11-21-2003

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Recommended Citation

Goldberg GS, Alexander DB, Pellicena P, Zhang ZY, Tsuda H, Miller WT. Src phosphorylates Cas on tyrosine 253 to promote migration of transformed cells. J Biol Chem. 2003 Nov 21;278(47):46533-40. Epub 2003 Sep 11. doi: 10.1074/jbc.M307526200. PMID: 12972425. PMCID: PMC2441571.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 47, Issue of November 21, pp. 46533–46540, 2003
Printed in U.S.A.

Src Phosphorylates Cas on Tyrosine 253 to Promote Migration of Transformed Cells*

Received for publication, July 14, 2003, and in revised form, September 10, 2003
Published, JBC Papers in Press, September 11, 2003, DOI 10.1074/jbc.M307526200

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Cas is a member of the focal adhesion complex. Phosphorylation of Cas by Src is an important event leading to cell transformation. Using mass spectrometry, we have mapped 11 sites in Cas that are phosphorylated by Src. These sites are all located between residues 132 and 414 of Cas, in a region that is required for binding to a number of other proteins including Crk. We tested synthetic peptides modeled on Cas phosphorylation sites, and found that the sequence containing tyrosine 253 was phosphorylated by Src most efficiently. Using cells derived from Cas-deficient mice, we confirmed that Cas greatly enhanced the ability of Src to transform cells. Phosphorylation of Cas on tyrosine 253 was not required for Src to increase growth rate, suppress contact inhibition, or suppress anchorage dependence. Yet, in contrast to these growth characteristics, phosphorylation of Cas on tyrosine 253 was required for Src to promote cell migration. Thus, a single phosphorylation site on this focal adhesion adaptor protein can effectively separate cell migration from other transformed growth characteristics.

Normal cells have intrinsic controls that limit their movement and growth, such as restraints imposed by cell cycle checkpoints. Cells must receive cues from their surrounding environment to override these restraints. Thus, cell growth normally relies on signals mediated from the extracellular matrix through integrins (1–3).

In contrast to normal cells, tumor cells ignore their surrounding environment, which allows them to overcome contact growth inhibition, lose anchorage dependence, and migrate to foreign tissues and organs. The degree to which tumor cells grow and migrate correlates with their aggressive potential (1, 2). Therefore, it is important to understand how extracellular signals guide cell behavior and how transformation ablates the need for integrin signaling.

* This research was funded in part by National Institutes of Health Grants CA58530 (to W. T. M.) and CA88805-01A2 (to G. S. G.), and a grant-in-aid and research fellowship from the Foundation for the Promotion of Cancer Research, part of the Second Term Comprehensive 10-Year Strategy for Cancer Control, from the Japanese Ministry of Health and Welfare (to G. S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Integrins transmit signals from the extracellular matrix to inside of the cell. Integrin signaling relies on a complex of associated kinases including focal adhesion kinase (FAK)[1] and Src and adaptor proteins including Grb2, Shc, paxillin, and Cas (4, 5). Cas is an important component of the integrin signaling network (6). Cas was originally identified as a protein phosphorylated in v-*src*-transformed cells (7).

Cas has several structural motifs including an SH3 domain, proline-rich regions, and a cluster of tyrosine phosphorylation consensus sites that act as SH2 binding motifs (6, 8). FAK binds to the SH3 domain in the amino-terminal part of Cas (9–11), whereas Src binds to a proline-rich region and a phosphorylated tyrosine residue at the carboxyl end of Cas (12, 13). Although FAK may phosphorylate Cas in certain cases (14, 15), the biological activity of Cas depends on its phosphorylation by Src (16–18). After phosphorylation, Cas associates with a number of proteins, including Crk, Src, phosphatidylinositol 3-kinase, Nck, and phospholipase Cγ, via SH2 binding motifs (8, 10, 19).

Phosphorylation of Cas by Src plays a critical role in cell transformation (20, 21). For example, Src must phosphorylate Cas to promote anchorage-independent growth and cell migration (20–23). Therefore, understanding the mechanistic relationship between Cas and Src should help elucidate mechanisms that underlie fundamental aspects of tumor cell growth.

To better understand the role of Cas in cell transformation, we have identified residues that are phosphorylated by Src. We then examined the effects of these phosphorylation events on cell behavior. We report here that a single phosphorylation site on Cas, tyrosine 253, can effectively separate the effects of transformation on cell migration from other hallmarks of transformation including anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Production of Cas and Src in Sf9 Cells—Wild type Cas (8) and v-Src (24) were tagged at the amino terminus with polyhistidine and produced by the Bac-to-Bac Baculovirus Expression System (Invitrogen) as described (16). Briefly, cells were grown at 27 °C, infected with baculovirus expression vectors, collected and rinsed in phosphate-buffered saline by centrifugation, and lysed in a French pressure cell in lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40 plus protease inhibitors). Lysates were clarified by centrifugation and applied to blue nickel-nitrilotriacetic acid resin to purify histidine-tagged Cas or Src according to the manufacturer's protocols (Qiagen) as described (16).

[1] The abbreviations used are: FAK, focal adhesion kinase; SH2 and SH3, Src homology 2 and Src homology 3, respectively; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; MS, mass spectrometry; LC-MS-MS, liquid chromatography-tandem mass spectrometry; MAP, mitogen-activated protein; MAPK, MAP kinase.

Mutagenesis and Production of Cas in Bacteria—A BamHI/NotI fragment containing Cas cDNA was subcloned into the complementary sites of the bacterial expression vector pET28A to produce the entire coding region of Cas with an amino-terminal polyhistidine tag as described by the manufacturer (Novagen). This construct was used as a template to produce site directed mutants with the QuikChange site-directed mutagenesis kit according to the manufacturer's protocols (Stratagene), which were verified by sequencing on an ABI373 automated DNA sequencer as described (16). The tyrosine residue at position 253 was mutated to phenylalanine to produce CasY253F with the forward primer 5′-GCTCCAGGTTCCCAGGACATCTTTGATGTGCCCCCTGTTCG-3′ and a complementary reverse primer. The amino acids between residues 118 and 423 were deleted to produce Cas118Δ423. EcoRI sites were introduced at the nucleotides encoding amino acids 118 and 423 with forward primers 5′-CCCCAACCTGACAATGAATTCCTGGTACCCACTCCC-3′ and 5′-CCAGCCGAGCGAGAATTCCCAACAGATGGCAAGCGC-3′, respectively, and complementary reverse primers. The construct was religated after excising the resulting EcoRI fragment, which contains the entire substrate binding region of the protein. Wild type and mutant Cas proteins were produced in *Escherichia coli* strain BL21 DE3RP codon+ (Stratagene) and purified as done for Sf9 cells described above.

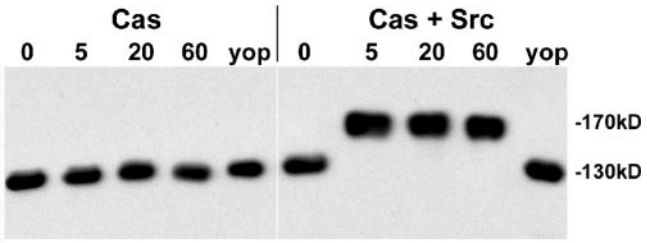

FIG. 1. **Phosphorylation of Cas by Src.** Full-length Cas was purified from baculovirus-infected Sf9 cells and incubated with ATP in the presence or absence of Src for 0, 20, or 60 min as indicated. After 60 min, one sample was incubated with *Yersinia* phosphatase (*yop*) for an additional 5 min. Samples were resolved by SDS-PAGE on 8% gels and examined by Western blotting with antibody directed against Cas. Phosphorylation of Cas was evident by a shift in migration from an apparent molecular mass of 130 kDa to about 170 kDa, which was reversed by phosphatase treatment.

Expression of Cas and Src in Mammalian Cells—To examine the effects of Cas and Src on a blank background, fibroblasts from homozygous null Cas knockout cells were used as described previously (20, 21). The BamHI/NotI fragments encoding wild type Cas, CasY253F, or Cas118Δ423 were excised from pET28A vectors and subcloned into the BamHI/SnabI sites of pBABEhygro (25). An EcoRI/Hind3 fragment containing the entire coding cDNA region of the pp60 Src kinase of the Schmitt-Rupin strain of v-Src (26) was inserted between the EcoRI and SnabI sites of pBABEpuro (25). For controls, cells were transfected with empty vectors (pBABEhygro and/or pBABEpuro) as appropriate so that all transfectants were selected for hygromycin resistance and puromycin resistance conferred by the transfection vectors. All cells were transfected with equal concentrations of both plasmids (1 μg/well of 6-well plate), and clones were not taken from the resultant cells, thus avoiding potential consequences of clonal variation.

In Vitro Kinase Reactions—*In vitro* kinase reactions on full-length Cas proteins were performed as described previously (16). Basically, 1 μg of Cas protein was incubated at 30 °C in 10 μl of kinase buffer (10

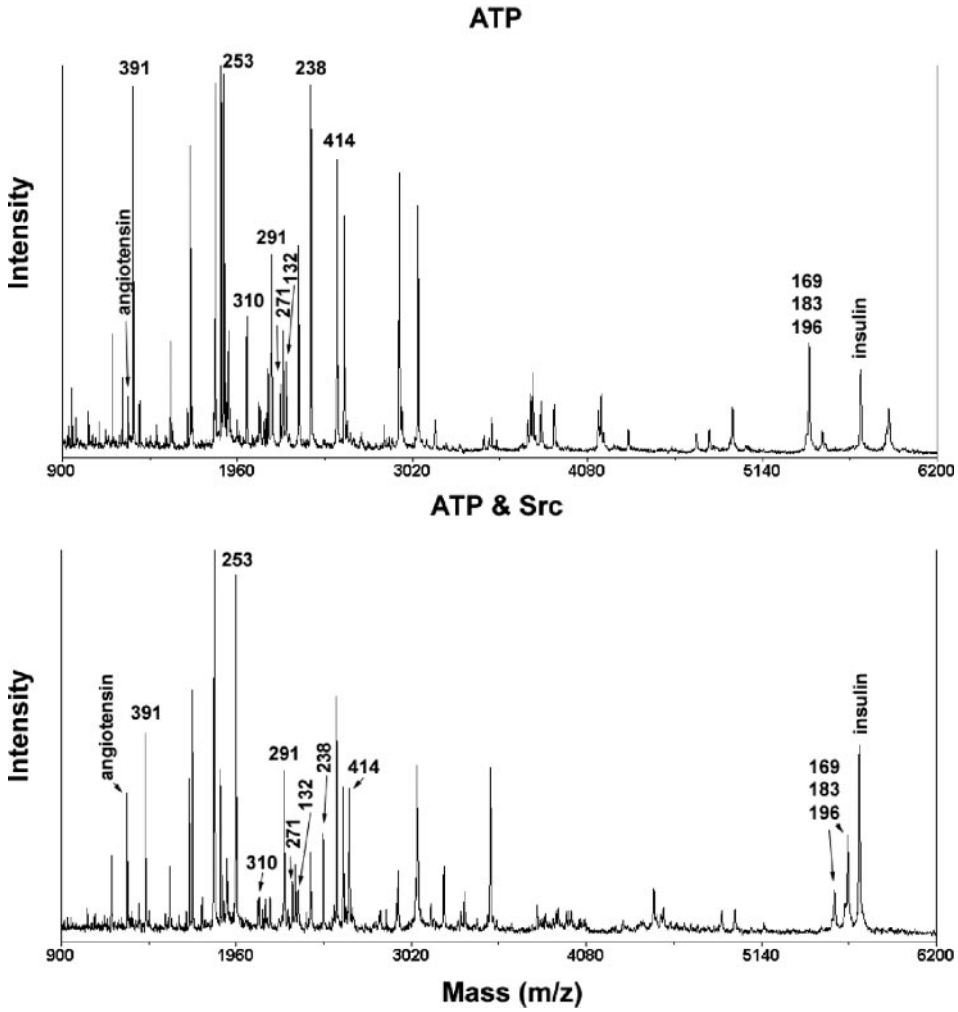

FIG. 2. **Mapping Src phosphorylation sites in Cas.** Cas was incubated with ATP and Src or ATP alone as indicated, resolved by SDS-PAGE, and digested with trypsin. Resulting peptides were then examined by MALDI-TOF MS. Mass and intensity are shown on the *x* and *y* axis, respectively. Phosphorylated peptides, detected by an increase in mass of 80 daltons in the Src + Cas sample, are designated by the apparent phosphorylation sites they contain. The amino acid sequences of these fragments are listed in Table I.

TABLE I

Identification of tyrosine residues in Cas that are phosphorylated by Src

Cas and Src were purified from Sf9 cells, utilized for *in vitro* kinase assays, and resolved by SDS-PAGE. All sites were identified by MALDI-TOF MS analysis of tryptic peptide fragments of Cas incubated in the presence or absence of Src.

Site[a]	Sequence[b]	MW[c]	
		ATP	Src
Tyr^{132}	^{127}TQQGLY*QAPGPNPQFQSPPAK^{147}	2255	2335
Tyr^{169}-Tyr^{183}-Tyr^{196}	^{155}QTPHHSFPSPATDLY*QVPPGPGSPAQDIY*QVPPSAGTGHDIY*QVPPSLDTR^{205}	5423	5583, 5663
Tyr^{238}	^{222}VGQGYVYEASQAEQDEY*DTPR^{242}	2406	2486
Tyr^{253}	^{243}HLLAPGSQDIY*#DVPPVR^{259}	1877	1957
Tyr^{271}	^{260}GLLPNQYGQEVY*DTPPMAVK^{279}	2221	2301
Tyr^{291}	^{280}GPNGRDPLLDVY*DVPPSVEK^{299}	2168	2248
Tyr^{310}	^{300}GLPPSNHHSVY*#DVPPSVSK^{318}	2018	2098
Tyr^{391}	^{384}RPGPGTLY*#DVPR^{395}	1328	1408
Tyr^{414}	^{398}VLPPEVADGSVIDDGVY*AVPPPAER^{422}	2564	2644

[a] Sites are indicated by residue number in primary sequence.
[b] Sequence of tryptic peptide fragments with phosphorylation sites indicated by asterisks; pound signs indicate sites verified by LC-MS-MS.
[c] Molecular weights of fragments after incubation with ATP alone or with ATP and Src, as indicated.

TABLE II

Evaluation of Cas peptides as Src substrates

Phosphorylation of peptides was evaluated by a spectrophotometric assay to detect kinase activity.

Site[a]	Sequence[b]	k_{cat}/K_m[c]
Tyr^{119}	^{114}QPDNVYLVPTPS^{125}	0.071
*Tyr^{132}	^{127}TQQGLYQAPGPN^{138}	NS
*Tyr^{169}	^{164}PATDLYQVPPGP^{175}	NS
*Tyr^{183}	^{178}PAQDIYQVPPSA^{189}	NS
*Tyr^{196}	^{191}TGHDIYQVPPSL^{202}	0.031
*Tyr^{253}	^{246}GSQDIYDVPPVR^{257}	0.232
*Tyr^{291}	^{286}PLLDVYDVPPSV^{297}	0.072
*Tyr^{310}	^{305}NHHSVYDVPPSV^{316}	0.091
Tyr^{366}	^{360}PAEDVYDVPPPA^{372}	0.145
*Tyr^{414}	^{409}IDDGVYAVPPPA^{420}	0.072
Tyr^{657}	^{652}SPDGQYENSEGG^{663}	NS
Tyr^{668}	^{663}GWMEDYDYVHLQ^{674}	NS

[a] Sites indicated by residue number in primary sequence with asterisks indicating sites identified by phosphorylation of full-length Cas.
[b] Sequence of peptide fragments.
[c] k_{cat}/K_m values for Src phosphorylation (min^1 μM^1). For several peptides (designated NS, no saturation), the values of K_m were too high to achieve saturation in these experiments, indicating that they were relatively poor substrates.

mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5 mM ATP) in the presence or absence of 0.15 μg of Src for varying lengths of time. After incubation with Src, some reactions were treated with 1 μg of *Yersinia* phosphatase for an additional 5 min. Reactions were stopped by the addition of SDS-PAGE sample buffer (to achieve a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromphenol blue).

A continuous spectrophotometric assay was also used to measure kinase activity on synthetic peptides shown in Table II as described by Porter *et al*. (27). Reactions contained 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM ATP, 1 mM phosphoenolpyruvate, 0.28 mM NADH, 89 units/ml pyruvate kinase, 124 units/ml lactate dehydrogenase, and varying concentrations of peptide substrates (75–2000 μM). The reactions were carried out in duplicate at 30 °C and were initiated by the addition of Src. Kinetic parameters were determined by fitting data to the Michaelis-Menten equation using nonlinear regression analysis of initial rates.

Western Blot Analysis of Protein Produced by Sf9 or Bacterial Cells—Protein (1 μg/lane) was resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Antisera were used to detect p130 Cas (rabbit antiserum C-20, catalog no. 860, Santa Cruz Biotechnology) and tyrosine-phosphorylated proteins (P-Tyr-100 monoclonal, catalog no. 9411, Cell Signaling Technology). Reactions were detected by chemiluminescence with Amersham ECL plus as described (28, 29).

Western Blot Analysis of Mammalian Cells—Western blot analysis was performed on cells grown for 24 or 72 h after plating. Cells were quickly aspirated, washed with phosphate-buffered saline, transferred to microcentrifuge tubes, pelleted by a brief spin (1 min at 8000 × *g*), aspirated, lysed in SDS-PAGE sample buffer (2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromphenol blue, and 62.5 mM tris-HCl (pH 6.8)), sheared through a 26-gauge needle, boiled for 5 min, and cooled on ice.

Protein (5 μg/lane) was resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Antisera were used to detect p130 Cas (rabbit antiserum C-20, catalog no. 860, Santa Cruz Biotechnology.), total pp60v-Src kinase (anti-avian clone E10 monoclonal, catalog no. 05-185, Upstate Biotechnology), active pp60 kinase (rabbit antiserum specific for Src phosphorylated at Tyr^{416}, catalog no. 2101, Cell Signaling Technology), total MAPK (rabbit antiserum against the p44 and p42 MAP kinase proteins, catalog no. 9102, Cell Signaling Technology), active MAPK (E10 monoclonal antibody specific for p42 and p44 MAP kinase protein phosphorylated at Thr^{202} and Tyr^{204}, catalog no. 9106, Cell Signaling Technology), other proteins phosphorylated on tyrosine residues (P-Tyr-100 monoclonal, catalog no. 9411, Cell Signaling Technology), and β-actin (monoclonal antibody AC15, catalog no. A5441, Sigma). Reactions were detected by chemiluminescence with Amersham ECL plus as described (28, 29).

Crk was immunoprecipitated to evaluate its association with wild type Cas and CasY253F in mammalian cells as described by Klemke *et al.* (23). Cells were washed twice with phosphate-buffered saline, lysed with a solution containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, protease inhibitor mixture (Sigma, P8340), and 1% Nonidet P-40, and clarified by centrifugation. 4 mg of total protein was precleared with protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences, catalog no. 17-0618-01), incubated overnight with monoclonal antibody against Crk (Transduction Laboratories, catalog no. 610035), immunoprecipitated with protein G-Sepharose beads, eluted in SDS-PAGE sample buffer, resolved by gel electrophoresis, and analyzed by Western blotting with antiserum against Crk and Cas as described above.

Phosphorylation Site Identification by MALDI-TOF—Cas protein (1 μg) produced by Sf9 cells was subjected to in an *in vitro* kinase reaction for 1 h either in the presence of 0.5 mM ATP alone or with ATP plus 0.15 μg of purified v-Src. Cas was then resolved by SDS-PAGE, and examined by MALDI-TOF MS as described previously (28). Briefly, protein was detected by Coomassie staining, excised, rehydrated, washed with 50 mM Tris (pH 8.0), 50% acetonitrile, dried, and completely digested with trypsin in 25 mM Tris (pH 8.5). Peptides were then extracted with 50% acetonitrile, 0.1% trifluoroacetate, dried, suspended in 10 mg/ml 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile, 0.1% trifluoroacetate containing bovine insulin and human angiotensin as internal standards, and applied to a MALDI sample plate. MALDI mass spectrometric analysis was performed on a PerSeptive Voyager DE-RP mass spectrometer in the linear mode. Profiles were queried against the expected Cas peptide products, and phosphorylated residues were apparent by an increase in molecular mass of 80 daltons (relative to the Cas + ATP samples).

Sequencing and Identification of Phosphorylation Sites by LC-MS-MS—Protein bands were subjected to in-gel digestion as for MALDI-MS. Peptide extracts were desalted using a C18 ZipTip (Millipore) followed by elution from the tip with 10 ml of 50% acetonitrile. Immediately prior to injection the acetonitrile was removed on a Speed-vac concentrator, and the volume was brought to ~2 ml. Analysis was done on a Micromass Q-Tof hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source. A fused silica tip mounting adapter from New Objective was fitted with a 75-mm inner dimension fused silica tip, also from New Objective, connected through 50-mm inner dimension fused silica tubing to the LC detector outlet. Nano-LC was performed with an LC Packings Ultimate micro pump and solvent

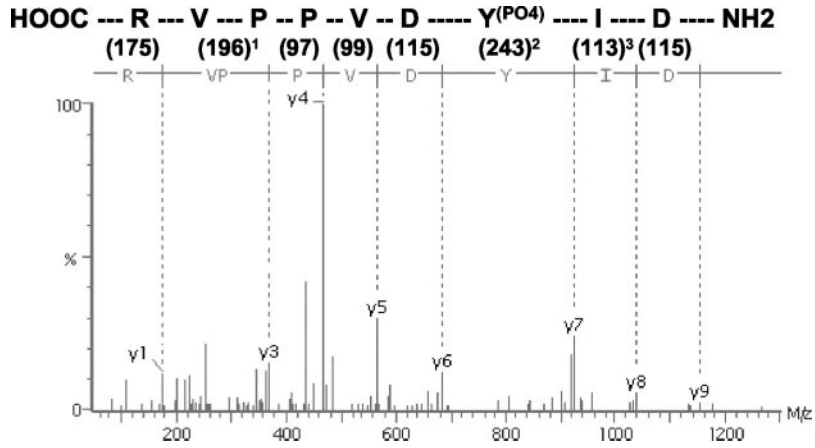

FIG. 3. **Phosphorylation of tyrosine 253 of Cas by Src.** Cas was phosphorylated by Src, resolved by SDS-PAGE, digested with trypsin, and analyzed by LC-MS-MS. A deconvoluted chromatogram of the peptide containing residues 251–259 (^{251}DIYDVPPVR^{259}) is shown. Peaks belonging to the y-type ion series are indicated along the *x* axis, with their relative intensity shown on the *y* axis. The sequence is given above the chromatogram with the phosphorylation site indicated. The monoisotopic mass of each amino acid residue is given in parentheses below the sequence. *Superscript 1*, Val (97 daltons) + Pro (99 daltons) = 196 daltons; *superscript 2*, Tyr (163 daltons) + phosphate (80 daltons) = 243 daltons. These data indicate that tyrosine 253 of Cas was phosphorylated by Src.

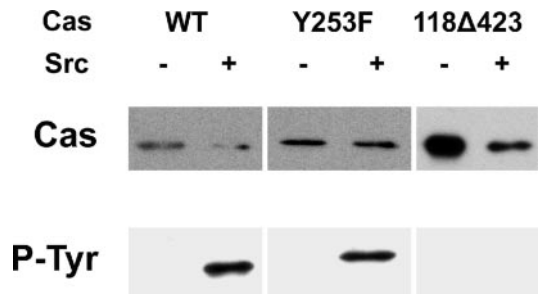

FIG. 4. **Src phosphorylation sites lie between residues 118 and 423 in Cas.** Wild type Cas (*WT*), CasY253F, and Cas118Δ423 were produced in bacteria and subjected to *in vitro* kinase assays with or without Src as indicated. Protein was then examined by Western blotting with anti-phosphotyrosine antibody (*P-Tyr*). Wild type Cas and CasY253F were phosphorylated by Src, but Cas118Δ423 was not, confirming that major Src phosphorylation sites were located between residues 118 and 423 in Cas.

organizer, and an Ultimate detector with a 10-nl flow cell and 214-nm wavelength. Sample was resolved through an LC Packings C18 PepMap column (5 mm, 75-mm ID, 15-cm length) at a flow rate of 200 nl/min and an injection volume of 1 ml (Solvent A: 2% acetonitrile, 0.1% formic acid; Solvent B: 80% acetonitrile, 0.1% formic acid; loading solvent: 0.1% formic acid).

Cell Growth Assays—For all assays, cells were grown in Dulbecco's modified Eagle's medium + 10% fetal bovine serum at 37 °C in 100% humidity. All comparisons were done in parallel to avoid any changes in culture conditions between experiments. 20,000 cells were seeded in 1 ml in each well of tissue culture-treated 12-well cluster plates (Falcon, catalog no. 3043) to examine anchored growth or in ultra low attachment 24-well cluster plates (Corning, catalog no. 3473) to examine nonanchored growth. Cell numbers were obtained by Coulter counter at the time points indicated in the Fig. 7.

To examine cell migration, 200,000 cells were plated in 6-well cluster plates on cell culture inserts with a 3-μm pore size as directed by the manufacturer (Transwell clear, Costar) and grown for 72 h. Cells were then separately released from the top of the membrane, the bottom of the membrane, and well beneath the membrane. Migration was then quantitated as the percent of cells found in the well over the total cell number.

RESULTS

Identification of Src Phosphorylation Sites in Cas—Src and Cas were purified from Sf9 cells and utilized for *in vitro* kinase assays. Phosphorylation of Cas by Src was evident in these experiments. The apparent molecular mass of Cas was shifted by Src phosphorylation from 130 to 170 kDa in SDS-polyacrylamide gels. This effect was reversed by treatment with a tyrosine phosphatase (Fig. 1).

To identify sites phosphorylated by Src, Cas was incubated with Src and ATP or with ATP alone, resolved by SDS-PAGE, cleaved with trypsin, and analyzed by MALDI-TOF MS. As shown in Fig. 2, several tryptic fragments were found in the unphosphorylated state in the ATP-treated sample, but these were shifted by the mass of a phosphate (+80) in the Src-phosphorylated sample. Based on these data, 11 tyrosine residues (132, 169, 183, 196, 238, 253, 271, 291, 301, 391, and 414) were phosphorylated by Src (Table I).

Src Preferentially Phosphorylates Cas on Tyrosine 253—Quantitative kinase assays were performed on peptides harboring several of the phosphorylation sites identified by MALDI-TOF MS. As shown in Table II, the peptide based on tyrosine 253 was the most efficient substrate of those tested, in terms of k_{cat}/K_m. Although individual sites might be phosphorylated differently in the context of the folded three-dimensional structure of Cas, the mass spectrometry and peptide data collectively point to tyrosine 253 on Cas as a primary target of Src phosphorylation.

Phosphorylation of full-length Cas on tyrosine 253 by Src was verified by LC-MS-MS. The calculated mass of the peptide sequence between residues 243 and 259 (HLLAPGSQDIYDVPPVR) is 1877 daltons. Phosphorylation of tyrosine 253 increased the mass of this fragment by 80 daltons to 1957 daltons (Fig. 2 and Table I). The carboxyl-terminal portion of this fragment, containing tyrosine 253, was clearly detected by LC-MS-MS in Fig. 3. The pattern of fragment ions confirmed the presence of phosphorylated tyrosine 253, with a mass of 243 daltons.

Src Phosphorylation Sites Reside in the "Src Substrate Region" of Cas—The phosphorylation sites identified in this study were all located between amino acids 132 and 414 of Cas. Expression vectors were created to produce full-length wild type Cas, a site mutant with tyrosine 253 changed to phenylalanine (Y253F), or a deletion mutant missing the entire substrate for Src substrate region between amino acids 118 and 423 (118Δ423). As shown in Fig. 4, wild type Cas and CasY253F were both phosphorylated by Src *in vitro*, whereas Cas118Δ423 was not. Thus, Src phosphorylated Cas at sites in addition to tyrosine 253, but they all resided within the Src substrate region as presented in Fig. 5.

Deletion of the Src Substrate Region Makes Cas Cytotoxic—Wild type Cas and mutant Cas constructs shown in Fig. 5 were transfected into fibroblasts derived from homozygous null Cas knockout mice to examine their effects on cell transformation by v-Src. However, in agreement with recent reports (21, 30, 31), we found Cas118Δ423 to be extremely toxic to mammalian cells. This construct appeared to cause rapid apoptosis, and we were unable to obtain stable transfectants for further study. Nonetheless, we were able to proceed with wild type Cas and CasY253F.

Cas Augments Src Transformation—As shown in Fig. 6, Src transfection caused cells to assume a spindly morphology com-

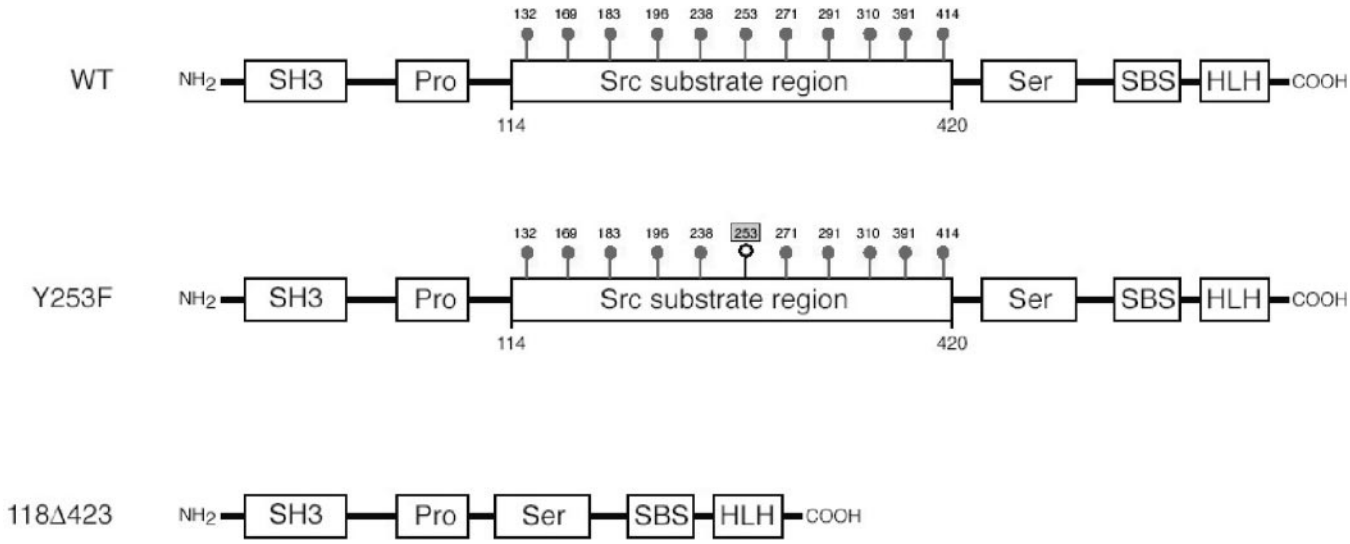

FIG. 5. **Diagram of Src phosphorylation sites and Cas transfection constructs.** Tyrosine residues phosphorylated by Src are *numbered* over a schematic representation of Cas. Positions of the SH3 domain, proline-rich region (*Pro*), kinase substrate region, serine rich-region (*Ser*), Src binding sequence (*SBS*), and helix-loop-helix (*HLH*) motifs are indicated. Schematic diagrams of two mutants made for further testing are also shown. Tyrosine 253 was changed to phenylalanine to create CasY253F, and the entire substrate for the Src kinase region between residues 118 and 423 was deleted to create Cas118Δ423. *WT*, wild type.

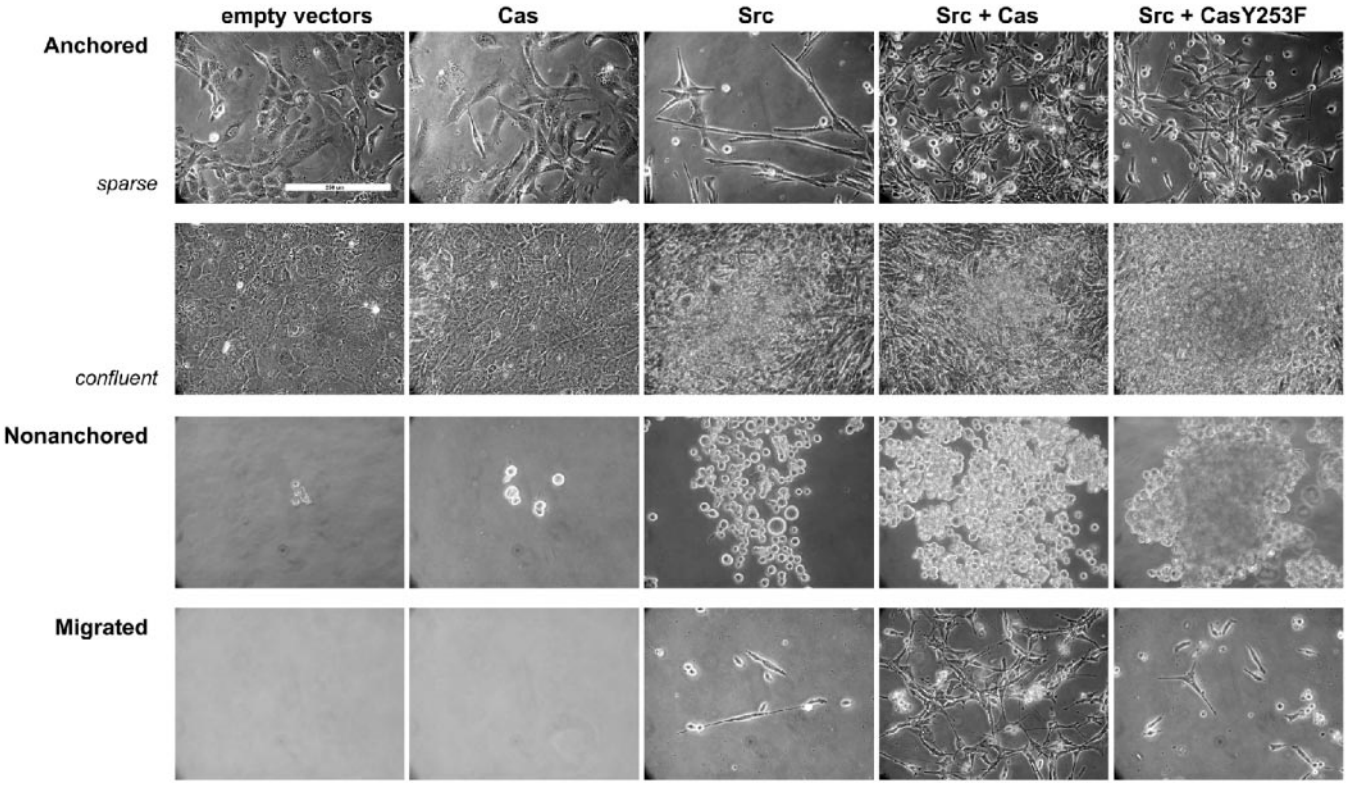

FIG. 6. **Effects of Cas and Src on cell morphology, growth, and migration.** Fibroblasts from homozygous null Cas knockout mice were transfected with wild type Cas or CasY253F in pBABEhygro, v-Src in pBABEpuro, or empty vectors and selected for resistance to puromycin and hygromycin. 20,000, 200, or 200,000 cells were plated on each well of 12-well tissue culture plates, 24-well low attachment plates, or Transwell inserts with 3.0-μm pores in 6-well cluster plates to examine anchored growth, nonanchored growth, or cell migration, respectively. Anchored cells grown for 2 days (subconfluent) and 6 days (confluent) are shown, along with nonanchored cells grown for 11 days and cells that migrated through a porous membrane to the bottom well of cluster plates over a 3-day period as indicated. Cas was not required for Src to cause transformation of cell morphology but was required for Src-transformed cells to achieve robust anchorage independence at these plating densities. Phosphorylation of Cas at tyrosine 253 was required for Src to induce cell migration, but not anchorage independence. (*Bar* = 250 μm)

pared with Cas or control transfectants. In contrast, coexpression of Cas and Src caused cells to assume a refractive and rounded morphology typical of transformed cells. Wild type Cas and CasY253F were indistinguishable in their ability to support Src-induced transformation (Fig. 6).

Eventually, Cas and control transfectants formed contact inhibited monolayers. At higher densities, cells transfected with Src formed multilayered foci. However, expression of Cas appeared to augment foci formation of postconfluent Src-transfected cells (Fig. 6).

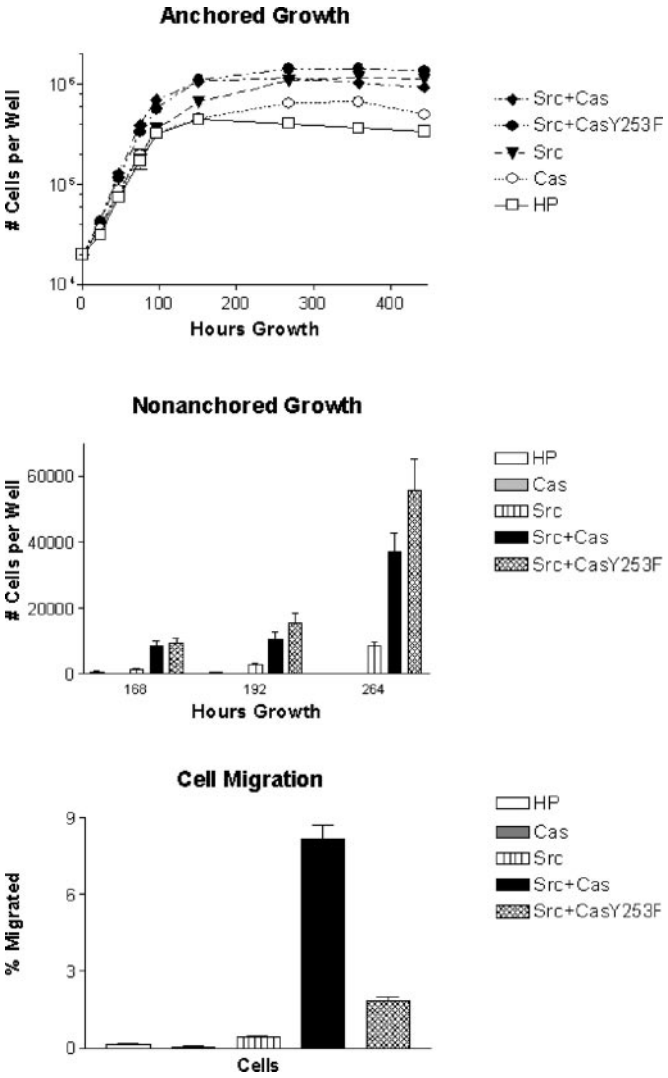

FIG. 7. **Phosphorylation of tyrosine 253 is important for cell migration, but not for anchored or nonanchored growth.** Cells were plated as described in the legend for Fig. 6 and counted at the indicated time points to examine anchored growth or nonanchored growth. To measure cell migration, the percent of cells that migrated through the membranes to the bottom well after 72 h was calculated as shown. Data are shown as mean ± S.E. with n = 2, 4, and 3 for anchored growth, nonanchored growth, and cell migration, respectively. Phosphorylation of Cas at tyrosine 253 was required for Src to induce cell migration but not to reduce contact growth inhibition or achieve anchorage independence. *HP*, empty hygro/puro vectors.

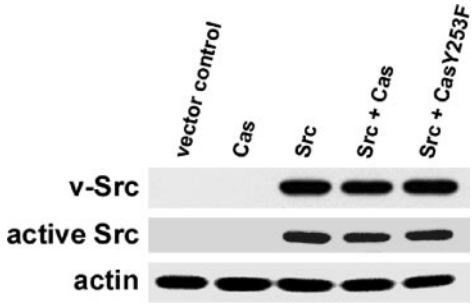

FIG. 8. **Src activity is independent of Cas.** Cells were examined by Western blotting for v-Src, active Src, and β-actin as indicated. Cas and Src were expressed in the appropriate transfectants. Src activity was not affected by Cas expression.

Reduced Contact Growth Inhibition by Src Is Independent of Cas Tyrosine 253—Foci formation of Src transfectants suggested a loss of contact growth inhibition. This was made evident by growth curves shown in Fig. 7. Control transfectants achieved an average saturation density of less than 1200 cells mm^2. Cas transfectants surpassed this by about 50%, reaching an average density of nearly 1800 cells/mm^2. Transfection with Src increased saturation density of these cells by about 300% to over 3000 cells/mm^2 or about 6-fold higher than the increase caused by Cas. Wild type Cas did not further increase the saturation density of Src transfectants. However, Cas did enable the Src-transfected cells to reach higher densities faster (in 11 days as opposed to 15 days). The same was true for CasY253F, which enabled cells to reach an even higher saturation density of over 3700 cells/mm^2 in the same amount of time (11 days). Thus, wild type Cas and CasY253F both augmented the ability of Src to decrease contact growth inhibition of these cells.

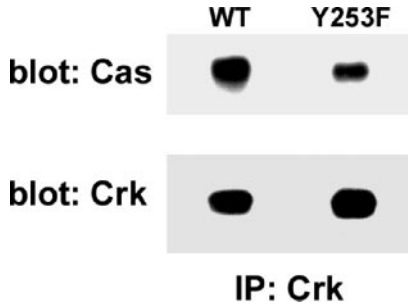

FIG. 9. **Phosphorylation of Cas at tyrosine 253 is not required for Crk binding.** Crk was immunoprecipitated (*IP*) from cells transfected with Src and wild type Cas (*WT*) or Src and CasY253F. Immunoprecipitated protein was then analyzed by Western blotting for Cas and Crk as indicated. Both wild type Cas and CasY253F coprecipitated with Crk.

Src Increases Growth Rate Independently of Cas Tyrosine 253—Cas also enabled Src to increase the growth rate of these cells. Control transfectants displayed a log phase doubling time of 24 h. Cells transfected by either Cas or Src alone displayed a similar log phase doubling time of 23 h. In contrast, cells transfected with both Cas and Src displayed a faster log phase doubling time of just 16 h. Thus, Cas potentiated the ability of Src to increase cell growth rate, as well as to suppress contact growth inhibition.

Loss of Anchorage Independence by Src Is Independent of Cas Tyrosine 253—Loss of anchorage dependence is a reliable hallmark of most tumor cells (3, 32). Consistent with earlier reports (20, 21), Cas augmented the ability of Src to promote anchorage-independent growth. As shown in Fig. 7, cells transfected with Cas or control vectors could not grow in suspension, whereas 200 Src transfectants grew to about 8500 in 11 days. In contrast, 200 Src transfectants expressing wild type Cas or CasY253F grew to an average of over 37,000 or 55,000, respectively, in the same period of time. This was significantly better than cells transfected with Src alone ($p < 0.005$ by t test). Thus, wild type Cas and CasY253F both potentiated the ability of Src to override anchorage dependence.

Src Requires Cas Tyrosine 253 to Increase Cell Migration—In addition to promoting anchorage-independent growth of transformed cells, Cas plays an important role in process of cell migration (21). As shown in Fig. 7, less than 0.5% of the cells transfected with control vectors, Cas, or Src were able to migrate through pores in a migration chamber. In contrast, more than 8% of cells transfected with both Src and wild type Cas migrated, representing a significant increase over cells transfected with Src alone ($p = 0.0001$ by t test). However, less than 2% of cells transfected with both Src and CasY253F migrated, representing a 4-fold decrease compared with Src-transformed cells expressing wild type Cas. Therefore, wild type Cas enabled Src transformed cells to migrate significantly better than

CasY253F ($p = 0.0003$ by t test).

Cas Does Not Affect Src Kinase Activity—We examined the effects of wild type Cas and CasY253F on Src activity in these cells. Consistent with previous reports (20), Cas did not affect v-Src activity (Cas has previously been shown to activate c-Src, the down-regulated cellular form of Src (16, 33)). As shown in Fig. 8, all cells transfected with Src had similar levels of total and active forms of the kinase. Therefore, Cas is likely to affect Src signaling downstream of its intrinsic kinase activity.

Phosphorylation of Cas at Tyrosine 253 Is Not Required for Crk Association—Previous reports indicate that binding of Crk to Cas is important for cell migration (21–23). Therefore, we investigated the ability of CasY253F to associate with Crk. As shown in Fig. 9, both wild type and mutant Cas associated with Crk in Src transformed cells.

DISCUSSION

Cas plays a critical role in cell growth and transformation (6). The importance of Cas in cell transformation is highlighted by investigations into the relationship between Cas and Src. For example, experiments with site-directed mutants revealed that the ability of Src and Crk to transform cells relies on their ability to bind Cas (34, 35). In addition, ablation of Cas with antisense constructs curtailed transformed characteristics imparted by agents including v-Src (36).

We identified 11 Src phosphorylation sites on Cas. These were all within the Src substrate region between amino acids 118 and 423 of Cas. These data are consistent with reports that sites outside of this region (*e.g.* tyrosine residues 668 and 670) are not phosphorylated by Src (37). These data are also consistent with reports that Src must phosphorylate tyrosine residues in this region to transform cells (21).

Our results also confirm reports that deletion of the Src substrate region causes Cas to induce cellular apoptosis (30). An apoptotic role for Cas may also be inferred from its cleavage by caspase-3 (38). However, mechanisms by which ablation of the Src substrate region turns Cas apoptotic are intriguing. In the absence of Cas (*e.g.* in Cas knockout cells), its homologues HEF1 and Efs/Sin may perform scaffolding functions needed for cell survival (6). HEF1 and Efs/Sin may not completely substitute for Cas because Cas knockout mice suffer from severe developmental disorders. However, some of this deficiency may be due to differences in expression patterns as opposed to function (20). Also, Cas is not required for formation of focal adhesions (20), whereas HEF1 is found in focal adhesion plaques of adherent cells (39). Therefore, deletion of the Src substrate region may cause Cas to act as a dominant negative protein that interferes with itself and its homologues, thus causing events leading to apoptosis. Nonetheless, the case does not seem simple because, instead of blocking apoptosis, transfection of HEF1 can actually increase apoptosis in some mammary carcinoma cells (39).

Regardless of how the Src substrate region in Cas affects apoptosis, the importance of its phosphorylation by Src in cell transformation is clear (20, 21). Our data confirm this in that Cas significantly enhanced the ability of Src to cause morphological transformation (Fig. 6). More importantly, Cas significantly increased the ability of Src to decrease contact growth inhibition and to increase anchorage-independent growth (Fig. 7).

Our data indicate that Src phosphorylates 11 sites within the Src substrate region of Cas. Once these sites are phosphorylated, they can bind to SH2 domains on different proteins (6). Tyrosine 253 was a preferred Src phosphorylation site in Cas. However, phosphorylation of this site was not required for Src to block contact growth inhibition or anchorage dependence. Instead, phosphorylation of tyrosine 253 was required for Src to augment the migration of transformed cells. Therefore, this site may target individual proteins to focal adhesions that are specifically required for cell migration to occur.

Binding of Crk to the Src substrate region of Cas is required for cell migration and invasion into collagen (21–23). In addition, Huang *et al.* (21) have recently reported that phosphorylation of this region is required for Crk binding to Cas, and for Src to induce cell migration but not for Src to promote nonanchored cell growth. Also, Cho and Klemke (40) have recently demonstrated that the association of Cas with Crk activates the small GTPase Rac1 to direct cell movement by causing the growth and retraction of pseudopodia. This same Cas/Crk/Rac1 association also blocks apoptosis (22, 23). However, Cas may regulate the binding of Crk to other proteins including Gab, Cbl, and the insulin receptor substrate IRS1 (41–43), which have all been implicated in cell migration (44–46).

Phosphorylation of Cas during cell migration differs from its phosphorylation profile during cell division. Cas is phosphorylated on serine and threonine, whereas tyrosine residues are dephosphorylated during mitosis (47). As opposed to the Cas/Crk/Rac1 cascade implicated in cell migration, phosphorylation of Cas at sites other than tyrosine 253 may enable Grb2 to associate with Shc and Shp2 to activate the Ras/MAPK cascade, which then activates genes required for mitogenesis via serum response elements (48).

Taken together, because phosphorylation of tyrosine 253 is not required for the effects of Cas on cell growth and anchorage independence but is required for Cas to enhance transformed cell migration, it may be proposed that phosphorylation of this site is especially suited to activate Rac, whereas other sites enable the activation of Ras. This system provides a striking example of how a single phosphorylation site on one protein can determine whether or not a tumor cell will migrate. Ultimately, this situation may be exploited to precisely target specific aspects of tumor cell growth, such as metastasis, without inhibiting the processes required for normal cell behavior.

Acknowledgments—We are very grateful to Drs. Amy Bouton (University of Virginia) and Hisamaru Hirai (University of Tokyo) who graciously supplied Cas knockout cells, Hyunna Song for kinetic experiments of Cas peptides, and Dr. Jim Bliska (State University of New York at Stony Brook) for helpful discussions.

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Src Phosphorylates Cas on Tyrosine 253 to Promote Migration of Transformed Cells

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J. Biol. Chem. 2003, 278:46533-46540.
doi: 10.1074/jbc.M307526200 originally published online September 11, 2003
