Subsets of the Major Tyrosine Phosphorylation Sites in Crk-associated Substrate (CAS) Are Sufficient to Promote Cell Migration*  

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Crk-associated substrate (p130CAS or CAS) is a major integrin-associated Src substrate that undergoes tyrosine phosphorylation at multiple YXXP motifs in its substrate domain (SD) to create docking sites for SH2-containing signaling effectors. Notably, recruitment of Crk adaptor proteins to the CAS SD sites is implicated in promoting cell migration. However, it is unclear which or how many of the 15 CAS SD YXXP tyrosines are critically involved. To gain a better understanding of CAS SD function, we assessed the signaling capacity of individual YXXP motifs. Using site-directed mutagenesis combined with tryptic phosphopeptide mapping, we determined that the ten tyrosines in YXXP motifs 6–15 are the major sites of CAS SD phosphorylation by Src. Phosphopeptide binding assays showed that all of these sites are capable of binding the Crk SH2 domain. To evaluate the requirement for CAS YXXP sites in stimulating cell migration, a series of phenylalanine substitution variants were expressed in CAS−/− mouse embryo fibroblasts. CAS expression enhanced the rate of cell migration into a monolayer wound in a manner dependent on CAS tyrosine phosphorylation. For example, SD deletion results in a loss of CAS tyrosine phosphorylation, and Crk family SH2 domains that mediate a stable interaction with CAS (5–7) have a strong binding preference for phosphorylated YXXP tyrosines. Three of the 15 CAS SD YXXP sites contain an aspartic acid residue in the Y + 1 position (YDXXP sites), predicted to further enhance Crk SH2 affinity (8). CAS tyrosine phosphorylation is achieved primarily by Src (or a related Src family kinase) (9–11) that is positioned to phosphorylate the YXXP through either direct binding of the Src SH3 domain to the CAS SBD or an indirect mechanism involving focal adhesion kinase interactions with the CAS SH3 and Src SH2 domains (4, 12).

Considerable evidence supports a role for CAS SD tyrosine phosphorylation in promoting cell migration. Thus, the CAS interaction with focal adhesion kinase is critical for the ability of focal adhesion kinase to stimulate cell migration (13, 14), and CAS overexpression in COS-7 cells promotes cell migration dependent on an intact SD (15). Moreover, CAS−/− mouse embryo fibroblasts (MEFs) exhibit cell spreading and migration defects that can be rescued by WT CAS but not by CAS SD deletion mutants (16). Recruitment of members of the Crk family to the CAS SD sites of tyrosine phosphorylation may be the primary function of CAS in integrin signaling affecting cell motility. Crk family proteins (reviewed in Ref. 17) are small SH2/SH3 adaptors that function in tyrosine kinase signaling events via SH2-mediated binding to phosphotyrosine sites while recruiting downstream effectors bound to an SH3 domain. The N-terminal Crk SH3 domain interacts with guanine nucleotide exchange factors including DOCK180. DOCK180 activates the small GTPase Rac1, which in turn can promote actin nucleating activity of the Arp 2/3 complex leading to plasma membrane protrusion and extension of lamellipodia (reviewed in Ref. 18). The ability of CAS to interact with Crk and promote Rac1 activity through DOCK180 has been linked to enhanced cell migration (15, 16). In addition to Crk, SH2/SH3 adaptors of the Nck family show strong SH2 binding preference for phosphorylated YDXP sites (8), have been shown to interact with CAS (19), and can promote Arp2/3 complex activation (20–22). Thus, by directly localizing binding of Crk (and possibly Nck) proteins, CAS SD tyrosine phosphorylation may serve as a switch for activating and/or sustaining the
protrusion step of cell motility. Supporting this, antibodies specific for phosphorylated CAS YXXP tyrosines prominently stain the leading edges of extending lamellipodia (12).

Questions remain regarding the signaling functions of CAS SD YXXP motifs and their role in cell migration. Previous studies employed large deletion mutants that remove all or substantial portions of the CAS SD and thus provided no insight into which or how many CAS SD tyrosines are critically involved. The large SD deletions also could not distinguish between phosphorylation-mediated functions versus other possible motility-related functions of the SD, such as its ability to interact with members of the ezrin family (23), and could impair proper protein folding and function of other CAS domains. Thus, to better understand the function of the CAS SD in cell migration, it is important to determine which CAS SD tyrosines are subject to phosphorylation by Src and to assess their roles in cell motility through analysis of mutants in which the phosphoacceptor tyrosines are changed to nonphosphorylatable phenylalanine residues. In the present study we used the strategy of site-directed mutagenesis combined with tryptic phosphopeptide mapping to identify CAS sites of tyrosine phosphorylation and found that the 10 most C-terminal SD YXXP tyrosines, including all of the YDXP sites, serve as the major sites of phosphorylation by Src. We further evaluated the involvement of the CAS YXXP tyrosines in promoting cell migration and found that a substantial number of the major Src sites are dispensable for this response.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Plasmid Construction—Using sequential PCR steps (24) with high fidelity amplification (Advantage-HF2; Clontech/BD Biosciences), point mutations were made in full-length mouse CAS cDNA (25) to convert individual codons for YXXP tyrosines to encode phenylalanines (FXXP mutations). To obtain specific products, 27–29 nucleotide primers were designed, and the annealing temperature was 59 °C. To generate variants with multiple FXXP mutations, sequential PCR was repeated using cDNA templates with existing FXXP mutations. Mutated cDNAs were then substituted for wild-type CAS cDNA in pRc/CMV-CASmyc (26) to generate a set of plasmids expressing Myc-tagged CAS variants with single and multiple phenylalanine substitutions. Mutated cDNAs were also subcloned into retroviral vector pLZR-MS-IRES-GFP (27) (generously provided by Al Reynolds, Vanderbilt University) for expression of untagged CAS variants in conjunction with GFP expressed from a bicistronic transcript. All cDNAs with FXXP mutations were sequenced in their entirety to verify final plasmid constructs.

Antibodies—Monoclonal antibody 9E10 against c-Myc epitope was a gift from Kathy Gould (Vanderbilt University). Monoclonal antibody against CAS (clone 24, here designated “CAS-1”) was obtained from BD Transduction Laboratories. The pCAS phosphospecific polyclonal antibody (12) was from Cell Signaling Technology, Inc. Anti-GST monoclonal antibody (clone GST-2) ascites fluid was from Sigma. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Cell Signaling Solutions. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch Laboratory, Inc. Western blotting and immunoblotting were performed essentially as described (27). Three successive rounds of infection were done to increase expression. Cells expressing CAS were then selected by two rounds of fluorescence-activated cell sorting for GFP. Gates were set to collect GFP-positive cells from a window between 70 and 85% of maximal expression. After expansion, CAS expression in the sorted cell population was roughly equivalent to levels of endogenous CAS in CAS+/−MEFs (12).

In Vivo Phosphorylation of CAS Variants and Phosphopeptide Mapping—Myc-tagged CAS variants were immunoprecipitated from radioimmunoprecipitation assay buffer lysates using 9E10 antibody and phosphorylated by the addition of recombinant Src in a kinase reaction buffer containing γ-[32P]ATP, as described previously (28). Phosphorylated CAS was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and visualized by autoradiography. The labeled region of the membrane was then excised, incubated with tosylphenylalanyl chloromethyl ketone-treated trypsin (Worthington), and cleaved peptides were subjected to two-dimensional separation on thin layer cellulose plates (26) followed by 30-min electrophoretic separation in pH 1.9 buffer and overnight chromatographic separation in phospho-chromatography buffer.

Immunoprecipitation, Immunoblotting, and SH2 Binding Assays—The cells were treated with 500 μM sodium orthovanadate for 3 h to increase CAS phosphorytrosine content and then lysed in Nonidet P-40 buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% aprotinin, 50 mM NaF, and 0.1 mM Na3VO4). For immunoblot analysis of total cell lysates, 15 μg of total protein/sample was used. For immunoprecipitation, 500 μg of total protein/ml of lysis buffer was incubated with 1 μg/ml CAS-TL antibody, and protein was recovered on protein G-agarose (Upstate). Immunoprecipitated proteins were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblot and Far Western analyses. The membranes were blocked in Tris-buffered saline containing 3% nonfat milk and 0.05% Tween 20. For immunoblot analysis, primary antibodies were used at 0.25–1.0 μg/ml with detection by horseradish peroxidase-conjugated secondary antibodies and development using ECL (Amersham Biosciences). For Far Western (blot overlay) analysis (29), the membranes were incubated with a GST-Crk-II SH2 fusion protein (0.1 μg/ml) for 20 min at room temperature, and binding was detected by a secondary incubation with anti-GST antibody (1:5,000 dilution of ascites) and ECL. For the peptide microassay of SH2 binding, cellulose membranes harboring 17-mer spot-synthesized peptides (30) were incubated with GST-SH2 fusion proteins (0.1 μg/ml for 30 min at 4 °C) and then overnight with a GST-Crk-II SH2 antibody as above for detection. GST-SH2 fusion proteins were prepared (31) using pGEX plasmids harboring SH2 domains of chicken Crk and Src and human Nck (32) kindly provided by Bruce Mayer (University of Connecticut Health Center).

Wound Healing Cell Migration Assay—Monolayer wounds were made using a pipette tip in confluent cell cultures grown on fibronectin-coated 35-mm dishes, and the cells were allowed to migrate into the denuded area over an 8-h period in DMEM containing 10% fetal bovine serum and buffered with 10 mM Hepes. During this time period, while cells were observed to maintain healthy morphology, differential interference contrast images were collected with a Nikon inverted microscope using a 10× objective lens and a 0.45 NA oil immersion objective. Automated collection of images using Metamorph imaging software (Universal Imaging) allowed collection of 12 fields of view at 10-min intervals. For each cell population analyzed (expressing either a CAS variant or vector-only control), the nuclear centers of at least 50 individual cells were plotted over a 6-h period beginning at the start of the second hour. Combined data from three independent experiments were plotted as “box and whisker” profiles showing the 10th, 25th, 50th, 75th, and 90th percentiles of cell migration distances. Analysis of variance indicated statistically significant differences in migration among the populations. Subsequent pairwise comparisons of mutants to either WT CAS or CAS F6–15 in post-hoc analysis by the Dunnett test revealed migration differences significant at the 0.05 level of probability.

RESULTS

Identification of CAS Phosphorylation Sites—To determine the capacity of individual CAS YXXP tyrosines to undergo phosphorylation by Src, site-directed mutagenesis combined with two-dimensional tryptic phosphopeptide mapping was employed. For mouse CAS, used in this analysis, the 15 SD YXXP tyrosines are contained in 12 predicted tryptic peptides (Fig. 1). The 15 YXXP tyrosines were individually substituted with phenylalanine, with the resulting variants designated F1–F15. In addition, two variants with either double (F12/F13) or triple (F3/F4/F5) phenylalanine substitutions were made to aid in the identification of tryptic phosphopeptides containing more than one YXXP site. Along with WT CAS, the variants

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were individually expressed as Myc epitope-tagged proteins in COS-7 cells, recovered by immunoprecipitation, and phosphorylated in vitro by incubating in the presence of Src and a kinase reaction buffer containing [γ-32P]ATP.

Maps of WT CAS, and CAS variants F1, F2, and F3/F4/F5 are shown in Fig. 2, A–D, respectively. Whereas the relative spot intensities vary somewhat, these maps all showed an essentially identical pattern of major phosphopeptide spots designated a–p (notation diagram in Fig. 2E). Because phenylalanine substitutions for any of the first five YXXP tyrosines did not result in loss of a consistently observed phosphopeptide spot, we conclude that none of these tyrosines are among the major sites of phosphorylation by Src. However, they could represent minor sites.

In contrast to the first five sites, maps of CAS variants F6–F15 indicated that each of the remaining 10 YXXP sites are subject to efficient phosphorylation by Src (Fig. 3). The F6, F7, F8, F9, F10, F11, F14, and F15 substitutions each resulted in a clear loss of one of the major phosphopeptide spots (d, e, o, f, k, g, and n, respectively), indicating that these phosphopeptides carry the respective YXXP tyrosines. These assignments are well supported by peptide mobility predictions (not shown). The closely migrating doublet of spots h and i is also missing from the F9 map, suggesting that one of these may also derive from the phosphopeptide containing the ninth YXXP tyrosine. In addition to phosphopeptide n, the F15 map is also missing phosphopeptide o, which was assigned to YXXP site 9. Thus, phosphorylation of site 9 could be dependent on prior phosphorylation of site 15. However, our analysis shows no other indication of such dependence.

YXXP tyrosines 12 and 13 reside on the same tryptic peptide, which could be singly phosphorylated at either site or doubly phosphorylated. The F12 and F13 individual substitutions generated similar maps characterized by chromatographic mobility shifts of phosphopeptides d and m (Fig. 3, G and H). Such shifts, because of the more hydrophobic character of phenylalanine in comparison with tyrosine, would be expected for a singly phosphorylated peptide containing two potentially phosphorylatable tyrosines. Thus, phosphopeptides d and m appear to represent two alternatively cleaved peptides containing YXXP tyrosines 12 and 13 where either, but not both, is phosphorylated. This was confirmed by the F12/F13 double substitution that eliminated both phosphopeptides d and m (Fig. 3I).

Peptides representing the doubly phosphorylated forms of d and m are not readily evident, indicating either that simultaneous phosphorylation of both sites is a low stoichiometric event or that the doubly phosphorylated peptides are poorly resolved in the maps.

To further evaluate CAS SD phosphorylation, additional CAS variants were constructed in which either all (F1–15) or substantial subsets (F1–9 and F10–15) of the YXXP tyrosines were changed to phenylalanines. Maps of these variants (Fig. 4) show the expected pattern of remaining spots and thus support the conclusions drawn from the individual site mutants. Phosphopeptide spot j is apparent even in the F1–15 map and thus must represent a non-YXXP tyrosine subject to phosphorylation by Src. Notably, all nine of the YDXP sites with potential for higher affinity binding to Crk or Nck proteins were identified as being efficiently phosphorylated by Src and thus likely to function in CAS SD signaling in vivo.

Analysis of Crk-, Nck-, and Src-SH2 Binding—To assess the capacity of the individual CAS SD YXXP tyrosines to interact with likely SH2-containing effector proteins, peptide microarray binding assays were performed. Membranes containing 17-residue synthetic phosphopeptides representing each of the

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**Fig. 1.** CAS domain structure and the predicted tryptic peptides containing SD YXXP tyrosines. The 15 YXXP motifs (designated sites 1–15 from the N terminus) of mouse CAS are shown in bold type, with the amino acid position of the tyrosine residue indicated. Note that two tryptic peptides contain multiple YXXP sites: 3/4/5 and 12/13.

**Fig. 2.** Two-dimensional tryptic phosphopeptide maps of WT CAS and CAS FXXP variants F1, F2, and F3/F4/F5 phosphorylated by Src in vitro. A, WT CAS. B, CAS F1 (Y119F). C, CAS F2 (Y132F). D, CAS F3/F4/F5 (Y169F/Y183F/Y196F). E, notation diagram indicating designations of the major phosphopeptides spots (a–p) observed in these maps.
Substitutions.

indicate additional missing spots resulting from the F9 and F15

15 YXXP motifs and two putative Src-SH2-binding sites in the SBD (Fig. 5A), as well as control nonphosphorylated peptides, were briefly incubated with GST fusion proteins containing the SH2 domains of Crk-II, Nck-I, or Src, and binding was assessed by secondary incubation with anti-GST antibody. The results indicate that each of the identified sites of Src phosphorylation (sites 6–15) can promote a stable interaction with Crk-SH2, with sites 9 and 10 having the highest apparent affinity (Fig. 5B, top panel). Sites 1, 4, and 5 also exhibited the capacity for Crk-SH2 binding. In contrast, only sites 9 and 10 were well recognized by Nck-SH2 (Fig. 5B, middle panel). As expected, the Src-SH2 control showed efficient binding only to the two SBD sites (Fig. 5B, bottom panel). No binding to the control nonphosphorylated peptides was observed.

Expression and Signaling Capacity of CAS Variants Lacking Subsets of SD YXXP Tyrosines—To evaluate functional requirements for CAS SD YXXP sites, MEF populations were established that stably express either WT CAS or one of several CAS variants in which different substantial subsets of the YXXP tyrosines were changed to phenylalanines (F1–9, F10–15, F6–15, or F1–15) (Fig. 6). These populations were established in CAS −/− MEFs using a retroviral vector that co-expresses GFP from a bicistronic transcript and then sorting the cells for high GFP expression. Immunoblotting demonstrated that the CAS variants are expressed to similar levels (Fig. 7, top panel).

To determine the capacity of the CAS variants to undergo cellular phosphorylation of SD YXXP tyrosines, the cells were treated with vanadate for 3 h to maximize phosphotyrosine content, and then immunoblotting was performed on total cell lysates using a set of CAS YXXP site phosphospecific antibodies (pCAS-165, pCAS-249, and pCAS-410). The pCAS antibodies were previously shown to specifically recognize phosphorylated CAS YXXP tyrosines, although none are strictly specific for a single site (12). As expected, the F1–15 variant was not recognized by any of the pCAS antibodies (Fig. 7). The F1–9 and F10–15 variants were both recognized by each of the three pCAS antibodies, indicating that the remaining YXXP tyrosines in these variants are still subject to significant phosphorylation. The F6–15 variant was weakly recognized by the pCAS-165 and pCAS-410 antibodies (Fig. 7), indicating that some or all of the first five YXXP tyrosines also undergo phosphorylation in the cells. To further assess the capacity of the CAS variants to undergo tyrosine phosphorylation, they were immunoprecipitated from lysates of vanadate-treated cells and analyzed by immunoblotting with the general anti-phosphotyrosine antibody 4G10 (Fig. 8A, top and middle panels). The F1–15 variant was not recognized by 4G10, indicating that all detectable CAS tyrosine phosphorylation occurs on YXXP tyrosines. Consistent with the pCAS antibody immunoblotting data, the F1–9, F10–15, and F6–15 CAS variants were all recognized by the 4G10 antibody with the F6–15 variant giving the weakest signal.

The capacity of the CAS variants to signal through Crk recruitment was then assessed by blot overlay (Far Western) assay. The SH2 domain of Crk-II fused to GST was incubated on a blot containing the CAS variants that had been immunoprecipitated from vanadate-treated cells, and binding was visualized by incubating with an antibody against GST. WT CAS

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**Fig. 3.** Two-dimensional tryptic phosphopeptide maps of other CAS FXXP variants phosphorylated by Src in vitro. A, CAS F6 (Y238F). B, CAS F7 (Y253F). C, CAS F8 (Y271F). D, CAS F9 (Y291F). E, CAS F10 (Y310F). F, CAS F11 (Y331F). G, CAS F12 (Y366F). H, CAS F13 (Y376F). I, CAS F12/F13 (Y366F/Y376F). J, CAS F14 (Y391F). K, CAS F15 (Y414F). The arrowheads point to positions of phosphopeptide spots that are either missing (−) or shifted (≠) as a result of the phenylalanine substitutions. The arrows in D and K indicate additional missing spots resulting from the F9 and F15 substitutions.

![Image](http://www.jbc.org/)

**Fig. 4.** Two-dimensional tryptic phosphopeptide maps of CAS variants lacking substantial subsets of YXXP tyrosines phosphorylated by Src in vitro. A, CAS F1–9 in which the first nine SD YXXP tyrosines (119, 132, 169, 183, 196, 238, 253, 271, and 291) are all changed to phenylalanines. B, CAS F10–15 in which the last six SD YXXP tyrosines (310, 331, 366, 376, 391, and 414) are all changed to phenylalanines. C, CAS F1–15 in which all fifteen YXXP tyrosines are changed to phenylalanines. The remaining major phosphopeptide spots are indicated.
had the highest capacity to interact with Crk-SH2, whereas the F1–9 and F10–15 variants also showed strong binding (Fig. 8A, lower panel). The F1–15 variant and the F6–15 variant exhibited little or no Crk-SH2 binding, indicating their poor signaling capacity.

Requirements for CAS SD Phosphorylation Sites in Cell Migration—The ability of the CAS variants to promote cell migration was assessed by monolayer wound healing assay. For this analysis cultures of confluent contact-inhibited cells grown on fibronectin-coated dishes were scratched with a pipette tip to create a denuded area, and then the cells at the wound edges were allowed to migrate into the denuded area over a 6-h period while being monitored by time lapse video microscopy. Representative images after 3 and 6 h of migration are shown in Fig. 9A for cells expressing WT CAS, the F6–15 variant, or the retroviral vector alone (also sorted for high GFP expression). It is evident that cells expressing WT CAS more efficiently migrated into the wound area than the cells expressing the F6–15 variant or vector alone. To quantify the wound healing migration, net movements of at least 50 individual cells present initially at a wound edge were determined. WT CAS expression significantly enhanced the migration of cells compared with the vector-only control cells or cells expressing the F6–15 variant (Fig. 9B). Thus, the ability of CAS to promote cell migration in this assay is dependent on the presence of the major sites of CAS SD phosphorylation by Src.

To determine whether all major Src sites are required for efficient wound healing migration, similar studies were carried out on cells expressing CAS variants in which either a single YDXP tyrosine (F7) or two YDXP tyrosines (F9/F10) were changed to phenylalanines. Both of these variants were extensively tyrosine-phosphorylated in vanadate-treated cells and exhibited a high capacity for Crk-SH2 binding (Fig. 8B). Wound healing assays indicated that the F7 and F9/F10 variants promoted cell migration to an extent not significantly different from WT CAS (Fig. 9B). Therefore, it appears that one or two YDXP tyrosines are largely dispensable for the CAS-enhanced cell migration response. The F1–9 and F10–15 variants were also analyzed and found to significantly promote wound healing migration to an extent approaching what was observed for the WT, F7, and F9/F10 variants (Fig. 9B). Thus, as few as four major Src phosphorylation sites (as present in the F10–15 variant) appear present initially at a wound edge to be sufficient for promoting wound healing migration. The ability of CAS to promote healing migration is dependent on the presence of the major sites of CAS SD phosphorylation by Src.

FIG. 5. Analysis of Crk-SH2 binding capacities of the phosphorylated YXXP tyrosines. A, peptides representing each of the 15 YXXP motifs (1–15) and two putative Src-SH2 binding sites in the SBD (S1 and S2). Each peptide was spot-synthesized on membranes in two separate forms in which the YXXP tyrosine was either phosphorylated or nonphosphorylated. B, SH2 domain binding capacities of the peptides. The membranes were incubated with GST fusion proteins of CrkII-SH2, NckI-SH2, or Src-SH2, and binding was assessed using anti-GST antibody. (pY-, phosphorylated peptides; Y-, nonphosphorylated peptides).

FIG. 6. CAS SD variants expressed in CAS −/− MEFs. WT CAS and six full-length CAS variants in which the indicated SD YXXP tyrosines were changed to phenylalanine were expressed in CAS −/− MEFs to study the functional requirements of CAS SD tyrosine phosphorylation. Y, YXXP tyrosine; F, phenylalanine substitution for YXXP tyrosines.

FIG. 7. Expression of CAS variants in CAS −/− cells and their recognition by pCAS antibodies. Total cell lysates were prepared from vanadate-treated cells expressing the CAS indicated variant and analyzed by immunoblotting with antibodies recognizing either total CAS protein (CAS-TL) or phosphorylated CAS SD YXXP tyrosines (pCAS-165, -249, and -410).

FIG. 8. Tyrosine phosphorylation of CAS variants and their capacity to associate with the Crk-II SH2 domain. CAS variants were immunoprecipitated from vanadate-treated cells and analyzed either for phosphotyrosine content by immunoblotting (IB) with 4G10 antibody (middle panels) or for the capacity to stably bind to the Crk-II SH2 domain by Far Western (FW) blot analysis (bottom panels). WT CAS, the F1–15 variant, and vector control were compared with either variants lacking substantial subsets of YXXP tyrosines (A) or only 1–2 YXXP tyrosines (B). Control immunoblotting with an anti-CAS antibody (top panels) that recognizes total CAS shows that comparable amounts of CAS proteins were immunoprecipitated.
signaling capacity may be required for a maximal response. Sufficient for CAS to promote cell migration, although additional signaling capacity may be required for a maximal response.

**DISCUSSION**

Tyrosine phosphorylation of the CAS SD by Src has been implicated as a major integrin signaling event promoting cell migration, but little is known regarding the capacity of individual SD tyrosine residues to undergo phosphorylation and participate in motility-related signaling. In this study we showed that Src extensively phosphorylates the CAS SD on YXXP tyrosines and identified 10 major sites of phosphorylation, including all nine YDXXP sites matching strong recognition consensus sequences for SH2 domains of likely downstream effectors of the Crk and Nck families. Phosphopeptide binding assays demonstrated the capacity of the Crk SH2 domain to stably interact with all identified CAS SD phosphorylation sites, whereas the Nck SH2 domain exhibited efficient binding to only two YDXXP sites. To study the requirements of CAS SD phosphoacceptor tyrosines in cell migration, we expressed, in CAS −/− MEFs, WT CAS versus a series of CAS variants containing phenylalanine substitutions for different subsets of YXXP tyrosines. Wound healing assays with these cells indicated that CAS promotes cell migration in a manner dependent on the major tyrosine sites of Src phosphorylation, that one or two sites are dispensable for the migration response, and that efficient migration is achieved by CAS variants containing as few as four of the major phosphorylation sites.

Our phosphorylation site mapping studies complement recent studies by Goldberg et al. (33) who used mass spectrometric analysis of tryptic peptides to identify 11 CAS SD YXXP tyrosines (sites 2–10, 14, and 15) that undergo in vitro phosphorylation by Src. Our experimental approach of site-directed mutagenesis combined with two-dimensional phosphopeptide mapping has potential to identify phosphopeptides not detected by mass spectrometry because of their poor ionization, high mass/charge ratio, or overcrowded spectra (34) while also providing useful quantitative information regarding relative phosphorylation site stoichiometry. In addition to sites identified by Goldberg et al., we determined that YDXXP tyrosines 331, 366, and 376 (sites 11–13) are among the sites efficiently phosphorylated by Src. On the other hand, Goldberg et al. reported that YXXP sites 2–5 are subject to Src phosphorylation, whereas we could not detect these as major phosphopeptides in our maps. Consistent with our observations, Goldberg et al. reported that peptides containing sites 2–5 are relatively poor Src substrates, and in support of the assignments made by Goldberg et al., we found that the CAS F6–15 variant containing only the first five YXXP tyrosines undergoes limited tyrosine phosphorylation in cells, as detected using pCAS and general phosphotyrosine antibodies. Notably the F1–15 variant lacking all 15 YXXP tyrosines was not detected using the general phosphotyrosine antibody, suggesting that all cellular tyrosine phosphorylation occurs on these sites, and although our mapping studies indicated that Src phosphorylates a non-YXXP tyrosine in vitro (represented by phosphopeptide spot i), we have no evidence that this yet-to-be identified site is phosphorylated in vivo. Thus, taken together, our study and that of Goldberg et al. show that the 10 most C-terminal CAS SD YXXP tyrosines (including all nine YDXXP tyrosines) are efficiently phosphorylated by Src, whereas the second, third, fourth, and fifth N-terminal YXXP tyrosines can also be phosphorylated. Of the 15 CAS SD YXXP tyrosines, then, only the most N-terminal site (Tyr-119) has not been identified as a Src site.

In addition to their ability to become phosphorylated, the signaling capacity of CAS SD YXXP tyrosines is governed by their ability to mediate stable interactions with downstream effector proteins. Therefore we addressed the capacity of each phosphorylated YXXP site to interact with Crk and Nck SH2 domains using a peptide array binding assay. Although this analysis should be considered as primarily qualitative in nature, signal intensities show good inverse correlation with dissociation constants (30, 35) and thus provide an estimate of binding affinities. From our analysis, it was evident that all the phosphorylation sites we identified can promote a stable interaction with the SH2 domain of Crk-II. Particularly strong binding was observed for YDXXP sites 9 and 10 in which, notably, four of the next five amino acid residues C-terminal to the YXXP motif are identical and could contribute to the binding affinity. Thus, in terms of their ability to undergo phosphoryl-

**Fig. 9.** Assessment of monolayer wound healing migration by cells expressing different CAS variants. A, representative data for cells expressing either WT CAS, CAS F6–15, or vector alone. Photomicrographs of the scratch wounds were taken immediately after their formation (0 h) and again at 3 and 6 h after incubation. B, box and whisker plots for migration distances of cells expressing CAS variants and vector-only control. The middle line of the box indicates median migration distance, the top of the box indicates 75th percentile, the bottom of the box indicates 25th percentile, and the whiskers indicate extent of 10th and 90th percentiles. Significant differences in cell migration distances compared with WT CAS (*) and CAS F6–15 (#) are indicated (p < 0.05, post-hoc Dunnett’s t tests). The numbers of cells analyzed were: vector only, 74; WT, 115; F7, 68; F9/F10, 58; F1–9, 152; F10–15, 126; and F6–15, 72.
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mediated signaling does not require a strict threshold of signaling that must be achieved through a majority of the major SD phosphorylation sites. The MEF cell populations we have generated should be useful for further studies investigating the involvement of CAS SD signaling in individual steps of cell motility including membrane protrusion, adhesion dynamics, and contractile force generation.

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