HHS Public Access

Author manuscript

*J Cancer Biol Res*. Author manuscript; available in PMC 2015 June 01.

Published in final edited form as:

*J Cancer Biol Res*. 2014 May 14; 2(1): .

# Cross-Phosphorylation and Interaction between Src/FAK and MAPKAP5/PRAK in Early Focal Adhesions Controls Cell Motility

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## Abstract

P38-regulated and activated kinase (PRAK/MAPKAPK5) is a serine/threonine kinase which lies downstream of the p38 and ERK3/4 MAP kinase pathways. PRAK plays diverse roles in the processes of cell growth, nutrient starvation response, programmed cell death, senescence and motility. PRAK has been shown to both promote and inhibit cell motility in different contexts. The pro-motility functions of PRAK are attributed mainly to cytoskeletal rearrangement occurring downstream of its phosphorylated substrate HSP27; however, it was recently shown that PRAK is required for motility in endothelial cells upstream of Focal adhesion kinase (FAK). Along with Src, FAK functions as a mediator of motility signaling through the phosphorylation of substrates in focal adhesions. Here, we show that PRAK, initially identified as a FAK substrate in an *in situ*/ kinase overlay assay, is a Src substrate, the phosphorylation of which directs PRAK to focal adhesions. Focal adhesion localization of PRAK was not found to affect cell motility, however transient over expression of PRAK inhibited motility in HeLa cells. This effect requires PRAK kinase activity and proceeds through an impairment of FAK activation via phosphorylation on Y861. Our studies demonstrate for the first time that PRAK is regulated by tyrosine phosphorylation, localizes to focal adhesions, and interacts physically with and can phosphorylate FAK/Src. Further we provide a novel mechanism for the inhibition of motility downstream of PRAK.

### Keywords

RAK; MAPKAPK5; MK5; Src; FAK

## INTRODUCTION

MAPKAPK5/PRAK is a serine/threonine kinase was originally identified based on homology with two other MAP kinase activated kinases, MK2 and MK3 [1, 2]. PRAK kinase activity is stimulated by phosphorylation of the active site, T182, by p38 [1] or the atypical ERK3/4 MAP kinases [3,4]. Both the kinase activation status and the subcellular localization of PRAK determine its function. Upon binding of p38 to a region overlapping PRAK's nuclear localization signal, PRAK is exported from the nucleus to the cytoplasm



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[5]. Through its phosphorylation of substrates such as p53, Rheb, FOXO3a, or HSP27, PRAK facilitates Ras-induced senescence [6], nutrient starvation responses [7], proliferation [8] and motility [9]. PRAK has been shown to either promote [10] or inhibit [11] cell motility in different studies. In many cell types, PRAK causes cytoskeletal rearrangement through the phosphorylation of HSP27 [12,13]. PRAK was shown recently to be required for the VEGF-induced enrichment of activated FAK in focal adhesions and cell motility in endothelial cells [9].

Focal Adhesion Kinase (FAK) and Src are non-receptor tyrosine kinases which regulate cytoskeletal rearrangement and focal adhesion turnover, as well as proliferation, survival and motility signaling. In response to integrin engagement, FAK and Src are recruited to areas of the interior cell membrane called focal adhesions, where they interact physically and activate one another. FAK autophosphorylation on Y397 creates a high-affinity binding site for the SH2 domain of Src [14], which releases it from pY527 of Src and in turn allows for autophosphorylation of Src on Y416 [15]. Activated Src further phosphorylates FAK on additional residues, namely Y576, Y577 [16,17], Y861 [18] and Y925 [19]. FAK/Src forms a platform for the docking and phosphorylation of additional molecules, including PI3K, Ras, and MAP kinase, which signal through downstream pathways. FAK/Src function is dependent on physical interaction with and phosphorylation of substrates such as Grb2 [19], paxillin [20], and $p130^{Cas}$ [17,21].

Although a role for PRAK in cell motility is indicated by several studies, it remains unclear whether PRAK is ultimately pro- or anti-motility. Further, the mechanism by which PRAK regulates motility through FAK [9] is unknown. Here we show that PRAK is a FAK/Src substrate which co-localizes with Src in focal adhesions; further, we demonstrate that PRAK is able to directly phosphorylate FAK and Src, and that it inhibits motility through a disruption of FAK phosphorylation on Y861. Our data therefore identify a novel role for PRAK in controlling adhesion and motility through interactions with FAK/Src.

## MATERIALS AND METHODS

### Antibodies and other reagents

The following rabbit (rb) or mouse (ms) Monoclonal (M) Antibodies (Ab) were used: GST (Abm); GAPDH, poY861-FAK, poY925-FAK, ms-PRAK (Santa Cruz); rb-PRAK (ProteinTech); poY416-Src, total Src, and poY118-paxillin (Cell Signaling); total phosphotyrosine (4G10) and total FAK (Millipore); GFP (Invitrogen); poY397-FAK (BD Transduction); vinculin (Sigma-Aldrich). Secondary Abs were from BioRad (HRP-conjugated) or Invitrogen (AlexaFluor-conjugated). Purified GST-FAK was from Invitrogen, purified GST-PRAK was from SignalChem, and purified His-Src was from ProQinase. Vitronectin was from Advanced BioMatrix; FN and soybean trypsin inhibitor were from Sigma-Aldrich. PF-573228 was from Pfizer. Dasatinib was from Watson International.

## Plasmids, cloning and site-directed mutagenesis

GST-347PRAK was generated by excising 347-PRAK, which encodes from bp 1,298 to the C-terminus of PRAK, from pBluescript and splicing into pGEX-5x-1 (GE) between EcoRI and XhoI. Full-length PRAK-1 from pBP-HA-hPRAK (gift of Peiquing Sun, Scripps Research Institute) was amplified using the primers, PRAK-01-F: 5'-TTACGCTGAATTCATGTCGGAGGAGA-3', PRAK-01-R: 5'-CCTAGAGCTCGAGTTATTGGGATTCG-3', and then fused to GST as an EcoRI/XhoI fragment in pGEX-5X-1. GST-PRAK-2 was generated by Site-Directed Mutagenesis (SDM) [22] using the primer: 5'-ATTCTCCCCCAGGCTGGTAAAGGAGAGAATGAA-GATGAGA-3'. GST-HSP27 was generated by excising the open reading frame of HSP27 from EST clone #2822325 (Open Bio-systems) and splicing into pGEX-5X-1 between EcoRI and XhoI. pGEX-5X-paxillin (chicken; a.a.1–151) was described previously [23]. His-PRAK was generated by excising the open reading frame from GST-PRAK between EcoRI and XhoI and ligating it into a version of pcDNA3.1 (Invitrogen) which was previously modified to contain a His6 sequence. T182A-His-PRAK was generated by SDM using the primer 5'-GACCAAGGTGACTTGATGGCACCCCAGT-TCACCCCT-3'. Y → F mutant versions of His-PRAK were generated using SDM with primers hPRAK-Y20F: 5'-TCCATTTTAGAAGAAT-TCAGTATCAATTGGACTCAGA-3', hPRAK-Y188F: 5'-ACCCCAGTTCACCCCTTTTTATGTAGCACCCCA-3', hPRAK-Y189F: 5'-CAGT-TCACCCCTTATTTTGTAGCACCCCAGGTA-3', hPRAK-Y216F: 5'-ACCTCACCGACGCCCTTCACTTACAACAAGA-3', hPRAK-Y218F: 5'-ACCGACGCCCTACACTTTCAACAAGAGCTGTGACTT-3', hPRAK-Y232F: 5'-CCTAGGGGTGATTATCTTTGTGATGCTGTGCGGATA-3', hPRAK-Y238F: 5'-TGTGATGCTGTGCGGATTCCCTCCTTTTTACTC-CAAA-3', hPRAK-Y242F: 5'-TGCGGATACCCTCCTTTTTTCTCCAAACACCACAGC-3', hPRAK-Y375F: 5'-GCCAAAGGACAGT-GTCTTTATCCACGACCATGAG-3', hPRAK-Y425F: 5'-GCAGGAGGCTTGGAAGTTTAACCGGGAATGCAAACTCC-3', msFAK-Y397F: 5'-CTGTGTCAGAGACAGATGACTTTGCAGAGATCATCGAT-GAGG-3'. HA-FAK and pm-v-Src were described previously [24]. c-Src and Src-Y527F were gifts of Margaret Frame (Edinburgh Cancer Research Centre). pLUdR-puro-YFP-FAK-Y180A/M183A (FAK180) was a gift of Michael Schaller (West Virginia University). pEGFP-MK5 was a gift of Ole Morten Seternes (University of Tromsø).

## Cell culture

Human embryonic kidney HEK293T and mouse NIH-3T3 fibroblast were obtained from the American Tissue Culture Collection (Manassas, VA). NIH-3T3/c-Src527F was a gift of Richard Jove (Beckman Research Institute). Src/Yes/Fyn-null (SYF) mouse embryonic fibroblasts (MEF) were a gift of Philippe Soriano (Mount Sinai School of Medicine). HEK293T and MEF were maintained in DMEM plus 10% FBS and penicillin/streptomycin; NIH-3T3 and NIH-3T3/c-Src527F were maintained in DMEM plus 10% BS and penicillin/streptomycin.

### Protein purification

Expression of GST, GST-347PRAK, GST-PRAK-2, GST-paxillin, GST-HSP27 in BL21-pLysS (Invitrogen) was induced with 1 mM IPTG for 2–4 h at 30°C. Bacteria were collected and lysed in GST binding buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, 100 mM NaCl) by freeze/thaw followed by sonication. Supernatants were incubated with GST-Bind resin (Novagen), and protein was eluted using GST Elution Buffer (50 mM Tris-Cl pH 8.0, 10 mM reduced glutathione). Eluted protein was dialyzed 1:1000 into PBS at 4°C using Slide-a-Lyzer cartridges (Pierce) or de-salted and concentrated using Amicon ultra centrifugal filters with 10 kDa molecular weight cutoff (Millipore).

### Immunoblotting (IB)

Cells were lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.1% SDS, 0.5% deoxycholic acid, 1 mM $Na_3VO_4$, 1 mM NaF, 1 mM PMSF, plus a protease inhibitor cocktail (Roche)), incubated on ice for 30 min and then centrifuged to remove debris. Protein concentration was measured using the Bio-Rad DC protein assay (Hercules, CA). Samples directly from lysate or those from IP were separated by SDS-PAGE using 7.5 or 10% gels. Proteins were transferred onto polyvinylidene fluoride membranes, which were then blocked for 1 h in 5% non-fat dry milk, and incubated with primary Ab in blocking solution overnight at 4°C. HRP- or Alexa Fluor-conjugated secondary Abs were applied for 1 h at room temperature, then immune complexes were visualized either by chemiluminescence using the Lumi-Light Western Blotting Substrate (Roche) or via scanning at 800 nm using the Licor Odyssey Infrared Imager (LiCor Biosciences).

### *In vitro* kinase reactions

*In vitro* kinase assays were performed using either purified active kinases or kinases immunoprecipitated from cells. For IP, lysates made from HEK293T cells expressing HA-FAK or GFP-MK5 were incubated with αHA or αGFP Ab at 4°C overnight, followed by incubation with 20 µl Protein A/G resin (Santa Cruz) for 1 h. Agarose beads were washed twice with RIPA buffer and twice with kinase buffer (20 mM HEPES pH 7.2, 5 mM $MnCl_2$, and 5 mM $MgCl_2$for FAK; 50 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM $Na_3VO_4$ for MK5), then resuspended in 20 µl of kinase buffer. 5 µl beads were then used in kinase reactions with purified substrate proteins and 10 µCi $^{32}$P-ATP. Where indicated, PF-573228 or Dasatinib were added to a final concentration of 10 µM. Reactions were incubated at 30° for 30 minutes, after which samples were subjected to SDS-PAGE and autoradiography. For non-radioactive IVK assays, purified kinases and substrates were incubated at 30°C for 30 min with 10 mM ATP, then separated by SDS-PAGE, and analyzed byanti-phosphotyrosine IB.

### Immunoprecipitation (IP)

100 to 500 µg of protein lysate was either bound to 1 µg Ab for 2 h and then incubated with 20 µl Protein A/G beads for 1 h, or incubated with 20 µl $Ni^{2+}$-NTA resin for 3 h at 4°C. Beads were washed twice in RIPA buffer and loaded onto denaturing polyacrylamide gels.

His-PRAK was precipitated using ms-αPRAK or $Ni^{2+}$-NTA agarose; endogenous PRAK was immunoprecipitated using Rb-αPRAK.

### Co-immunoprecipitation (co-IP)

Cells were lysed in either RIPA buffer (co-IP of His-PRAK and v-Src; co-IP of HA-FAK and GFP-MK5) or a low-salt NP40 buffer (co-IP of FAK and Src/His-PRAK) (Polte & Hanks). For co-IP of FAK and Src/His-PRAK, lysates were precleared by incubating with protein A/G agarose beads for 1 h at 4°C. 450 ug of protein lysates were incubated at 4°C either with $Ni^{2+}$-NTA resin (co-IP of His-PRAK and v-Src) for 4 h, with αHA Ab overnight (co-IP of HA-FAK and GFP-MK5), or with αFAK Ab-conjugated agarose beads for 2 h (co-IP of FAK and Src/His-PRAK). For co-IP of HA-FAK and GFP-MK5, this was followed by incubation with protein A/G agarose for 1 h at 4°C. Beads were washed twice in the respective buffers, separated by SDS-PAGE and subjected to IB.

### Immunofluorescence (IF)

Transfected cells (HeLa or MEF with WT or mutant His-PRAK) were serum-starved overnight in media containing 0.5 % serum, trypsinized, incubated with soybean trypsin inhibitor, and resuspended in DMEM. Cells were adhered to coverslips pre-coated with 10 μg/mL FN and then fixed in a solution of 60% acetone and 0.37% formaldehyde for 20 min at −20°C. Coverslips were washed thrice in PBS, then blocked in 5% FBS in PBS for 30 min. Primary Abs were diluted 1:100 in blocking solution and applied for 2 h at room temperature. Coverslips were then washed thrice and secondary FITC- or Texas Red-conjugated Ab, diluted 1:1000, were applied for 1 h. Coverslips were washed overnight in PBS at 4°C, then mounted onto slides using ProLong Antifade (Invitrogen). Fluorescent images were captured using a TE2000-E inverted microscope (Nikon) equipped with a charge-coupled CoolSNAP HQ camera (Photometrics). Focus was maintained between different images to ensure capture in the same plane. Images were acquired using MetaVue software (v6.2, Molecular Devices).

### Adhesion

HeLa cells were transfected with pcDNA3.1, WT or mutant versions His-PRAK, then serum-starved overnight in medium containing 0.5% FBS. After trysinization, trypsin was neutralized using soybean trypsin inhibitor (0.5 μg/mL). Cells were resuspended in medium containing 0.5% FBS, and either held in suspension at 37°C for 1 h or adhered to culture dishes which had been pre-coated with 10 μg/mL fibronectin (FN) or 1 μg/mL vitronectin (VN) for 30 or 60 minutes. Cells were lysed in RIPA buffer, and lysates were used for direct IB and for IP/IB, or cells on FN-coated coverslips were fixed and analyzed by IF.

### Densitometry and IF quantification

IB autoradiographs were scanned and individual band intensities were quantified using Image J [25]. IF slides were quantified at 60× magnification:cells showing PRAK overexpression were scored for peripheral punctate PRAK structures and for the number of individual puncta/cell. 5 fields containing >20 cells/fields were used for each analysis.

PRAK and Src co-staining was scored based on 3 or more overlapped PRAK-Src puncta/cell.

### shRNA silencing

PRAK shRNAs PK-sh3 (5'-AGGACTGTGTAGTTGTATA-3') and PK-sh6 (5'-GCCCGACTCTTAATTGTAA-3') plus a control non-targeting sequence in the pGIPZ vector were obtained from the RPCI shRNA Core (I.H. Gelman, Director), and packaged into virus by co-transfecting HEK293T cells with pGIPZ vectors, pCMVR8.74 and pMD2.G. High infection efficiency was verified by GFP expression and puromycin selection, and stable knockdown was verified by IB analysis.

### Chemotaxis

Cells were serum-starved in medium containing 0.5% FBS for either 4h or overnight, then rinsed, collected in PBS containing 0.5 μg/mL soybean trypsin inhibitor, and resuspended in serum-free DMEM. $1.5 – 2 \times 10^5$ cells in 0.5 mL were seeded in the tops of 24-well Boyden chambers containing polycarbonate membranes with 8 μm pores. Cells were allowed to migrate towards 0.75 mL of complete medium (containing 10% FBS) for 12 or 16 h at 37°C. Cells were removed from the inside of the upper chamber were removed by scrubbing with a cotton swab, and those adhered to insert bottoms were stained using Diff-Quik solutions (Seimens). Eight fields containing >40 cells/field were counted for each condition at 20× magnification, and used to calculate average migration.

## RESULTS AND DISCUSSION

### PRAK is tyrosine phosphorylated *in vitro* and *in vivo*

A partial cDNA encoding the C-terminal portion (a.a. 347–473) of *MAPKAPK5* (*PRAK*) was identified in a FAK *in situ* kinase overlay assay [26] (Figure 1a). The cDNA sequence represented the PRAK-2 isoform which includes six extra base pairs generating a lysine-glycine insertion at amino acids 407–408 [27]. When expressed as a GST-fusion protein, the truncated PRAK-2, designated 347-PRAK, was well phosphorylated by FAK *in vitro* (Figure 1b). In contrast, the full-length versions of PRAK-1 and PRAK-2 were poorly phosphorylated by FAK *in vitro* (Figure 1c). Src phosphorylated 347-PRAK (data not shown) as well as full-length PRAK-1 (Figure 1d) and -2 (data not shown) in *in vitro* kinase (IVK) assays. The ability of FAK to phosphorylate the truncated version, 347-PRAK, may reflect the existence of cleaved PRAK products or isoforms which have not yet been identified. Alternatively, full length PRAK may be phosphorylated by FAK under specific biological circumstances which were not tested here.

To test whether phosphorylation of PRAK occurred in cells, HEK293T cells were transfected with His-tagged PRAK alone or in combination with v-Src and/or a constitutively active version of FAK, FAK180 [28]. In line with the results from *in vitro* kinase reactions, full-length PRAK was phosphorylated by Src but not by FAK180 (Figure 2a). Although FAK180 could not induce PRAK phosphorylation itself, its expression augmented PRAK phosphorylation by v-Src (Figure 2b), suggesting that it may play a role in scaffolding PRAK/Src or in activating Src's kinase activity towards PRAK. Due to the

fact that His-PRAK and Src have similar molecular weights, we confirmed that Src is able to phosphorylate GFP-tagged MK5, whose motility in SDS-PAGE is notably slower than that of Src (Figure 1c). Endogenous MK5 was also found to be tyrosine phosphorylated in an NIH-3T3 cell line stably expressing Y527F-Src [29] (Figure 2d). These results suggest that full-length PRAK is a substrate of Src, whereas truncated PRAK (i.e., 347PRAK), whose biological significance is unclear, can serve as a substrate for FAK.

In order to identify the sites on PRAK which are targeted by Src, a series of mutants were generated in which individual tyrosines were mutated to phenylalanine. When co-expressed in HEK293T cells with Src, Y188F-, Y189F- and Y216F-PRAK were strongly impaired in tyrosine phosphorylation (Figure 2e), suggesting that these are either substrate sites or required for Src-induced phosphorylation. Moreover, we showed that Src and FAK could co-immunoprecipitate with His-PRAK when co-expressed, suggesting that PRAK complexes with FAK and Src notwithstanding their abilities to use it as a substrate (Figure 2f,2g).

## PRAK is tyrosine phosphorylated during cell adhesion

Based on the finding that PRAK can be phosphorylated by Src and forms complexes with FAK and Src, we tested whether cell adhesion, which activates FAK/Src kinases, could induce PRAK phosphorylation. HeLa cells were held in suspension or adhered to FN-coated plates for increasing amounts of time, a condition sufficient to induce FAK and Src activation, based on the surrogate autophosphorylation markers, poY397-FAK and poY416-Src (Figure 3a). IP and anti-phosphotyrosine IB revealed adhesion-induced tyrosine phosphorylation of endogenous PRAK, with maximal induction between 30 min and 1 h (Figure 3a).

We also detected adhesion-based tyrosine phosphorylation of exogenously expressed His-PRAK in HeLa cells which were adhered for 1 h (Figure 3b,3c, inset). Co-expression of Y527F-Src enhanced the levels of phosphorylation achieved by adhesion to either FN or vitronectin (Figure 3b). In order to determine which tyrosines are targeted during adhesion, individual Y F His-PRAK mutants transiently transfected into HeLa cells were tested by IP/IB analysis for phosphorylation during attachment. All mutants showed some level of tyrosine phosphorylation (Figure 3c), suggesting that more than one site is targeted during adhesion.

## Phosphorylation by Src induces PRAK relocalization to focal adhesions

Because PRAK was found to be tyrosine phosphorylated during cell adhesion and to complex with FAK and Src, we hypothesized that PRAK may localize to focal adhesions. To test this, HeLa cells were transfected with His-PRAK and adhered to FN for 1 h, a time at which His-PRAK was shown to be phosphorylated (Figure 3c). IF staining identified pools of PRAK enriched in peripheral puncta, in addition to cytoplasmic and nuclear pools of PRAK (Figure 4a). The proportion of cells showing punctate PRAK structures increased during early adhesion, with the greatest degree of relocalization occurring 45 to 90 minutes after adhesion (Figure 4b).

The transient appearance and location of PRAK puncta during adhesion is consistent with PRAK enriching in immature focal adhesions. To confirm this, we performed co-IF studies with PRAK and several known focal adhesion molecules. PRAK puncta appeared in areas adjacent to, but not coincident with vinculin and FAK, whereas many puncta overlapped with phosphorylated paxillin (poY118) and Src (Figure 4a). There was a strong and consistent co-staining of PRAK and Src in puncta during adhesion, lasting 2 h after cell adhesion, with the strongest co-localization between 30 min and 1 h, after which both PRAK and Src egressed from focal adhesion structures (Figure 5a).

In order to test whether Src phosphorylation is required for adhesion-induced PRAK localization to focal adhesions, IF studies were performed in SYF MEF. His-PRAK expressed in SYF MEF failed to relocalize to puncta following cell adhesion; instead PRAK appeared to be retained in the nucleus (Figure 5b). Re-expression of the constitutively-active mutant Y527F-Src, but not c-Src, restored adhesion-based punctual relocalization of PRAK (Figure 5b), indicating that constitutive Src kinase activity is required for PRAK relocalization. To further investigate whether Src-mediated tyrosine phosphorylation of PRAK directs its relocalization to focal adhesions, we tested the ability of PRAK Y F mutants to enrich in puncta during adhesion. Compared to WT-PRAK, the Y188F, Y216F and Y238F mutants showed significantly fewer cells with puncta (Figure 6a), whereas the Y188F, Y216F and Y218F mutants showed fewer (Figure 6b) and smaller (Figure 6d) puncta/cell. We further tested the extent to which Y F mutants co-stained with Src in puncta, and found that only Y216F-PRAK showed significantly impaired colocalization (Figure 6c). These data strongly suggest that PRAK phosphorylation by Src on Y188 and Y216 drives the relocalization of PRAK to focal adhesion structures during cell adhesion. Altogether our findings suggest that whereas multiple sites on PRAK are tyrosine phosphorylated during adhesion, the phosphorylation of specific sites is required for relocalization.

## PRAK inhibits cell motility through inhibition of Y861 FAK phosphorylation

Within focal adhesions, FAK and Src activation control the signals required for cell adhesion and motility. We therefore hypothesized that focal adhesion-localized PRAK may have an effect on motility through modulation of FAK/Src activities. In contrast to previous studies, we found that transient PRAK over expression consistently caused a decrease in chemotaxis (Figure 7a). In order to determine whether this decrease could be attributed to changes in FAK/Src activation, we surveyed adhesion-induced phosphorylation of key activation sites on FAK and Src in HeLa cells over expressing PRAK. Levels of FAK and Src autophosphorylation (poY397 and poY416, respectively) as well as a known Src substrate site on FAK, Y925 [30], were similar in cells expressing empty vector or PRAK; however, exogenous PRAK diminished levels of FAK phosphorylated on Y861 (Figure 7b), a known Src substrate site [18] involved in recruitment of cell motility proteins such as DOCK180 and ELMO via association with a $p130^{Cas}$-Crk complex [31].

To test whether pools of focal adhesion-localized PRAK were responsible for impaired motility, we tested chemotaxis in HeLa cells expressing Y216F-PRAK. Y216F-PRAK was equally capable of suppressing chemotaxis as WT PRAK (Figure 7c). In contrast, the kinase-deficient mutant T182A-PRAK did not decrease cell motility (Figure 7d), suggesting

that PRAK kinase function is essential for chemotaxis attenuation. Furthermore, over expression of Y216F-, but not T182A-PRAK, impaired phosphorylation of FAK on Y861 during adhesion (Figure 7e). Altogether, these results suggest that high levels of PRAK inhibit chemotaxis through interference with FAK phosphorylation on Y861.

Somewhat surprisingly, silencing of endogenous PRAK in HeLa cells did not cause the opposite effect of overexpression but rather decreased chemotaxis (Figure 7f). IB analysis of these cells revealed that knockdown of PRAK did not interfere with levels of poY861 (data not shown); therefore the loss of endogenous PRAK and the over expression of exogenous PRAK seem to both impair motility through different mechanisms; alternatively, PRAK may control motility by acting as a biphasic concentration-dependent scaffold.

Since PRAK's kinase activity was required for inhibition of Y861 phosphorylation, we tested whether FAK and Src could be phosphorylated directly by PRAK in IVK assays. FAK or Src kinase activity was suppressed by PF-573228 or Dasatinib, respectively, neither of which inhibited PRAK serine/threonine kinase activity (Figure 8a,8b). Recombinant PRAK was capable of phosphorylating FAK (Figure 8a), Src (Figure 8b), and paxillin (Figure 8a,8c), as well as the known PRAK substrate, HSP27 (Figure 8c). We then tested whether PRAK could affect Src binding to FAK, which is known to occur through an SH2-dependent interaction with FAK-poY397 [14]. Neither WT- nor T182A-PRAK affected FAK association with Src in co-IP assays (Figure 8d). Furthermore, WT- and T182A-PRAK associated with FAK, and this association decreased during adhesion to FN (Figure 8d). The decrease in the amount of PRAK found in complex with FAK during short-term adhesion corresponds with PRAK puncta staining near but not coincident with FAK puncta during adhesion (Figure 4a). This observation is consistent with the idea that a dynamic dissociation of PRAK from FAK is required for cell motility, such that PRAK over expression and knockdown may have the same effect. The notion that different effects on cell motility are achieved through finely tuned levels of PRAK may explain previous data showing that PRAK over expression enhanced HeLa chemotaxis [10]. Taken together, these data correlate PRAK-mediated chemotaxis attenuation with PRAK binding to FAK/Src, PRAK kinase activity, and its ability to suppress phosphorylation of FAK-Y861.

## CONCLUSION

Our studies identify a novel set of interactions and cross-phosphorylations between MAPKAP5/PRAK, FAK and Src, that control chemotaxis and the transient co-staining of PRAK with Src in early, adhesion-induced focal adhesions. PRAK also seems to regulate initial focal adhesion formation by antagonizing FAK-Y861 phosphorylation. In sum, our data elaborate on several non-nuclear roles for PRAK in adhesion and cell motility.

## ACKNOWLEDGEMENT

This work is supported by grants (I.H.G.) CA94108, CA116430 (National Institutes of Health/National Cancer Institute), PC074228, PC101210 (Department of Defense), and in part, through National Cancer Institute Comprehensive Cancer funds (P30-CA016056).

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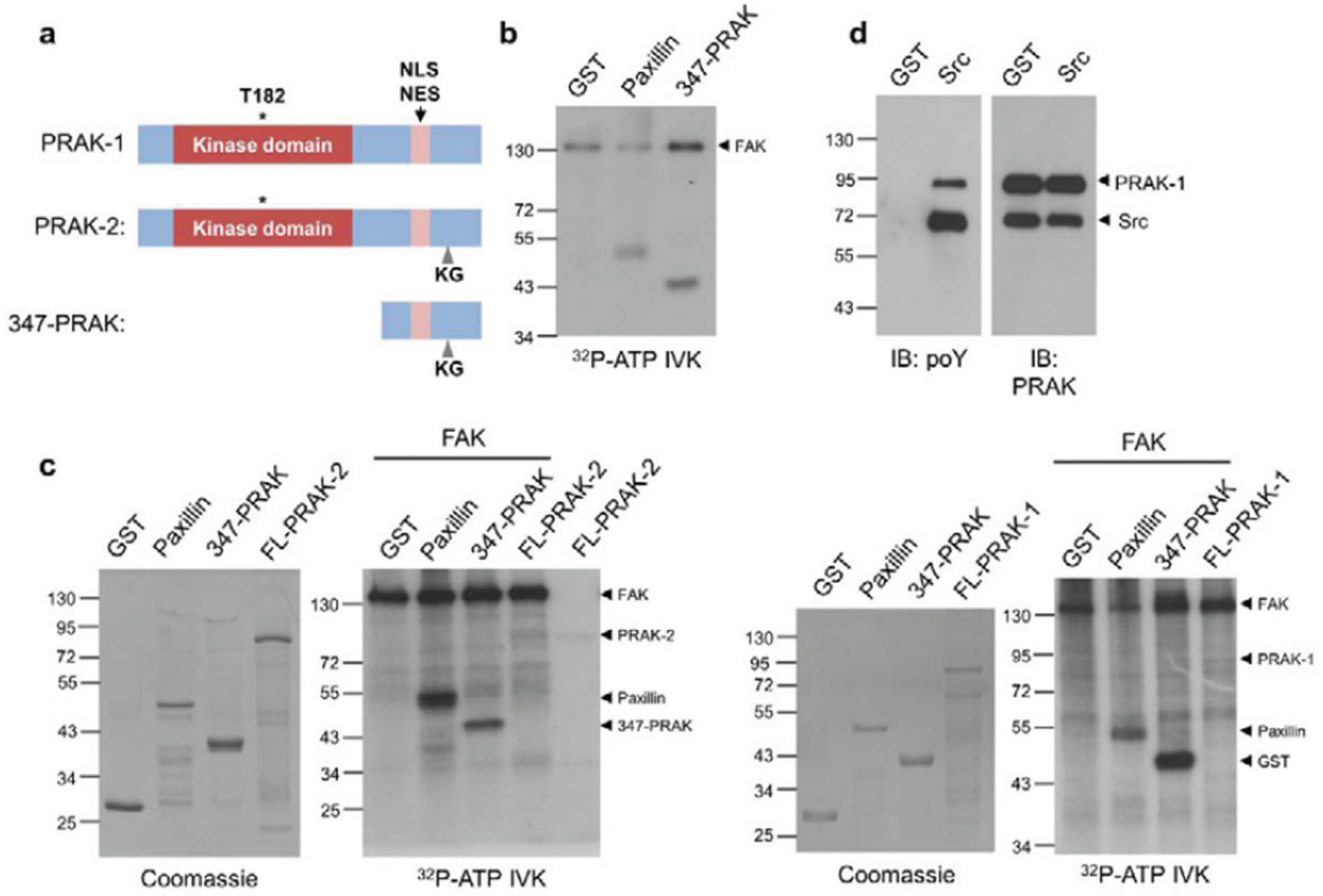


**Figure 1. PRAK is phosphorylated by FAK and Src *in vitro***

**a.** PRAK isoforms and 347-PRAK cDNA isolated in a FAK kinase overlay screen. T182 activation site, nuclear localization (NLS) and nuclear export sequences (NES) are indicated.

**b.** *In vitro* kinase (IVK) reactions with immunoprecipitated HA-FAK, purified proteins and $^{32}$P-ATP.

**c.** IVK reactions with immunoprecipitated HA-FAK and $^{32}$P-ATP, purified full-length GST-PRAK-2 (left) or PRAK-1 (right) compared to substrate proteins, including GST-paxillin and GST in Coomassie stained gels (at left).

**d.** IVK reaction with purified Src, full-length PRAK-1 and unlabeled ATP, analyzed by IB for phosphotyrosine and PRAK.

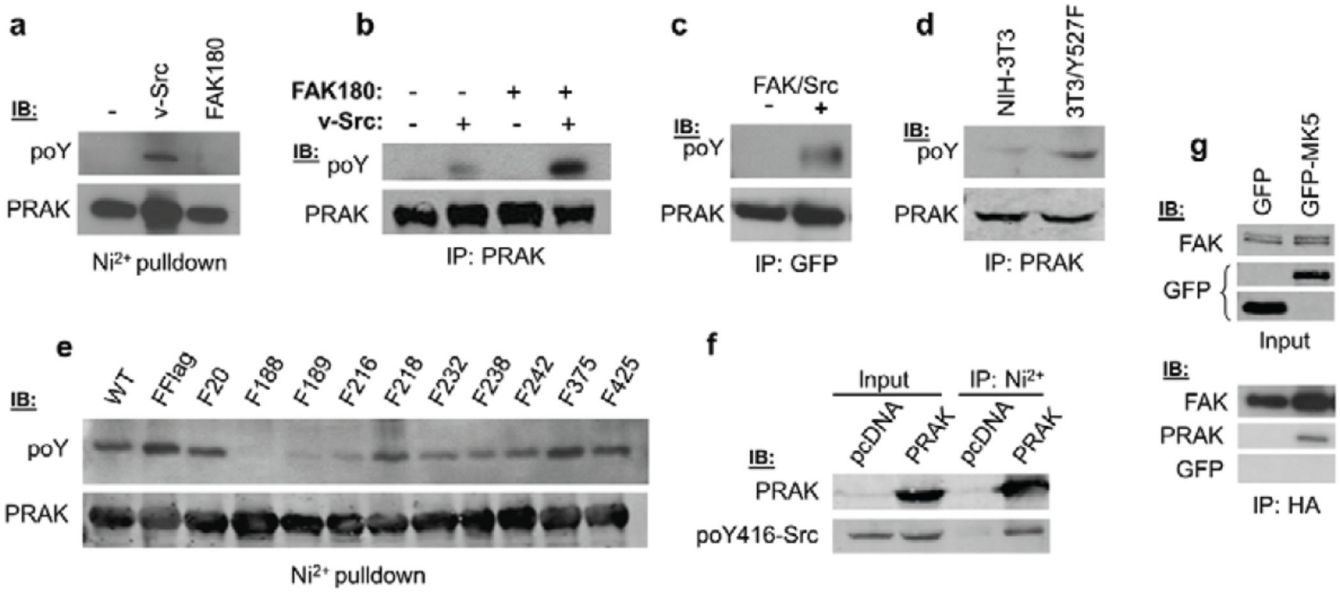


**Figure 2.**

PRAK is a Src substrate which complexes with FAK and Src. His-PRAK was co-expressed with v-Src and/or FAK180 in HEK293T cells, then precipitated using Ni2+ beads **(a)** or αPRAK **(b).** Phosphotyrosine was detected using MAb-4G10 (anti-poY). (**C).** GFP-MK5 was co-expressed with HA-FAK and v-Src in HEK293T cells, then immunoprecipitated and subjected to MAb-4G10 IB. **(d).** Endogenous MK5 was immunoprecipitated from NIH-3T3 or NIH-3T3/Y527F-Src; phosphotyrosine was detected by MAb-4G10 IB. **(e)**. WT and Y◊F mutant versions of His-PRAK were co-expressed with v-Src in HEK293T cells, precipitated using Ni2+ beads and analyzed by IB using MAb-4G10 or PRAK Ab. FFlag is a control mutant in which the single tyrosine within the partial FLAG sequence contained in His-PRAK was mutated from Y to F. **(f).** His-PRAK was co-expressed with v-Src in HEK293T cells, precipitated with Ni2+ beads, then subjected to IB. Input represents 1/10 of lysate used for IP. **(g)**. HA-FAK was co-expressed with GFP-MK5 in HEK293T cells, immunoprecipitated with αHA, and subjected to IB. Input represents 1/40 of lysate used for IP.

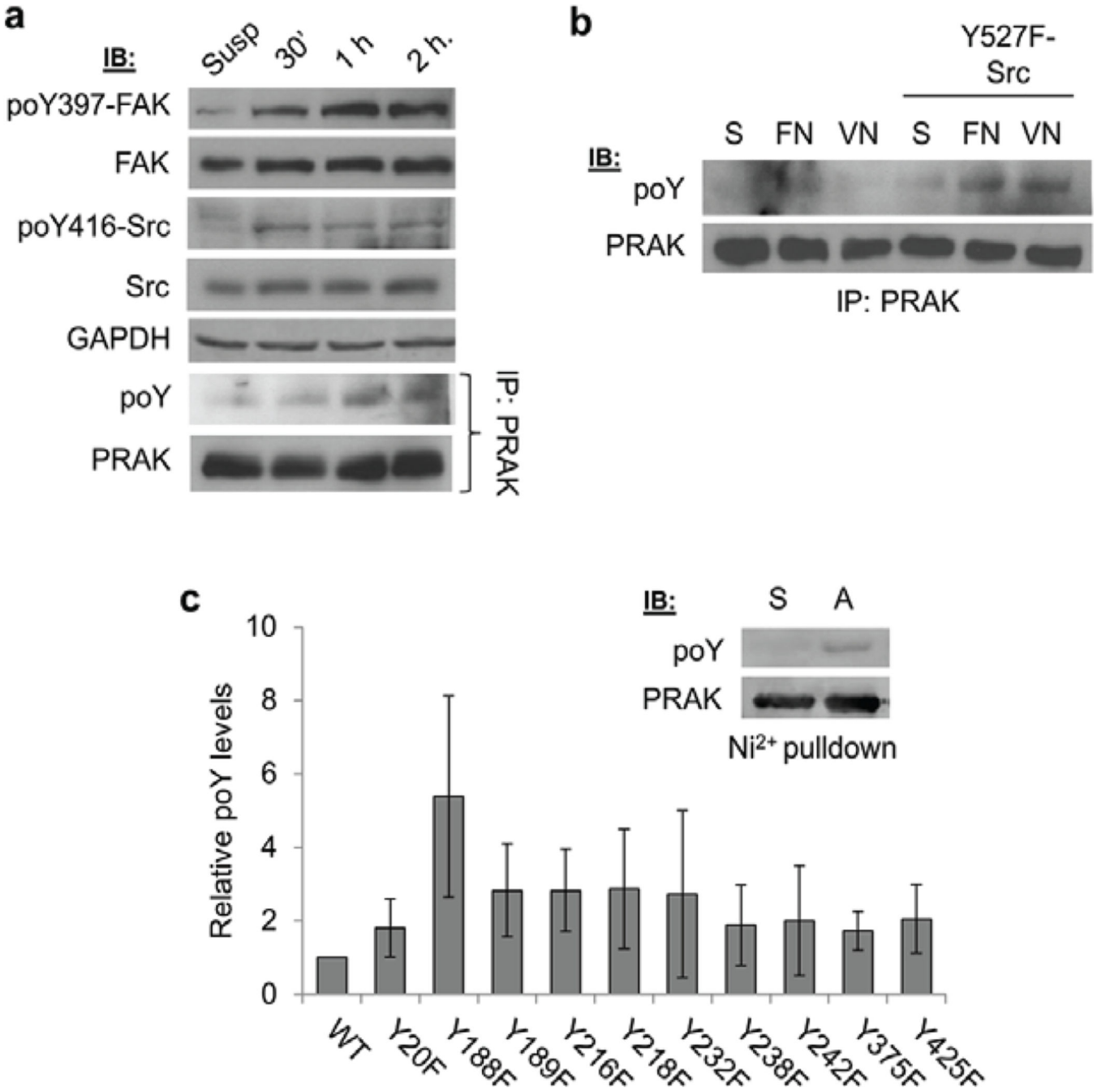


**Figure 3. PRAK is phosphorylated during adhesion**

**a.** HeLa cells adhered to FN-coated plates for the times indicated, after which endogenous PRAK was immunoprecipitated and probed for phosphotyrosine using MAb-4G10 or for total PRAK. Relative levels of FAK, FAK$^{poY397}$, Src, Src$^{poY416}$ and GAPDH (protein loading control) are shown at top.

**b.** His-PRAK was expressed alone or in combination with Y527F-Src in HeLa. Cells were adhered to FN- or vitronectin (VN)-coated plates for 1 h, then PRAK was immunoprecipitated and analyzed by IB with MAb-4G10 or PRAK Ab.

**c.** Phosphorylation of Y→F PRAK mutants during adhesion. HeLa transiently expressing His-PRAK mutants were adhered to FN for 1 h, and lysates precipitated with $Ni^{2+}$ beads followed by IB analysis with MAb-4G10 or PRAK Ab. Western blots were then analyzed by densitometry; data shown represents the average of three experiments; Error bars, SEM. Inset: phosphorylation of WT His-PRAK in HeLa adhered to FN for 1h.

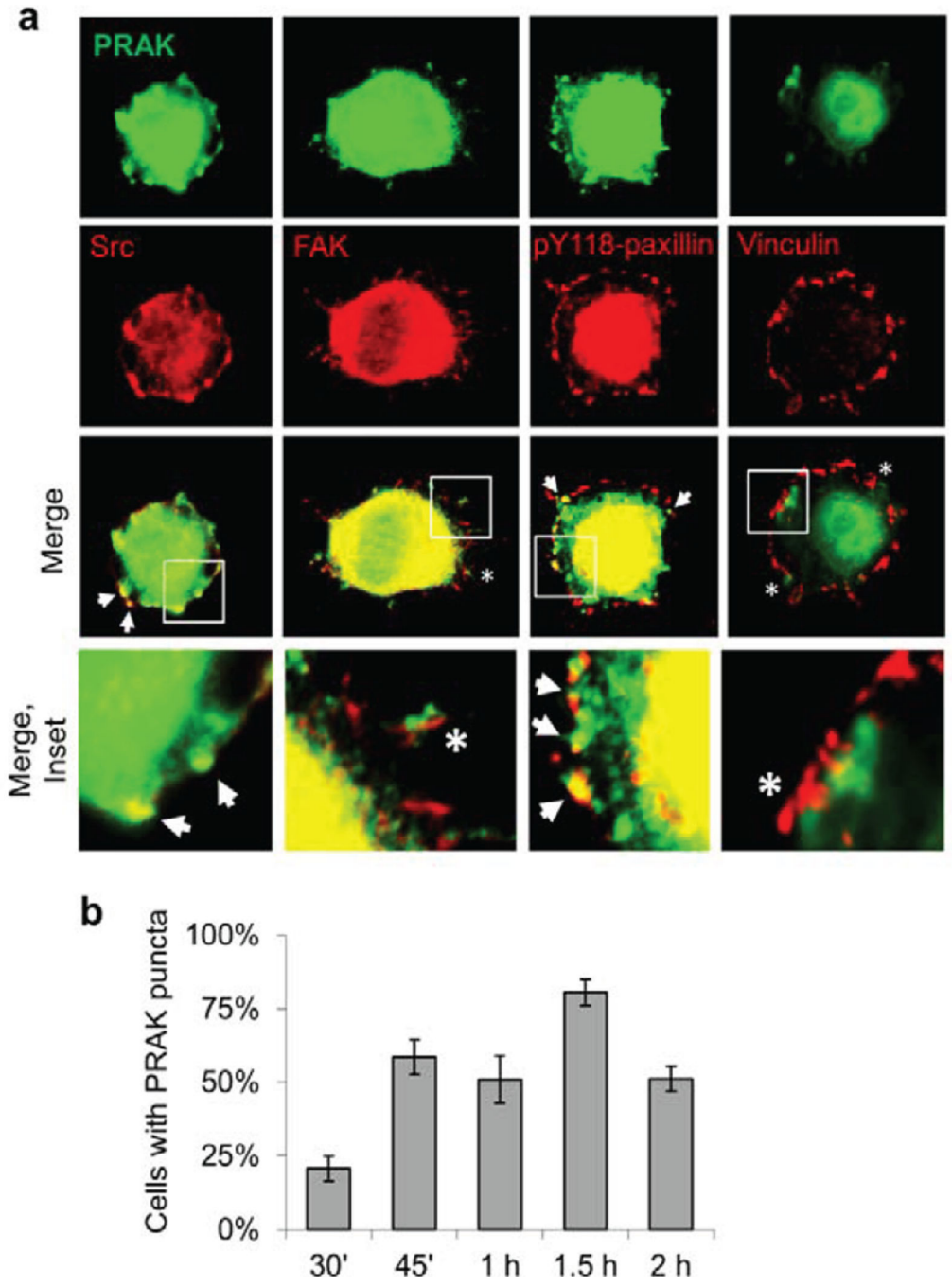


**Figure 4.**

PRAK localizes to focal adhesions. **a.** HeLa cells transiently expressing His-PRAK were adhered to FN-coated coverslips for 1 h, then stained for PRAK and the indicated focal adhesion proteins by IF. Row 3 shows merged images; row 4 shows enlargements of boxed areas. Arrowheads, areas of coincident staining; asterisks, areas of adjacent staining. **b.** Quantification of the percent of HeLa cells with PRAK puncta during adhesion to FN for the indicated times. A single experiment is shown. Error bars, SEM.

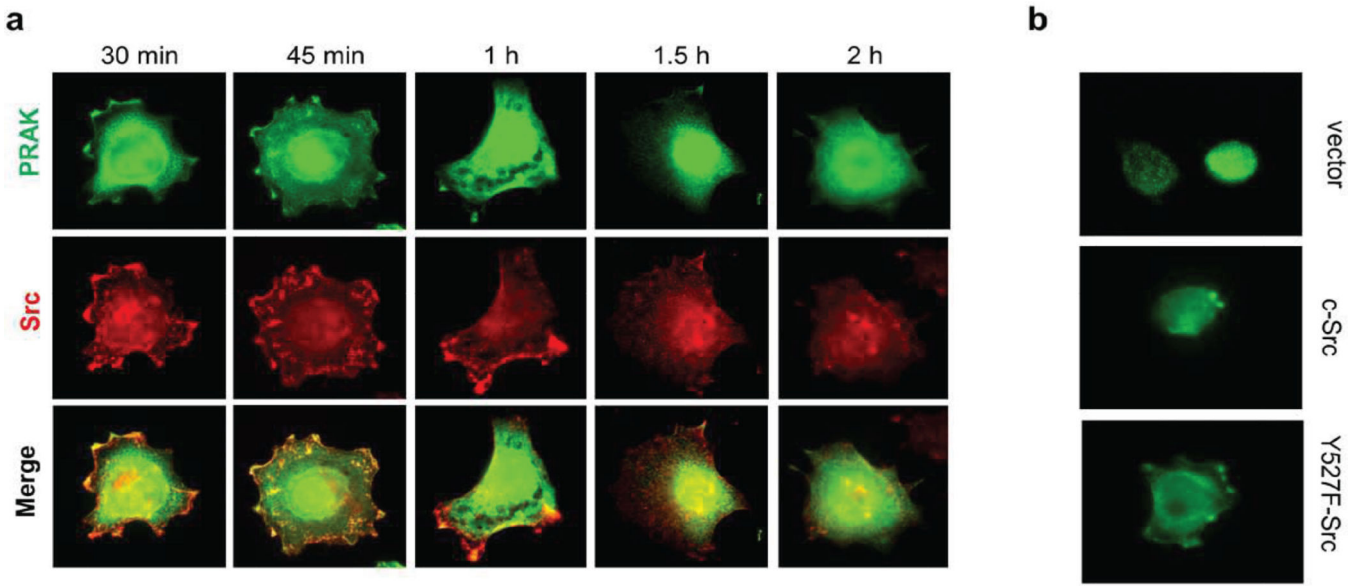


**Figure 5.**

Colocalization of PRAK and Src in focal adhesions during cell attachment. a. HeLa cells transiently expressing His-PRAK were adhered to FN-coated coverslips for the indicated amounts of time, then stained with Ab against PRAK and Src and analyzed by IF. b. SYF cells expressing His-PRAK, c-Src, $Src^{527F}$ or vector were adhered to FN for 1 h and analyzed for PRAK by IF as in panel a.

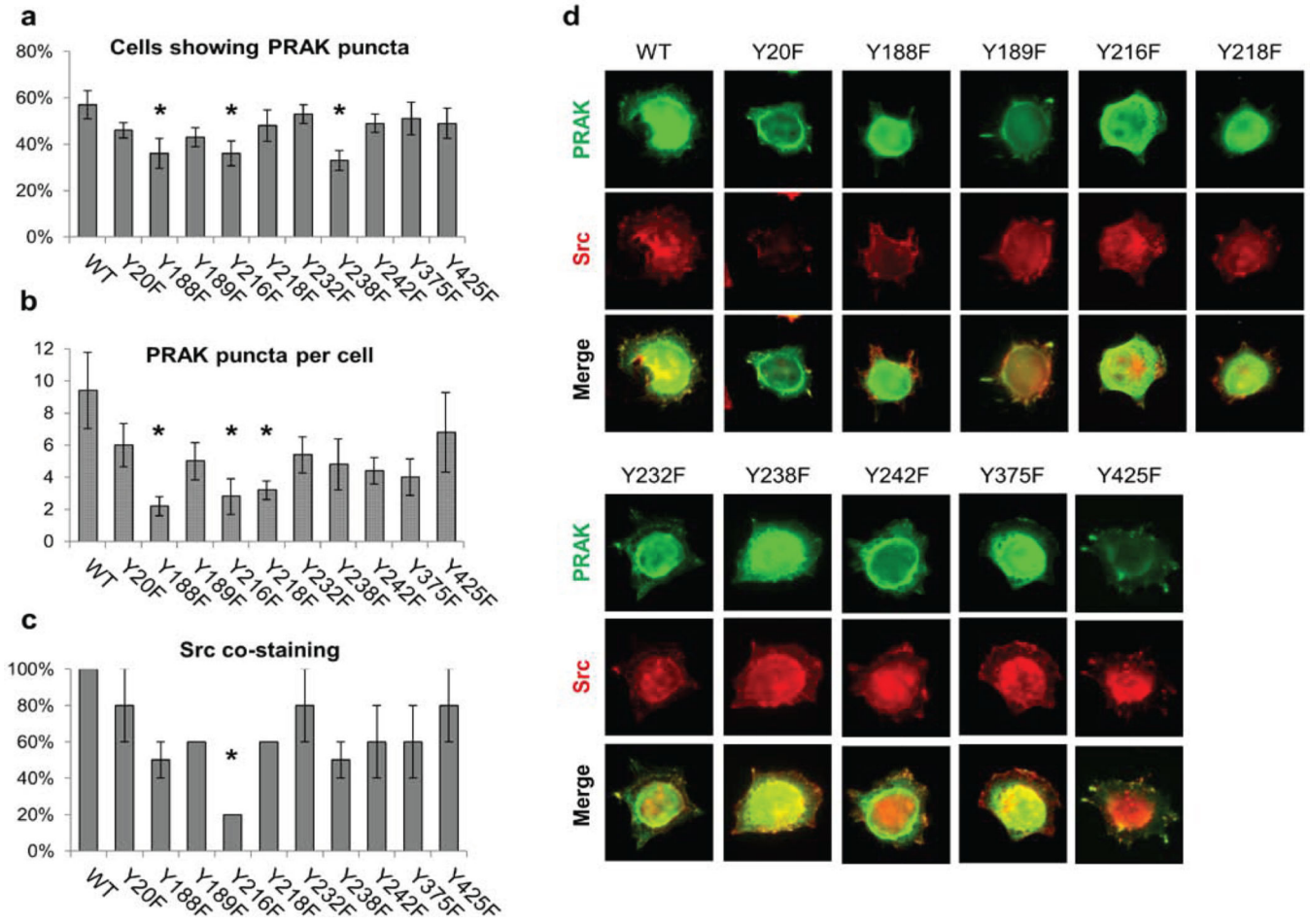


**Figure 6.**

Y188 & Y216 facilitate Src-mediated PRAK relocalization to focal adhesions. HeLa cells were transfected with WT or Y→F mutant versions of His-PRAK, adhered to FN for 75 min, fixed and stained for PRAK and Src by IF. Images were used to quantify the proportion of cells showing PRAK puncta (**a**), the number of puncta per cell (**b**), and the degree of puncta co-staining with Src (**c**). Error bars, SEM of cells scored in 5 slide regions; *, $p<0.05$ as determined by unpaired *t* test. **d.** Representative IF images of individual cells expressing each mutant during adhesion.

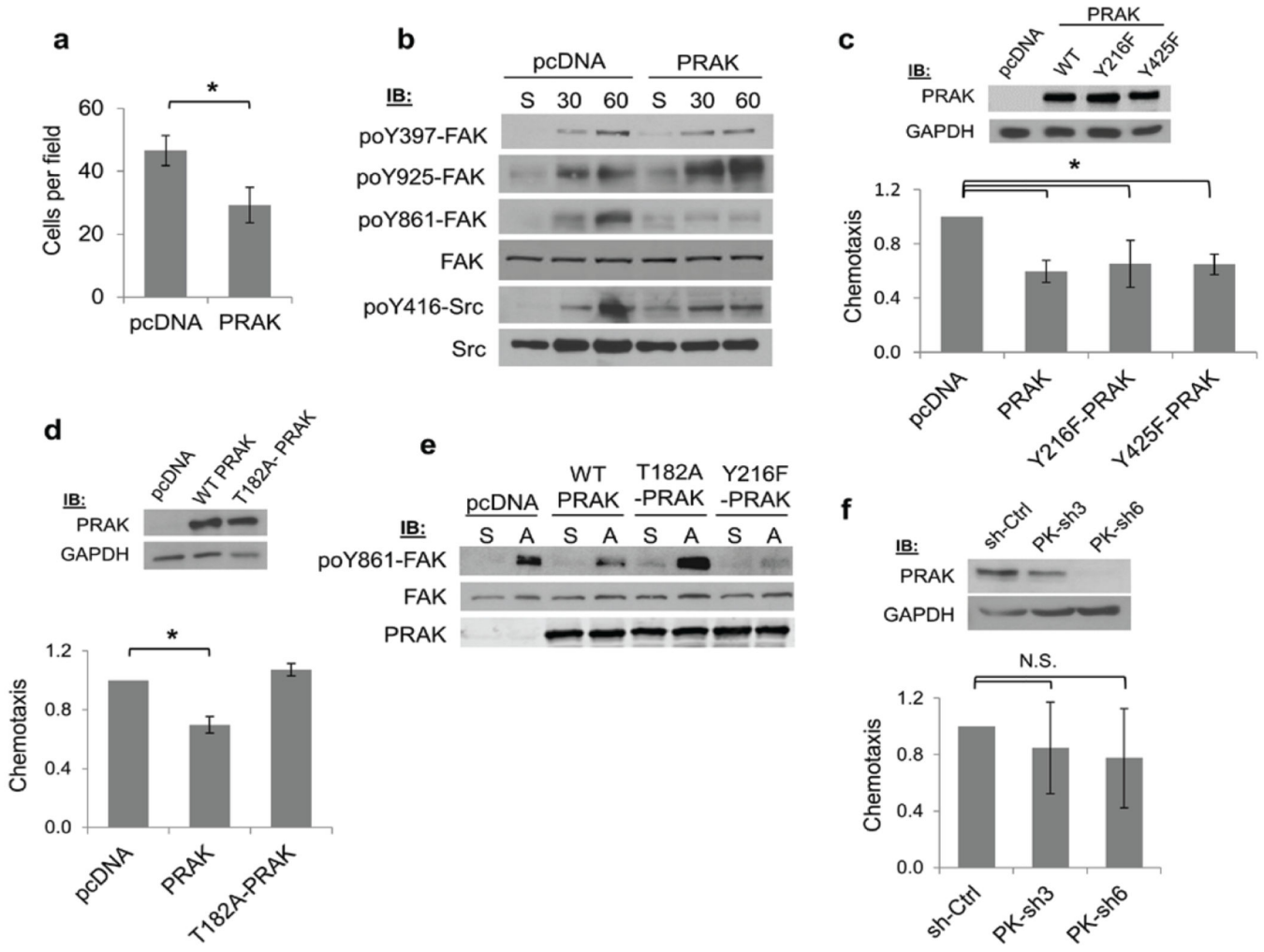


**Figure 7.**

Overexpression of PRAK inhibits chemotaxis. **a.** Chemotaxis of HeLa cells transiently transfected with pcDNA or His-PRAK. $1.5 \times 10^5$ cells were seeded into Boyden chambers and migrated towards 10% FBS for 16 h. Numbers shown represent the average of at least 20 cells/field in eight fields counted at 20× × SD. Error bars, SD; *, $p<0.05$. **b.** HeLa cells transfected with pcDNA or His-PRAK were held in suspension or adhered to FN-coated dishes for 30 or 60 min; lysates were analyzed by IB. **c.** Upper panel: IB showing equivalent transient expression of WT and mutant His-PRAK in HeLa cells. Lower panel: Chemotaxis of HeLa transiently transfected with pcDNA, WT or mutant His-PRAK, performed as in panel **a**. Error bars, SD of three independent experiments. *, $p<0.05$. **d.** Upper panel: IB showing expression of WT and T182A-His-PRAK in HeLa cells. Lower panel: chemotaxis of HeLa cells transiently transfected with pcDNA, WT or mutant His-PRAK, performed as in panel **a**. Error bars, SD of three independent experiments. *, $p<0.05$. **e.** HeLa cells transfected with pcDNA, WT His-PRAK or mutant His-PRAK were held in suspension or adhered to FN-coated dishes for 1 h; lysates were analyzed by IB. **f.** Upper panel: IB showing PRAK knockdown in HeLa cells expressing PRAK shRNA clones. Lower panel:

chemotaxis of HeLa cells stably expressing shRNAs. Error bars, SD from three independent experiments. * $P < 0.05$ as determined by unpaired *t* test. N.S., no significance.

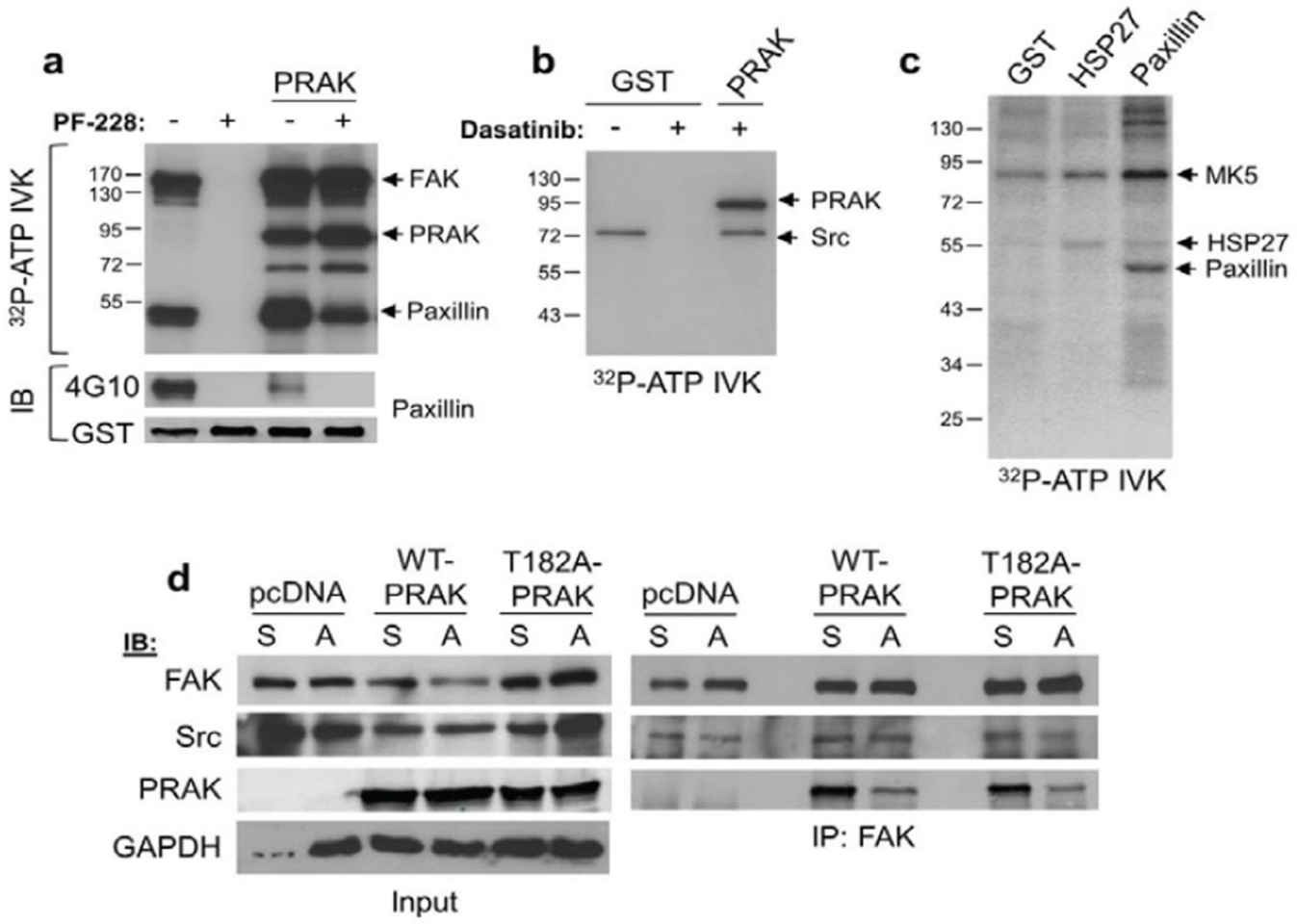


**Figure 8.**

PRAK directly phosphorylates focal adhesion components, and dissociates from FAK during adhesion. **a.** Upper panel: IVK reactions with purified FAK, His-PRAK and GST-paxillin and $^{32}$P-ATP, with or without 10 μM PF-573228. Lower panel: IVK reactions with purified FAK, His-PRAK and GST-paxillin and unlabeled ATP, with or without PF-573228; phosphotyrosine was detected by MAb-4G10 IB. **b.** IVK reactions with purified Src, GST and PRAK and $^{32}$P-ATP, with or without 0.1 μM Dasatinib. **c.** IVK reactions following GFP-MK5 IP, HSP27 or GST-paxillin and $^{32}$P-ATP. **d.** HeLa cells transfected with WT or mutant His-PRAK were held in suspension or adhered to FN-coated dishes for 1 h, then lysed and subjected to IP with αFAK followed by IB. Input represents 1/80 of lysate used for IP.