains *per se* were clearly not required to facilitate phosphorylation by Src and subsequent detection since none of the Tks cDNAs encode an intact SH3 domain (data not shown).

The Tks-5 clone, encoding Fish, is characterized further here. We identified Fish as an *in vitro* substrate for Src both when expressed as a β -galactosidase fusion protein (Figure 1) and as a GST fusion protein when incubated with a Src immunoprecipitate (data not shown). Fish was also tyrosine-phosphorylated in 293 cells when co-expressed with an activated form of Src, and endogenous Fish was phosphorylated in Src-transformed NIH 3T3 cells. Furthermore, tyrosine phosphorylation of Fish increased within minutes of transferring cells containing a temperature-sensitive form of v-Src from non-permissive to permissive temperature. These findings suggest that Fish is indeed a direct substrate of Src in Src-transformed cells. We are currently analysing several other proteins identified by this method, and have found that at least two others are substrates for activated Src in mammalian

cells (C.Abram and S.A.Courtneidge, unpublished data). All of the cDNA products that were identified contained at least one consensus Src phosphorylation site (Songyang and Cantley, 1995), suggesting that enzyme specificity is retained under the conditions we used to phosphorylate the cDNA library. This is perhaps not surprising, since substrate recognition by tyrosine kinases, like SH2 domain specificity, appears to be dictated by very few residues surrounding the phosphorylation site and is therefore likely to be independent of substrate conformation (Songyang and Cantley, 1995). We believe that this method will be generally applicable, using expression libraries from different tissues and the tyrosine kinase of choice. Indeed, an analogous method to ours for identifying substrates of serine/threonine as well as tyrosine kinases was reported recently (Fukunaga and Hunter, 1997). The MAP kinase ERK1 was used to phosphorylate a HeLa cell cDNA library in the presence of [γ -³²P]ATP and positive clones were detected by autoradiography. Using this method a number of known substrates of MAP kinases, as well as the novel kinase MNK1, were identified. In contrast to this method, we used enriched rather than purified protein kinase for the phosphorylation step. In addition, we used a commercial λ gt11 cDNA library while Fukunaga and Hunter (1997) constructed a library using a novel expression vector.

Fish—a new adaptor protein

Fish is a new, broadly expressed adaptor protein, which appears to associate with several proteins in

Fig. 8. Src is activated by cytochalasin D treatment. (A) Src is activated by cytochalasin D treatment. Lysates of NIH 3T3 cells treated with either DMSO alone or 10 μ M cytD for 10 min were immunoprecipitated with anti-Src antibodies. The immunoprecipitates were subjected to an *in vitro* kinase assay using enolase as a substrate. Proteins were analysed by SDS-PAGE and autoradiography. (B) The level of Src is not altered by cytochalasin D treatment. Lysates of NIH 3T3 cells treated with either DMSO alone, or 10 μ M cytD for 10 min, were immunoprecipitated with anti-Src antibodies. Proteins were analysed by SDS-PAGE, transferred to nylon and immunoblotted with anti-cst.1.

Src-transformed cells. Three forms of Fish were detected in NIH 3T3 cells. These forms are unlikely to be proteolytic breakdown products because they were detected even when cells were lysed in hot extraction buffer containing SDS. They may occur as a result of post-translational modifications, although this cannot be accounted for by tyrosine phosphorylation since Fish is not detectably phosphorylated in non-transformed NIH 3T3 cells. In addition, preliminary experiments suggest that neither serine/threonine phosphorylation nor glycosylation can account for the three forms. The most likely explanation is that they are encoded by different RNA splice products. Indeed we identified two distinct cDNAs for Fish in the embryonal cDNA library, the smaller of which encoded a protein of ~150 kDa when expressed in COS cells. The p140 and p130 forms of Fish may be generated by other splice variations. The complexity of Fish isoforms is also evident in different cell types. For example in human platelets a single band of 150 kDa was detected, whereas all three forms were found in human fibroblasts and vascular smooth muscle cells (data not shown). Rat1 fibroblasts have p150 and p140 forms (Figure 6). The fact that in all of these cases two different antibodies raised against the original murine Fish sequence recognized each of these proteins confirms that they are indeed isoforms of Fish.

Fish contains many potential binding sites for other proteins; the most notable are the five SH3 domains. Strikingly, all five are most related to each other and to the p47^{phox} SH3 domains, suggesting that they may all

bind to the same or related ligands, although the binding specificities of the Fish SH3 domains remain to be determined. Interestingly, the Fish PX domain is also most related to that of p47^{phox} suggesting that these proteins may be the products of genes which arose from a common ancestor. The region of Fish encoded by the Tks-5 cDNA contained three consensus Src phosphorylation sites, designated Y552, Y557 and Y619 (see Figure 2A). These sites conform to consensus binding sites for the SH2 domains of Abl (Y-E-E-P), Nck or Crk (Y-D-X-P), and Grb2 (Y-X-N-X), respectively (Songyang *et al.*, 1993). Conceivably, tyrosine-phosphorylated forms of Fish, such as those found in Src-transformed fibroblasts, could associate with one or more of these proteins *in vivo*. As well as SH3 domains, Fish also has three polyproline segments which encompass one or more P-X-X-P motif that could allow it to bind to SH3 domains (Pawson, 1995). Fish also has many potential sites of serine phosphorylation.

Perhaps the most unusual feature of Fish is the presence at the N-terminus of a phox homology or PX domain. This domain, which is ~100–110 amino acids in length, has only recently been described (Ponting, 1996), and its function is not yet known. To our knowledge Fish is the first protein containing a PX domain to be implicated in a tyrosine-kinase signalling pathway. PX domains have several well conserved residues, many of them hydrophobic, and two almost invariably conserved basic residues, which may be important for function. The pattern of conserved hydrophobic residues strongly suggests that the PX domain includes an N-terminal segment predominantly composed of β -strand-like sequences and both central and C-terminal α -helical structures that are separated by a conserved proline-rich sequence of indeterminate secondary structure (Ponting, 1996; data not shown). It has been proposed that the proline-rich sequence might constitute a binding site for SH3 domains although this has not been tested directly (Ponting, 1996).

Where studied, PX domain-containing proteins are often associated with processes involving the actin cytoskeleton, membranes and/or GTP-binding proteins. For example the *Saccharomyces cerevisiae* protein Bem1, and Scd2, its orthologue in *Shizosaccharomyces pombe*, appear to coordinate rearrangement of the cortical cytoskeleton during cell polarization in response to mating factors (Chenevert *et al.*, 1992; Chang *et al.*, 1994). Bem1 interacts directly with Cdc24, a guanine nucleotide exchange factor for the Rho-family GTPase Cdc42, and probably indirectly with Cdc42 itself (Peterson *et al.*, 1994). Similar interactions have been reported for Scd2 in *S.pombe* (Chang *et al.*, 1994). Bem1 has also been shown to interact with Ste20p, a serine/threonine kinase belonging to the p21-activated kinase (PAK) family, as well as to actin (Leeuw *et al.*, 1995). Another bud emergence gene, Bem3, also has a PX domain (Zheng *et al.*, 1994). Interestingly, Bem3 is a GTPase activating protein (GAP) for certain Rho-family proteins indicating that, like Bem1, it also interacts with GTP binding proteins. Two components of the neutrophilic NADPH oxidase complex, p40^{phox} and p47^{phox}, each have a PX domain very similar to that of Fish. Activation of the oxidase, and production of superoxide and peroxide in response to invading microorganisms, or to agonists such as fMLP, involves recruitment of the cytoplasmic proteins p40^{phox} and p47^{phox} (and other

proteins including p67^{phox} and the small GTP-binding protein Rac2) to the plasma membrane, where they associate with membrane-bound flavocytochrome b₅₅₈ (Segal and Abo, 1993; Dorseuil *et al.*, 1996). Significantly, oxidase activation is concomitant with phagocytosis, a membrane process requiring rearrangement of the cortical actin cytoskeleton (Hall, 1992). Recently a new PI 3-K was described in mouse and *Drosophila* (variously known as Cpk, 68 D or p170) that also contains a PX domain (MacDougall *et al.*, 1995; Molz *et al.*, 1996; Virbasius *et al.*, 1996). Another enzyme which contains a PX domain and is involved in membrane-associated phospholipid metabolism is phospholipase D1 (Ponting and Parker, 1996). Finally, other PX domain-containing proteins with known functions include Vam7, which is involved in vacuolar morphogenesis (Wada and Anraku, 1992), Mvp1 and Vps17, which are involved in sorting of proteins to vacuoles (Kohrer and Emr, 1993; Ekena and Stevens, 1995) and Mdm1, which is involved in organelle inheritance (McConnell and Yaffe, 1992). It is hard to predict from the sequence of the PX domain what it might interact with; both phospholipids and proteins are candidate ligands. It is now possible to undertake functional studies of PX domain-containing proteins bearing targeted mutations, as well as binding studies of the isolated domains *in vitro*, in order to resolve this issue.

Fish was tyrosine-phosphorylated at cytochalasin D concentrations that disrupt the cortical actin cytoskeleton, and also in Src-transformed fibroblasts, which have a dramatically altered actin cytoskeleton. Furthermore, Fish was tyrosine-phosphorylated in response to treatment of Rat1 fibroblasts with either PDGF, LPA or bradykinin. Each of these mitogens activates the Rho family of small GTP binding proteins and causes changes in the actin cytoskeleton such as stress fibre, lamellipodia and filopodia formation (Hall, 1998). These data suggest that Fish may play a role in cytoskeletal changes.

Many of the Src substrates identified so far are cytoskeletal proteins, including p110 AFAP, paxillin, vinculin and cortactin, which has led to the suggestion that in transformed cells Src can regulate cytoskeletal structures (Brown and Cooper, 1996). In normal cells, Src is activated by mitogens such as PDGF, and is required for DNA synthesis in response to several growth factors (Erpel and Courtneidge, 1995). More recently, it has also been reported that PDGF stimulation of cells results in the translocation of Src to regions of the cell periphery that also show dense actin staining. This effect was seen within 30–60 min of PDGF addition, and required an intact actin cytoskeleton and the activity of Rho family proteins (Fincham *et al.*, 1996), suggesting that the activity of Src may be under the control of the actin cytoskeleton. By this mechanism, the cytoskeleton could control the access of Src to substrates such as Fish, which might explain the rather slow kinetics of Fish phosphorylation following PDGF stimulation. Alternatively, Fish could be on a pathway downstream of Src that controls the activity of Rho family proteins and their effect on the actin cytoskeleton. In this regard, it is interesting that tyrosine kinases are proposed to participate both upstream and downstream of Rho proteins (Ridley and Hall, 1994; Nobes *et al.*, 1995; Kranenburg *et al.*, 1997).

An alternative potential function for Fish can be pro-

posed from the observation that it is most closely related to the cytosolic NADPH oxidase components of neutrophils, p47^{phox} and p40^{phox}. Interestingly, a recent report described the identification of Posh, a Rac-interacting adaptor protein with four SH3 domains, which has a striking similarity to p67^{phox} (Tapon *et al.*, 1998). The expression of the p47 and p67 proteins is restricted to phagocytic cells (Segal and Abo, 1993), whereas both Posh and Fish are more broadly expressed. In neutrophils, the respiratory burst can be initiated by the chemoattractant and G-protein-coupled receptor agonist fMLP (Segal and Abo, 1993). However, superoxide formation has also been observed in many other cell types, and has indeed been linked to carcinogenesis (Burdon, 1995). One exciting possibility is that Fish forms part of the superoxide-generating machinery in non-phagocytic cells, a hypothesis which is currently being tested.

Materials and methods

Phosphorylation screening

The phosphorylation screening method we used to identify Src substrates was adapted from a technique designed to identify binding partners of the Shc phosphotyrosine binding (PTB) domain (Kavanaugh *et al.*, 1995). In the primary screen for Src substrates, 2 × 10⁵ individual clones from a λgt11 library containing mouse Swiss 3T3 fibroblast cDNA (Clontech; ML1023b) were plated on four 150 mm Petri dishes. Plates were overlaid with nitrocellulose filters impregnated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 37°C for 16 h to induce expression of recombinant lacZ fusion proteins. Replica filters were washed extensively in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100) then equilibrated for 1 h in kinase buffer (TBST containing 10 mM MgCl₂ and 2 mM MnCl₂). Filters were blocked for 1 h at room temperature in kinase buffer containing 3% bovine serum albumin (BSA). Recombinant proteins were phosphorylated by incubating filters at 30°C for 60 min in kinase buffer supplemented with one tenth volume of an Sf9 extract containing baculovirus-derived human Src, 250 μM ATP and 100 μM sodium orthovanadate. Filters were washed briefly with kinase buffer alone then incubated in stripping buffer (62.5 mM Tris-HCl pH 7.0, 2% SDS, 100 mM 2-β-mercaptoethanol) at 50°C for 30 min to remove possible associated phosphoproteins, including Src itself or Sf9 cell-derived proteins, which might interfere with the screening. Tyrosine-phosphorylated proteins were detected with an anti-phosphotyrosine monoclonal antibody, 4G10, using standard immunoblotting methodology (see below). Twelve positive clones were plaque-purified by successive rounds of phosphorylation screening. The cDNA inserts were amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase (Boehringer Mannheim) and subcloned into pCRII (Invitrogen Corp.). The cDNA inserts were sequenced with the aid of T7 and SP6 oligonucleotide primers.

Isolation of a full-length Fish cDNA

To identify the full-length Fish cDNA, a murine day 11.5 embryo cDNA library (Clontech, ML1027b) (a generous gift of G.Plowman, SUGEN) was screened by hybridization with a 1 kb ³²P-labelled DNA fragment corresponding to the Tks-5 cDNA that was isolated during the initial screen for Src substrates. Two cDNA clones were identified, λME14 and λME5E, which contained ~1.3 kb of additional 5' sequence and 0.5 kb of 3' sequence relative to Tks-5, respectively. Radiolabelled DNA probes corresponding to fragments at the 5'-end of λME14 and the 3'-end of λME5E were then used to rescreen the embryo cDNA library. A cDNA designated λMEA3C was identified which contains an additional 0.3 kb of sequence at the 5'-end relative to λME14, including a presumptive translational initiation codon. Two non-identical clones, λME14(3') and λME5A, were found to contain ~0.6 kb of additional 3'-sequence relative to λME5E including two stop codons at the 3'-end of the Fish coding sequence. λMEA3C and λME14 were shown to differ with respect to putative alternative exons of 45 and 84 bp which are present in λME14 but absent from λMEA3C, indicating the existence of at least two alternatively spliced transcripts. Two Fish cDNA sequences were predicted on the basis of the overlapping cDNAs identified. The amino acid sequence of the corresponding Fish isoforms, which include

both the 15 and the 28 residue insertions, or lack these sequences, respectively, was deduced on the basis of these cDNAs.

Analysis of RNA expression

A multiple tissue Northern blot containing 2 µg of poly(A) RNA from various adult mouse tissues (Clontech) was hybridized with a ³²P-labelled DNA probe corresponding to the 1 kb Tks-5 cDNA (shown in Figure 2A) under high stringency conditions (65°C, 0.1× SSC, 0.1% SDS). The blot was stripped according to the suppliers protocol and rehybridized with a ³²P-labelled β-actin probe.

Constructs

Expression plasmids encoding wild-type and epitope-tagged versions of the smaller Fish isoform (see above) were constructed. For the wild-type construct, the following DNA fragments were subcloned into the *NotI* and *XbaI* sites of pBluescript II KS (pBSIIKS, Stratagene): a 1423 bp *EagI*–*EcoRI* fragment from λMEA3C which contains 54 bp of 5′-untranslated region (UTR) and sequences encoding residues 1–412; a 566 bp *EcoRI*–*SacI* fragment from λME14 which encodes residues 413–602; and a 1548 bp *SacI* partial *XbaI* fragment originating from λME14(3′) which codes for amino acids 603–1081, contains 80 bp of 3′-UTR and an additional 36 bp of polylinker sequences from pBSIIKS. The resulting plasmid, pBS-Fish, was digested with *EagI* and *XbaI*, releasing a 3414 bp fragment encompassing the complete Fish coding region. Recessed 3′-termini were filled using Klenow enzyme (Boehringer Mannheim), and *BstXI* adaptors (Invitrogen) were ligated onto the ends. The fragment was subcloned into the *BstXI* sites of pEF-BOS (Mizushima and Nagata, 1990) in the forward and reverse orientations to generate the plasmids pEF-BOS Fish(+) and pEF-BOS Fish(–), respectively. The plasmid pEF-BOS NmycFish, encoding two copies of the myc epitope recognized by the 9E10 antibody fused to the N-terminus of Fish, was generated by ligating the following fragments into the *BamHI* and *XbaI* sites of pEF-BOS Nmyc (a gift from P.Orban, EMBL, Germany): a 294 bp *BglIII*–*ClaI* fragment derived by PCR using Vent polymerase (New England Biolabs), which encodes Fish residues 1–96; a 950 bp *ClaI*–*EcoRI* fragment encoding residues 97–412; and a 2119 bp *EcoRI*–*XbaI* fragment encoding residues 413–1081 and including 80 bp of 3′-UTR plus 36 bp of polylinker sequences.

Antibodies and peptides

The Fish-specific polyclonal antiserum, Fish.1, was generated by immunising rabbits with a purified glutathione *S*-transferase (GST) fusion protein containing residues 457–787 of Fish. A second polyclonal anti-Fish antibody, Fish.2, was generated by immunising rabbits with a GST fusion protein containing residues 807–908. Fish.2 antiserum was affinity purified by passing over a column containing the GST fusion protein, coupled to 3M Emphaze™ Biosupport Medium AB1 (Pierce) according to manufacturer's instructions. The anti-phosphotyrosine (α-pTyr) monoclonal antibody 4G10 was from Upstate Biotechnology Incorporated (UBI). The mouse monoclonal antibodies, EC10 (UBI) and 327 (vSrc-1, Calbiochem) and the rabbit polyclonal antibody, anti-cst.1 (Courtneidge and Smith, 1984) were used to detect Src.

Cell culture, baculoviral infection and DNA transfection

COS cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics at 37°C in 5% CO₂. 293 cells, Rat1 cells, ts68.10 cells which express a temperature-sensitive mutant of Src (Courtneidge and Bishop, 1982), NIH 3T3 cells and their derivative, 527 cells, which stably express a mutant form of chicken Src with an activating Y527F substitution, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and antibiotics at 37°C in 10% CO₂. Sf9 insect cells were maintained in Grace's medium containing 7.5% FCS at 27°C. COS cells were transfected by electroporation. Briefly, 2×10⁷ cells were transfected with 10 µg of pEF-BOS Fish(+) or pEF-BOS Fish(–). Transfectants were analysed ~40 h post-transfection. 293 cells were transfected with Lipofectamine™ (Gibco-BRL) according to manufacturer's instructions. Ten centimetre dishes of cells at ~70% confluence were transfected with a total of 6 µg of DNA (pEF-BOS NmycFish alone, or in combination with pSG5-SrcK+, pSG5-SrcK- or pSG5-SrcY527F) in serum free medium OptiMEM (Gibco) for 12–24 h. Cells were lysed 24 h later. For baculoviral infections, adherent cells were infected with recombinant baculovirus containing the human Src cDNA at a multiplicity of infection (MOI) of 1 and cell lysates prepared after ~72 h.

Cell treatment, lysis, immunoprecipitation and kinase assay

Cells were treated with Cytochalasin D (Sigma or Calbiochem) at 10 µM for 10 min at 37°C unless stated otherwise. The permissive and non-

permissive temperatures used in experiments with ts68.10 cells were 34.5 and 39.5°C, respectively. Cells were treated with 50 ng/ml PDGF (UBI) at 37°C for the times stated. Extracts were prepared from NIH 3T3, 527, 293, ts68.10, Rat1 or COS cells, by washing the adherent cells with ice cold TBS (25 mM Tris–HCl pH 7.5, 150 mM NaCl) containing 100 µM Na₃VO₄ and 2 mM dithiothreitol (DTT). Cells were lysed in 0.5 to 2.0 ml of either NP40 lysis buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 1% Nonidet P40) or RIPA lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 100 µM Na₃VO₄, 10 mM NaF, 2 mM DTT, 10 µg/ml aprotinin, 20 µM leupeptin and 100 µM phenylmethylsulfonyl fluoride (PMSF) for 10–15 min at 4°C. Extracts were clarified by centrifugation at 10 000 g for 10 min and the protein concentration determined. Extracts of Sf9 cells expressing human Src baculovirus were prepared essentially as described above except that the cells were harvested by centrifugation in 50 ml tubes, prior to lysis. For immunoprecipitations, samples containing 150–400 µg of total protein were incubated with either 1–2 µl of crude pre-immune serum or antiserum, or 1 µg of affinity-purified antibody, and ~7 µl packed volume of protein A–Sepharose beads (Pharmacia) or protein A–agarose beads (Santa Cruz) for 1 h at 4°C. Immunocomplexes were washed four times in ice cold RIPA buffer containing 100 µM Na₃VO₄ and 1–2 mM DTT. Samples were resuspended in SDS sample buffer (80mM Tris pH 6.8, 2% SDS, 75 mM DTT, 10% glycerol, 1.25% Bromophenol Blue), heated to 95°C for 5 min and subjected to SDS–PAGE using 7.5 or 9% polyacrylamide gels. Kinase assays were carried out as described previously (Kypta *et al.*, 1990). Briefly, 50 µg lysate was immunoprecipitated with 1 µg of anti-Src antibody (327) and 20 µl protein A/G plus agarose (Santa Cruz) followed by washing four times with ice cold RIPA buffer containing 100 µM Na₃VO₄ and 1 mM DTT, and once with kinase buffer (20 mM HEPES pH 7.4, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT). The reactions were carried out in 20 µl kinase buffer containing 15 µM unlabelled ATP, 3 µg acid-denatured enolase and 10 µCi [γ-³²P]ATP for 10 min at 30°C, and stopped with SDS-sample buffer. The samples were analysed by SDS–PAGE and autoradiography using Fuji RX film.

Immunoblotting

SDS–PAGE gels were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) or PVDF (Millipore) using a Millipore semi-dry blotting apparatus. Filters were incubated in blocking solution (either PBS or TBS containing 0.1% Tween 20 and 2–3% BSA) for 1 h at room temperature. Filters were washed three times in wash solution (PBS or TBS containing 0.1% Tween 20) and incubated with blocking solution plus the primary antibody for 1 h at room temperature using the following antibody dilutions: α-pTyr, 1:1000–1500; α-Fish.1, 1:7500–10 000; α-Fish.2, 0.5 µg/ml; EC10, 2 µg/ml; α-cst.1, 1:200. Filters were washed three times in wash solution and incubated with the secondary detection reagent for 1 h at room temperature. For blots probed with a rabbit polyclonal antibody, filters were incubated with blocking solution containing either protein A-horseradish peroxidase (HRP) conjugate (Amersham) at a dilution of 1:1000–2500 or donkey anti-rabbit HRP (Amersham) at 1:20 000. For blots probed with mouse monoclonal antibodies, filters were incubated with sheep anti-mouse HRP conjugate (Amersham) at a dilution of 1:5000. Filters were washed three times in wash solution and the protein bands detected using enhanced chemiluminescence (ECL, Amersham or Supersignal, Pierce) in conjunction with Fuji RX film.

EMBL Accession Number

The accession number for the Fish cDNA sequence is AJ007012.

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