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Interactions between Two Cytoskeleton-associated Tyrosine Kinases: Calcium-dependent Tyrosine Kinase and Focal Adhesion Tyrosine Kinase*

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The calcium-dependent tyrosine kinase (CADTK), also known as Pyk2/RAFTK/CAKβ/FAK2, is a cytoskeleton-associated tyrosine kinase. We compared CADTK regulation with that of the highly homologous focal adhesion tyrosine kinase (FAK). First, we generated site-specific CADTK mutants. Mutation of Tyr^{402} eliminated autophosphorylation and significantly decreased kinase activity. Mutation of Tyr^{881}, a putative Src kinase phosphorylation site predicted to bind Grb2, had little effect on CADTK regulation. Src family tyrosine kinases resulted in CADTK tyrosine phosphorylation even when co-expressed with the Tyr^{402}/Tyr^{881} double mutant, suggesting that Src/Fyn etc. phosphorylate additional tyrosine residues. Interestingly, CADTK tyrosine-phosphorylated FAK when both were transiently expressed, but FAK did not phosphorylate CADTK. Biochemical experiments confirmed direct CADTK phosphorylation of FAK. This phosphorylation utilized tyrosine residues other than Tyr^{397}, Tyr^{925}, or Tyr^{576}/Tyr^{577}, suggesting that new SH2-binding sites might be created by CADTK-dependent FAK phosphorylation. Last, expression of the CADTK carboxyl terminus (CRNK) abolished CADTK but not FAK autophosphorylation. In contrast, FAK carboxyl terminus overexpression inhibited both FAK and CADTK autophosphorylation, suggesting that a FAK-dependent cytoskeletal function may be necessary for CADTK activation. Thus, CADTK and FAK, which both bind to some, but not necessarily the same, cytoskeletal elements, may be involved in coordinate regulation of cytoskeletal structure and signaling.

In rat liver epithelial cells, epidermal growth factor-dependent tyrosine phosphorylation occurs in two waves, the second wave is attributable, in part, to an epidermal growth factor-stimulated rise in intracellular calcium (1, 2). G protein-coupled receptor agonists initiate a greater intracellular calcium signal and more robust tyrosine phosphorylation (1, 3, 4). Purification and peptide sequencing of the responsible soluble tyrosine kinase revealed a novel sequence highly related to the focal adhesion tyrosine kinase, FAK[1] (5, 6). We named this second member of the cytoskeleton-associated tyrosine kinase family, the calcium-dependent tyrosine kinase (CADTK) to denote a principle mechanism of regulation. At the same time, four other groups identified this kinase and named it Pyk2 (7), CAKβ (8), RAFTK (9), and FAK2 (10). CADTK is activated by a wide variety of hormones and other G protein-coupled receptor agonists as well as pharmacological agents that raise intracellular calcium or activate protein kinase C (6, 7, 11–15). Additionally, growth factors (16–20), chemokines (21–24), cytokines (25, 26), cell stress signals (6, 27, 28), and in some instances cell adherence (23, 29–31), have all been shown to activate CADTK in some cell types. Once activated, CADTK has been implicated in the regulation of ion channels (7), extracellular signal regulated kinase (7, 12–14), c-Jun N-terminal kinase (JNK) (6, 18, 22, 25, 27, 28, 32), and p70 S6 kinase ($p70^{S6K}$) (33). It has also been reported that CADTK may play a role in Fyn-mediated T cell receptor (34, 35), Syk-mediated FcεRI receptor (36), and JAK3-mediated interleukin-2 receptor signaling pathways (26). CADTK physically associates with cytoskeletal proteins, such as paxillin and $p130^{Cas}$ (37–40), as well as their homologues Hic5 (41), leupaxin (42), and $p105^{HEF1}$ (37), and may directly regulate their tyrosine phosphorylation in response to agonists as diverse as growth factors and cell adherence (16, 23).

CADTK and FAK are highly homologous, sharing an overall 45% amino acid sequence identity with 60% identity in the catalytic domain. Several tyrosine residues appear to be conserved between CADTK and FAK, including a Src family tyrosine kinase SH2-binding site. Furthermore, CADTK, like FAK, contains proline-rich motifs capable of SH3 domain interaction and a putative focal adhesion targeting domain. Immunostaining of CADTK and expression of GFP-tagged CADTK revealed that CADTK localizes to the focal adhesion region. In cells expressing both FAK and CADTK, both proteins appear to co-localize near the focal adhesion membrane attachment sites, however, CADTK extends further into the cell, *e.g.* CADTK extends onto actin stress fibers in smooth muscle cells (16).[2]

Given the high degree of structural and amino acid sequence similarity, CADTK and FAK may well have some similar or even interchangeable biological functions. In fact, well studied mouse fibroblast cell lines appear to express only FAK and not CADTK. In contrast, we and others have recently shown that normal circulating monocytes, as well as T and B cells, express

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[1] The abbreviations used are: FAK, focal adhesion tyrosine kinase; CADTK, calcium-dependent tyrosine kinase; CRNK, calcium-dependent tyrosine kinase related non-kinase; FRNK, focal adhesion tyrosine kinase related non-kinase; kd, kinase deficient; PAGE, polyacrylamide gel electrophoresis; SH2, Src homology domain 2; GST, glutathione *S*-transferase.

[2] T. Harding, X. Li, L. Grave, and S. Earp, manuscript in preparation.

only CADTK (23, 24). Thus certain cells can function with only one of these two cytoskeleton-associated kinases. Conversely, since both proteins are often co-expressed in mesenchymal cells, multiple epithelial cells, neural cells and tissues, and endothelial cells (6, 7, 14, 16, 39, 43), they presumably have distinct functions as well. When expressed together the major difference between the two enzymes was easily detected, FAK is constitutively active in resting, adherent cells while CADTK is dephosphorylated and inactive until stimulated by agonists, *i.e*. CADTK regulation is more dynamic in the sense that it rapidly responds to extracellular signals.

In this report, we examined the tyrosine autophosphorylation and tyrosine kinase activity of CADTK and variants constructed by site-directed mutagenesis and compare them to previous structure/function data obtained with FAK. CADTK and FAK were tyrosine phosphorylated by Src family tyrosine kinases; the sites of CADTK are not necessarily those previously thought to be Src targets on FAK. Intriguingly, we showed that CADTK tyrosine phosphorylated FAK both *in vivo* and *in vitro* while FAK did not phosphorylate CADTK. Again, sites other than major autophosphorylation or putative Src target site appeared to be involved. Last, by transiently co-expressing the carboxyl terminus of CADTK (CRNK) and the carboxyl terminus of FAK (FRNK) with wild type CADTK or FAK, we demonstrated that FRNK inhibited both FAK and CADTK tyrosine autophosphorylation, while CRNK inhibited only CADTK autophosphorylation. Our results demonstrate that although CADTK and FAK are regulated differentially in cells expressing both proteins, they may, additionally, influence signal transduction from the other family member.

EXPERIMENTAL PROCEDURES

Materials—Yes and Y527F Src were kindly provided by Drs. Nancy Rabb-Traub, and Channing Der (University of North Carolina at Chapel Hill), respectively. Fyn, Lck, Syk, and Zap70 were generously provided by Dr. Andre Veillette (McGrill University). Polyclonal anti-CADTK antibody was described previously (6), anti-HA monoclonal antibody and anti-FAK (C-20, A-17) were purchased from Boehringer Mannheim and Santa Cruz Biotechnology, respectively. Human 293(T) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum as described previously (39).

Site-directed Mutagenesis—Rat wtCADTK cDNA was used as a template to generate a series of CADTK mutants based on polymerase chain reaction site-directed mutagenesis strategy (Stratagene). Mutated CADTK cDNAs were amplified by Pfu DNA polymerase (Stratagene) with complementary DNA mutagenic oligonucleotides for designed mutations (K457A, D567N, Y402F, Y881F, K457A/Y402F, Y579F/Y580F CADTK). The human FAK cDNA was used to make the mutants Y397F, Y576F/Y577F, and Y925F FAK by the same method. All the mutants were confirmed by DNA sequence analysis (University of North Carolina, Sequence Facility).

Cell Lysate Preparation—Cell lysates were prepared essentially as described previously (5). Briefly, cells treated with agonists were scraped into ice-cold cell lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM sodium fluoride, and 10%(v/v) glycerol with freshly added 1 mM Na_3VO_4, 20 μg/ml phenymethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin/ml). Cell lysates were clarified by centrifugation and their protein content determined by Coomassie protein assay reagent (Pierce).

cDNA Transient Expression in Human 293(T) Cells—pcDNA3 vector (Invitrogen), pcDNA3-CADTK, pcDNA3-wtCADTK, pcDNA3-FAK, pCMV-FRNK, pcDNA3-Yes, PXM139-Fyn, Lck, Syk, and ZAP70 were transfected or co-transfected into human 293(T) cells with Fugene6 according to the manufacturer's procedure (Boehringer Mannheim). After 48 h, transfected cells were harvested and lysed, and the lysates were analyzed by immunoprecipitation, followed by immunoblotting with anti-Tyr(P), anti-CADTK, anti-FAK, or by a tyrosine kinase activity assay.

Immunoprecipitation and Immunoblotting—In a typical experiment, ~500 μg of cell lysate was immunoprecipitated by incubation with the antibody for 2 h at 4 °C. 20 μl of protein A-agarose beads were then added for 1 more hour. Immune complexes were collected by centrifugation, washed three times with lysis buffer, and resuspended in SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE, transferred to Immobilon (Millipore), and incubated with the selected antibody. Immunoblots were developed with ECL according to the manufacturer's procedure (Amersham). Immunoblots were stripped in buffer (62.5 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol) at 50 °C for 30 min and reprobed with another antibody.

Tyrosine Kinase Activity Assay—Immune complex tyrosine kinase and autokinase assays were performed as previously reported (3). Briefly, immune complex suspensions were preincubated for 5 min at 4 °C with 160 μg of the synthetic tyrosine kinase substrate poly(Glu_4:Tyr) (Sigma) or the control substrate poly(Glu). Additional experiments were performed using a GST NH_2 terminus of human FAK fusion protein encompassing amino acids 1 to 426 (5 μg per assay) as a substrate. Reactions (80 μl of total reaction volume) were initiated by adding of 5 μCi of [γ-^{32}P]ATP (5 μM) After 15 min at 25 °C, 50 μl of the reaction mixture was spotted on P81 Whatman paper. The papers were washed once with 10 mM sodium pyrophosphate in 10% trichloroacetic acid and twice with 5% trichloroacetic acid, air-dried, and assayed by liquid scintillation counting. For GST NH_2-terminal FAK phosphorylation, the assays were stopped with SDS stop solution, run on 8% SDS-polyacrylamide gels, and subjected to autoradiography. Assessment of the ability of CADTK and FAK to directly cross-phosphorylate was performed using an *in vitro* tyrosine phosphorylation assay. Briefly, CADTK-GFP, kdCADTK, kdFAK, and control vector were transiently expressed in 293(T) cells, independently. Equal amounts of cell lysates were mixed before adding specific antibodies. Cell lysate mixtures were incubated at 4 °C for 2 h followed by addition of protein A/G-agarose beads. Immunocomplexes were washed three times with lysis buffer and once with kinase assay buffer before providing [γ-^{32}P]ATP. Reaction mixtures were incubated at 25 °C for 15 min. After removing the supernatant, SDS-PAGE sample loading buffer was added to the immunocomplexes and they were subjected to SDS-PAGE and autoradiography. These experiments were repeated using wild type CADTK and separating phosphorylated CADTK (p115) and FAK (p125) on 8% low bis-acrylamide gels and by immunoprecipitating both CADTK-GFP and kdFAK, using buffers with 0.1% SDS in the washes.

RESULTS

Site-directed Mutagenesis: Analysis of Tyrosine Autophosphorylation and Tyrosine Kinase Activity of CADTK Mutants—Tyr^{397} is a major FAK tyrosine autophosphorylation site (44). Mutation of this site dramatically inhibited FAK tyrosine autophosphorylation and decreased tyrosine kinase activity by 50% (45). Autophosphorylated Tyr^{397} serves as an SH2 domain docking site for the recruitment of Src family tyrosine kinases (44, 46–48), which in turn may tyrosine phosphorylate FAK on Tyr^{925} (49) or other tyrosine residues (45). Guided by amino acid sequence similarity to FAK, we mutated homologous CADTK tyrosine residues to investigate their effects on CADTK tyrosine phosphorylation and kinase activity. In addition, to create "kinase deficient" CADTK, we separately mutated two CADTK residues important in Mg^{2+}-ATP binding, Lys^{457} and Asp^{567}, changing them to Ala and Asn, respectively. For comparison, we also made similar mutations in the human FAK cDNA.

Both wild type and mutant cDNAs were transiently expressed in 293(T) cells and their tyrosine autophosphorylation and kinase activity was assessed. As shown in Fig. 1*A*, mutation of the CADTK Mg^{2+}-ATP-binding site residues (K457A or D567N) abolished tyrosine kinase activity as assessed by poly(Glu_4:Tyr) phosphorylation as well as CADTK tyrosine autophosphorylation (Fig. 1*B*), suggesting that these two residues are each required for CADTK kinase activity. Mutation of Tyr^{402} (corresponding to the Src family binding site, Tyr^{397}) also abolished CADTK tyrosine autophosphorylation, severely depressing but not completely inhibiting tyrosine kinase activity (~70% decrease). These data suggest that Tyr^{402}, like Tyr^{397} in FAK, is the major tyrosine autophosphorylation site. Its phosphorylation is important but not absolutely required for tyrosine kinase activity. Mutation of adjacent tyrosine residues Tyr^{579}/Tyr^{580} in the catalytic domain of CADTK (corre-

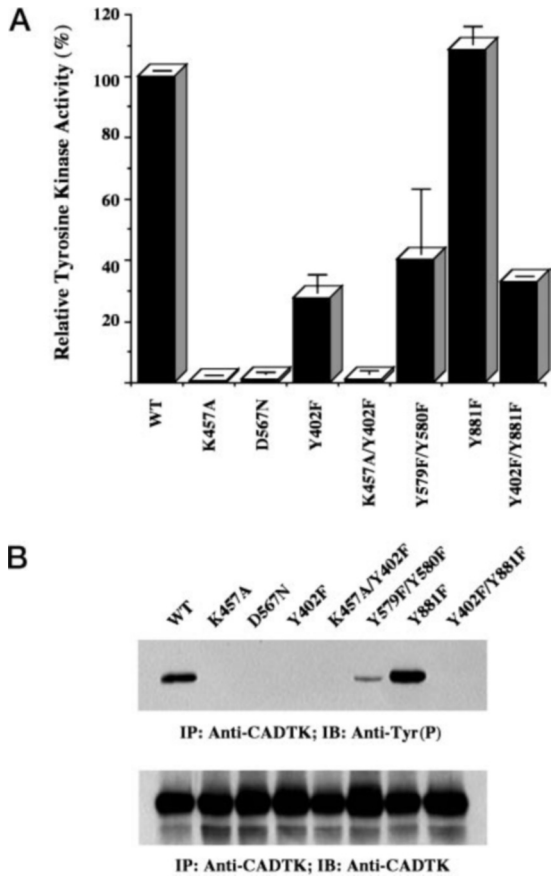

FIG. 1. **Tyrosine kinase activity and autophosphorylation of CADTK and its mutants.** Wild type CADTK and its mutants were independently transfected into 293(T) cells. After 48 h, each was immunoprecipitated with anti-CADTK antibody. *A,* tyrosine kinase activity assays were performed using poly(Glu_4:Tyr) as a substrate and measuring ^{32}P incorporation as described under "Experimental Procedures." *B,* immunoprecipitated samples were subjected to SDS-PAGE and immunoblotted with anti-Tyr(P) antibody. The blot was then stripped and reblotted with anti-CADTK antibody. These data are representative of three independent experiments.

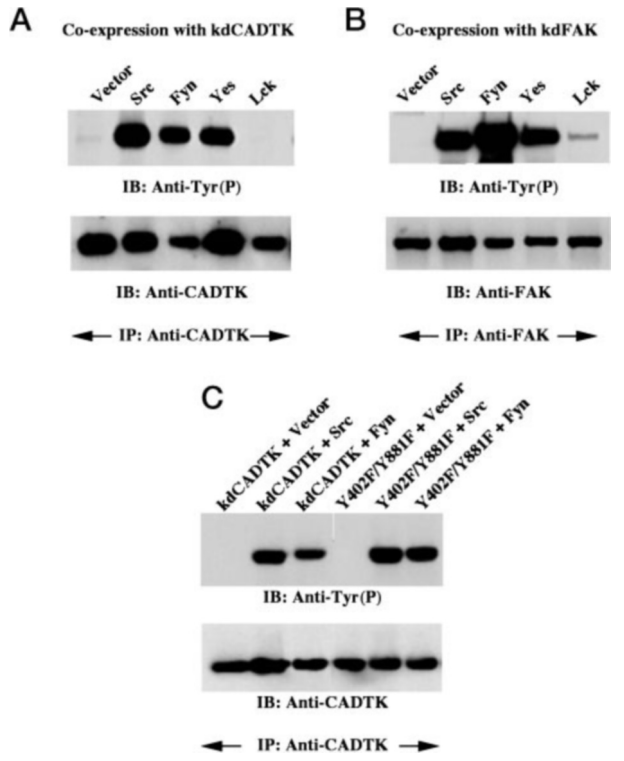

FIG. 2. **Src family tyrosine kinases phosphorylate both CADTK and FAK *in vivo*.** Src family tyrosine kinases (Src, Fyn, Yes, and Lck) were co-transfected with kinase-deficient CADTK (*kdCADTK*) or kinase-deficient FAK (*kdFAK*), respectively. kdCADTK (*A*) and kdFAK (*B*) were immunoprecipitated, subjected to SDS-PAGE, and immunoblotted with an anti-Tyr(P) antibody. Blots were stripped and reblotted with anti-CADTK and anti-FAK antibodies, respectively. *C,* kdCADTK or Y402F/Y881F CADTK were co-expressed with Src or Fyn in 293(T) cells. CADTK was immunoprecipitated with anti-CADTK antibody and subjected to anti-Tyr(P) followed by anti-CADTK immunoblotting.

sponding to FAK Tyr^{576}/Tyr^{577}) significantly decreased CADTK tyrosine autophosphorylation and reduced kinase activity by ~60%. These data, taken together with the Y402F mutant data, suggest that Tyr^{579}/Tyr^{580} are not major tyrosine autophosphorylation sites but that transient tyrosine phosphorylation of these sites may be required for full or continued CADTK activation.

Mutation of Tyr^{881} (corresponding to FAK Tyr^{925}, the site of Grb2 SH2 domain association) had little or no effect on CADTK tyrosine autophosphorylation or tyrosine kinase activity. Thus, autophosphorylation appears to involve Tyr^{402} but not Tyr^{881}. Finally, double mutants, K457A/Y402F and Y402F/Y881F, produced the same effect as the single mutants, K457A and Y402F, respectively, suggesting that the single mutation of K457A and Y402F produced the dominant effect. CADTK tyrosine kinase activity and tyrosine autophosphorylation data were generally concordant, indicating that tyrosine phosphorylation correlates with kinase activity. Comparison of the effects of corresponding CADTK and FAK mutations (45), suggests that the structure-function regulatory mechanisms of these two tyrosine kinases are similar.

To further examine the regulation of these two kinases, the kinase-deficient CADTK (kdCADTK), K457A CADTK, and kinase-deficient FAK (kdFAK) K454A FAK were transiently co-expressed with the Src family tyrosine kinase members, Src, Fyn, Yes, and Lck. As shown in Fig. 2, Src, Fyn, and Yes dramatically increased CADTK (Fig. 2*A*) and FAK (Fig. 2*B*) tyrosine phosphorylation, while Lck had little effect (particularly on CADTK). These data suggest that CADTK and FAK signaling may be similarly affected by Src family tyrosine kinases, presumably by phosphorylation of conserved tyrosine residues. However, Src and Fyn were also capable of substantially phosphorylating the Y402F/Y881F CADTK double mutant (Fig. 2*C*), suggesting that other tyrosine residue(s) must be significant targets for Src family tyrosine kinases. Moreover, at least when co-expressed, neither activated Src, wild type Fyn, nor Yes required a Tyr^{402} docking site on CADTK to tyrosine phosphorylate CADTK. Last, transient co-expression of two important hematopoietic cytoplasmic tyrosine kinases, Syk or ZAP70, with kdCADTK did not increase their tyrosine phosphorylation (data not shown), suggesting that Syk and ZAP70 cannot directly phosphorylate CADTK under these experimental conditions.

CADTK Tyrosine Phosphorylates FAK in Vivo and in Vitro—Receptor tyrosine kinases from the epidermal growth factor receptor family form heterodimers and cross-phosphorylate each other (50). To test whether CADTK and FAK can also associate and cross-phosphorylate, kdCADTK and kdFAK were transiently co-expressed in human 293(T) cells with wild type FAK or CADTK, respectively. Immunoprecipitation with specific antibodies followed by phosphotyrosine immunoblotting showed that CADTK significantly increased kdFAK tyrosine phosphorylation (Fig. 3*A*). Similar results were obtained with co-expression of either wtCADTK or CADTK-GFP (a fusion protein with a green fluorescence protein fused to the CADTK carboxyl terminus) and kdFAK (Fig. 3*A*). In contrast, FAK

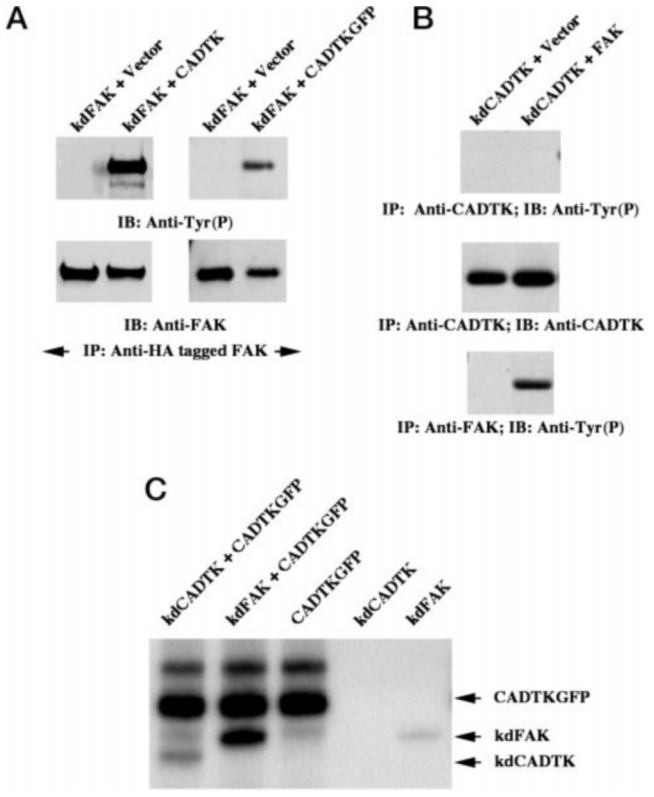

FIG. 3. **CADTK phosphorylated FAK *in vivo* and *in vitro*, but not *vice versa*.** *A,* CADTK or CADTK-GFP were co-expressed with kdFAK in 293(T) cells. kdFAK was immunoprecipitated with anti-FAK antibody. Immunocomplexes were subjected to SDS-PAGE and immunoblotted with anti-Tyr(P) antibody. The blot was stripped and reblotted with anti-FAK antibody. *B,* wtFAK were co-transfected with kdCADTK in 293(T) cells. kdCADTK was immunoprecipitated with anti-CADTK antibody and analyzed for anti-Tyr(P) and anti-CADTK. The samples were also immunoprecipitated with anti-FAK antibody and blotted with anti-Tyr(P) antibody, showing that FAK was tyrosine phosphorylated by co-transfection. *C,* CADTK-GFP, kdFAK, kdCADTK, and pcDNA3 vector were transfected into 293(T) cells, respectively. After lysis, equal amounts of cell lysates were mixed as indicated, and immunoprecipitated with NH_2-terminal anti-CADTK and anti-FAK antibodies. After washing, *in vitro* immunocomplex kinase assays were performed labeling with [γ-^{32}P]ATP. Phosphotyrosine was analyzed by SDS-PAGE, followed by autoradiography.

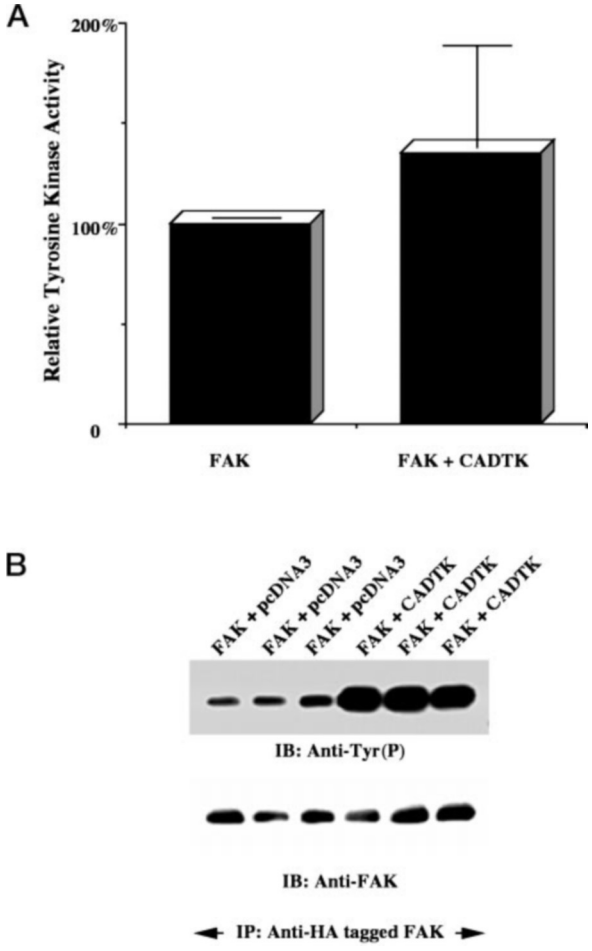

FIG. 4. **CADTK-dependent stimulation of FAK tyrosine kinase activity and phosphorylation.** In three separate transfections, wt-FAK was co-expressed with vector or CADTK in 293(T) cells. FAK was immunoprecipitated with anti-FAK antibody. *A,* FAK kinase activity was measured by using poly(Glu_4:Tyr) as a substrate as described under "Experimental Procedures." *B,* the immunocomplexes were subjected to SDS-PAGE and blotted with anti-Tyr(P) antibody, then stripped and reblotted with anti-FAK antibody.

failed to phosphorylate kdCADTK (Fig. 3*B*). Interestingly, we could not detect physical association between these two tyrosine kinases (data not shown), *i.e.* immunoprecipitation (even using reduced ionic strength buffer) of either enzyme from the dual transfected cells did not reveal the other protein. Under the same conditions we have easily detected complexes of CADTK and cytoskeletal proteins or other SH3 containing proteins (39). Therefore, CADTK results in tyrosine phosphorylation of kdFAK without forming lasting CADTK:FAK heterodimers.

Since the co-expression of CADTK could have activated an endogenous third tyrosine kinase resulting in kdFAK phosphorylation, we investigated CADTK:FAK cross-phosphorylation *in vitro*. To separate CADTK and FAK on SDS-PAGE, we used CADTK-GFP, which has a molecular mass of ~145 kDa, and similar tyrosine autophosphorylation and kinase activity when compared with wtCADTK (data not shown). Transient expression of CADTK-GFP, kdFAK, kdCADTK, and control vector were performed individually in 293(T) cells. Equal amounts of cell lysate from the indicated pair of transfected cells were combined and incubated with two antibodies, one directed against the NH_2 terminus of CADTK and one against the NH_2 terminus of FAK. Subsequently, protein A/G-agarose beads were added and the indicated pairs of transfected proteins or transfected protein and control were precipitated, washed, and *in vitro* phosphorylation was performed using [γ-^{32}P]ATP. Following SDS-PAGE, autoradiography showed that CADTK-GFP facilitated the incorporation of ^{32}P into kdFAK, suggesting direct *in vitro* phosphorylation of FAK by CADTK-GFP (Fig. 3*C*). Interestingly, CADTK-GFP only weakly phosphorylated kdCADTK *in vitro*, suggesting that, at least for intermolecular phosphorylation, FAK may be a preferred substrate. Thus, CADTK can tyrosine phosphorylate FAK both *in vivo* and *in vitro* suggesting that agonist-dependent (*e.g.* angiotensin II, lysophosphatidic acid, etc.) increases in FAK phosphorylation in cells expressing and co-localizing both enzymes may be attributed, in part, to CADTK-dependent FAK tyrosine phosphorylation.

We next examined whether CADTK-dependent FAK tyrosine phosphorylation resulted in increased FAK tyrosine kinase activity as measured by phosphorylation of poly(Glu_4:Tyr). First, co-expression of FAK and CADTK followed by immunoprecipitation of FAK showed that CADTK can increase tyrosine phosphorylation of wtFAK (Fig. 4*B*) in addition to its phosphorylation of kdFAK (Fig. 3). Second, assessment of *in vitro* kinase activity showed an increase in FAK tyrosine kinase activity (30–40%) (Fig. 4*A*). This was clearly less than the ~5–10-fold CADTK-dependent increase in FAK tyrosine phos-

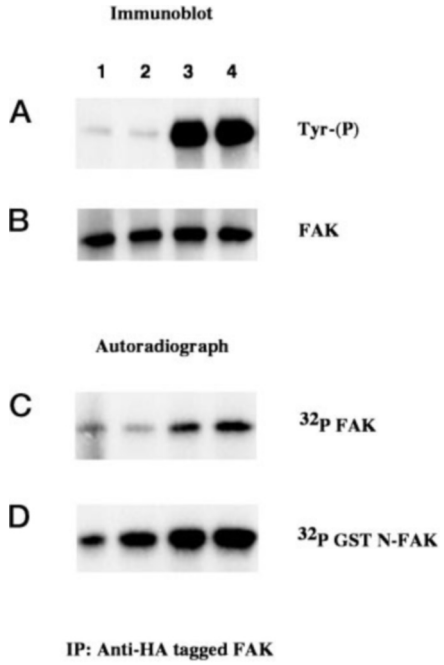

FIG. 5. **CADTK-dependent phosphorylation and FAK autokinase activity.** Following duplicate transfections (vector and wtFAK (*lanes 1* and *2*) and CADTK and wtFAK (*lanes 3* and *4*)), FAK was immunoprecipitated and the immune complex analyzed. *A,* phosphotyrosine immunoblotting demonstrated substantial CADTK-dependent FAK tyrosine phosphorylation. *B,* similar amounts of FAK were immunoprecipitated in each sample as demonstrated by stripping and reprobing with anti-FAK (C20) antibody. *C,* another portion of each immune complex was incubated with [γ-^{32}P]ATP and GST NH_2-terminal FAK, (1–423) as described under "Experimental Procedures. The autoradiograph shows FAK autophosphorylation was increased slightly by a CADTK-dependent action. *D,* FAK kinase activity toward an exogenous substrate, GST NH_2-terminal FAK was also minimally increased. FAK parallel FAK-dependent poly(Glu_4:Tyr) phosphorylation by these immune complexes showed an average of 14,366 cpm from samples 1 and 2 and 19,964 cpm from samples 3 and 4.

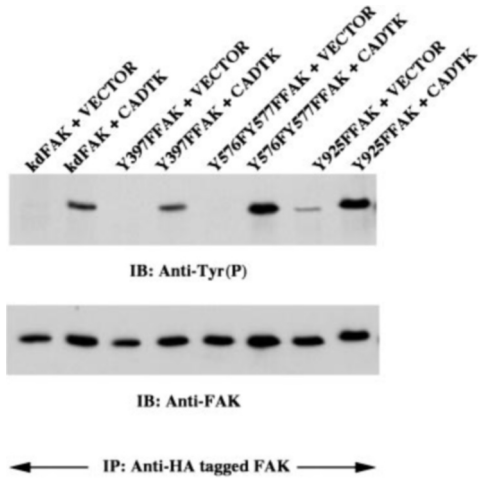

FIG. 6. **CADTK phosphorylated FAK on tyrosine phosphorylation sites other than Tyr^{397}, Tyr^{576}/Tyr^{577}, and Tyr^{925}.** Several mutant FAK constructs were co-transfected with CADTK or pcDNA3 vector in 293(T) cells as indicated. FAK was immunoprecipitated and subjected to SDS-PAGE. After transfer and immunoblotting with anti-Tyr(P), the blot was stripped and reprobed with anti-FAK antibody.

phorylation (Fig. 4*B*). This result (Fig. 4) obtained with triplicate, independent transfections was typical of multiple experiments in which the increase in FAK tyrosine kinase activity was much less than the increase in CADTK-dependent FAK tyrosine phosphorylation. Since poly(Glu_4:Tyr) is not a a physiologic substrate, the experiment, was repeated, using a GST FAK NH_2-terminal fusion protein that included Tyr^{397} as a substrate for wild type FAK precipitated from vector or CADTK transfected cells. The CADTK-dependent tyrosine phosphorylation of FAK in co-transfected cells was again increased dramatically (Fig. 5*A*), but autokinase activity (^{32}P incorporation into FAK *in vitro*, Fig. 5*C*) and substrate phosphorylation (^{32}P incorporation into GST NH_2-terminal FAK, Fig. 5*D*) was only increased ~50–100%. Thus using 2 substrates, poly(Glu_4:Tyr) and GST NH_2-terminal FAK, CADTK-dependent FAK phosphorylation is greater than the activation of FAK kinase. Because the kinase activity of FAK in adherent cells has seldom been reported to change dramatically, it is unclear what an ~50% change in kinase activity means. However, the substantial increase in kdFAK or wtFAK tyrosine phosphorylation upon co-transfection suggests that CADTK may indeed regulate FAK tyrosine phosphorylation *in vivo*. Because FAK is in part a structural protein whose function is to localize SH2-containing domain proteins, increased FAK tyrosine phosphorylation may have physiologic consequences.

We investigated the potential CADTK-dependent tyrosine phosphorylation sites on FAK by co-transfecting wtCADTK and FAK mutants, kdFAK, Y397F, Y576F/Y577F, and Y925F FAK in 293(T) cells (Fig. 6). Transiently expressed CADTK phosphorylated all FAK mutants *in vivo* suggesting CADTK-dependent FAK tyrosine phosphorylation may occur on other sites in addition to the well studied tyrosine residues. Taken together, these data suggest that CADTK may potentially function as an upstream regulator of FAK by directly phosphorylating FAK, slightly increasing its tyrosine kinase activity, and generating more or even new sites for the recruitment of SH2 containing proteins.

Overexpression of FRNK Inhibited FAK and CADTK Tyrosine Autophosphorylation while Overexpression of CRNK Blocked Only CADTK Tyrosine Phosphorylation—Alternative RNA splicing of the FAK primary transcript can yield a 43-kDa species encoding the carboxyl terminus of FAK initiated just beyond the tyrosine kinase domain (51). Overexpression of this protein, termed FRNK (FAK-related non-kinase), inhibits FAK activity, presumably by displacing FAK (52) and in some manner blocking the formation of focal adhesions on fibronectin (53, 54). FRNK probably acts by competing for FAK binding partners through FRNK's focal adhesion targeting domain, the sequence both necessary and sufficient for FAK recruitment to the focal adhesion (55). CADTK shares 60% homology with FAK in the carboxyl terminus. While not yet detected as an expressed protein, CADTK has a methionine residue as a potential start site for an alternatively spliced product analogous to FRNK. This has been termed CRNK (CADTK or CAKβ-related non-kinase, pronounced "crank") by our colleague, Michael Schaller (56). Since CADTK activation, like FAK, requires an intact engaged cytoskeleton (23, 39), we created a CRNK construct in pcDNA3 to investigate whether CRNK overexpression inhibited CADTK tyrosine autophosphorylation. When co-expressed CRNK blocked CADTK tyrosine autophosphorylation (Fig. 7*A*); *i.e.* CRNK functions similarly to FRNK and negatively regulates CADTK autophosphorylation. In contrast, co-expression of FAK and CRNK did not alter FAK tyrosine autophosphorylation (Fig. 7*B*), suggesting that under these conditions of co-expression of CRNK, inhibition was specific for CADTK, presumably because its sequence is not similar enough to FRNK to displace FAK. As expected, FRNK inhibited FAK activity when co-expressed (Fig. 7*B*) but, unexpectedly, FRNK co-expression dramatically inhibited CADTK tyrosine autophosphorylation (Fig. 7*A*). Thus, FRNK regulates both CADTK and FAK activation under these conditions; however, the mechanisms by which FRNK acts may not be the

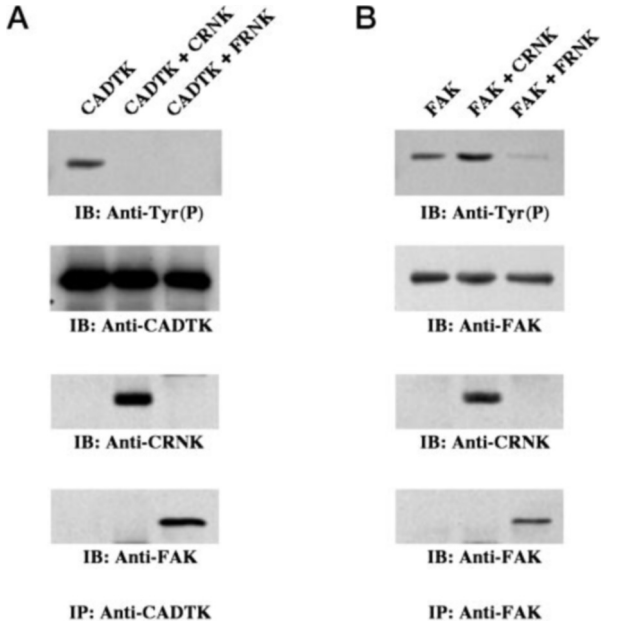

FIG. 7. **Overexpression of CRNK only inhibited CADTK autophosphorylation, while overexpression of FRNK inhibited both CADTK and FAK tyrosine phosphorylation.** wtCADTK or FAK were co-expressed with CRNK, FRNK, or pcDNA3 vector in 293(T) cells, respectively. *A,* CADTK was immunoprecipitated with anti-CADTK antibody and analyzed with anti-Tyr(P) and anti-CADTK antibodies to determine CADTK autophosphorylation, CADTK and CRNK expression. Samples were also analyzed for FRNK expression. *B,* FAK was immunoprecipitated with anti-FAK antibody, and its phosphorylation and expression level was analyzed using anti-Tyr(P) and anti-FAK antibodies.

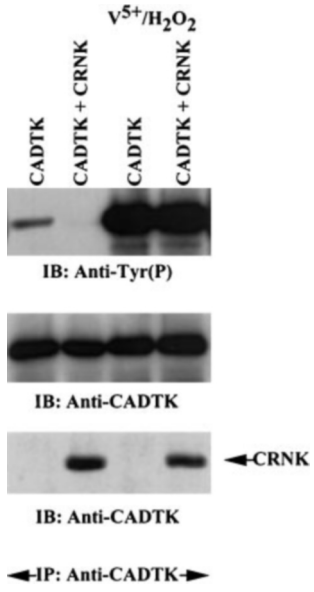

FIG. 8. **CADTK activation by pervanadate pretreatment is not inhibited by CRNK.** wtCADTK was co-expressed with vector or CRNK in 293(T) cells. 48 h after transfection, cells were treated with or without pervanadate (100 mM H_2O_2/Na_3VO_4) for 5 min. Cell lysates were prepared and immunoprecipitated with anti-CADTK antibody. Immunocomplexes were analyzed with anti-Tyr(P) and anti-CADTK antibodies.

same for the two cytoskeleton-associated kinases.

The specific mechanisms of CADTK or FAK activation remain elusive. In resting, adherent cells, FAK is constitutively activated by the process of adherence, while CADTK is "off" in resting adherent cells, but is rapidly activated by agonists. Transient overexpression of CADTK in 293(T) and other cells using a strong promoter results in CADTK activation in resting cells without the addition of an agonist. This presumably occurs by one of several mechanisms potentially including: (i) overexpression-dependent movement of CADTK into an activating binding site or (ii) by overcoming a saturable negative regulation system such as a constitutively activated tyrosine phosphatase that removes CADTK activating tyrosine phosphorylation. To investigate this, we co-expressed CADTK and vector or CADTK and CRNK and pretreated cells with pervanadate (5 min), a technique known to inhibit most, if not all, tyrosine phosphatases. Treatment with pervanadate resulted in a substantial increase (10–20-fold) in CADTK tyrosine phosphorylation (Fig. 8), indicating that there is a vast pool of underphosphorylated CADTK in the transfected cells. CRNK, as previously shown, inhibited the autophosphorylation of CADTK stimulated by overexpression but did not significantly depress CADTK phosphorylation in cells pretreated with pervanadate. That CRNK does not block activation by pervanadate suggests that at least two mechanisms of CADTK activation, one dependent on cellular binding and localization (this "site" can be accessed by transient CADTK overexpression and blocked by CRNK co-expression) and another mechanism dependent on suppression of phosphatase activity. The latter is not detectably influenced by CRNK.

DISCUSSION

The discovery and isolation of CADTK, the second member of the FAK family of tyrosine kinases, raised questions about how this kinase is regulated in comparison to FAK, and whether the two family members interact as do some receptor tyrosine kinase family members. In this report, we show that, 1) the structure-function relations governing CADTK tyrosine autophosphorylation and kinase activity are similar to those of FAK, at least superficially (Fig. 1); 2) CADTK and FAK are both direct targets of Src family tyrosine kinases; and CADTK contains Src-phosphorylated tyrosine residue(s) in addition to the Tyr^{881}, identified as a FAK Tyr^{925} equivalent (Fig. 2); 3) CADTK directly cross-phosphorylates FAK slightly increasing FAK tyrosine kinase activity and perhaps creating novel phosphotyrosine sites as targets for SH2 group-containing proteins (Figs. 3–6); 4) FAK does not cross-phosphorylate CADTK under the conditions tested (Fig. 3B); 5) the carboxyl terminus of CADTK (CRNK) can inhibit CADTK but not FAK tyrosine autophosphorylation, presumably by displacing CADTK from its cytoskeleton-binding sites (Fig. 7*A*); and 6) overexpression of FRNK abolishes both FAK and CADTK tyrosine autophosphorylation (Fig. 7*B*).

The major CADTK tyrosine autophosphorylation site is Tyr^{402}; mutation of this site abolished CADTK tyrosine autophosphorylation and substantially inhibited its kinase activity (Fig. 1). The role of Tyr^{402} phosphorylation in full activation of the kinase may result from two (or more) potential mechanisms. First, tyrosine autophosphorylation of Tyr^{402} may modify CADTK structure by opening or keeping the catalytic cleft open thereby promoting substrate access. Second, phospho-Tyr^{402} may recruit Src family tyrosine kinases to further increase CADTK kinase activity. We doubt that these results (the difference between wtCADTK and Y402F CADTK) are confounded by associated Src family tyrosine kinases (or other co-precipitated endogenous kinases) because these experiments have been repeated using RIPA buffer containing SDS, which reverses the association with other molecules, *e.g.* Src family tyrosine kinases. We attempted to test the first hypothesis by introducing a permanent negative charge at this site by mutating Tyr^{402} to aspartic acid (Y402D). As expected, the Y402D mutation was not autophosphorylated, but the reduction of CADTK immunocomplex kinase activity was similar to

that of the Y402F mutant (data not shown). Whether this is an adequate test of this hypothesis is unclear. The negatively charged aspartate which has been shown to mimic activating phosphoserines in some kinases (*e.g.* MEK) (57) may not be spatially equivalent to the negative charge provided by phosphate on the tyrosyl ring.

In addition to Tyr^{402}, full activation of CADTK requires tyrosine phosphorylation of two other tyrosine residues (Tyr^{579} and Tyr^{580}) in the catalytic domain. Mutation of these two residues decreased CADTK tyrosine autophosphorylation and kinase activity by 60%. Unlike the Y402F mutation, the Y579F/Y580F CADTK mutant exhibited partial tyrosine autophosphorylation when overexpressed, suggesting that the residual Y579F/Y580F CADTK kinase activity autophosphorylates Tyr^{402}. Surprisingly, there is no Tyr^{579}/Tyr^{589} tyrosine phosphorylation in the Y402F mutant despite detectable tyrosine kinase activity (Fig. 1*A*). Tyrosines 579 and 580 lie within the catalytic domain at a position that is conserved among many but not all protein tyrosine kinase families (58). In some instances, phosphorylation of these residues is obligatory for tyrosine kinase activation (*e.g.* insulin receptor) (59, 60). As noted above, it has been proposed that tyrosine phosphorylation of FAK Tyr^{397} provides an SH2-binding site for Src family tyrosine kinases (44, 46–48) which in turn phosphorylate Tyr^{576} and Tyr^{577} and further increase tyrosine kinase activity (45). While CADTK Y579F/Y580F does have slightly higher autophosphorylation and kinase activity than Y402F, it seems unlikely that the difference is due to endogenous Src/Fyn activation of overexpressed CADTK. It seems more likely that both Tyr^{402} and Tyr^{579}/Tyr^{580} tyrosine phosphorylation are independently important for opening and/or maintaining the active conformation of the kinase. Construction and assessment of a $Tyr^{402}/Tyr^{579}/Tyr^{580}$ triple mutant will help to resolve this issue.

CADTK phosphorylates FAK but does not form heterodimers *in vivo*. These data suggest that CADTK may be an agonist-dependent regulator of FAK. Agonist stimulation in most cells slightly increases FAK tyrosine phosphorylation (by ~50% in rat liver epithelial and smooth muscle cells) (5, 16, 39). In addition, agonist-dependent tyrosine phosphorylation of paxillin, tensin, and $p130^{Cas}$) is better correlated with CADTK than FAK activation (39). The increase in FAK phosphorylation complicates the analysis of which enzyme is responsible for agonist-dependent paxillin tyrosine phosphorylation. It may even be that increased CADTK (Tyr^{402}) and FAK (Tyr^{397}) phosphorylation induces Src activation which is primarily responsible for agonist-dependent cytoskeletal protein phosphorylation. CADTK can phosphorylate FAK on sites in addition to Tyr^{397} and Tyr^{925} (Fig. 6) raising the possibility that new docking sites for SH2 containing proteins are generated on FAK. The increase in CADTK-dependent FAK phosphorylation is clearly greater than its effect on FAK kinase activity. In summary, agonist-dependent CADTK activation may alter FAK activity and function, adding more complexity to cytoskeleton regulation in cells expressing both enzymes. While, we cannot rule out that CADTK-dependent FAK tyrosine phosphorylation *in vivo* may be due to another tyrosine kinase, it is a direct effect *in vitro* in an immune complex. In addition, the fact that autophosphorylated wtFAK, which associates with Src family tyrosine kinases, fails to induce CADTK tyrosine phosphorylation *in vivo*, suggests, that the role of Src family tyrosine kinases in CADTK and FAK cross-phosphorylation is limited.

Like FAK, CADTK tyrosine phosphorylation can be regulated by its CRNK (Fig. 7*A*). Neither finding (FRNK inhibiting FAK or CRNK inhibiting CADTK) can explain the physiological mechanism of activation by adhesion or agonists, but these results suggest that access to a specific binding site is crucial for activation. While CRNK only inhibited CADTK autophosphorylation, FRNK inhibits both FAK and CADTK autophosphorylation. This initially suggested that the FRNK sequence is better able to displace both FAK and CADTK from crucial sites. However, we previously showed that cytoskeleton engagement is necessary for agonist-dependent CADTK activation; *i.e.* CADTK is not activated in suspended monocytes, rat liver epithelial, or smooth muscle cells (23). Thus, FRNK could influence CADTK through its established action, displacing FAK from focal contacts. This might change focal contact function or structure, preventing CADTK activation by inhibiting a crucial "permissive step." CADTK and CRNK have a higher affinity for paxillin than does FAK (39); talin, which physically associates with FAK (61), does not interact with CADTK (62). This reinforces a model in which CRNK and FRNK, which have different target affinities, may act differently to regulate CADTK. Said simply, CRNK may displace CADTK from its site of activation, while FRNK may displace FAK, making the cells behave as if they were in suspension.

It is unclear why overexpression of CADTK results in its activation, but it does so in 293(T) (Fig. 8), as well as in HeLa, MCF10, and NIH3T3 cells (data not shown). Our best explanation is that overexpressed CADTK molecules access activation sites or effectors that overcome the physiologic proclivity to be "off" in resting adherent cells. Substantial additional CADTK activation by phosphatase inhibition (pervanadate) demonstrates that only a small proportion of CADTK is activated by overexpression. A large inactive pool of overexpressed CADTK, perhaps located in the cytoplasm, can be activated by pervanadate (Fig. 8). The small pool of activated CADTK can be completely inhibited by CRNK, presumably by displacement from activating protein-protein interactions. Although we cannot rule out a mechanism by which CRNK activates a CADTK-specific phosphatase, we favor the above explanation.

In summary, our results suggest that CADTK and FAK, at some level, participate in "cross-talk." CADTK directly phosphorylates FAK, possibly generating more SH2 docking sites. This may enhance physiologic control mechanisms in response to agonists in some cells. In turn, FAK may be necessary for CADTK activation in some adherent cells. This requirement cannot be absolute since FAK is not present when adherence activates CADTK in monocytes and the T cell receptor activates CADTK in T cells. Nevertheless, epithelial, neural, smooth muscle, and endothelial cells, may use these two mechanisms of CADTK-FAK interaction to orchestrate cytoskeleton regulation of morphology and signaling in response to agonist stimulation and extracellular matrix.

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