ARTICLE

ACS chemical biology

Synthesis of Functional Signaling Domains by Combinatorial Polymerization of Phosphorylation Motifs

Parag Patwardhan[†], Kiyotaka Shiba[‡], Chris Gordon[†], Barbara P. Craddock[†], Minamisawa Tamiko[‡], and W. Todd Miller[†,*]

[†]Department of Physiology and Biophysics, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794 and [‡]Department of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan

ABSTRACT The adaptor protein Cas contains a core substrate domain with multiple YXXP motifs that are phosphorylated by Src and other tyrosine kinases. Here, we used a synthetic strategy to determine the importance of the arrangement, spacing, and identity of the YXXP motifs. By polymerizing short DNA sequences encoding two phosphorylation motifs, we created a panel of Cas mutants in which the entire substrate domain was replaced by synthetic domains containing random numbers and arrangements of the motifs. Most of these synthetic Cas variants were recognized and phosphorylated by Src *in vitro* and in intact mammalian cells. The random polymer mutants also restored migration activity to Cas knockout cells; even artificial proteins containing a single motif retained some biological function. Our results suggest that the arrangement of Cas motifs is not critical for signaling. This method could be used to identify the minimal functional units in other signaling proteins.

The adaptor/docking protein Cas (Crk-associated substrate or p130^{Cas}) plays a key role in cell migration signaling (*1–3*). Cas is localized to focal adhesions, and the protein undergoes extensive tyrosine phosphorylation by Src-family kinases in response to integrin-mediated adhesion (*4–6*). Cas contains an N-terminal SH3 domain, a central substrate domain with 15 YXXP motifs, and a C-terminal Src-binding region (*7–9*). Previous work from our laboratory (*10*) and by others (*11*) has shown that the YXXP motifs in the substrate domain of Cas constitute the major sites of Src phosphorylation. The phosphorylated YXXP motifs bind to the SH2 domains of Crk-family adaptor proteins (*1, 9*). Cas-Crk coupling promotes activation of the GTPase Rac1 and has been described as a "molecular switch" for the induction of cell migration (*12*). The YXXP motifs of Cas can be divided into YDXP and YQXP motifs (Figure 1). The YDXP motifs have been shown to be particularly important in cell migration; *en bloc* deletion of the region containing the YDXP motifs eliminated the ability of Cas to promote migration (*13*). On the other hand, mutant forms of Cas with reduced numbers of YXXP motifs have been shown to retain some function in cell migration assays (*11*). We showed previously that Src phosphorylates Cas by a processive mechanism in which the enzyme phosphorylates all available sites before dissociating (*14, 15*). Mutants containing single or multiple YXXP mutations were phosphorylated processively by Src, indicating that individual sites are dispensable for Src recognition and that there is no defined order to Cas phosphorylation by Src (*15*).

*Corresponding author, todd.miller@stonybrook.edu.

Received for review February 13, 2008 and accepted July 23, 2009.

Published online July 23, 2009

10.1021/cb900059f CCC: $40.75

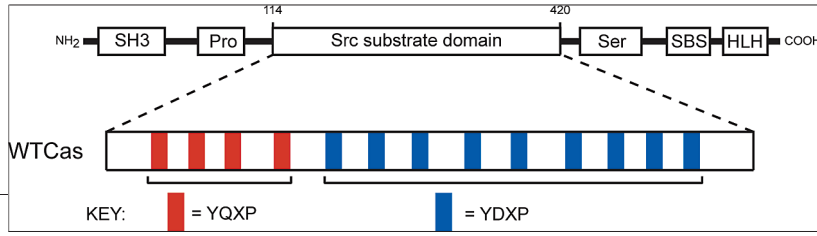

Figure 1. Domain architecture of Cas. From N- to C-terminus, Cas contains an SH3 domain, Pro-rich region, substrate domain, Ser-rich region, Src-binding sequence (SBS), and a helix–loop–helix region (HLH). The central substrate domain of Cas contains four YQXP motifs and nine YDXP motifs. The substrate domain was replaced by synthetic substrate domains derived from microgene polymers.

On the basis of these observations, we sought to determine the importance of the arrangement and identity of the YXXP motifs in the substrate domain of Cas. One possibility is that each of the YXXP motifs might have a unique identity and serve a unique signaling function. An alternative possibility is that any collection of YXXP motifs, arranged in any random order, might be phosphorylated by Src and be functional when expressed in cells. We adopted a synthetic strategy to distinguish between these possibilities. We created a library of Cas mutants in which the substrate domain was replaced by artificial domains that contain YDVP and YQVP motifs in various numbers and in various orders. We observed that synthetic variants containing as few as one motif were phosphorylated by Src *in vivo* and *in vitro* and restored cell migration activity to $Cas^{-/-}$ fibroblasts.

RESULTS AND DISCUSSION

Creating Cas Random Polymer Mutants. The YXXP motifs in the substrate domain of Cas play crucial roles in a variety of cellular functions, particularly in cell migration (*10*–*12*). However, it is not clear whether there is a threshold number of tyrosine residues that is needed for Src recognition and biological function or whether the motifs need to be present in a certain order. To address these issues, we created a panel of artificial Cas proteins in which the entire substrate domain was replaced by synthetic substrate domains that contain random numbers and arrangements of the YXXP motifs (Figure 2). To prepare the synthetic substrate domains, we used the MolCraft method (*16*), in which a single short DNA sequence (a microgene) is subjected to a microgene polymerization reaction (MPR) (*17*). Figure 2, panel a shows the microgenes used in this study. The first reading frames of the microgenes encode 12 amino acid peptides containing YQXP and YDXP motifs. Previous synthetic peptide studies showed that a substrate with the Cas Y253 sequence had the highest value of k_{cat}/K_m for Src phosphorylation among the YDXP motifs, while the Y196 peptide had the highest k_{cat}/K_m among the YQXP motifs (*10*). Y253 was also shown to play a critical role in migration of Src transformed cells. These sites were also phosphorylated in "add-back" mutants of Cas, in which Y253 or Y196 were added to a Cas^{F17} mutant lacking all other tyrosines in the substrate domain (*15*). Therefore, we focused on two motifs, YDVP and YQVP, and prepared two microgene sequences, one of which encodes aspartic acid in its seventh position, whereas the other encodes glutamine. By mixing two MPR forward primers and one reverse primer (Figure 2, panel b) that can form base pairs in their 3′ ends, we prepared polymers of the microgenes.

Previous studies have revealed that the use of multiple MPR primers resulted in the random polymerization of the different microgenes (*17*–*19*). Therefore, the three primers shown in Figure 2, panel b were mixed in a single reaction to produce combinatorial polymers of YDXP and YQXP motifs. Because the MPR conditions allow random insertions, deletions, and mutations at the junctions of microgenes (*17*), the translational products of the resultant microgene polymers make up a library of combinatorial polymers of three peptides coded by three different reading frames of the microgene (Figure 2). As expected, MPR with three primers created artificial proteins, in which the YDVP and YQVP motifs were arranged in various numbers, in various orders, and at various intervals (Figure 2 and Supplementary Figure 1). We substituted the wild-type substrate domain of Cas with the 19 synthetic substrate domains as described in Methods and characterized the mutant forms of Cas *in vitro* and *in vivo*.

Cas Random Polymer Mutants Are Phosphorylated by Src. We expressed the random polymer mutants of Cas, as well as wild-type Cas (WTCas) and ΔCas (mutant lacking the entire substrate domain), using either BL21(DE3) or BL21-CodonPlus(DE3) *E. coli* cells. We then purified the proteins using Ni-NTA affinity chromatography. We carried out *in vitro* kinase assays using these purified proteins and v-Src (purified from Sf9 cells) in the presence of [γ-^{32}P] ATP. Our results showed that many of the mutant forms of Cas are phosphorylated by Src, albeit to lower levels than WTCas (Supplementary Figure 2). Surprisingly, mutants containing as few as one YDXP motif (such as 660) were also phosphorylated by Src. The most highly phosphorylated mutants in these studies were 622, 660, 661, 663, 664, and 665, while others such as 643, 650, 651, 654, 655, 656, and 657 were phosphorylated to very low levels. On the basis of these results, it is clear that some alternative motif arrangements can be recognized by Src *in vitro*. There

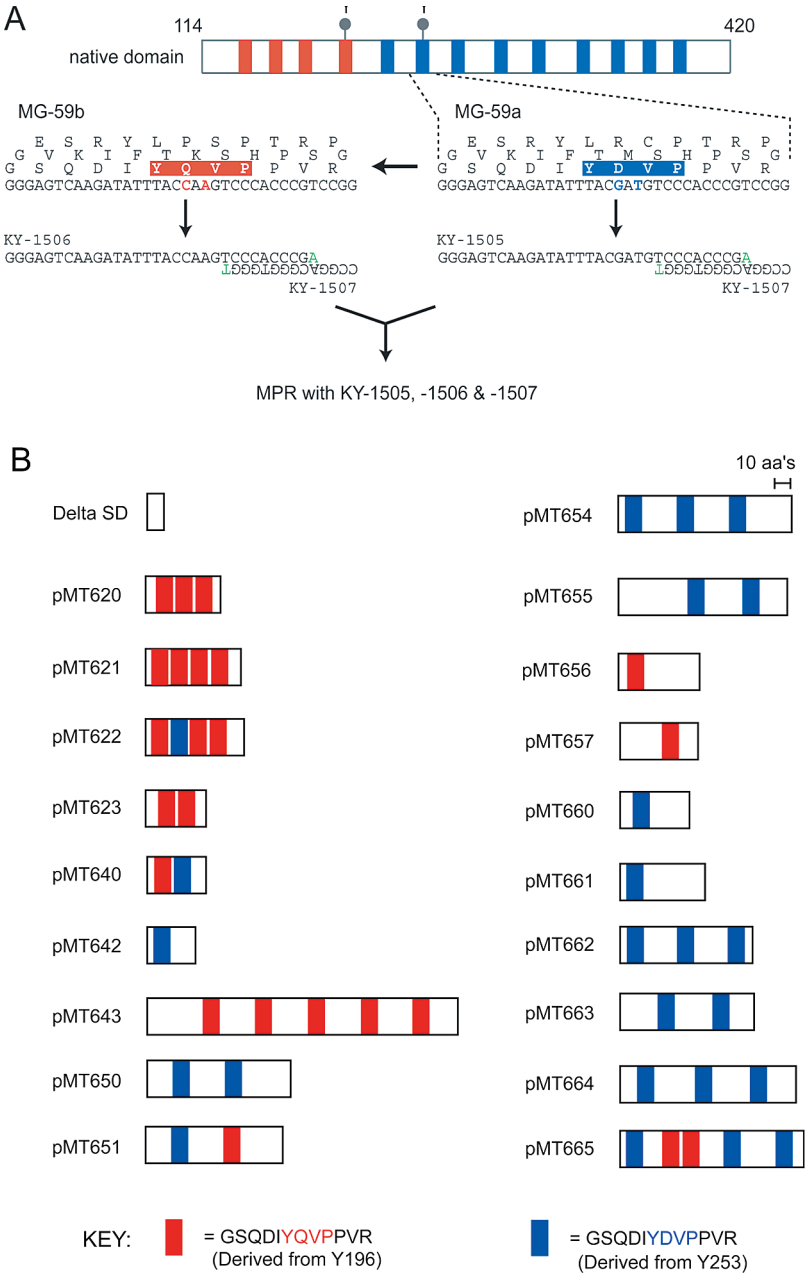

Figure 2. Synthesis of random polymers containing YDXP and YQXP motifs. A) On the top, a schematic diagram of the natural Cas substrate domain (residues 114–420) is shown. Two phosphorylation sites that were discussed in the text are shown (Y196 and Y253). The second YDXP motif (containing Y253) was used to design the microgene, MG-59a, from which the YQXP derivative, MG-59b was obtained. The three MPR primers, KY-1505, -1506, and -1507, can recreate MG-59a and -59b through stochastic association. Terminal mismatched nucleotides (shown in green) have been shown to enhance polymer formation (*17*). For details, see Methods. B) Schematic representation of 19 synthetic substrate domains. Note that 5 pairs of mutants (pMT654 and 655; pMT656 and 657; pMT660 and 661; pMT662 and 663; pM664 and 665) were derived from the same microgene polymers using two different reading frames. All synthetic domains were reinserted into the 114–420 region of Cas protein.

were no obvious correlations between the strength of the signal and the arrangement of motifs, although all the mutants showing high levels of phosphorylation had at least one YDXP motif in their substrate domain. This lack of correlation was consistent with observations in previous motif-programming experiments, in which the functions of motifs were greatly influenced by their context in artificial proteins (*18, 20, 21*). In agreement with our previous studies using "add-back" mutants of Cas (*15*), these data suggest that there is no defined order to Cas phosphorylation by Src.

Next, we wished to study whether the random Cas mutants can be recognized by Src in mammalian cells. We expressed Cas (wild-type or the 19 mutants) together with v-Src in Cas-deficient ($Cas^{-/-}$) fibroblasts. We immunoprecipitated the proteins using anti-Cas antibody and analyzed the phosphorylation status by using anti-phosphotyrosine antibody (Figure 3). Our results showed that WTCas as well as all mutant forms of Cas but one (662) were expressed in $Cas^{-/-}$ cells. By anti-phosphotyrosine immunoblotting, it was apparent that all the mutants that were expressed were also phosphorylated

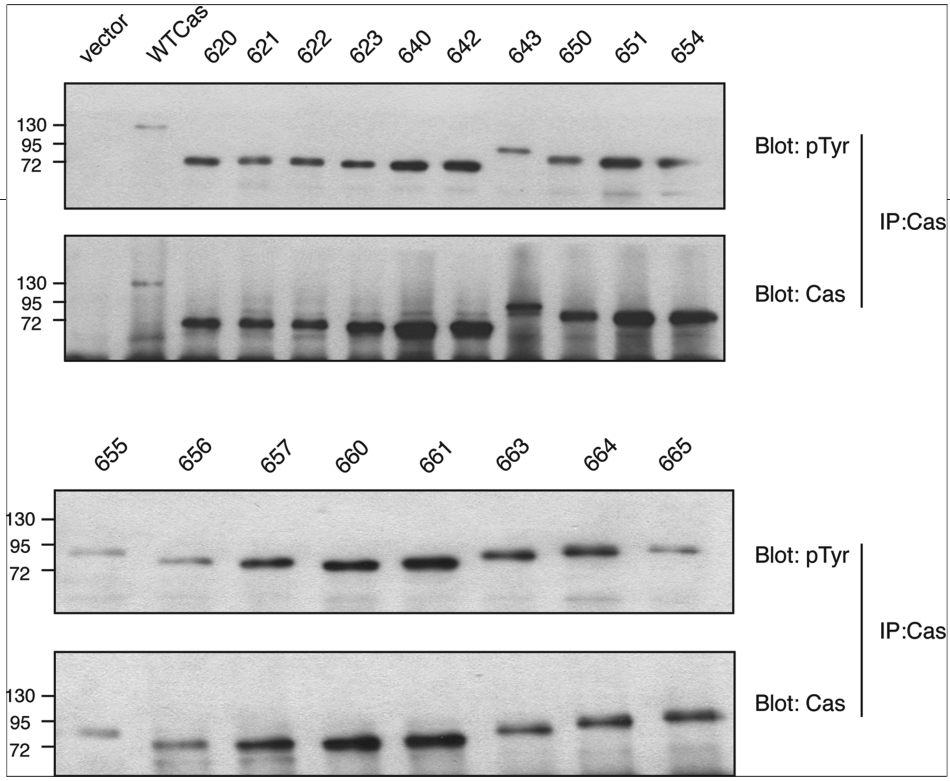

Figure 3. Expression and phosphorylation of random polymer mutants of Cas in Cas$^{-/-}$ fibroblasts. Anti-Cas immunoprecipitation reactions were performed, and immunoprecipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine and anti-Cas antibodies, using appropriate horseradish peroxidase conjugated secondary antibodies. The exposure times for these Western blots were 10 s; a wider range of exposure times is presented in Supplementary Figure 3.

in these cells. In order to normalize the levels of phosphorylation to the levels of expression for each of these proteins, we carried out densitometry studies on the Western blots (Supplementary Figures 3 and 4). The levels of expression for all of the mutants were much higher than that of WTCas, for reasons that are not clear. Most of the mutants were phosphorylated to higher levels than WTCas, even after normalizing for protein expression (Supplementary Figure 4). We observed more than 2-fold higher levels of phosphorylation for mutants 651, 657, 660, 661, 663, and 664 when compared to that of WTCas. Interestingly, mutants containing only a single YXXP motif, such as 642, 656, 657, and 660, also showed a much stronger phosphorylation signal than WTCas. Mutants 623, 660, 661, 663, 664, and 665 showed higher levels of phosphorylation than the other mutants both *in vitro* and in cells. On the other hand, mutants 640, 642, 643, 650, 651, 654, 655, 656, and 657 showed very low levels or no phosphorylation in the *in vitro* studies but were phosphorylated to significantly higher levels when expressed in Cas$^{-/-}$cells. It is possible that in the *in vitro* experiments using purified proteins Src did not bind efficiently to these mutants and therefore did not phosphorylate them significantly. In intact cells, the presence of other proteins such as FAK that can bind to the SH3 domain of Cas could help tether Src to Cas, thereby allowing higher phosphorylation of these mutants in cells (*1*).

To confirm that Src was responsible for promoting phosphorylation of the Cas mutants, we carried out experiments in the presence and absence of Src. In the absence of Src, phosphorylation of wild-type Cas and of the mutants was virtually undetectable (Figure 4). Our data from the expression and phosphorylation studies of these mutants *in vitro* as well as in mammalian cells clearly suggests that the artificial proteins created by microgene polymerization can serve as Src substrates.

Cas Random Polymer Mutants Can Promote Migration of Cas$^{-/-}$ Cells. Phosphorylation of the YXXP motifs plays a crucial role in cell migration (*1, 11, 13*), and Cas-deficient fibroblasts show defects in cell migration (*22, 23*). Although there is no conclusive evidence for a threshold number of tyrosine residues required for cell migration, it is generally believed that phosphorylation of multiple YXXP motifs is necessary. Since we observed that the random polymer mutants were expressed and phosphorylated in Cas$^{-/-}$ fibroblasts, we wished to determine whether these mutants can promote migration. We transiently expressed Cas (WT or mutant forms) together with v-Src in Cas$^{-/-}$ fibroblasts and examined the ability of these transfectants to migrate through pores in a migration chamber. Cells expressing WTCas and Src showed a significant increase in migration when compared to the vector-only control (Figure 5). Cas$^{-/-}$ cells expressing ΔCas migrated approximately 10% as well as cells expressing WTCas. The reason for this residual substrate domain-independent level of migration is unknown, but it may be related to the ability of the C-terminal portion of Cas to activate Src (*24*). Mutant forms of Cas supported cell migration at levels significantly above ΔCas but not as high as WTCas. Mutants containing multiple YXXP motifs such as 620, 622, 623, 654, 655, and 661 supported cell migration better than the others, whereas mutants 651, 657, 663, and 664 did not promote migration significantly (Figure 5). In addition, our data showed

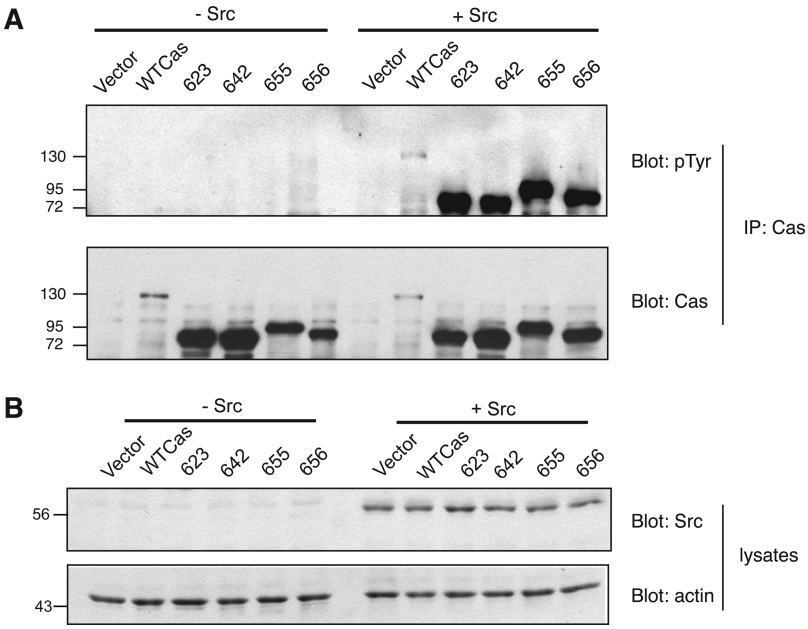

Figure 4. Src dependence of Cas phosphorylation. Selected Cas mutants were expressed in Cas-deficient fibroblasts alone or together with Src. A) Cas phosphorylation was analyzed as in the legend to Figure 3. B) Cell lysates were analyzed by Western blotting with anti-Src antibody, and anti-actin as a loading control.

that mutants containing even a single YDXP motif (642) or a YQXP motif (656) could support migration significantly higher than ΔCas. There was no clear correlation between the ability to support migration and the phosphorylation status of these proteins, except for mutants 623, 661, and 665, which were phosphorylated at high levels both *in vitro* and in mammalian cells and also supported migration at levels better than ΔCas. Thus, our results indicate that the random polymer mutants are functional and can promote migration when expressed in $Cas^{-/-}$ cells, although to a lower extent than WTCas.

Significantly, some Cas mutants containing a single substrate motif supported cell migration. To examine the dependence of migration on substrate domain phosphorylation in this system, we mutated the single Tyr residue of 656 to Phe. Src-mediated migration of cells expressing 656YF was reduced relative to 656 but not eliminated (Supplementary Figure 5). This suggests that some of the cell migration activity is independent of Cas substrate domain phosphorylation, consistent with the finding on ΔCas. We also tested whether cell migration activity correlated with Cas-Crk binding. Crk was immunoprecipitated from $Cas^{-/-}$ cells expressing wild-type Cas or selected mutants, and the levels of associated Cas were analyzed by Western blotting (Supplementary Figure 6). Wild-type Cas and mutant 655 showed high levels of Cas-Crk binding, consistent with their cell migration activity (Figure 5). Mutants 651 and 623 showed low levels of associated Cas, while Cas-Crk binding was undetectable for mutant 657. Mutants 651 and 657 displayed levels of cell migration that were comparable to that of ΔCas (Figure 5), consistent with the Crk binding data. On the other hand, mutant 623 showed strong cell migration activity, raising the possibility of Crk-independent migration in this case.

We also examined the subcellular localization of selected Cas mutants. When re-expressed in $Cas^{-/-}$ cells, wild-type Cas has previously been shown to have a diffuse cytoplasmic distribution, with several areas of dense immunostaining around the plasma membrane (*13*). We confirmed this observation by immunofluorescence microscopy using anti-Cas antibodies (Supplementary Figure 7). For comparison, we selected four Cas mutants (661, 656, 654, and ΔCas). Mutant 661 contains one YDXP motif, 656 contains one YQXP motif, and 654 contains multiple YDXP motifs; all were phosphorylated by Src in cells (Figure 3). We also studied ΔCas, the mutant lacking the entire substrate region (but retaining the Src binding region and other domains of Cas). The localization of these Cas mutants was very similar to that of wild-type Cas (Supplementary Figure 7).

Our previous data using the "add-back" mutant Cas^{F17}+Y253 showed that this mutant containing only one YDXP motif could not support Src-mediated cell migration (*15*). Thus, the context in which the YDXP motifs

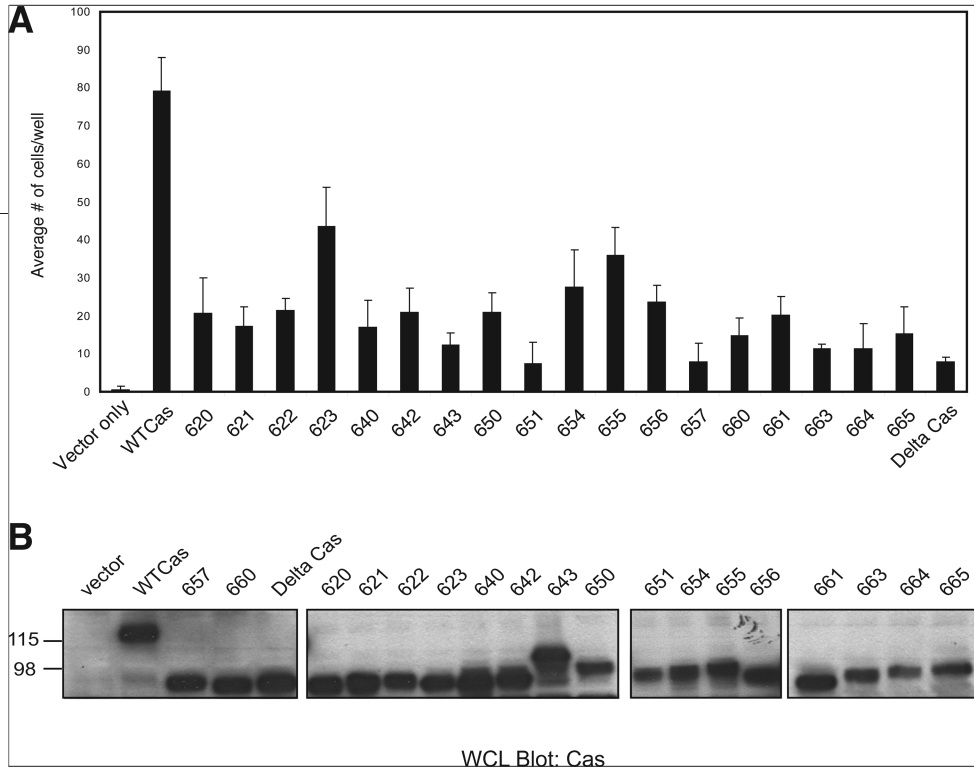

Figure 5. Mutant forms of Cas can promote Src mediated cell migration. A) Cas$^{-/-}$ cells were transiently transfected with Cas (10 μg of WT or 1 μg of mutant) and v-Src (0.5 μg) or with appropriate vector controls. Cells were allowed to migrate for 48 h, fixed, and counted. The graph shows the average number of cells migrated per well for each cell type. WTCas showed the highest levels of migration compared to the vector-only controls ($p < 0.000002$ as determined by single factor ANOVA) or each of the 19 mutant forms of Cas ($p < 0.002$). B) Cas$^{-/-}$ cells used for migration experiments were analyzed by anti-Cas Western blotting.

are embedded plays a role in biological function. An important difference between our previous "add-back" mutants (e.g., Cas^{F17}+Y253) and the random polymer mutants used in this study is that the single site in the "add-back" mutants was in the middle of a large number of mutated YXXP motifs. There are 302 residues in the substrate domain of full length Cas, as compared to 28 residues in mutant 642 and 49 residues in mutant 656. Furthermore, we intentionally avoided sequences with high propensities to form α-helical or β-sheet structures in the mutants. Since the substrate domain is shorter in the case of random polymer mutants, it is possible that the rate of phosphorylation is much higher for the random polymer mutants than for the "add-back" mutants. This could give rise to a larger pool of phosphorylated substrate that can bind to the downstream effectors, thereby allowing cell migration to occur.

An important question then arises: if the mutant forms of Cas containing as few as one YXXP motif can support cell migration, why are there so many phosphorylation sites present in WT-Cas? Our study does not provide a final answer to this question. We note that while mutants with single sites are active in the migration assays, none is as active as wild-type Cas (Figure 5); thus, the strength of the biological response does appear to be related to the large number of phosphorylation sites. Appropriate mechanisms of multisite phosphorylation have presumably evolved to regulate different systems. For example, the nonprocessive phosphorylation of MAPKs by MAPKKs introduces a steep dependence on enzyme concentration, resulting in switch-like behavior (*25, 26*). In contrast, the processive phosphorylation of WTCas by Src is independent of enzyme concentration, ensuring that all YXXP sites are phosphorylated each time Src binds to Cas. Processivity may be better suited to biological systems where rapid signaling is paramount, rather than switch-like behavior. It is possible that the maximal output is achieved by enabling a larger pool of Crk molecules to bind to the numerous sites on wild-type Cas, resulting in a higher migration response than could be achieved with a single site.

Previous mutagenesis studies on Cas by our laboratory and others have had the limitation that they cannot distinguish whether the number of phosphorylation sites is important in a biological effect, or the identity and arrangement of those sites. The "bottom-up" synthetic strategy employed here allows us to show that the arrangement of Cas phosphorylation motifs is not crucial for proper signaling. Similar methods could be used in other multidomain proteins to identify minimal signaling motifs.

METHODS

Reagents. ^{32}P-labeled ATP was purchased from PerkinElmer. Dulbecco's Modified Eagle Medium (low glucose, containing L-glutamine, HEPES buffer, and sodium pyruvate) was purchased from Invitrogen. Anti-Cas and anti-Crk monoclonal antibodies were obtained from BD Transduction Laboratories. Monoclonal antibody against avian pp60v-Src kinase (Clone EC10) and anti-phosphotyrosine antibody (Clone 4G10) were obtained from Upstate Biotechnology. Anti-Cas polyclonal antibody (C-20) and anti-phosphotyrosine antibody (PY99) were obtained from Santa

Cruz Biotechnology. Anti-actin antibody (produced in rabbit) was obtained from Sigma.

Src Production in Sf9 Cells. N-Terminal polyhistidine-tagged v-Src was expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen) as previouslydescribed (*14*). Sf9 cells were grown at 27 °C in Excel 401 (JRH Biosciences) in the presence of 2.5% heat-inactivated fetal bovine serum (Sigma) and 1% penicillin/streptomycin/amphotericin B (Invitrogen). Approximately 500–600 mL of cells at a density of 1.5×10^6 cells mL^{-1} were infected with recombinant baculovirus at a multiplicity of infection of 5–10 plaque-forming units $cell^{-1}$ for a period of 72 h. The cells were centrifuged and stored at −70 °C until needed. Src was purified using nickel-nitrilotriacetic acid resin (Qiagen), as described previously (*14*). The enzyme was stored at −20 °C.

Construction of Cas Random Polymer Mutants. The 36-bp microgene, MG-59, was designed as follows. First, the 12-mer peptide GSQDIYDVPPVR that corresponds to residues 248–259 of Cas (including Y253) was selected as a representative of the YDXP motif. Among approximately 6×10^5 DNA sequences that can code for this peptide, we identified a sequence with the following properties: (i) it does not contain any termination codons in its second and third reading frames, and (ii) it encodes peptides in the second and third reading frames with the lowest propensities to form secondary structures. The latter property was taken into account because the formation of helix or sheet in the peptides between motifs could potentially prevent the kinase from accessing the substrate motif. This *in silico* evolution was performed by using the CyberGene program (*16*). On the basis of the selected DNA sequence, we then designed a derivative so that the D residue in the YDXP motif would be substituted with Q to encode YQXP, which was achieved by changing the 19th and 21st nucleotides (Figure 2). We named these two sequences MG-59a and MG-59b, respectively.

On the basis of the DNA sequence of MG-59ab, we designed two forward primers (KY-1505, 5′-GGG AGT CAA GAT ATT TAC GAT GTC CCA CCC GA-3′, and KY-1506, 5′-GGG AGT CAA GAT ATT TAC CAA GTC CCA CCC GA-3′) and one reverse primer (KY-1507, 5′-CCG GAC GGG TGG GT-3′) (Figure 2). The primer pairs KY-1505:KY-1507, and KY-1506:KY-1507 overlap at their 3′-regions, forming eight base pairs and containing a mismatched base at their 3′-OH ends, which enables efficient polymerization of the microgenes by MPR (microgene polymerization reaction (*17*)). Previous studies have also shown that stochastic association of multiple (≥2) MPR primers resulted in the random polymerization of the microgenes having different sequences (*17*–*19*).

Polymerization of MG-59a and MG-59b was performed by mixing the three MPR primers, and the resultant microgene polymers were size-fractionated from agarose gel as described previously (*17, 27*). The polymers were once cloned in the *Sma*I site of pTZ19R (*28*) and their sequences were determined. Plasmid pET-Cas expresses the N-terminal poly-His-tagged wild-type Cas in pET28a (*10*). Because the microgene polymers in pTZ19R can be excised by digestion with *Asp*718 and *Spe*I in the multiple cloning site, we made derivatives of pET-Cas that have *Asp*718 and *Spe*I at sites corresponding to both ends of the substrate domains (residues 114 and 420 in Figure 1). To adjust the reading frames of the N-terminal region, synthetic substrate domain, and C-terminal region, we prepared a series of three vectors in which the microgene polymers could be ligated in the +0, +1, and +2 reading frames. In addition, because the polymers were inserted in pTZ19R in both directions, we prepared derivatives with the arrangement *Asp*718-(insert)-*Spe*I and *Spe*I-(insert)-*Asp*718. In all, we made six derivatives of pET-Cas: pKS606-pKS611. These vectors can be cleaved into two large fragments by *Pst*I digestion. By using the 12 DNA fragments from these vectors, the microgene polymers could be inserted in the Cas protein as a synthetic substrate domain in any reading frame. Using this cloning system, we made 19 mutants of Cas from 14 polymers (the pMT series of plasmids). In 5 cases, the same microgene polymer was used in two different reading frames (see Figure 2 legend).

Expression of Cas Random Polymer Mutants in Bacteria. pET-Cas, pMTs, and pET-ΔCas (lacking the entire substrate domain) (*10*) were expressed using either BL21 (DE3) or BL21 (DE3) *Codon plus E. coli* cells. Briefly, an overnight 2 mL culture started from a single isolated colony was inoculated into 200 mL of LB (Luria Broth) medium containing kanamycin. When A_{600} reached ~0.8 (after 2–2.5usb h), the cultures were induced with 0.2 mM of IPTG at 30 °C. Cells were harvested after 4.5 h of induction, and the cell pellets were stored at −20 °C until needed. WTCas and mutant forms of Cas were purified using nickel-nitrilotriacetic acid resin (Qiagen), as described previously (*14*). All purified proteins were stored at −20 °C.

***In Vitro* Kinase Assays.** For *in vitro* kinase assays using Cas random polymer mutants, 0.2 μM Cas was incubated in the presence of 100nM v-Src in kinase assay buffer (150 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, no BSA). Reactions were started by the addition of 0.1 mM unlabeled ATP plus [γ-^{32}P]-ATP (10 μCi) and were carried out at 30 °C. The reactions were stopped by mixing with Laemmli Buffer and boiling and were analyzed by SDS–PAGE followed by autoradiography.

Transient Expression of Cas and Src in Mammalian Cells. The *Eco*RI/*Not*I fragment encoding mutant Cas was excised from the pMT vector and subcloned into the *Eco*RI/*Not*I sites of pcDNA6-V5HisB. WTCas was subcloned into the *Bam*HI/NotI sites of pcDNA6-V5HisB. Cas-deficient cells (~70–80% confluent) were transfected using TransIT-LT1 transfection reagent (Mirus Bio Corporation) with a DNA:TransIT ratio of 1:3. Cells were cotransfected with either WTCas (5 μg) or mutant Cas (1 or 2.5μg) and 1 μg of pBabeSrc (*15*). Cells were allowed to grow for 40–45 h after transfection.

Immunoprecipitation and Western Blot Analysis of Mammalian Cells. Immunoprecipitation reactions were carried out using transiently transfected Cas deficient cells. Transfected cells were quickly aspirated, washed with phosphate-buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 5 mg L^{-1} aprotinin, 5 mg L^{-1} leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4) at 4 °C for 1 h. The cell lysates were centrifuged at 14,000*g* for 10 min at 4 °C. Protein concentrations were determined using the Bio-Rad protein assay. Lysates (125–250 μg) were used for immunoprecipitations with 2 μg mL^{-1} Cas C-20 antibody. After the addition of 50 μL of protein A/G Plus-agarose (Santa Cruz), incubations were continued overnight at 4 °C. The resin was collected and washed three times with modified RIPA buffer. The precipitated proteins were separated by 10% SDS–PAGE, transferred to PVDF membrane, and analyzed by Western blotting with appropriate antibodies. Blots were visualized using horseradish peroxidase-conjugated secondary antibody and detected by ECL plus (Amersham Biosciences).

Immunofluorescence. $Cas^{-/-}$ cells were seeded onto 100-mm dishes containing several uncoated cover glasses and grown to ~50% confluency. Cells were transfected with Cas or vector DNA using Mirus TransIT-LT1 transfection reagent. At 24 h post transfection, the medium was removed and replaced with DMEM with 10% heat inactivated FBS, 1x antibiotic/antimycotic. At ~48 h post-transfection, the cover glasses were removed, and the cells were fixed with 3.7% formaldehyde at 4 °C. Leftover cells on the dish were collected for Western blot analysis. The fixed cells were washed with 1x PBS and permeabilized with 0.2% Triton X-100 in 1x PBS for 3 min. The cells were rinsed with

1x PBS and then blocked with 3% BSA (Sigma) in 1x PBS for 30 min at RT. The cells were incubated with 1:100 diluted Cas(C20) antibody and 1:40 diluted monoclonal anti-vinculin antibody (Sigma) in blocking solution at 37C for 1 h in a humidified chamber. The cells were washed in 1x PBS then incubated with 1:100 diluted fluorescein (FITC)-conjugated Affini-Pure goat anti-rabbit IgG (Jackson Immuno Research) and 1:100 diluted Texas Red dye conjugated Affini-Pure goat anti-mouse IgG (Jackson Immuno Research) in blocking solution for 1 h at 37 °C in a humidified chamber. Cells were washed with 1x PBS, and the cover glasses were mounted onto clean slides with Thermo IMMU-MOUNT. Cells were observed with a Zeiss Axiovert 200 M inverted microscope. Image data was acquired using a Zeiss AxioCam digital camera and the Zeiss AxioVision version 4.5 software.

Cell Migration Assays. To measure cell migration of transiently transfected cells, 48 h after transfection, cells were washed once with PBS, trypsinized, suspended in DMEM (low glucose, 10% FBS), and counted using a Neubauer chamber. Next, 100,000 cells were plated on cell culture inserts (3.0 μm pore size, Transwell clear, Costar) in 24-well plates (5 wells for each cell type). After 48 h of migration, cells migrated to the bottom of the well were washed once with PBS, fixed with 1:1 acetone/methanol at 4 °C for 20 min, washed twice with PBS, and counted under the microscope.

Acknowledgment: P.P. gratefully acknowledges the support of Dr. Marilyn Resh (Sloan-Kettering). This work was supported by National Institutes of Health Grant CA 58530 to W.T.M.

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Bouton, A. H., Riggins, R. B., and Bruce-Staskal, P. J. (2001) Functions of the adapter protein Cas: signal convergence and the determination of cellular responses, *Oncogene 20*, 6448–6458.
2. Chodniewicz, D., and Klemke, R. L. (2004) Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold, *Biochim. Biophys. Acta 1692*, 63–76.
3. Vuori, K., and Ruoslahti, E. (1995) Tyrosine phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix, *J. Biol. Chem. 270*, 22259–22262.
4. Sakai, R., Nakamoto, T., Ozawa, K., Aizawa, S., and Hirai, H. (1997) Characterization of the kinase activity essential for tyrosine phosphorylation of p130Cas in fibroblasts, *Oncogene 14*, 1419–1426.
5. Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996) Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases, *Mol. Cell. Biol. 16*, 2606–2613.
6. Ruest, P. J., Shin, N. Y., Polte, T. R., Zhang, X., and Hanks, S. K. (2001) Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src, *Mol. Cell. Biol. 21*, 7641–7652.
7. Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. (1996) Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase, *J. Biol. Chem. 271*, 8959–8965.
8. Burnham, M. R., Harte, M. T., Richardson, A., Parsons, J. T., and Bouton, A. H. (1996) The identification of p130cas-binding proteins and their role in cellular transformation, *Oncogene 12*, 2467–2472.
9. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner, *EMBO J. 13*, 3748–3756.
10. Goldberg, G. S., Alexander, D. B., Pellicena, P., Zhang, Z. Y., Tsuda, H., and Miller, W. T. (2003) Src phosphorylates Cas on tyrosine 253 to promote migration of transformed cells, *J. Biol. Chem. 278*, 46533–46540.
11. Shin, N. Y., Dise, R. S., Schneider-Mergener, J., Ritchie, M. D., Kilkenny, D. M., and Hanks, S. K. (2004) Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration, *J. Biol. Chem. 279*, 38331–38337.
12. Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresh, D. A. (1998) CAS/Crk coupling serves as a "molecular switch" for induction of cell migration, *J. Cell Biol. 140*, 961–972.
13. Huang, J., Hamasaki, H., Nakamoto, T., Honda, H., Hirai, H., Saito, M., Takato, T., and Sakai, R. (2002) Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate, *J. Biol. Chem. 277*, 27265–27272.
14. Pellicena, P., and Miller, W. T. (2001) Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions, *J. Biol. Chem. 276*, 28190–28196.
15. Patwardhan, P., Shen, Y., Goldberg, G. S., and Miller, W. T. (2006) Individual Cas phosphorylation sites are dispensable for processive phosphorylation by Src and anchorage-independent cell growth, *J. Biol. Chem. 281*, 20689–20697.
16. Shiba, K. (2004) MolCraft: a hierarchical approach to the synthesis of artifical proteins, *J. Mol. Catal. B 28*, 145–153.
17. Shiba, K., Takahashi, Y., and Noda, T. (1997) Creation of libraries with long ORFs by polymerization of a microgene, *Proc. Natl. Acad. Sci. U.S.A. 94*, 3805–3810.
18. Saito, H., Kashida, S., Inoue, T., and Shiba, K. (2007) The role of peptide motifs in the evolution of a protein network, *Nucleic Acids Res. 35*, 6357–6366.
19. Saito, H., Minamisawa, T., and Shiba, K. (2007) Motif programming: a microgene-based method for creating synthetic proteins containing multiple functional motifs, *Nucleic Acids Res. 35*, e38.
20. Shiba, K., and Minamisawa, T. (2007) A synthesis approach to understanding repeated peptides conserved in mineralization proteins, *Biomacromolecules 8*, 2659–2664.
21. Saito, H., Honma, T., Minamisawa, T., Yamazaki, K., Noda, T., Yamori, T., and Shiba, K. (2004) Synthesis of functional proteins by mixing peptide motifs, *Chem. Biol. 11*, 765–773.
22. Cho, S. Y., and Klemke, R. L. (2000) Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix, *J. Cell Biol. 149*, 223–236.
23. Honda, H., Nakamoto, T., Sakai, R., and Hirai, H. (1999) p130(Cas), an assembling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts, *Biochem. Biophys. Res. Commun. 262*, 25–30.
24. Burnham, M. R., Bruce-Staskal, P. J., Harte, M. T., Weidow, C. L., Ma, A., Weed, S. A., and Bouton, A. H. (2000) Regulation of c-SRC activity and function by the adapter protein CAS, *Mol. Cell. Biol. 20*, 5865–5878.
25. Burack, W. R., and Sturgill, T. W. (1997) The activating dual phosphorylation of MAPK by MEK is nonprocessive, *Biochemistry 36*, 5929–5933.
26. Ferrell, J. E., Jr., and Bhatt, R. R. (1997) Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase, *J. Biol. Chem. 272*, 19008–19016.
27. Shiba, K., Takahashi, Y., and Noda, T. (2002) On the role of periodism in the origin of proteins, *J. Mol. Biol. 320*, 833–840.
28. Mead, D. A., Szczesna-Skorupa, E., and Kemper, B. (1986) Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering, *Protein Eng. 1*, 67–74.