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A Chemical Genetic Screen for Direct v-Src Substrates Reveals Ordered Assembly of a Retrograde Signaling Pathway

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Summary

Using an ATP analog that is a specific substrate for an analog-specific allele of v-Src, we identified several novel cytoskeletal substrates that control actin assembly processes. A screen for less abundant v-Src substrates revealed the scaffolding protein Dok-1 as a direct substrate of v-Src. Further studies suggest that v-Src phosphorylation sites on Dok-1 are critical for its binding to RasGAP and Csk, negative regulators of Src signaling. This results in the downregulation of growth-promoting signals of the Src family kinases and the Ras pathway. Identification of the direct substrates of v-Src leads to a model for the precise order of assembly of a retrograde signaling pathway in v-Src-transformed cells and has provided new insight into the balance between those signals that promote cell transformation mediated by v-Src catalyzed tyrosine phosphorylation and those that inhibit it.

Background

Signaling networks regulated by tyrosine kinases are complex, highly interconnected, and consequently difficult to dissect [1]. Tyrosine kinases are critical components of the proximal intracellular signaling machinery. Yet the precise connectivity between protein kinases and their downstream substrates has not been elucidated in the case of many protein kinases. This is because many tyrosine kinases display overlapping substrate specificities in vitro and can functionally compensate for each other in single-gene knock-out experiments [2]. Various techniques have been used to define the precise substrate phosphorylation events in signaling cascades by cell lysis followed by immunoprecipitation of relevant kinase complexes [3, 4], cDNA library screening [5, 6, 7], and in vitro determination of optimal peptide phosphorylation sequences [8]. Recently, mass spectrometry and functional proteomics have been used to probe the phosphorylation of substrates in intact cells by the use of a specific MKK1/2 inhibitor [9]. However, the fundamental question of which kinase is responsible for a given phosphorylation event remains elusive because of the presence of many kinases in the same pathway that exhibit overlapping substrate specificities [10] and because of the downstream effects of inhibiting one kinase in a cascade [9].

In contrast to the poorly defined direct substrates for protein kinases, much is known about the downstream effects of phosphoproteins [11, 12]. Two basic mechanisms for conversion of a phosphorylation event into a molecular switch have been revealed. These are regulation of the catalytic activity of an enzyme through a phosphorylation-induced conformational change or the induction of a protein-protein interaction via a phospho-specific recognition domain such as an SH2 domain. Recently, scaffolding proteins, which are an extreme example of the latter class of phosphoproteins, have been cloned and functionally characterized in various signaling networks. These proteins typically are membrane associated, lack enzymatic activity, and contain multiple domains and phosphorylation sites. Examples include Dok-1 (Downstream of kinase-1) in a variety of tyrosine kinase cascades [13–17], Lat-1 (Linker for T cell activation-1) in T cell signaling complexes [18], and Cbp (Csk binding protein) in neurons [19]. Studies of the phosphorylation sites in these proteins and the subsequent association of SH2-containing proteins have revealed the importance of scaffolding proteins in providing docking sites for relocalization of cytoplasmic components of the signaling apparatus to specific membrane regions, such as lipid rafts or T cell receptor complexes. However, the presence of multiple protein kinases in such complexes has made assignment of the kinases that are responsible for the phosphorylation of scaffolding proteins impossible to determine [1]. The ability to define the precise sites on scaffolding proteins phosphorylated by one kinase would allow for an understanding of the role of particular kinases in recruiting specific downstream effectors to these critical targets.

Although v-Src has been the focus of many studies since it was shown to possess tyrosine kinase activity, its direct substrates and mechanism of cell transformation remain to be fully described [20]. Here we use a chemical method for specifically radiolabeling the direct cellular substrates of the oncoprotein v-Src. The method relies on a v-Src mutant that is capable of accepting a non-natural phosphate donor substrate (A*TP = N^6 (benzyl) ATP) that is poorly accepted by wild-type protein kinases in the cell [21–23]. The modification of v-Src's active site (I338G v-Src) so that it accepts a structurally modified nucleotide analog provides a unique tool by which the direct substrates of any particular kinase (v-Src) can be traced in the presence of other protein kinases for the first time.

A broad screen for v-Src targets in NIH 3T3 cells led to the identification of several heretofore unknown candidate substrates of v-Src; these substrates are known to be involved in cellular processes critical for cell transformation. A focused screen was carried out with protein

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preparations enriched for potential direct substrates of v-Src. This analysis revealed a highly restricted set of candidate direct v-Src substrates. One protein substrate in particular, Dok-1, contains multiple phosphorylatable tyrosine residues (14 sites) and is known to serve as a scaffold protein for the recruitment of downstream signaling components [24, 25]. Only two of the Dok-1 phosphorylation sites were directly phosphorylated by v-Src. The role of phosphorylation at these sites was analyzed in a transformation assay, and the sites were found to be involved in the negative regulation of both Ras and cytoplasmic tyrosine kinases in the Src family. This analysis has allowed us to generate a model linking a specific sequence of v-Src phosphorylation events to two different retrograde signaling mechanisms in two distinct Dok-1 complexes. Since the presence of multiple protein kinases in signaling complexes has made assignment of the kinases responsible for site-specific phosphorylation of scaffolding proteins all but impossible to determine, this model represents the first view of multiprotein complexes at this resolution.

Results and Discussion

Global Analysis of Direct v-Src Targets Reveals New Substrates

v-Src-induced transformation of fibroblasts leads to the tyrosine phosphorylation of more than 50 proteins [20]. However, it is not clear if all of these proteins are direct v-Src substrates or if they are phosphorylated as a consequence of the v-Src activation of other kinases. We carried out a global analysis of direct v-Src substrates by using *v-Src-as1(v-Src-analog-sensitive-1)* NIH 3T3 cells and [γ-^{32}P] N^6 (benzyl) ATP. The use of [γ-^{32}P] N^6 (benzyl) ATP allows the exclusive labeling of v-Src-as1 substrates because the ATP analog is not a substrate for wild-type protein kinases [21]. Furthermore, [γ-^{32}P] N^6 (benzyl) ATP is an efficient substrate for v-Src-as1 [22]. Whole-cell kinase assays with both *v-Src* and *v-Src-as1* NIH 3T3 cell lysates with [γ-^{32}P] ATP showed an indistinguishable pattern of phosphoproteins (Figure 1A, lane 1 versus lane 3) and confirmed similar substrate specificities of both the wild-type and mutant v-Src. The similarity in total cellular phosphoprotein content agrees well with previous data showing that as1 mutant kinases exhibit the same phosphoacceptor specificity and regulatory properties of the corresponding wild-type kinases [21, 22] (Witucki et al., this issue of *Chemistry & Biology*, pp. 25–33). Labeling of *v-Src-as1* NIH 3T3 cell lysates with [γ-^{32}P] N^6 (benzyl) ATP showed a different phosphorylation pattern, as expected, since only the direct substrates of v-Src-as1 are radiolabeled (Figure 1A, lane 4). As a control, identical kinase assays with *v-Src* NIH 3T3 cell lysates containing [γ-^{32}P] N^6 (benzyl) ATP were also performed. Minimal labeling occurred in this case (Figure 1A, lane 2).

To further resolve the direct v-Src substrates from other phosphorylated proteins, we carried out 2D gel electrophoresis on *v-Src-as1* NIH 3T3 cell lysates phosphorylated with either [γ-^{32}P] ATP or [γ-^{32}P] N^6 (benzyl) ATP. More than 20 proteins were phosphorylated with [γ-^{32}P] N^6 (benzyl) ATP (Figure 1C) but were not detected in the presence of [γ-^{32}P] ATP (Figure 1B). This result is expected because all cellular kinases utilize [γ-^{32}P] ATP, and thus Figure 1B is a composite labeling of all kinase substrates in the cell. On the other hand, Figure 1C reveals only the kinase activity of v-Src-as1 and consequently required a longer exposure time to reveal proteins not visible in Figure 1B. Importantly, a high concentration of nonradiolabeled ATP was included in all reactions to ensure similar overall stoichiometry of phosphorylation and thus conservation of protein mobility.

Mass spectrometry was performed on eight Coomassie-stained protein spots that corresponded to radioactively labeled spots in Figure 1C. Five proteins were identified from a database search and are listed in Table 1. Three substrates, tubulin α-2, tubulin β-5, and α enolase, have been previously identified as substrates of v-Src based on studies in several cell types [26–28]. Our direct labeling data support these earlier studies. The remaining two proteins, Cofilin and Calumenin, are novel candidate substrates of v-Src.

One caveat of our whole-cell screen for direct v-Src substrates is the possibility that substrate phosphorylations in the cell lysate may not accurately recapitulate phosphorylation events in the intact cell. Two facts argue against this being a substantial issue with our study. Firstly, our method is able to efficiently radiolabel direct substrates without the need to overexpress the kinase v-Src at a level that was not achieved in the intact cell. Such a level might cause aberrant or incorrect substrates to become labeled. Secondly, our screen revealed three proteins previously identified by other methods as v-Src substrates.

Cofilin and Calumenin, the remaining proteins identified by mass spectrometry, are novel candidate substrates, not previously suggested to be substrates of v-Src. Cofilin is a 21 kDa actin-depolymerizing protein and is essential for cytokinesis, endocytosis, T cell stimulation, and other cellular processes that require rapid actin dynamics [29]. Regulation of Cofilin depends on several factors, including phosphorylation and dephosphorylation [30]. To date, S3 is the only Cofilin phosphorylation site that has been shown to be critical for actin binding [30]. According to our mass spectrometry data, Y117 is likely to be the direct v-Src phosphorylation site on Cofilin.

Since Cofilin has not been previously shown to be tyrosine phosphorylated and is not known to be a direct substrate of v-Src, we wondered if our mass spectrometry analysis could have misidentified Cofilin because of misalignment of the two-dimensional Coomassie-stained gel with the autorad. To ask if v-Src is likely to be the kinase responsible for the phosphorylation of Cofilin, we immunoblotted the v-Src NIH 3T3 cell lysate with the anti-phosphotyrosine-specific antibody 4G10 (Figure 1D, lane 1). No band was observed at the expected MW of Cofilin (21 kDa). To enrich for the presence of cellular Cofilin, we used the anti-Cofilin antibody (sc8442) to immunoprecepitate Cofilin from the same cells, which revealed intense phosphotyrosine staining of Cofilin. We next isolated v-Src immune complexes and found the same intense phosphotyrosine-containing 21 kDa protein, suggesting that Cofilin is both

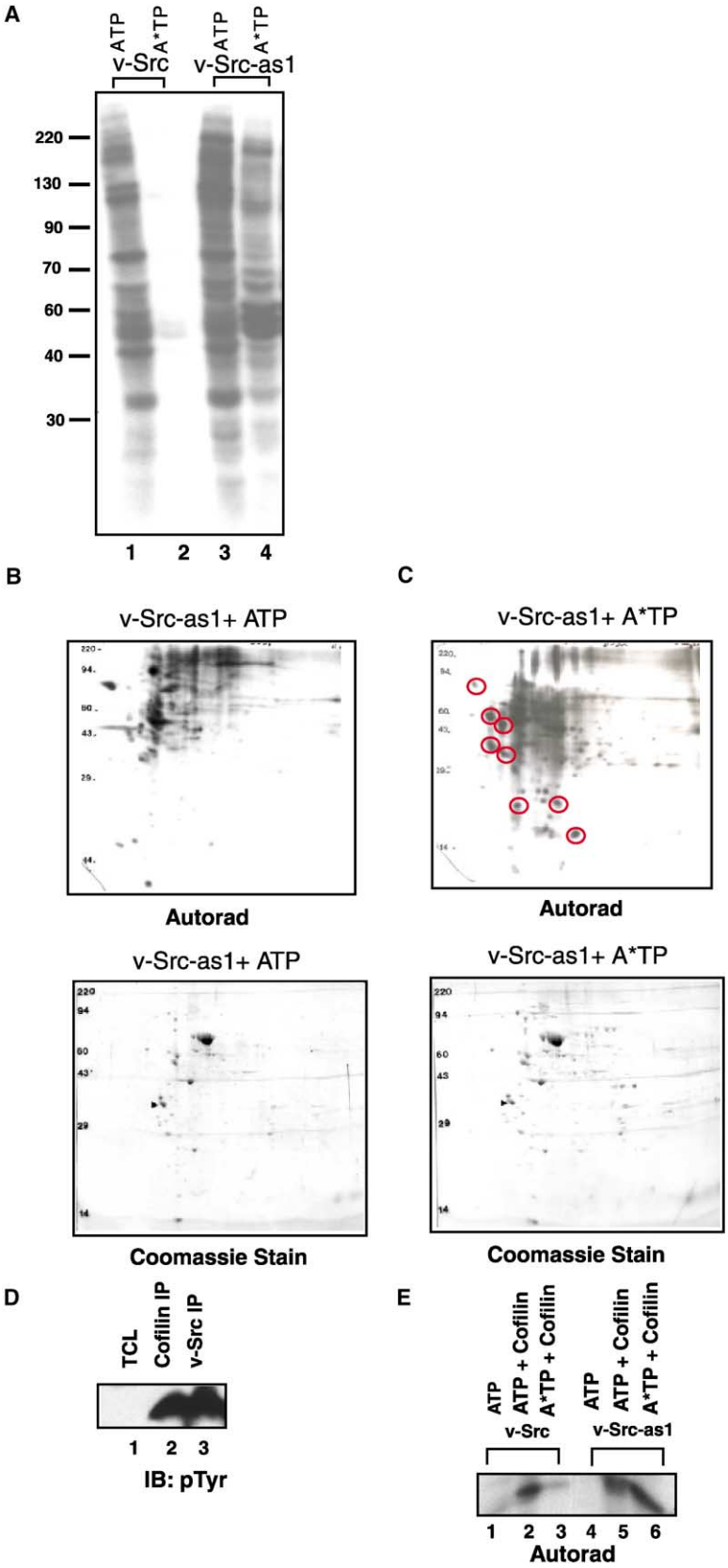

Figure 1. Differential Phosphorylation of Direct v-Src Substrates in *v-Src-as1* NIH 3T3 Cell Lysate with [γ-^{32}P]N^6(Benzyl)ATP

(A) Kinase reactions of *v-Src* and *v-Src-as1* NIH 3T3 cell lysate in the presence of 100 μM ATP and either [γ-^{32}P] ATP (lanes 1 and 3) or [γ-^{32}P]N^6(benzyl)ATP (lanes 2 and 4) for 45 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography.

(B) 2D gel electrophoresis of the kinase reaction of *v-Src-as1* NIH 3T3 cell lysate and [γ-^{32}P] ATP in the presence of 100 μM ATP for 45 min. The internal standard (tropomyosin) is shown as an arrow at molecular weight 33 K and pI 5.2. The top image is Autorad. In the bottom image, the gels were stained with Coomassie blue, dried, and scanned.

(C) 2D gel electrophoresis of the kinase reaction of *v-Src-as1* NIH 3T3 cell lysate and [γ-^{32}P] A*TP in the presence of 100 μM ATP for 45 min. The top image is Autorad. In the bottom image, the gels were stained with Coomassie blue, dried, and scanned.

(D) The lysates from wild-type *v-Src* were subjected to immunoprecipitation either with anti-Cofilin antibody (lane 2) or anti-Src (mAb 327) antibody (lane 3). Lane 1 represents whole-cell lysate. Proteins were separated on 10% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-p-Tyr (4G10).

(E) Kinase reactions of *v-Src* and *v-Src-as1* immunoprecipitates either in the absence (lanes 1 and 4) or in the presence of Cofilin (lanes 2, 3, 5, and 6). Reactions were initiated either with [γ-^{32}P] ATP (lanes 1, 2, 4, and 5) or [γ-^{32}P] N^6(benzyl)ATP (lanes 3 and 6) for 20 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography.

Table 1. Direct v-Src Substrates Identified by 2D Gel Electrophoresis and Mass Spectral Analysis

Substrates	Accession Number
Calumenin	O35887
Cofilin	P18760
Tubulin alpha-2 chain	P05213
Tubulin beta-5	NP_035785
Alpha enolase	P17182

In brief, gel spots were digested with trypsin, followed by MALDI mass spectrometric analysis with a PerSeptive Voyager DE-RP mass spectrometer in the linear mode. These peptides were analyzed by peptide mass fingerprinting and data base searching with Protein Prospector.

present and tyrosine phosphorylated in complex with v-Src, consistent with it being a substrate of v-Src. The fact that Cofilin was not visible in a whole-cell phosphotyrosine immunoblot is consistent with it being present at low concentration or at low phosphorylation stoichiometry, a situation not uncommon for critical kinase substrates [24–25].

To confirm that v-Src is capable of direct phosphorylation of Cofilin, we asked if purified Cofilin is phosphorylated by v-Src in an in vitro kinase reaction. When Cofilin was added to a v-Src immune complex kinase assay with [γ-^{32}P] ATP, a new 21 kDa band is intensely labeled (Figure 1D, lane 2). Furthermore, this same band is labeled when the v-Src-as1 immune complex is provided with [γ-^{32}P] N^6 (benzyl) ATP, confirming that Cofilin phosphorylation is a direct consequence of v-Src-as1 activity. Together, the studies of the intracellular tyrosine phosphorylation state of Cofilin (Figure 1D) and the ability of v-Src to phosphorylate purified Cofilin in vitro strongly support the identification of Cofilin as a direct cellular substrate of v-Src. Cytoskeletal reorganization is the hallmark of v-Src transformed fibroblasts; direct phosphorylation of Cofilin by v-Src may reveal a new mechanism for this regulation.

The other novel candidate direct v-Src substrate, Calumenin, is localized to the ER. Other ER resident proteins, such as BiP, have been suggested to be Src kinase substrates [31], which would suggest that this cellular compartment may contain other v-Src substrates as well. Calumenin belongs to a newly identified family of multiple EF-hand proteins called the CREC (Cab45, Reticulocalbin, ERC-45, Calumenin) family [32]. The exact function of CREC family proteins are not known, but recent evidence shows that Reticulocalbin is overexpressed in highly invasive breast cancer cell lines [33], and ERC-55 is implicated in cervicical cancer [34]. To date, there have been no reports of tyrosine phosphorylation of any of the CREC family members. In view of the fact that some of the CREC family members are overexpressed in breast and cervical cancer, phosphorylation of Calumenin by v-Src may have important consequences related to metastasis. We are currently eliciting antibodies against a C-terminal peptide sequence in Calumenin to facilitate studies to assess the intracellular tyrosine phosphorylation status of this protein.

v-Src Has a Restricted Repertoire of Direct Targets

Association of the novel v-Src substrate Cofilin in a protein complex with v-Src (Figure 1D) suggested an alternative to whole-cell lysate screening for direct v-Src substrates. We predicted that the use of v-Src immunoprecipitations to enrich for v-Src substrates prior to ^{32}P labeling would allow the identification of substrates present at low concentration and those substrates that are phosphorylated at low stoichiometry. This technique has been used by many investigators to enrich for kinase substrates [35, 36]. v-Src-as1-containing complexes were immunoprecipitated by GD11 from v-Src-as1 NIH 3T3 cells, and [γ-^{32}P] N^6 (benzyl) ATP was added to the immune complex. As controls, we carried out identical kinase assays with immune complexes isolated from *v-Src* NIH 3T3 cells and incubated with [γ-^{32}P] N^6 (benzyl) ATP (negative control) or [γ-^{32}P] ATP (positive control). When v-Src-as1 was immunoprecipitated from pooled *v-Src-as1* NIH 3T3 and incubated with [γ-^{32}P] N^6 (benzyl) ATP, the three most prominently phosphorylated proteins observed were v-Src, p62, and p120 (Figure 2A, lane 4). The proteins were identified based on previous studies of v-Src-associated proteins as well as by use of antibodies to the candidate proteins [20]. The p62 protein was identified as the scaffolding protein Dok-1, a component of the RasGAP complex in transformed fibroblasts [24, 25]. Further confirmation of the identity was carried out by phospho-tryptic mapping of the radiolabeled protein (see below). The p120 protein was identified as focal adhesion kinase (Fak), a kinase activated in response to both integrin-ECM (extracellular matrix) interactions [37] and the engagement of certain tyrosine kinase growth factor receptors [38] as well as certain G protein-coupled receptors [39]. Fak also functions as a scaffolding protein that binds several signaling cytoskeletal molecules, after its full activation by Src (reviewed in [40–42]).

Because we identified Fak as a direct v-Src substrate, and because it is known to bind to certain cytoskeletal elements, we used Fak-specific antibodies and antibodies against cortactin, another putative Src substrate that regulates cytoskeletal reorganization in vivo [43], to enrich for other potential v-Src substrates in the cytoskeletal compartment. Fak and cortactin immune complexes were isolated from both *v-Src* and *v-Src-as1* NIH 3T3 cells. Kinase assays with the *v-Src-as1* NIH 3T3 lysates in the presence of [γ-^{32}P] N^6 (benzyl) ATP revealed that both Fak and cortactin were phosphorylated (Figures 2B and 2C), verifying that they are indeed direct Src targets. The only other protein identified in these complexes as a direct v-Src-as1 substrate was Dok-1. The fact that Dok-1 is present in multiple immune complexes (anti-v-Src, anti-Fak, anti-cortactin), and that it is a direct substrate of v-Src in each case, led us to focus on the role of Dok-1 phosphorylation as a potential central regulator of cellular transformation. Furthermore, since Dok-1 is present in low abundance, we suspected it might be a limiting mediator of v-Src signaling [24, 44].

Dok-1 Is Not a Direct v-Src Substrate When Dok-1 Is in the RasGAP Complex

Dok-1 was initially cloned as a RasGAP-associated protein. Previous data [45] have shown that the RasGAP immune complex contains a tyrosine kinase(s) that is capable of phosphorylating Dok-1 and other proteins. However, this kinase is present in very low abundance

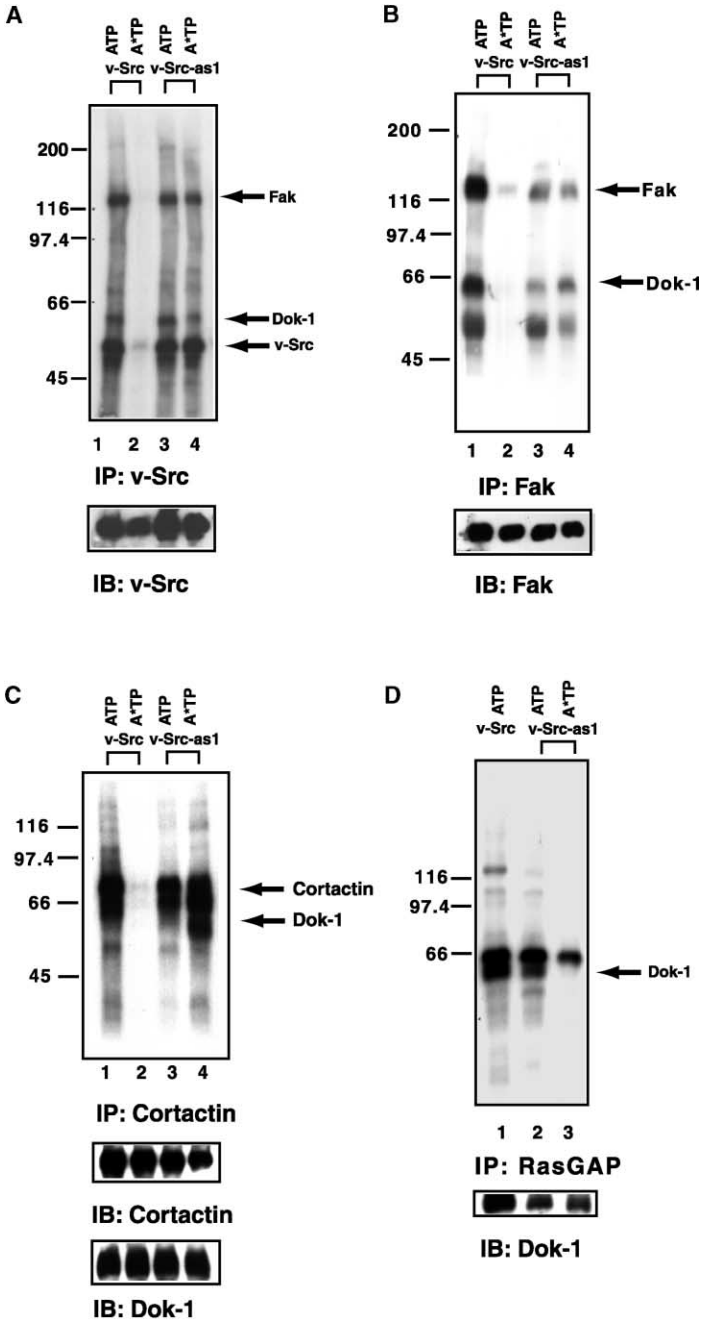

Figure 2. Different Immune Complexes Isolated from *v-Src* and *v-Src-as1* NIH 3T3 Cells Were Reacted with ATP or [γ-^{32}P]N^6(Benzyl)ATP

(A) Anti-v-Src immune complex kinase reaction in *v-Src* and *v-Src-as1* NIH 3T3 cells. In the top image, the lysates from wild-type *v-Src* (lanes 1 and 2) and *v-Src-as1* NIH 3T3 cells (lanes 3 and 4) were subjected to immunoprecipitation with anti-Src (GD11) antibody. The immune complexes were incubated in the presence of [γ-^{32}P] ATP (lanes 1 and 3) or [γ-^{32}P]N^6(benzyl)ATP (lanes 2 and 4) for 10 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography. In the bottom image, the same PVDF membrane was analyzed by Western blotting with anti-Src antibody (EC10) as a loading control.

(B) Anti-Fak (2A7) immune complex kinase reaction in *v-Src* and *v-Src-as1* NIH 3T3 cells. In the top panel, the lysates from *v-Src* (lanes 1 and 2) and *v-Src-as1* NIH 3T3 cells (lanes 3 and 4) were subjected to immunoprecipitation with anti-Fak antibody. The immune complexes were incubated in the presence of [γ-^{32}P] ATP (lanes 1 and 3) or [γ-^{32}P]N^6(benzyl)ATP (lanes 2 and 4) for 15 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography. In the bottom image, the same PVDF membrane was probed with anti-Fak antibody as a loading control.

(C) Anti-cortactin immune complex kinase reaction in *v-Src* and *v-Src-as1* NIH 3T3 cells. In the top image, the lysates from *v-Src* (lanes 1 and 2) and *v-Src-as1* NIH 3T3 cells (lanes 3 and 4) were subjected to immunoprecipitation with anti-cortactin (4F11) antibody. The immune complexes were incubated in the presence of [γ-^{32}P] ATP (lanes 1 and 3) or [γ-^{32}P]N^6(benzyl)ATP (lanes 2 and 4) for 15 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography. In the bottom images, the PVDF membrane was analyzed by immunoblotting with the anti-cortactin antibody (4F11) and an anti-Dok anti-sera (M-276) as loading controls.

(D) Anti-RasGAP immune complex kinase reaction in *v-Src* and *v-Src-as1* NIH 3T3 cells. In the top image, the lysates from *v-Src* (lane 1) and *v-Src-as1* NIH 3T3 cells (lanes 2 and 3) were subjected to immunoprecipitation with anti-RasGAP (B4F8) antibody. The immune complexes were incubated in the presence of [γ-^{32}P] ATP (lanes 1 and 2) or [γ-^{32}P]N^6(benzyl)ATP (lanes 3) for 10 min at room temperature. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by autoradiography. In the bottom image, the PVDF membrane was subjected to immunoblotting with the anti-Dok anti-sera (M-276) as a loading control.

and, therefore, could not be identified by immunoblotting [46]. Since this RasGAP-associated kinase activity was only found in *v-Src*-transformed cells, v-Src was thought to be the responsible kinase. To determine if v-Src was directly responsible for the phosphorylation of Dok-1 in the RasGAP complex, we immunoprecipitated RasGAP from *v-Src-as1* NIH 3T3 cells. Strikingly, Dok-1 is not phosphorylated when v-Src-as1 is provided with the orthogonal substrate [γ-^{32}P] N^6 (benzyl) ATP (Figure 2D, lane 3). Instead, an unidentified 66 kDa protein (p66) is phosphorylated in a v-Src-as1-dependent manner. This p66 band is not a hyperphosphorylated form of Dok-1, as determined by immunoblotting with polyclonal sera capable of detecting phosphorylated Dok-1 [47] (data not shown). This result indicates that v-Src is present in the RasGAP complex and confirms the suggestion by McCormick and coworkers [45]. Thus, Dok-1 is phosphorylated not by v-Src, but by an as-yet-undefined kinase in the complex. Unfortunately, neither the other kinase responsible for the phosphorylation of

Dok-1 nor the identity of p66 could be identified (data not shown). It is likely that v-Src phosphorylates Dok-1 prior to its binding to RasGAP and thus fails to further phosphorylate Dok-1 in a RasGAP complex. Another possibility is that RasGAP binding to Dok-1 somehow dislodges v-Src, so that relevant Dok-1 phosphorylation sites are no longer accessible to v-Src. It is intriguing that although v-Src is present and active in multiple complexes (anti-Fak, anti-cortactin, and anti-RasGAP), it is not a promiscuous kinase that always phosphorylates a given protein (Dok-1) in all signaling complexes. Other kinases in these complexes are responsible for highly specific phosphorylation events, revealing such protein complexes to be more dynamic and specifically regulated by individual kinases than previously appreciated.

Dok-1 Is a Key Kinase Target and Scaffold for Adaptor Proteins

The finding that Dok-1 is frequently a direct substrate of v-Src in multiple signaling complexes (v-Src, Fak, and cortactin) and yet is not phosphorylated in other complexes (RasGAP) led us to investigate the role of v-Src in the production of highly phosphorylated forms of Dok-1. The difficulty in answering this question is that almost every tyrosine kinase tested has been reported to phosphorylate Dok-1. It is the major tyrosine phosphorylated protein in cells transformed by oncogenic tyrosine kinases such as v-Abl, v-Src, v-Fps, and v-Fms [13]. Furthermore, Dok-1 has been shown to become tyrosine phosphorylated after platelet-derived growth factor (PDGF) [14, 15], epidermal growth factor (EGF) [16], and insulin stimulation [17], providing a link between growth factor receptors and MAP kinase activation. Upon phosphorylation, Dok-1 has been shown to interact with multiple signaling proteins, including Grb2 [48], Nck [49, 50], RasGAP [24, 25], and PLCγ [48]. Interestingly, Dok-1 has emerged as a key negative regulator of several receptor and cytoplasmic tyrosine kinase cascades. In particular, the pleckstrin homology (PH) domain and the phosphotyrosine binding (PTB) domain as well as the oligomerization domain of Dok-1 have been recently shown to be required for this negative regulatory activity [44]. We sought to study the role of the tyrosine kinase v-Src in formation of specific phosphoforms of Dok-1 and to determine if these phosphorylation events were important in the regulation of cellular function.

v-Src Phosphorylates Only a Subset of the Dok-1 Phosphorylation Sites

Since Dok-1 contains multiple phosphorylation sites, we sought to map the specific sites that are directly phosphorylated by v-Src via two-dimensional phosphopeptide mapping of v-Src-as1's direct phosphorylation sites on Dok-1 [51]. Four phosphotyrosine-containing peptides from a tryptic digest of Dok-1 are radiolabeled (Y1-Y4) with [γ-^{32}P] ATP and either v-Src (Figure 3, map a) or v-Src-as1 (Figure 3, map b). The identities of the tryptic peptides derived from Dok-1 were determined by a combination of mobility prediction [51] and an analysis of Dok-1 mutants in which specific tyrosine residues were replaced by nonphosphorylatable phenylalanine residues (Figure 4). The Y1 peptide corresponds to a tryptic peptide containing one phosphorylatable tyrosine (Y295). Due to weak phosphorylation of Y295 in v-Src and v-Src-as1 immune complexes with [γ-^{32}P] ATP (Figure 3, maps a and b), it cannot be ruled out that v-Src phosphorylates this site directly but at very low efficiency.

v-Src-as1 directly phosphorylates sites corresponding to peptides Y2 (Dok-1, Y361) and Y3 (Dok-1, Y450) (Figure 3, map c, lower right). Surprisingly, one Dok-1 phosphorylation site (peptide Y4) does not contain a v-Src phosphorylation site. We were unable to identify the Dok-1 site corresponding to the Y4 peptide, although it was confirmed by phosphoamino acid analysis that it contained a phosphotyrosine residue (data not shown). One of the v-Src-as1 phosphorylation sites (Y2 = I**Y**$_{361}$DEP) agrees quite well with the known in vitro specificity of Src as determined by peptide library screening [52, 53]. However, the other site (Y3 = L**Y**$_{450}$QSV) was not predicted to be an optimal consensus phosphorylation site for Src (see below) [52]. The identification of Y450 as a direct phosphorylation site for v-Src highlights the ability of protein tyrosine kinases to phosphorylate sites that are not predictable purely based on their optimal peptide specificity.

v-Src Specifically Phosphorylates Dok-1 at RasGAP and Csk Docking Sites

Tyrosine-phosphorylated Dok-1 acts as a scaffold for proteins containing SH2 or PTB domains (17, 48, 49). In order to identify these proteins, HA-tagged Dok-1 phosphorylation site mutants (Y295F, Y361F, and Y450F) were immunoprecipated from *v-Src* NIH 3T3 cells and probed with antibodies for known Dok-1-associated proteins (v-Src, RasGAP, and Csk). The pY361 site on Dok-1 has been shown to bind Nck upon insulin stimulation [50]. The Y314F Dok-1 mutant was included as a negative control because it contains the identical YXXP motif found at Y295 and Y361 yet was not found to be phosphorylated by v-Src. All Dok-1 mutants had similar expression levels, as confirmed by immunoblotting with a Dok-1-specific antibody (data not shown).

We found that the v-Src binding site on Dok-1 is Y361. Anti-v-Src immune complexes were isolated, and the binding of the HA-Dok-1 mutants was determined by immunoblotting with an anti-HA antibody. Dok-1 mutants Y295F, Y314F, and Y450F bound efficiently to v-Src, whereas Y361F Dok-1 binding to v-Src was severely decreased (Figure 5A, lane 4). This suggests that Y361 is the site most responsible for Dok-1's binding to v-Src. The weak v-Src binding observed to the Y361F Dok-1 mutant (Figure 5A, lane 4) is probably mediated by v-Src's SH3 domain [54].

Dok-1 contains a YXXP motif at Y295, Y314, and Y361, suggesting that all three sites could provide a site for RasGAP binding [52, 53]. Probing the HA-Dok-1 immune complexes for RasGAP binding revealed that all four mutants are capable of binding to RasGAP, although Dok-1(Y295F) and Dok-1(Y361F) bind much less efficiently, suggesting that both of these sites may be involved in binding to RasGAP (Figure 5B). When these

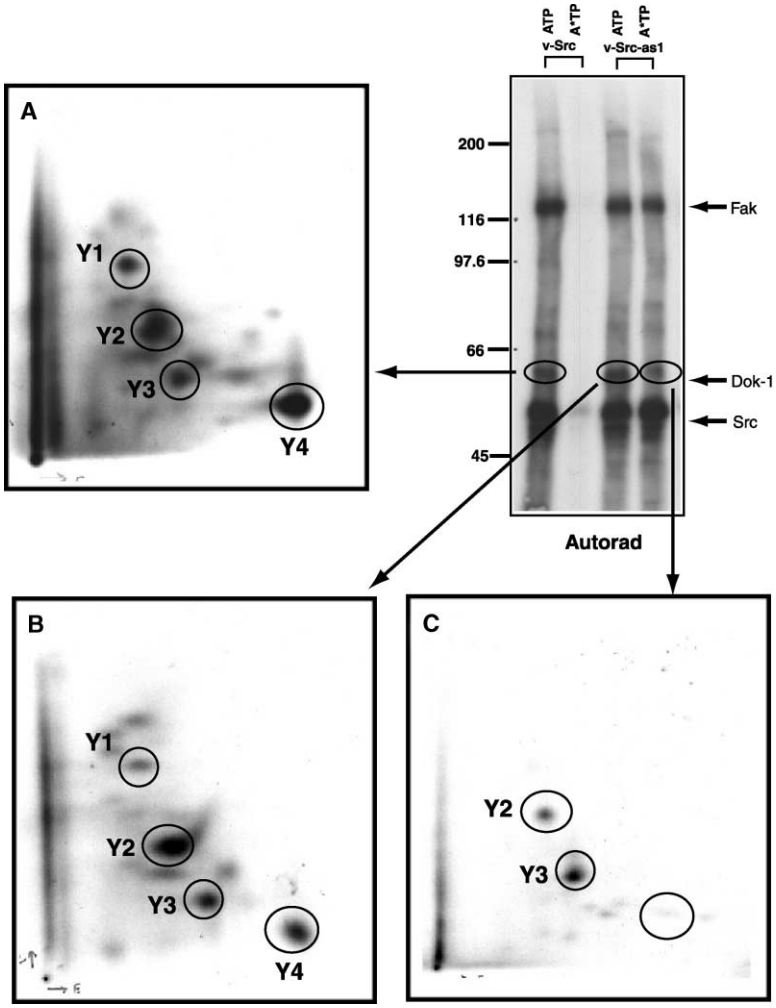

Figure 3. Phosphotryptic Maps of Dok-1

Phosphotryptic digests of Dok-1 protein isolated from v-Src immunoprecipitates from *v-Src* and *v-Src-as1* NIH 3T3 cells after kinase reactions with either [γ-^{32}P] ATP (lanes 1 and 3) or [γ-^{32}P]N^6(benzyl)ATP (lanes 2 and 4).

two sites were mutated simultaneously (Y295/361F), faint binding to RasGAP was still observed, implying that additional sites on Dok-1 may be involved in RasGAP binding. Since the Y361 site on Dok-1 is directly phosphorylated by v-Src, v-Src may be responsible in part for the recruitment of RasGAP to the Dok-1 scaffold. This provides the first direct link between a specific phosphorylation event on Dok-1 and recruitment of a component of the Ras signaling machinery.

Our analysis of the Y450 phosphorylation site on Dok-1 suggests that Csk binds to this site. The Y450 site on Dok-1 contains a Val at the Y + 3 position (YSQV) and the pYXXV motif has been shown to be an optimal binding site for the Csk SH2 domain [55]. Neet and Hunter [3] proposed that the adaptor protein Dok-1 mediates the localization of Csk to subcellular membrane and cytoskeletal regions in v-Src- and activated c-Src-transformed cells. Probing anti-Csk immune complexes with an HA-specific antibody revealed that the binding of Dok-1 to Csk is completely abolished by the mutation of Y450 (Figure 5C), suggesting that Y450 is the major site of interaction between Csk and Dok-1. These results suggest that the mechanism responsible for Csk's recruitment from the cytosol to the membrane is the v-Src-dependent phosphorylation of Dok-1 at Y450.

Phosphorylation of Dok-1 Y361 Reduces Src-Dependent Transformation

Phosphorylation of Dok-1 at Y361 results in the binding of RasGAP to Dok-1 and subsequent inhibition of cellular transformation. The functional relevance of v-Src phosphorylation of Dok-1 in terms of cellular transformation was probed with a genetic screen to measure the transforming potential of individual phosphorylation site mutants of Dok-1. An initial screen of Dok-1 mutants with v-Src-transformed NIH 3T3 cells revealed no positive or negative effects of Dok-1 phosphorylation (data not shown). We then used a mutant [*c-Src (T338I, Y527F)*] that causes partial (5%–10%) transformation relative to v-Src isolates [56], which allowed the assessment of both positive and negative effects of Dok-1 mutants on Src-dependent cellular transformation. Four single phosphorylation site mutants of *HA-dok-1 (Y295F, Y314F, Y361F, and Y450F)* as well as wild-type *dok-1* were introduced into *c-Src (T338I, Y527F)* cells by retroviral infection. Immunoblotting with a Dok-1-specific antibody confirmed that all Dok-1 mutants had similar expression levels (data not shown). Soft agar growth assays were performed with each of the *dok-1*-expressing *c-Src (338I, 527F)*-transformed cell lines (each as a pooled population). Expression of wild-type Dok-1 has

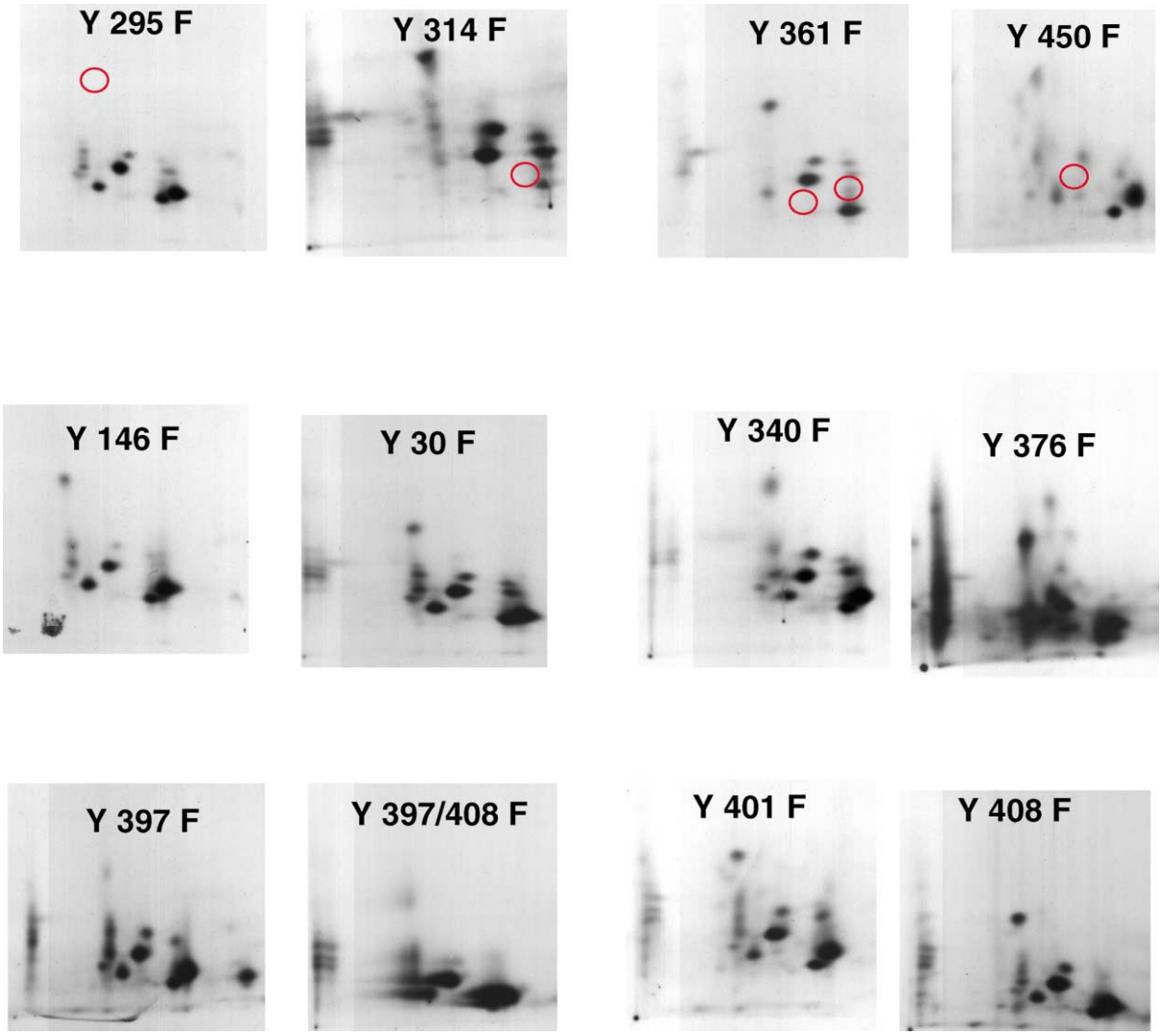

Figure 4. Phosphotryptic Maps of Different Y-to-F Mutants of Dok-1

Different GST-Dok-1 mutant proteins were expressed in *E. coli* and were phosphorylated with v-Src immunoprecipitates from *v-Src* NIH 3T3 cells with [γ-^{32}P] ATP. Proteins were resolved on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. ^{32}P-labeled GST-Dok-1 bands were excised from the nitrocellulose membrane, and phosphotryptic peptide mapping procedures were carried out as described in the Experimental Procedures.

an inhibitory effect on transformation (85% inhibition), confirming that Dok-1 is involved in the negative regulation of *Src*-dependent transformation [44]. When either *Y295F* or *Y361F HA-dok-1* was expressed, 80%–85% of this inhibition was lost in comparison to wild-type *dok-1*-expressing *c-Src (338I, 527F)* cells (Figure 6). Because Y295 and Y361 are RasGAP binding sites on Dok-1, this suggests that phosphorylation of these sites plays a role in inhibition of the Ras pathway. Expression of the *dok-1(Y450F)* allele, which is defective in binding Csk, showed little effect on cellular transformation. The role of Csk is to phosphorylate Y527 of Src family kinases [57], and so it is expected that Csk recruitment had no effect on transformation by a c-Src allele lacking the C-terminal tyrosine 527. Our analysis reveals that 80%–85% of the negative regulatory function of Dok-1 on cell transformation is controlled by direct phosphorylation of a single site on Dok-1, which recruits RasGAP to the Dok-1 scaffold. Recent studies by Baltimore and coworkers have shown that the PH domain as well as a multimerization domain present in Dok-1 are also necessary for negative regulation of Src-dependent transformation [44]. Together, these results suggest that Dok-1 requires multiple inputs, including phosphorylation, membrane localization, and multimerization, in order to serve as a negative regulator of tyrosine kinase-dependent cell transformation.

Significance

Global analysis of direct v-Src substrates in v-Src-transformed fibroblasts led to the identification of sev-

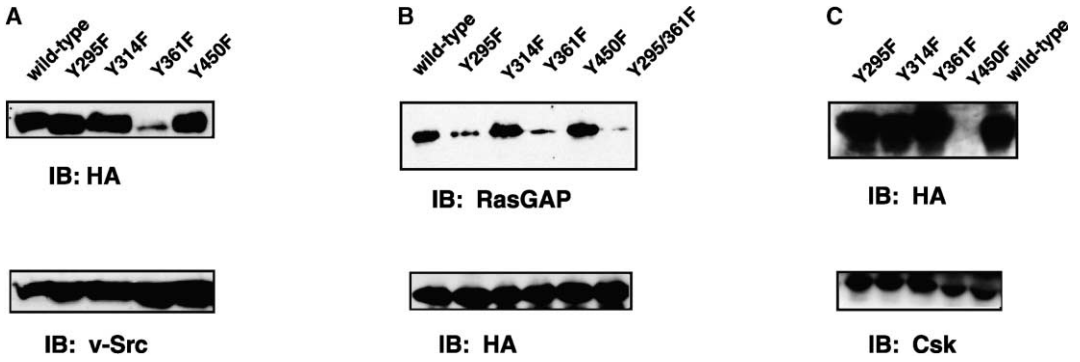

Figure 5. Identification of Downstream Proteins Recruited on Dok-1 as a Result of v-Src-Mediated Phosphorylation

(A) Anti-v-Src immunoprecipitation from *v-Src* NIH 3T3 cells coexpressing either *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, or *dok-1(Y450F)*. In the top image, the lysates from *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, or *dok-1(Y450F)*-expressing cells (lanes 1–5) were subjected to immunoprecipitation with anti-Src antibody (GD11). Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with an anti-HA antibody. In the bottom image, the PVDF membrane was subjected to immunoblotting with an anti-Src antibody (EC10) as a loading control.

(B) Anti-HA immunoprecipitation from *v-Src* NIH 3T3 cells coexpressing either *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, *dok-1(Y450F),* or *dok-1(Y295F/Y361F)*. In the top image, the lysates from *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, *dok-1(Y450F),* or *dok-1(Y295F/Y361F)* (lanes 1–5) expressing *v-Src* NIH 3T3 cells were subjected to immunoprecipitation with anti-HA antibody. Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with the anti-RasGAP antibody B4F8. In the bottom image, the PVDF membrane was subjected to immunoblotting with anti-HA antibody as a loading control.

(C) Anti-Csk immunoprecipitation from *v-Src* NIH 3T3 cells coexpressing either *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, or *dok-1(Y450F)*. In the top image, the lysates from *v-Src* NIH 3T3 cells coexpressing either *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, or *dok-1(Y450F)* were subjected to immunoprecipitation with the anti-Csk antibody (C-20). Reaction products were separated on 10% SDS-PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with an anti-HA antibody. In the bottom image, the PVDF membrane was subjected to immunoblotting with anti-Csk antibody as a loading control.

eral novel direct v-Src targets, including Cofilin and Calumenin. A more focused screen for v-Src substrates revealed a model for the assembly of negative regulatory proteins onto the scaffolding protein Dok-1. These studies revealed a number of surprising features of v-Src substrates that contradict currently accepted models of tyrosine kinase specificity. In one example, we found that out of four phosphorylation sites on Dok-1, only two were definitively sites phosphorylated by v-Src, even though three of the four sites shared a similar consensus sequence. Moreover, the site that was predicted to provide a nonoptimal v-Src phosphorylation site was one of the directly phosphorylated sequences. One of the v-Src phosphorylation sites (Y361) on Dok-1 produces an optimal binding site for v-Src. This association may allow v-Src to phosphorylate the suboptimal phosphorylation site. Such a processive phosphorylation model has been proposed by Cantley [52] and by Scott and Miller [58]. The role of Dok-1 phosphorylation by v-Src is to recruit negative regulators of cellular transformation. Mutation of v-Src phosphorylation sites Y295 or Y361 abrogated the inhibitory effect of Dok-1 on cellular transformation, indicating that these sites are necessary

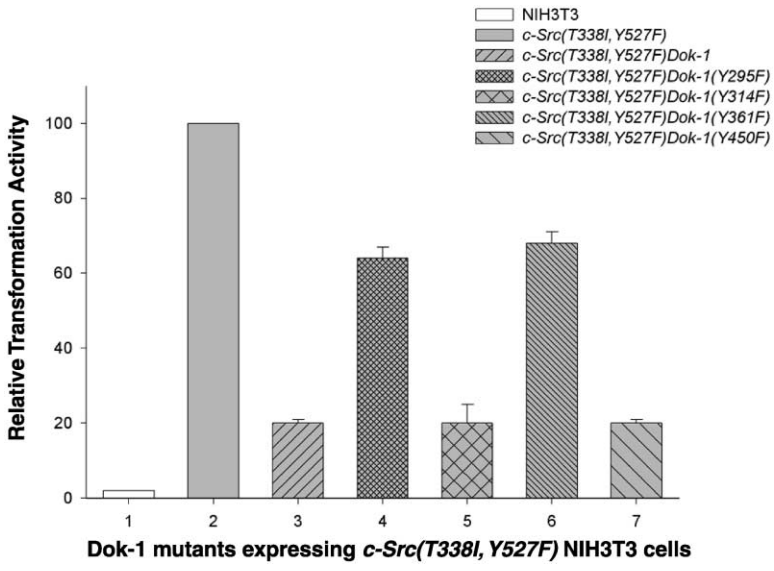

Figure 6. Functional Relevance of Dok-1 Phosphorylation in Cellular Transformation

Soft-agar colony formation assays were performed with either *c-Src(T338I/Y527F)* NIH 3T3 cells alone or *c-Src(T338I/Y527F)* NIH 3T3 cells coexpressing *dok-1, dok-1(Y295F)*, *dok-1(Y314F)*, *dok-1(Y361F)*, or *dok-1(Y450F)*. Relative transformation efficiencies show the mean of three independent experiments, and the bars represent standard deviations from the mean value.

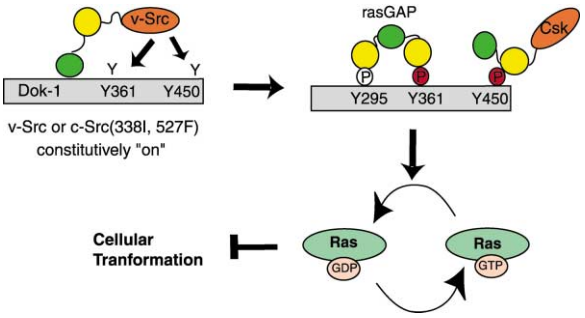

Figure 7. v-Src-Mediated Phosphorylation of Dok-1 Negatively Regulates Both Ras and Growth Factor-Stimulated Pathways

Proposed model for a retrograde signaling pathway for *c-Src(Y527F, T338I)* or *v-Src*. The pathway is mediated by the adaptor protein Dok-1 and its effect in cellular transformation. Red circles represent sites directly phosphorylated by v-Src. Red ovals represent kinase domains; yellow and green ovals represent SH2 and SH3 domains, respectively.

for the repression of transformation activity. The protein that associates with Dok-1 at pY295 and pY361 is RasGAP, a negative regulator of the Ras signaling pathway that is critical for cellular transformation by *v-Src* [59]. Thus, recruitment of RasGAP as a result of v-Src-mediated phosphorylation on Dok-1 highlights another arm of retrograde signaling that is controlled by v-Src itself (Figure 7). The chemical genetic approach used here to specifically tag the direct substrates of one kinase in the cell has revealed an unexpected level of substrate specificity. Since the mutation that renders v-Src able to utilize [γ-^{32}P] N^6 (benzyl) ATP can be identified in *all* protein kinases by simple sequence alignment, this method can be used to decipher complex cascades one kinase at a time [21].

Experimental Procedures

Synthesis of [γ-^{32}P] N^6(Benzyl) ATP ([γ-^{32}P] A*TP)

N^6(benzyl)adenosine was synthesized by refluxing 6-chloropurine riboside (1 mmol) (Aldrich) with benzylamine (5 mmol) in ethanol (10 ml) overnight. Ethanol was removed in vacuo, and the resulting oily residue that was obtained was crystallized from ethanol (yield 90%). N^6 (benzyl) ADP was synthesized by sequential phosphorylation according the method of Hecht and Kozarich [60]. To an ice-cooled suspension of N^6(benzyl) adenosine (68 mg, 0.2 mmol) in trimethyl phosphate (0.5 mmol), $POCl_3$ (0.025 ml) was added, and the reaction mixture was stirred at 0°C for 1 hr, after which the reaction was quenched with 5 ml of 1 M triethylammonium bicarbonate (TEAB buffer [pH 7.5]). Solvent was removed in vacuo at <40°C by rotary evaporation. The resulting slurry was purified on a DEAE (A-25) Sephadex (Pharmacia) column with TEAB (pH 7.5) (0.1–0.5 M gradient). The purified N^6(benzyl) AMP shows a retention time of 7.5 min on a strong anion exchange HPLC column (SAX, catalog # 83-E03-ETI, Varian) with a gradient of 5–750 mM ammonium phosphate (pH 3.9) for 10 min at a flow rate of 0.5 ml/min.

In the second step, a solution of N^6(benzyl) AMP (44 mg, 0.1 mmol) and carbonyl diimidazole (81 mg, 0.5 mmol) in DMF (5 ml) was stirred at room temperature for 20 hr, after which methanol (35 μl) was added. After 1 hr, a solution of tributyl ammonium phosphate (1 mmol) was added in DMF (1 ml). The reaction was stirred for an additional 24 hr. After the reaction mixture was quenched with 2 ml of TEAB buffer (pH 7.5), solvent was removed in vacuo at <40°C, and the residue was purified as described above (retention time 9.7 min).

N^6(benzyl) ADP (2.5 μmol, molar absorbancy (ϵ_{max}) 15.4 $\times$ 10^3 at 265 nm at pH 7.00) was dissolved in DMF (200 μl), and carbonyl diimidazole (8 mg, 10 μmol) was added to it. The reaction mixture was stirred for 24 hr at room temperature, then methanol (4 μl) was added and the reaction was stirred for an additional 1 hr. ^{32}P orthophosphoric acid (5 mCi, 8500 Ci/mmol) was dried in vacuo, dissolved in DMF (100 μL), and added to the reaction mixture. After the mixture was stirred for 24 hr, DMF was removed in vacuo, and the radiolabeled analog was purified by ion-exchange chromatography with DEAE (1.5 cm $\times$ 7 cm packed volume) and a gradient of 0.1–1 M TEAB buffer (pH 7.5) at a flow rate of 1.5 ml/min. The purified product was concentrated in vacuo at <40°C by rotary evaporation. The concentrated triphosphate was redissolved in 200 μl water, and the concentration was determined by scintillation counting (yield 20%). The [γ-^{32}P] N^6(benzyl) ATP was characterized by coinjection of the radiolabeled material with an authentic sample of N^6(benzyl) ATP [8] on a strong anion exchange-HPLC column (retention time 11.2 min).

Antibodies

Anti-v-Src antibodies GD11 and EC10 (1 μg/μl) were a generous gift from Sally Parsons (University of Virginia, Charlottesville). Tom Parsons (University of Virginia, Charlottesville) provided Fak antibody 2A7 and cortactin antibody 4F11. Anti-RasGAP antibody B4F8, anti-Csk (C-20), anti-Cofilin (sc8442), and anti-Dok antibody M-276 were purchased from Santa Cruz Biotech. Anti-HA (12CA5) was from Roche Biochemicals; mAb 327 was purchased from Oncogene Research Products. Goat antimouse HRP was purchased from ICN (catalog number 55550), and goat antirabbit HRP was purchased from VWR (catalog number RL611-103-122). FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies were purchased from Jackson Immunoresearch.

Expression Plasmids

Babe puro vector and Babe hygro vector were gifts from Alex Muller and H. Land. Murine HA-tagged *dok-1* cDNA was a gift from Yuji Yamanashi (University of Tokyo, Japan). Wild-type *v-Src* was cloned into the pBabe puro vector [61] at BamHI and HindIII sites as follows. Wild-type *v-Src* DNA was amplified by PCR with primers 5′-GCTACAGT**GGATCC**ATGGGGAGTAGCAAGAGCAAGC-3′ (primer 1) and 5′-GCTACAGT**AAGCTT**CTACTCAGCGACCTCCAACACAC-3′ (primer 2), Pfu polymerase (Stratagene), and 100 ng of template; 30 amplification cycles (94°C for 45 s, 55°C for 45 s, and 72°C for 3 min) were used. The *I338G v-Src* mutant (*v-Src-as1*) was produced by the overlap extension PCR mutagenesis method with primers 1, 2, 3, and 4. Primers 3 (5′-CTACATCGTC**GGC**GAGTACATGAG-3′) and 4 (5′-CTCATGTACTC**GCC**GACGATGTAG-3′) contain the nucleotide sequence changes that introduce the I338G mutation (shown in bold). Wild-type *v-Src* pBabe Hygro was constructed by cloning at EcoRI and SalI sites with the following primers: 5′-GCTACAGT**GAATTC**ATGGACGGGGGCTGT-3′ (primer 5) and 5′-GCTACAGT**G TCGAC**TCAGGTGGAACCCTCAGA-3′ (primer 6). HA-tagged *GST-dok-1* was constructed by PCR with murine *dok-1* cDNA as a template [22] and the following phosphorylated primers: 5′-GGC CATGGCTTACCCATACGATGTTCCAGATTACGCTGACGGGGCTG TGATGGAGGGTCCG-3′ and 5′-CCCACTCAGACCAGTGGGCCGC TTC-3′. The PCR product was cloned into the Sma1 site in pGEX-KT1 (Pharmacia). All mutants of *dok-1* were constructed by QuickChange protocol with 100 ng of wild-type *dok-1* as a template. The *dok-1 Y397F* mutant was used as a template for Y397/401F and Y397/408F double mutants, whereas the *dok-1 Y401F* mutant was used for the Y401/408F double mutant.

Expression of wild-type and mutant *GST-dok-1* was carried out in DH5α cells. A single colony was inoculated into 25 ml of LB superbroth (Bio 101) liquid media with 100 μg/ml ampicillin. The culture was grown at 37°C overnight. This culture was added to 250 ml LB superbroth (Bio 101). After 2 hr at 37°C (O.D.$_{600}$ = 0.5), IPTG was added to a final concentration of 1 mM. The culture was shaken for 5–6 hr at 37°C and then was centrifuged at 2500 $\times$ g for 15 min. The pellet was resuspended in 5 ml of 25 mM Tris (pH 8.0), 1 mM EDTA, and 1 mM DTT buffer and lysed twice at 8000 psi in a French press at 4°C. The lysate was centrifuged again at 2500 $\times$ g for 10 min. The supernatant was added to 1 ml of reconstituted glutathione

beads (Sigma) and was gently shaken for 30 min on ice. The slurry was added to a column (Fisher Scientific # 11-387-50), and the beads were washed with 10 ml of 25 mM Tris (pH 8.0) and 10 mM EDTA buffer followed by 50 mM tris (pH 8.0) (5 ml). The glutathione fusion protein was eluted with 4 ml of 10 mM free glutathione (Sigma), 50 mM Tris (pH 8.0), and 150 mM NaCl solution.

Cell Culture

NIH 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum (Hyclone-SH30072.03). Wild-type and *I338G v-Src (v-Src-as1)* NIH 3T3 cells were routinely grown in DMEM with 10% bovine calf serum and 2.5 μg/ml puromycin (Calbiochem).

Transfection and Retroviral Infection

Both *v-Src* and *v-Src-as1* pBabe puro plasmids were transiently transfected into Bosc 23 cells by the calcium phosphate transfection method [62]. Culture medium containing the retroviruses was harvested from 72 hr to 5 days after transfection. Viral supernatants were stored at –80°C. Negative control retroviruses lacking *v-Src* were prepared by using pBabe puro or hygro vectors alone. 5×10^5 NIH 3T3 cells were seeded on a 10 cm culture dish and were allowed to attach to the plate for several hours. Infection was carried out by adding the retrovirus supernatant (3 ml) in the presence of polybrene (4 μg/ml). After 3 hr the viral supernatant was removed and replaced with fresh media (10% bovine calf serum/DMEM). After 48 hr the infected cells were selected in the presence of 2.5 μg/ml of puromycin. After another 24 hr, more than 95% of the cells were viable by trypan blue staining. The expression of wild-type and *v-Src-as1* in NIH 3T3 cells was confirmed by immunoprecipitation (GD11) and immunoblotting (EC10). Both wild-type and v-Src-as1 proteins were present in equal amounts (data not shown). Accordingly, for Dok-1 coexpression in *c-Src (T338I, Y527F)* NIH 3T3 cells or in NIH3T3 cells, viral supernatants either of the wild-type or of Dok-1 mutants were added to v-Src (hygro) and NIH 3T3 cells, respectively, and were selected in the presence of 2.5 μg/ml of puromycin.

Kinase Assays and 2D Gel Electrophoresis of v-Src and v-Src-as1 NIH 3T3 Cells

Cells were lysed at 4°C in modified RIPA buffer (1% NP 40, 50 mM Tris [pH 7.5], 150 mM NaCl, 0.25% sodium deoxycholate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, and 10 μg/mL aprotinin) and cleared by centrifugation at 13,000 rpm for 15 min at 4°C. Cleared lysates were mixed with 10 $MgCl_2$, 10 mM $MnCl_2$, 1 mM sodium orthovanadate, 100 μM ATP, 5 μCi of [γ-^{32}P] ATP (6000 Ci/mmol) (Figure 1, lanes 1 and 2) or [γ-^{32}P] N^6 (benzyl) ATP (8500 Ci/mmol) (lanes 3 and 4) and were incubated for 45 min at room temperature. Cell lysates were boiled for 5 min in sample buffer (62.5 mM Tris (pH 6.8), 2.5% SDS, 10% glycerol, 2.5 mg/ml DTT, 2.5% β-mercaptoethanol) and separated on 10% SDS-PAGE gels. Separated proteins were electrophoretically transferred to immobilon polyvinylidene membranes (Millipore, Bedford, Massachusetts) with a semidry apparatus (Owl Scientific) for 1 hr at 10 V. For autoradiography, the membranes were exposed to Kodak Biomax MS film (Kodak) with a Biomax MS intensifying screen at −70°C.

2D gel electrophoresis of radiolabeled samples was carried out by Kendrick Labs (Madison, Wisconsin) with 2% pH 3.5–10 ampholines (Pharmacia) for 9600 volt hr. One mg of an IEF internal standard, tropomyosin, was added to each sample. Ten percent SDS slab electrophoresis was carried out for about 4 hr at 12.5 mA/gel. The gels were stained with Coomassie blue, dried, and exposed to Kodak X-OMAT AR film.

Immunoprecipitation and Kinase Assays of v-Src and v-Src-as1 NIH 3T3 Cells

Unless otherwise specified, cells were lysed at 4°C in modified RIPA buffer with 0.1% SDS and 2 mM sodium orthovanadate. After centrifugation, the cleared lysate was incubated with 1–5 μg of the desired antibody at 4°C for 2 hr with gentle rotation, followed by incubation with 50 μl of 50% protein A Sepharose slurry (Pharmacia) precoated with rabbit IgG (except for the Dok-1 immunoprecipitation) for another 2 hr at 4°C. The immune complexes were washed three times (500 μl each) in modified RIPA buffer without SDS and twice in kinase buffer (30 mM Tris [pH 7.5], 10 mM $MgCl_2$, and 10 mM $MnCl_2$). Kinase reactions were initiated by the addition of 0.5 μCi of [γ-^{32}P] ATP alone or 1 μCi of [γ-^{32}P] N^6(benzyl) ATP in the presence of 10 μM ATP and were incubated for 10–15 min (as indicated in the figure legends) at room temperature. After boiling for 5 min in sample buffer, immune complex proteins were separated on 10% SDS-PAGE gels, electrophoretically transferred, and exposed as described before.

Kinase Assays with GST Fusion Proteins

The Dok-1 protein was expressed as a GST fusion protein in *E. coli* to facilitate rapid mutagenesis and protein purification. These mutant proteins were phosphorylated with the same v-Src immune complex, and GST-fused Dok-1 was gel purified based on its larger molecular weight as compared to that of endogenous Dok-1. The recombinant GST-Dok-1 showed a phosphotryptic pattern of phosphorylation identical to that of endogenous Dok-1.

Purified wild-type and mutant GST-Dok-1 proteins were added to v-Src immune complexes in the presence of [γ-^{32}P] ATP, and the kinase assay was carried out for 15 min at room temperature. The reaction mixture was centrifuged (13,000 rpm), and the supernatants were boiled in the sample buffer for 5 min. Proteins were resolved on 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane, and visualized as described above.

Kinase Assays with Recombinant Cofilin

Cofilin (Sigma) was added (2 μg) to v-Src and v-Src-as1 immune complexes in the presence of [γ-^{32}P] ATP or [γ-^{32}P] N^6(benzyl) ATP, and the kinase assay was carried out for 20 min at room temperature. The reaction mixture were boiled in the sample buffer for 5 min. Proteins were resolved on 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane, and visualized as described above.

Immunoblotting

PVDF membranes were blocked for varying amounts of time (2 hr to overnight) in 5% BSA in TBS-Tween (50 mM Tris [pH 8.00], 150 mM NaCl, 0.1% Tween 20) and then probed with antibodies in 1% BSA in PBS. Horseradish peroxidase (HRP)-conjugated anti-mouse antibody (VWR # 55550) was used for the detection of monoclonal antibodies, and HRP-conjugated goat anti-rabbit antibody (VWR # RL 611-103-122) was used for the detection of p62 Dok. Enhanced chemiluminescence (Pierce) was used for antibody detection as described in the manufacturer's instructions.

Phosphotryptic Peptide Mapping

Immunoprecipitations and in vitro kinase assays with GST-Dok proteins were performed as described above. ^{32}P-labeled Dok-1 bands were excised from the nitrocellulose membrane, and phosphotryptic peptide mapping procedures were carried out according to the methods of Boyle et al. [45]. The nitrocellulose membrane carrying the protein was soaked in 0.5% PVP-360 in 100 mM acetic acid at 37°C for 30 min, after which it was washed extensively with 0.05 M NH_4HCO_3. Ten micrograms of TPCK-treated trypsin (Worthington, Freehold, New Jersey) in 0.1 mM HCl (1 mg/ml) was added to the membrane soaked in 200 μl of NH_4HCO_3 and was incubated at 37°C for 2 hr. At the end of the digestion, 300 μl of water was added, and the supernatant was lyophilized. Chilled performic acid (100 μl) was then added, and the oxidation was carried out at 4°C for 1 hr, followed by the addition of 400 μl of water and lyophilization. Phosphotryptic peptides were resolved on cellulose TLC (thin-layer chromatography) plates by electrophoresis in pH 1.9 buffer (1000 V for 70 min) in the first dimension, followed by ascending chromatography in phosphochromo buffer (25% pyridine, 7.5% glacial acetic acid, 37.5% 1-butanol in water) in the second dimension. Labeled peptides were detected by autoradiography by exposing the plate to Kodak Biomax MS film (Kodak) with a Biomax MS intensifying screen at −70°C.

Soft-Agar Colony Formation Assay

Wild-type and I338G v-Src-expressing NIH 3T3 cells and pBabe vector-infected NIH 3T3 cells were plated in DMEM (10^3, 10^4, and 10^5 cells per dish, each in triplicate), 0.3% agar, and 10% bovine calf serum in 35 × 10 mm dishes. Transformed colonies were counted after 3 weeks.

Mass Spectral Analysis

Gel spots were digested by adding 0.05 μg modified trypsin in the minimum amount of 0.025 M Tris (pH 8.5) and leaving the gel overnight at 32°C. Peptides were extracted with 2 × 50 μl of 50% acetonitrile/2% TFA, and the combined extracts were dried and resuspended in matrix solution prepared by making a 10 mg/ml solution of 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile/0.1% TFA and adding two internal standards, angiotensin and bovine insulin, to the matrix solution. MALDI mass spectrometric analysis was performed on the digest by using a PerSeptive Voyager DE-RP mass spectrometer in the linear mode. These peptides were analyzed by peptide mass fingerprinting and database searching with Protein Prospector.

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