The N Termini of Focal Adhesion Kinase Family Members Regulate Substrate Phosphorylation, Localization, and Cell Morphology*

Jill M. Dunty and Michael D. Schaller‡

From the Department of Cell and Developmental Biology, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina 27599

The focal adhesion kinase (FAK) and cell adhesion kinase β (CAKβ, PYK2, CADTK, RAFTK) are highly homologous FAK family members, yet clearly have unique roles in the cell. Comparative analyses of FAK and CAKβ have revealed intriguing differences in their activities. These differences were investigated further through the characterization of a set of FAK/CAKβ chimeric kinases. CAKβ exhibited greater catalytic activity than FAK *in vitro*, providing a molecular basis for differential substrate phosphorylation by FAK and CAKβ *in vivo*. Furthermore, the N terminus may regulate catalytic activity since chimeras containing the FAK N terminus and CAKβ catalytic domain exhibited a striking high level of catalytic activity and substrate phosphorylation. Unexpectedly, a modulatory role for the N termini in subcellular localization was also revealed. Chimeras containing the FAK N terminus and CAKβ C terminus localized to focal adhesions, whereas chimeras containing the N and C termini of CAKβ did not. Finally, prominent changes in cell morphology were induced upon expression of chimeras containing the CAKβ N terminus, which were not associated with apoptotic cell death, cell cycle progression delay, or changes in Rho activity. These results demonstrate novel regulatory roles for the N terminus of FAK family kinases.

Focal adhesion kinase (FAK)[1] and cell adhesion kinase β (CAKβ, also known as PYK2, CADTK, or RAFTK) constitute the FAK family of cytoplasmic tyrosine kinases. The structural features of this family include large N- and C-terminal domains that flank a central tyrosine kinase domain (1). The sequences of FAK and CAKβ are 45% identical and 65% similar. The greatest homology exists between the catalytic domains (60% identity) and the extreme C termini, which corresponds to the region of FAK that directs subcellular localization (62% identity) (2). Regions within the N termini of both kinases have homology with the band 4.1/ERM family of proteins within a region known as the FERM domain (3). In addition, FAK and CAKβ share conserved phosphorylated tyrosines and C-terminal proline-rich regions that mediate interactions with SH2- and SH3-containing proteins (4).

FAK is expressed in nearly all tissues and cell types, and in a wide variety of adherent cells FAK is discretely localized to focal adhesions (5, 6). In contrast, CAKβ expression is restricted mainly to the brain and hematopoetic cells, and its subcellular localization is cell type-specific (4). In some cells, CAKβ is localized to focal adhesions or focal adhesion-like structures (7, 8). CAKβ has also been localized to specialized actin-containing structures such as the podosomes of macrophages, the sealing zone of osteoclasts, and along stress fibers in smooth muscle cells (9–11). It is also targeted to membrane ruffles and lamellipodia in some spreading and motile cells (12, 13). Alternatively, CAKβ staining has been described as diffuse, perinuclear, or colocalized with the Golgi (7, 14–17). The differential subcellular localization of FAK and CAKβ may underscore important differences in biological function.

Focal adhesion targeting of FAK is mediated by conserved sequences within the C terminus, designated the Focal Adhesion Targeting (FAT) sequence (18), which shares extensive homology with the C terminus of CAKβ. The C-terminal non-catalytic domain of CAKβ localizes discretely to focal adhesions when autonomously expressed (7, 19). Since full-length CAKβ exhibits focal adhesion localization in only a subset of cells, a functional FAT sequence in the C terminus of CAKβ appears to be masked in some cell types. This suggests that focal adhesion localization may be regulated on multiple levels.

FAK is primarily activated through integrin-mediated cell adhesion to an insoluble extracellular matrix. To a lesser extent, FAK is activated by growth factors, neuropeptides, and bioactive lipids (20). Conversely, activation of CAKβ occurs largely in response to soluble extracellular factors, including signals that act through G-protein-coupled receptors, cytokines, antigen receptors, and stress signals (4). CAKβ is maximally activated in response to integrin-mediated cell adhesion in only a subset of cells including hematopoetic cells (8, 21, 22). However, in many other cells, adhesion induces a slight increase in tyrosine phosphorylation (7, 14, 23). Many of the stimuli that activate CAKβ also elevate intracellular calcium levels, and in fact, CAKβ activation is dependent upon the presence of calcium (24–26). Interestingly, a chimeric protein consisting of the N terminus and catalytic domain of CAKβ fused to the C terminus of FAK was driven to focal adhesions and was strongly regulated by adhesion to fibronectin (14). The positive correlation between focal adhesion localization and activation by integrin-mediated adhesion has been reported in the literature and highlights a potential mechanism for differential regulation of FAK and CAKβ activity (27, 28).

Although the signals that lie upstream of FAK family kinases may differ, many of the immediate consequences of activation are conserved. These include recruitment of SH2 do-

‡ To whom correspondence should be addressed: Dept. of Cell and Developmental Biology CB7090 University of North Carolina-Chapel Hill, Chapel Hill, NC 27599. Tel.: 919-966-0391; Fax: 919-966-1856; E-mail: crispy4@med.unc.edu.

[1] The abbreviations used are: FAK, focal adhesion kinase; BrdUrd, 5-bromo-2′-deoxyuridine; CAKβ, cell adhesion kinase β; CE, chick embryo; DAPI, 4′-6-diamidino-2-phenylindone dihydrochloride; ERM, Ezrin/Radixin/Moesin; FAT, focal adhesion targeting; SH, Src homology; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione *S*-transferase.

main containing signaling molecules such as Src and Grb2 into complex with the kinase, and tyrosine phosphorylation of cytoskeleton-associated adaptor proteins, such as paxillin and p130[cas] (1). Signaling via FAK and CAKβ is also involved in the activation of MAP kinase family members (4, 20). These data suggest that differential regulation of common signaling events downstream of FAK and CAKβ are important for biological function.

Since FAK and CAKβ trigger tyrosine phosphorylation of some common substrates, it may be predicted that the biological outcomes of FAK/CAKβ signaling would be similar as well. In fact, both kinases have been implicated in the processes of cell spreading, focal adhesion turnover, and migration (1, 9, 13, 29, 30). Despite this commonality, FAK and CAKβ clearly have divergent functions as well. The two kinases have opposing effects on cell cycle progression, whereas FAK accelerates progression into S phase, CAKβ delays this transition (31, 32). In addition, FAK has been implicated in adhesion-dependent cell survival (33, 34), while CAKβ has been implicated in cell death pathways (35, 36). In neurons, the integration of FAK and CAKβ-mediated signals may promote neurite outgrowth and differentiation, and CAKβ may play a unique role in maintenance of plasticity through modulation of ion channels (25, 37, 38). These results suggest that FAK and CAKβ may have both common and distinct functions.

Although FAK and CAKβ are highly homologous, bind to a common subset of proteins, and are capable of initiating a subset of common signaling pathways, they clearly have unique and perhaps complementary roles in the cell. Subtle differences in mode of activation, regulation, subcellular localization, catalytic activity, substrate preference, and/or scaffolding activity appear to be critical determinants of differential signaling. In order to define the molecular basis of these subtle yet critical differences, we have constructed, expressed, and characterized a complete set of six chimeric FAK/CAKβ proteins in chick embryo cells. CAKβ exhibited higher catalytic activity than FAK in an *in vitro* kinase assay. Furthermore, chimeric kinases revealed that coupling of the FAK N terminus and CAKβ catalytic domain yielded a highly active kinase, suggesting that the N terminus of FAK family kinases may be involved in regulation of catalytic activity. Substrate phosphorylation by chimeric kinases *in vivo* correlated perfectly with catalytic activity *in vitro*, providing a molecular basis for differential substrate phosphorylation by FAK and CAKβ *in vivo*. This analysis revealed a potential role for the N terminus in modulating subcellular localization, since chimeras with the FAK N terminus and CAKβ C terminus targeted to focal adhesions, whereas chimeras with both the N- and C termini of CAKβ did not. The CAKβ N terminus also mediated striking changes in cell morphology that were independent of changes RhoA activity. Although it was not strictly required, targeting to focal adhesions enhanced the ability of this domain to alter morphology.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Plasmids—Chick embryo (CE) cells were isolated from 9-day-old embryos and maintained as described previously (39). For expression of exogenous proteins in CE cells, constructs were subcloned into a replication competent avian retroviral vector, RCAS A (40). RCAS A constructs were transfected into CE cells using the calcium phosphate method as described (39) or LipofectAMINE Plus (Invitrogen) according to the manufacturer's recommended protocol. Expression of RCAS A-encoded exogenous proteins was evaluated 7–9 days post-transfection. For co-expression with c-Src, cells expressing wild type or chimeric FAK family kinases were infected with RCAS B c-Src virus on day 5 post-transfection and lysed on day 10 post-transfection as described previously (41). pCMV-Myc RhoA Q63L and pCMV-Myc RhoA T19N plasmids, which were the generous gifts of Dr. Krister Wennerberg, were transfected using LipofectAMINE Plus and analyzed

3 days post-transfection. Cell cultures were viewed using a Nikon TMS inverted microscope and imaged using a Nikon CoolPix 950 digital camera (×100 magnification).

PCR Mutagenesis and Cloning—The DNA sequence corresponding to the FAK N terminus (amino acids 1–332), catalytic domain (332–690), and C terminus (690–1038), and the CAKβ N terminus (1–336), catalytic domain (336–693), and C terminus (693–1009) were amplified using PCR. At the junctions of these domains, restriction sites were created. The N termini of FAK and CAKβ were amplified with N- and C-terminal *Bam*H1 sites. The catalytic domains were flanked by an N-terminal *Bam*H1 site and C-terminal *Eco*R1 site. The C-terminal fragments were flanked by an N-terminal *Eco*R1 site and C-terminal *Sal*I site. pBluescript KS+/FAK and pBluescript SK−/CAKβ (7, 42) were used as templates, and VENT polymerase (New England Biolabs, Beverly, MA) was used for PCR. Both template constructs had a C-terminal KT3 epitope tag, and therefore all resultant chimeric molecules were also C-terminal tagged. Fragments were ligated to generate sequences that encoded eight chimeric proteins, each maintaining the three-domain structure. The clones were named using a three-letter system corresponding to the three domains in the chimeric protein. The FFF and CCC clones were reconstituted FAK and CAKβ molecules that contained the restriction sites that were engineered using PCR. Chimeric constructs were subcloned into RCAS A for expression in CE cells. All amplified fragments were sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (PerkinElmer, Applied Biosystems Division) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PerkinElmer). Two unintended substitutions in FAK led to amino acid changes: amino acid substitutions R724W and A872G. These mutations, as well as the mutations engineered to create restriction sites, were inconsequential in our analysis since the FFF and CCC chimeras behaved like wild type FAK and CAKβ, respectively.

Antibodies—The following antibodies were used for immunoprecipitation, immunoblotting, and/or indirect immunofluorescence. The KT3 monoclonal antibody (43), which recognizes the C-terminal KT3 epitope tag on FAK, CAKβ, and chimeric proteins, was the kind gift from Dr. J. T. Parsons (University of Virginia, Charlottesville, VA). A commercially available KT3 antibody was used for indirect immunofluorescence (Covance, Princeton, NJ). Monoclonal antibody RC20 (Transduction Labortatories, San Diego, CA) and polyclonal phosphospecific antibodies anti-PYK2 (pY[402]) and anti-PYK2 (pY[579/580]) (BIOSOURCE International,Camarillo, CA) were used to detect phosphotyrosine. Monoclonal antibody anti-RhoA was purchased from Transduction Laboratories. Anti-Src monoclonal antibody EC10 was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-Myc antibody (clone 9E10) was purchased from Sigma. Polyclonal antibody 8605, which recognizes paxillin, was previously described (44). Rhodamine-conjugated goat anti-mouse and fluorescein-conjugated donkey anti-rabbit antibodies were used for indirect immunofluorescence (Jackson ImmunoResearch Laboratories, West Grove, PA). The AlexaFluor 488-conjugated anti-BrdUrd antibody (Molecular Probes, Eugene, OR) was used for analysis of cell cycle progression.

Cell Lysis, Protein Analysis, and Immunoprecipitation—Confluent monolayers of cells were lysed in ice-cold Triton X-100/radioimmune precipitation assay buffer as described (44). Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce). For immunoprecipitations, the paxillin antibody (2 ug), the KT3 antibody (6 ug), or the EC10 antibody (7 ug) were incubated with 0.2–0.8 mg of cell lysate at 4 °C for 1 h. Immune complexes were precipitated at 4 °C for 1 h with protein A-Sepharose beads (Sigma), anti-mouse IgG-agarose beads (Sigma), or protein A-Sepharose beads coated with AffiniPure rabbit anti-mouse IgG (Jackson ImmunoResearch Labs). Immune complexes were washed twice with ice-cold lysis buffer, and once with ice-cold PBS. Beads were resuspended in Laemmli sample buffer and boiled to elute the proteins (45), and the samples were analyzed by Western blotting.

In Vitro Kinase Assays—Kinases were overexpressed in CE cells and immunoprecipitated as described above with minor modifications. To compensate for differences in expression level and to promote recovery of comparable amounts of each kinase, some chimeric proteins were immunoprecipitated from more lysate. Specifically, FAK, CAKβ, FFF, FFC, FCF, FCC, and CCC were immunoprecipitated from 0.4 mg of lysate, whereas mock, CCF, CFC, and CFF were immunoprecipitated from 0.8 mg of lysate. Immune complexes were washed two times with ice-cold lysis buffer, once with ice-cold PBS, and once with kinase reaction buffer (20 mM PIPES, pH 7.2, 3 mM MnCl$_2$). Each reaction was resuspended in 20 μl of kinase reaction buffer supplemented with 10 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences). The reactions were
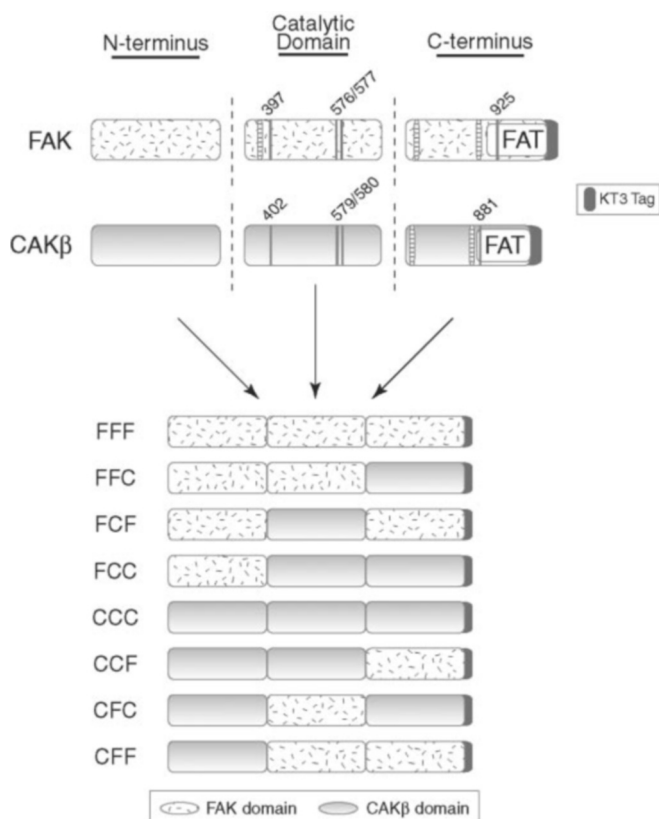
FIG. 1. **Schematic diagram of FAK/CAKβ chimeric proteins.** Chimeric molecules were built as described under "Experimental Procedures." *Upper panel,* N-terminal, catalytic, and C-terminal domains of FAK (*stippled boxes*) and CAKβ (*shaded boxes*) were individually amplified using PCR. The catalytic domains of the chimera contained the catalytic domains and Src binding sites (FAK pY^{397} and adjacent proline-rich region; CAKβ pY^{402}). Each construct had a C-terminal KT3 epitope tag. *Lower panel,* chimeric molecules were named using a three-letter system. The three letters correspond to the three domains that constitute the chimeric protein. *F* represents a FAK domain (*stippled*) and *C* represents a CAKβ domain (*shaded*). FFF and CCC are reconstituted FAK and CAKβ molecules that also contain the engineered restriction sites at the domain joints. *Gray boxes,* phosphotyrosine; *striped boxes,* proline-rich regions; FAT, focal adhesion targeting domain.

incubated at room temperature with periodic mixing for 10 min. The reaction was stopped by adding 20 μl of 2× Laemmli sample buffer and boiled to elute the protein. Samples were resolved on an SDS-polyacrylamide gel. The gel was dehydrated and exposed to film to reveal phosphorylated species.

Immunofluorescence—Glass coverslips were coated with 50 μg/ml bovine plasma fibronectin (Sigma) in PBS for 1 h at 37 °C. Cells were plated onto the coated coverslips and maintained at 37 °C for 16 h. Cells were fixed in 3.7% formaldehyde in Universal Buffer (UB; 20 mM Tris, pH 7.6, 150 mM NaCl) for 8 min, washed twice in UB, permeabilized with 0.5% Triton X-100 in UB for 5 min, and washed twice more in UB. Coverslips were incubated with primary antibody (KT3, 1:1000; 8605, 1:800) diluted in UB for 1 h at 37 °C in a humidified chamber, washed twice, and incubated with secondary antibody (anti-mouse rhodamine and anti-rabbit fluorescein, 1:1000) diluted in UB for 1 h at 37 °C in a humidified chamber. Coverslips were washed twice in UB and once with distilled water, and mounted on slides for visualization using a Leitz Orthoplan fluorescence microscope. Images were captured using a Hamamatsu digital camera and Metamorph imaging software (Universal Imaging Corporation, West Chester, PA).

RhoA Activity Assay—The level of RhoA activity in CE cells was assessed as previously described (46). Briefly, lysates made from confluent mock-transfected and CCF-expressing cells were incubated with a GST-Rhotekin RBD fusion protein bound to glutathione-agarose beads (Sigma). Complexes were washed and Western blotted with anti-RhoA and anti-Myc antibodies to reveal the amount of precipitated RhoA. As controls, CE cells were transfected with Myc-tagged RhoA
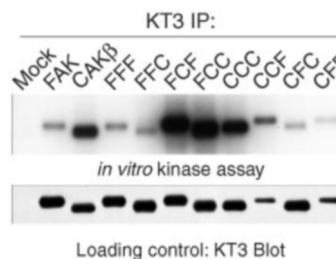

FIG. 2. **Catalytic activity of FAK/CAKβ chimeric kinases *in vitro*.** Cell lysates were made from mock-transfected CE cells or cells expressing FAK, CAKβ, or FAK/CAKβ chimeric proteins. Kinases were immunoprecipitated with the KT3 antibody. To compensate for differences in levels of protein expression, FAK, CAKβ, FFF, FFC, FCF, FCC, and CCC were immunoprecipitated from 400 μg of lysate and mock, CCF, CFC, and CFF were immunoprecipitated from 800 μg of lysate. *Upper panel*, one-half of each sample was subjected to an *in vitro* kinase assay as described under "Experimental Procedures." Kinase reactions were separated by SDS-PAGE, and incorporation of ^{32}P into the kinases was visualized by autoradiography. *Lower panel*, the second half of each immunoprecipitation was separated by SDS-PAGE and immunoblotted with the KT3 antibody to show the amount of kinase present in each reaction.

Q63L (constitutively active) or RhoA T19N (dominant negative) and analyzed in parallel.

Apoptosis Assay—In order to assess apoptosis, the Apoptag Plus Fluorescein *in situ* apoptosis detection kit was used as recommended by the manufacturer (Intergen Company, Purchase, NY). Briefly, CE cells expressing wild type and chimeric kinases were plated on fibronectin-coated coverslips as described above and grown to confluence over 2 days. As a positive control for apoptosis, mock-transfected cells were treated with 1 μM staurosporine (Sigma) for at least 4 h. Cells were fixed in 3.7% formaldehyde in PBS and permeabilized in ethanol/acetic acid (2:1) at −20 °C. Coverslips were then incubated with a fluorescein-conjugated anti-digoxigenin antibody, and 4′-6-diamidino-2-phenylindone dihydrochloride (DAPI, 1 μg/ml, Molecular Probes, Eugene, OR) to counterstain the nuclei. Coverslips were mounted onto glass slides and visualized as described for immunofluorescence experiments. In each experiment, 200 cells were counted, and the percentage of apoptotic cells was determined.

Cell Cycle Progression Assay—Differences in the rate at which starved CE cells expressing wild type and chimeric constructs entered S-phase following serum stimulation was assessed. Briefly, cells were plated onto fibronectin-coated coverslips at low density (∼25% confluence) and incubated overnight in complete media. Cells were then starved in Dulbecco's modified Eagle's medium (DMEM) +0.2% chick serum (Invitrogen) for 48 h. After rinsing twice with DMEM, complete CE media (DMEM+5% fetal bovine serum (Sigma), 1% chick serum) supplemented with 100 μM BrdUrd (5-bromo-2′-deoxyuridine, Sigma) was added back to the cells. As a control, one coverslip was incubated in serum-free media supplemented with BrdUrd to verify that serum starvation had arrested the cell cycle. After 16 h, cells were fixed in 3.7% formaldehyde in PBS and permeabilized in 0.5% Triton X-100 in PBS. Coverslips were blocked in 2% bovine serum albumin in PBS for 1 h and subsequently treated with 0.1 unit/μl DNase (Promega, Madison, WI) for 30 min at 37 °C. Slips were incubated serially with anti-BrdUrd antibody (1:20), KT3 (1:500), and finally, a mixture of rhodamine-conjugated anti-mouse antibody (1:500) and DAPI (1 μg/ml) for 1 h each at 37 °C, with washing following each antibody step. Coverslips were then rinsed in water, mounted onto glass slides, and visualized as described for indirect immunofluorescence. In each experiment, 200 cells were counted, and the percentage of cells that had entered S phase was determined.

RESULTS

Construction of Chimeric Molecules—In order to determine the molecular basis of the functional differences between FAK and CAKβ, a set of chimeric FAK/CAKβ molecules was constructed (Fig. 1). The three major domains of FAK and CAKβ were individually amplified by PCR. Restriction sites were created at the N and C termini of each domain to allow for assembly of the domains into chimeric molecules (Fig. 1, *upper panel*). The central domains, which are referred to as the catalytic domains in this manuscript, include both the catalytic
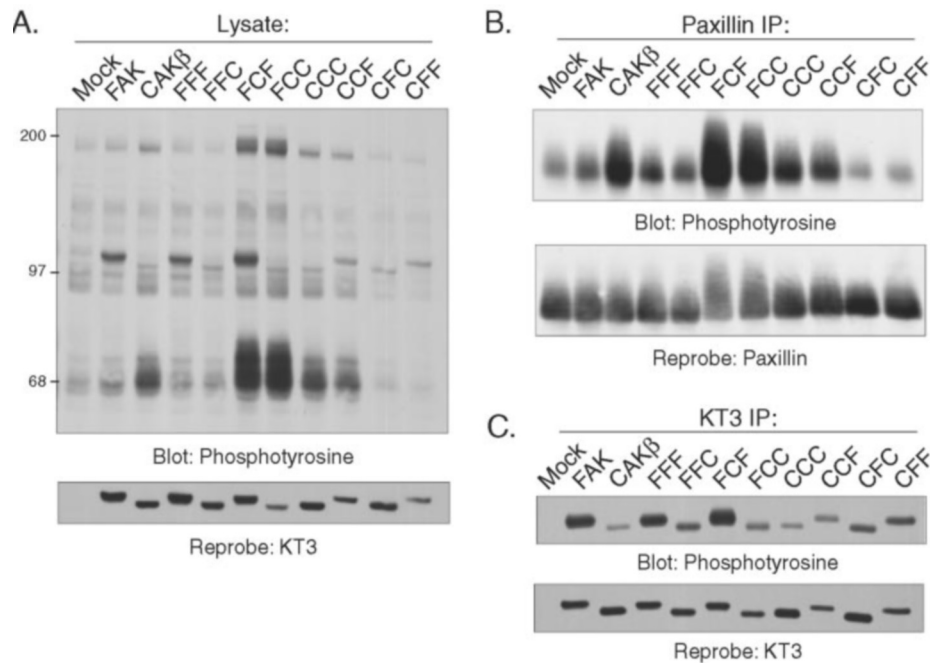
FIG. 3. **Tyrosine phosphorylation of substrates and FAK/CAKβ chimeric kinases *in vivo*.** Cell lysates were made from mock-transfected CE cells or cells expressing FAK, CAKβ, or FAK/CAKβ chimeric proteins. *A*, whole cell lysates (30 ug) were separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted to reveal tyrosine-phosphorylated species (*upper panel*). The membrane was stripped and reprobed with the KT3 antibody to show the level of kinase expression (*lower panel*). *B*, paxillin was immunoprecipitated and Western blotted for phosphotyrosine (*upper panel*). The membrane was stripped and reprobed to show the amount of paxillin in each lane (*lower panel*). *C*, wild type and chimeric kinases were immunoprecipitated from cell lysates using the KT3 antibody. Immune complexes were Western blotted with an anti-phosphotyrosine antibody (*upper panel*) and stripped and reprobed with the KT3 antibody to reveal the amount of kinase in each lane (*lower panel*).

domains and the binding sites for the Src SH2 and SH3 domains (8, 47–49). A KT3 epitope tag was engineered at the C terminus of each construct. Domains were assembled to generate a full set of chimeric proteins that retain the overall size and structure of the kinase. Chimeric proteins were named using a three-letter system (Fig. 1, *lower panel*) corresponding to the three domains, where an *F* represents a FAK domain and a *C* represents a CAKβ domain. Creation of restriction sites resulted in amino acid substitutions at the joints of the chimeric proteins. To control for the presence of these substitutions, two constructs, FFF and CCC, were built. These correspond to wild type FAK and CAKβ except that they contain the engineered restriction sites at the domain joints. The chimeric constructs were subcloned into RCAS A, a replication-competent avian retroviral vector, for expression in chick embryo (CE) cells. In order to verify the domain structure of chimeric proteins, a panel of antibodies that recognized specific domains of FAK and CAKβ was used to probe Western blots of chimera-expressing CE cell lysates.[2] The results confirmed the predicted domain structure of chimeric FAK/CAKβ proteins.

Catalytic Activity of FAK/CAKβ Chimeric Molecules in Vitro—CAKβ expression in CE cells results in greater substrate phosphorylation than expression of FAK (7). To explore whether differences in intrinsic catalytic activity could explain this result, the catalytic activity of FAK, CAKβ, and FAK/CAKβ chimeric kinases was assessed in an *in vitro* immune complex kinase assay. From lysates made from confluent mock-transfected CE cells and cells expressing FAK, CAKβ, and the chimeric proteins, kinases were immunoprecipitated using the KT3 epitope tag antibody. One-half of each immune complex was incubated with $[\gamma$-^{32}P]ATP in kinase reaction buffer for 10 min and separated by SDS-PAGE. Incorporation of ^{32}P into the kinases was detected by autoradiography (Fig. 2, *upper panel*). The other half of the immune complex was separated by SDS-

PAGE and Western blotted with the KT3 antibody to reveal the amount of kinase in each reaction (*lower panel*). Under these assay conditions, CAKβ exhibited higher catalytic activity than FAK. This result is accentuated by the fact that less CAKβ was present in the reaction than FAK. The catalytic activity of control chimeric proteins FFF and CCC were similar to FAK and CAKβ, respectively, demonstrating that amino acid substitutions in the chimeric kinases did not alter catalytic activity. Overall, chimeric proteins that contained the catalytic domain of CAKβ (including FCF, FCC, and CCF) exhibited higher autophosphorylation activity than those that contained the FAK catalytic domain (including FFC, CFC, and CFF). Unexpectedly, juxtaposition of the FAK N terminus and catalytic domain of CAKβ (*i.e.* FCF, FCC) resulted in an enhancement of catalytic activity above that of CAKβ itself. However, there was no apparent change in activity when the N terminus of CAKβ was combined with the FAK catalytic domain (compare FAK, CFC, and CFF). These data implicate the catalytic domain as the principal determinant of catalytic activity *in vitro* and suggest that CAKβ may have higher intrinsic catalytic activity relative to FAK. Furthermore, these results revealed a potential modulatory role for the N terminus. This is the first report of a direct comparison of FAK and CAKβ catalytic activity *in vitro*, and identifies a biochemical basis for differential substrate phosphorylation *in vivo*.

Tyrosine Phosphorylation of Chimeric Kinases and Substrates in Vivo—It was previously shown that in adherent CE cells, CAKβ expression elicited higher levels of whole cell phosphotyrosine than FAK expression. The major substrates that were differentially phosphorylated were paxillin, p130[cas], and tensin. Interestingly, the basal level of FAK phosphorylation was high, whereas that of CAKβ was very low (7). These phosphorylation events were investigated further using FAK/CAKβ chimeric kinases. Lysates were made from confluent cultures of mock-transfected CE cells and cells expressing FAK, CAKβ, and chimeric FAK/CAKβ proteins. To compare cellular phos-

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[2] J. Dunty and M. Schaller, unpublished observations.

photyrosine profiles, whole cell lysates (30 μg) were Western blotted with an anti-phosphotyrosine antibody (Fig. 3*A, upper panel*). The membrane was stripped and reprobed with the KT3 antibody to reveal the relative amount of exogenous kinase in each lysate (*lower panel*). Paxillin was also immunoprecipitated from these lysates. The immune complexes were Western blotted for phosphotyrosine (Fig. 3*B, upper panel*) and stripped and reprobed for paxillin to verify equal protein loading (*lower panel*). As previously described (7), FAK expression had little effect on cellular phosphotyrosine, whereas CAKβ expression induced the phosphorylation of several species (Fig. 3*A*). Likewise, paxillin phosphorylation was only slightly elevated in cells expressing FAK but was dramatically increased in CAKβ-expressing cells (Fig. 3*B*). Importantly, FFF and CCC behaved as wild type FAK and CAKβ in these experiments, suggesting that amino acid substitutions within the chimeric proteins did not alter biochemical activity *in vivo*. Similar to FAK, expression of chimeric proteins that contained the FAK catalytic domain resulted in little change in cellular phosphotyrosine or paxillin phosphorylation (Fig. 3, *A* and *B*; *FFC, CFC*, and *CFF*). Chimeric kinases that contained the CAKβ catalytic domain were as efficient or more efficient than CAKβ in elevating cellular phosphotyrosine and paxillin phosphorylation (Fig. 3, *A* and *B*; *FCF, FCC,* and *CCF*). Interestingly, the N terminus modified substrate phosphorylation *in vivo*, since chimeras containing the FAK N terminus induced greater levels of cellular phosphotyrosine and paxillin phosphorylation than their counterparts containing the CAKβ N terminus (Fig. 3, *A* and *B*, compare *FFF* and *CFF*, *FFC* and *CFC*, *FCF* and *CCF*, *FCC* and *CCC*). Furthermore, the combination of the FAK N terminus and the CAKβ catalytic domain resulted in the most potent induction of substrate phosphorylation (Fig. 3, *A* and *B*; *FCF* and *FCC*). Tyrosine phosphorylation of p130^{cas} and tensin was also examined by immunoprecipitation and Western blot analysis, and the results were similar to those seen with paxillin.[2] Therefore, levels of whole cell phosphotyrosine and paxillin phosphorylation were determined primarily by the catalytic domain, whereby the CAKβ catalytic domain was more efficient than that of FAK in elevating cellular phosphotyrosine. In addition, the N terminus of the kinase may modify catalytic activity *in vivo*. Significantly, the level of substrate phosphorylation induced by the kinases *in vivo* correlates well with catalytic activity *in vitro* (refer to Fig. 2).

In CE cells growing in culture, FAK is heavily phosphorylated on tyrosine while CAKβ exhibits very low phosphorylation (7). To define the domains that mediate this dissimilarity, the phosphorylation state of chimeric FAK/CAKβ proteins *in vivo* was determined. Kinases were immunoprecipitated using the KT3 antibody and Western blotted for phosphotyrosine (Fig. 3*C, upper panel*). The membrane was stripped and reprobed with the KT3 antibody to reveal the amount of protein in each lane (Fig. 3*C, lower panel*). As previously reported, FAK was heavily phosphorylated in cells growing in culture, whereas the basal level of tyrosine phosphorylation on CAKβ was low (7). Likewise, FFF phosphorylation was high, and CCC phosphorylation was low. Phosphotyrosine on FCF and CFF was equivalent to FAK. FCC phosphorylation was similar to CAKβ. The remaining chimeric proteins, including FFC, CCF, and CFC, exhibited an intermediate level of phosphorylation. Tyrosine phosphorylation *in vivo* correlated best with the C terminus; chimeras containing the FAK C terminus (*i.e.* FFF, FCF, CCF, and CFF) were more highly phosphorylated on tyrosine than their counterparts that contained the CAKβ C terminus (*i.e.* FFC, FCC, CCC, and CFC, respectively). In addition, chimeric proteins containing the FAK catalytic domain were phosphorylated slightly better than those containing the CAKβ cata-
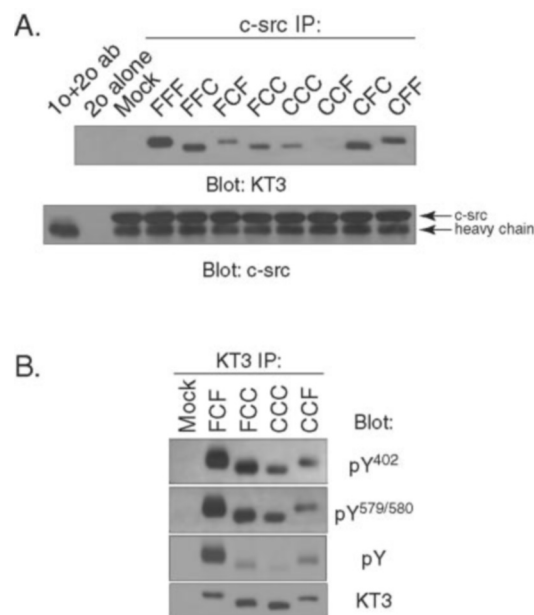

FIG. 4. **Coupling of chimeric kinases with c-Src.** *A*, cell lysates were made from CE cells expressing c-Src alone (*mock*) and cells coexpressing c-Src and FAK, CAKβ, or FAK/CAKβ chimeric proteins. From these lysates, c-Src was immunoprecipitated, and the immune complexes were split in half. One-half was separated on an 8% gel and Western blotted with the KT3 antibody to reveal co-precipitated wild type and chimeric kinases (*upper panel*). The second half was separated on a 12% gel and Western blotted for c-Src (*lower panel*). As a control, FFF lysate was incubated with secondary antibody conjugated to beads alone (2° alone). To distinguish between c-Src and the antibody heavy chain, antibodies used for the immunoprecipitation were incubated with buffer and analyzed in parallel (1° + 2° Ab). *B*, cell lysates were made from mock-transfected CE cells or cells expressing FCF, FCC, CCC, or CCF. Kinases were immunoprecipitated with the KT3 antibody. To compensate for differences in levels of protein expression, FCF and FCC were immunoprecipitated from 400 μg of lysates, and mock, CCC and CCF were immunoprecipitated from 800 μg of lysate. The immune complexes were split into thirds and Western blotted for phosphorylated Tyr^{402} (pY^{402}), phosphorylated Tyr$^{579/580}$ (pY$^{579/580}$), and phosphotyrosine (pY) as indicated. The pY blot was stripped and reprobed with the KT3 antibody to reveal the amount of immunoprecipitated kinase.

lytic domain. Thus, the C terminus and catalytic domains are the primary and secondary determinants, respectively, of tyrosine phosphorylation *in vivo*. It is noteworthy that the level of tyrosine phosphorylation on chimeric FAK/CAKβ kinases correlates with neither the relative catalytic activity *in vitro* nor the ability to induce substrate phosphorylation *in vivo*.

Coupling of Chimeric Kinases with Src in Vivo—FAK and Src family kinases act coordinately to send downstream signals in the cell. Therefore, the ability of FAK kinases to send strong signals downstream *in vivo* may be attributable to both high intrinsic catalytic activity and increased coupling to Src. In order to study this interaction, the ability of wild type and chimeric kinases to co-immunoprecipitate with exogenously expressed c-Src was determined. Lysates were made from confluent cultures of CE cells expressing c-Src alone (Mock) or co-expressing c-Src and FAK/CAKβ chimeric kinases. From these lysates, c-Src was immunoprecipitated, and the immune complexes were split in half. One-half was Western blotted with the KT3 antibody to reveal the amount of co-immunoprecipitated chimeric kinases (Fig. 4*A, upper panel*). The other half was blotted with the EC10 antibody to verify equal recovery of Src in the immunoprecipitates (*lower panel*). A greater amount of FAK was found in complex with c-Src than CAKβ, and likewise more FFF was found in complex with c-Src than CCC. Chimeras containing the FAK catalytic domain (FFF,
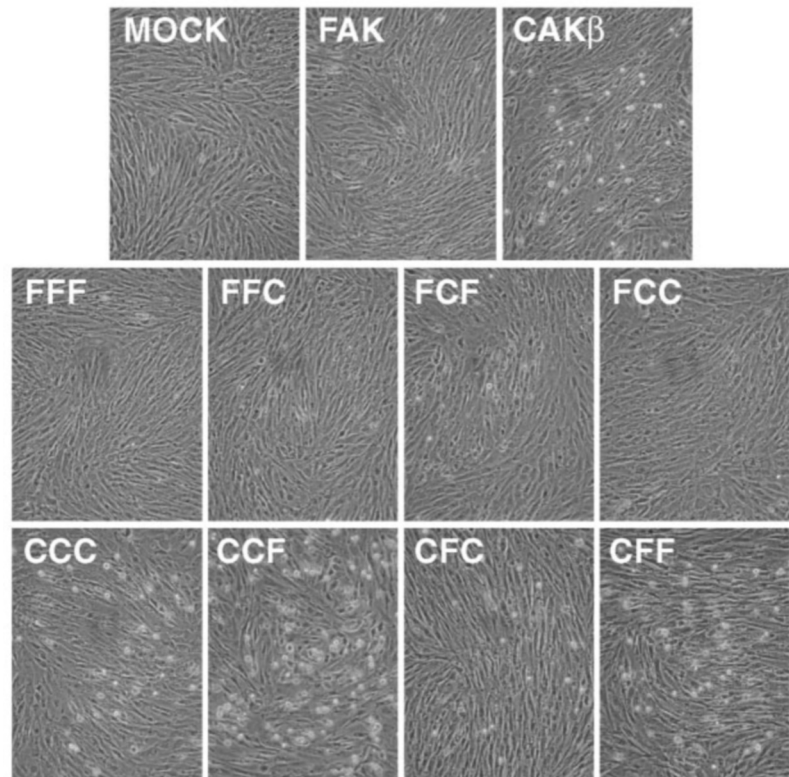
F<small>IG</small>. 5. **Changes in cell morphology induced by kinase expression.** Mock-transfected CE cells and cells expressing FAK, CAKβ, or FAK/CAKβ chimeric proteins were imaged using a Nikon TMS inverted microscope and a Nikon CoolPix 950 digital camera (×100 magnification).

FFC, CFC, CFF) bound more efficiently to c-Src than those bearing the CAKβ catalytic domain (FCF, FCC, CCC, CCF). Note that the chimeras were built such that the central catalytic domains included all c-Src binding sites. Thus, the catalytic domain was the primary determinant of c-Src binding.

Among those kinases containing the CAKβ catalytic domain, those that also contained the FAK N terminus (FCF, FCC) bound better to c-Src than those containing the CAKβ N terminus (CCC, CCF). This suggested that the N terminus of FAK was permissive for c-Src binding in comparison to the CAKβ N terminus. The major autophosphorylation site on FAK family kinases also serves as the Src SH2-domain binding site. Therefore, increased c-Src binding may be associated with elevated phosphorylation at this site as well as sites that serve as Src targets, such as the activation loop regulatory tyrosines. To further examine their tyrosine phosphorylation, phosphospecific antibodies were used to determine the level of phosphorylation on the major autophosphorylation site (Tyr^{402}) and regulatory tyrosines in the activation loop (Tyr$^{579/580}$) of chimeric kinases containing the CAKβ catalytic domain (FCF, FCC, CCC, and CCF). Kinases were immunoprecipitated using the KT3 antibody, and immune complexes were split into thirds and Western blotted for phosphorylated Tyr^{402} (pY^{402}), phosphorylated Tyr$^{579/580}$ (pY$^{579/580}$), and phosphotyrosine (pY) as indicated in Fig. 4*B*. The phosphotyrosine blot was stripped and reprobed with the KT3 antibody to reveal the amount of recovered kinase (Fig. 4*B*, *lower panel*). FCF and FCC exhibited relatively higher levels of autophosphorylation than their counterparts CCF and CCC, respectively (Fig. 4*B*, *pY^{402}*), providing a molecular basis for increased Src binding to these chimeras. Furthermore, FCF and FCC exhibited relatively higher levels of activation loop phosphorylation than CCF and CCC (Fig. 4*B*, *pY$^{579/580}$*). Phosphorylation on activation loop tyrosines is associated with increased catalytic activity (50, 51).

Induction of Morphological Changes by Selected FAK/CAKβ Chimeric Proteins—Expression of CAKβ in CE cells results in sporadic cell rounding within the confluent monolayer. Treat-

ment of these cells with sodium orthovanadate, a tyrosine phosphatase inhibitor, results in severe disruption of the monolayer as evidenced by abundant cell rounding and loss of confluence (7). Significantly, FAK expression has no effect on the integrity of the monolayer, nor does treatment of FAK-expressing cells with sodium orthovanadate. In order to identify the unique features of CAKβ that mediate changes in cell morphology, the morphology of CE cells expressing FAK, CAKβ, and FAK/CAKβ chimeric kinases was examined (Fig. 5). As predicted, expression of FAK or FFF had no effect on cell morphology, whereas expression of CAKβ or CCC induced mild cell rounding, in agreement with the previous report (7). FFC, FCF, or FCC expression had little or no effect on cell morphology. Surprisingly, in the absence of vanadate treatment, expression of several chimeric proteins had striking effects on cell morphology that were reminiscent of vanadate-treated CAKβ-expressing cells. Cells expressing CFC were extremely elongated and spindly, and some cell rounding was also induced. Expression of CCF and CFF caused dramatic cell rounding, where CCF was more potent than CFF. Furthermore, at the time when changes in morphology were most striking, monolayers of CCF, CFC, and CFF-expressing cells seemed to take longer to reach confluence than other chimera-expressing cell cultures. These data implicate the N terminus of CAKβ in the induction of changes in cell morphology. Furthermore, the C terminus of FAK may cooperate with the N terminus of CAKβ to promote the most dramatic alterations in morphology. Morphological changes did not correlate with high catalytic activity *in vitro* or an overall elevation of tyrosine phosphorylation *in vivo*.

Further Characterization of Cells that Exhibit Altered Cell Morphology—The morphological changes that were observed were intriguing, and could be due to alteration of a number of cellular processes. Cells with altered morphologies, *i.e.* cells expressing CCF, CFC, and CFF, were further analyzed to determine the basis of these changes. Since CAKβ expression promotes apoptosis in some cell types, (35, 36) the viability of
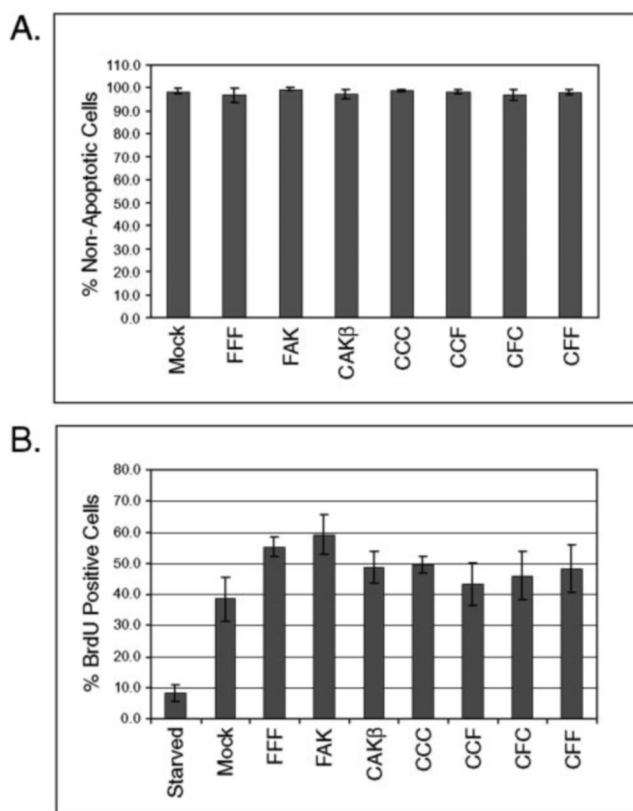
FIG. 6. **Analysis of apoptotic cell death and cell cycle progression.** *A*, mock-transfected CE cells and cells expressing FAK, CAKβ, FFF, CCC, CCF, CFC, and CFF were plated onto fibronectin-coated coverslips and grown to confluence at approximately day 7 post-transfection. The percentage of non-apoptotic cells was assessed using the Apoptag system as described under "Experimental Procedures." The data are a summation of four independent experiments. *Error bars* represent S.D. *B*, mock-transfected CE cells and cells expressing FAK, CAKβ, FFF, CCC, CCF, CFC, and CFF were adhered to fibronectin-coated coverslips overnight and subsequently starved for 48 h in low serum-containing media. Cells were then stimulated with complete media containing 100 μM BrdUrd for 16 h. Nuclei were scored for BrdUrd incorporation as described under "Experimental Procedures." The data is expressed as percentage of BrdUrd-positive cells and are a summation of three independent experiments. *Error bars* represent S.D.

these cells was determined (Fig. 6*A*). At 7 days post-transfection, cells were plated onto fibronectin coverslips and allowed to reach confluence over 2 days. Similar to cells grown on tissue culture-treated dishes, dramatic morphological changes were evident. As a positive control for apoptosis, mock-transfected cells were treated with staurosporine. The percentages of apoptotic and non-apoptotic nuclei were calculated. The number of non-apoptotic mock-transfected cells was quite high (98.5 ± 1.1%). FAK (96.8 ± 2.9%), FFF (99.4 ± 0.7%), CAKβ (97.3 ± 2.1%), and CCC (98.7 ± 0.6%) exhibited similar high levels of non-apoptotic cells. Importantly, expression of chimeric kinases that induced the greatest morphology changes did not increase the rate of apoptotic cell death (CCF, 98.4 ± 0.7%; CFC, 97.1 ± 2.3%; and CFF, 98.1 ± 1.2%). Therefore, cell rounding induced by chimera expression was not associated with an elevation in the rate of apoptosis.

While FAK expression has been reported to accelerate cell cycle progression, CAKβ expression had an opposing effect (32). In order to assess whether expression of morphology-altering chimeras was associated with a decreased rate of cell cycle progression, a BrdU incorporation assay was employed (Fig. 6*B*). Serum-starved cells were stimulated with complete media containing 100 μM BrdUrd for 16 h, and the percentage of

BrdUrd-positive nuclei was determined. A low level of BrdUrd incorporation was seen in serum-starved cells (8.3 ± 2.8%). Stimulation of mock-transfected cells with serum-containing media prompted 38.5 ± 7.2% of cells to enter S phase. At the same time point, more FAK-expressing cells (55.3 ± 3.1%) had incorporated BrdUrd than mock cells, suggesting that, in accordance with previous reports, FAK expression accelerated S phase entry (31). However, in contrast to reports that it slows cell cycle progression (32), CAKβ (48.7 ± 5.1%) accelerated S phase progression almost as well as FAK in CE cells. FFF and CCC expression (59.3 ± 6.4% and 49.5 ± 2.6%, respectively) mimicked the effects of FAK and CAKβ expression. Although each chimeric kinase had a slightly different affect on cell cycle progression, all cells entered S phase at a rate that was greater than mock-transfected cells (CCF, 43.3 ± 6.8%; CFC, 46.0 ± 7.8%; CFF, 48.3 ± 7.6%). Therefore, altered cell morphology and the apparent disruption of the monolayers were not due to a cell cycle delay.

Subcellular Localization of Chimeric Proteins—In CE cells, FAK is localized discretely to focal adhesions via its C-terminal focal adhesion targeting (FAT) sequence. Although highly homologous to FAK within its putative FAT sequence region, CAKβ localization is mainly diffuse in CE cells (7). In order to elucidate the molecular mechanisms of differential subcellular localization of FAK and CAKβ, indirect immunofluorescence was used to study the localization of FAK/CAKβ chimeric proteins in CE cells. Cells were plated onto fibronectin-coated glass coverslips at low density and allowed to adhere and spread for 16 h in complete media. Cells were then processed and labeled with the KT3 antibody to reveal kinase localization (Figs. 7 and 8). For the purposes of this analysis, only well spread cells were studied.

Mock-transfected cells exhibited a nearly undetectable level of nonspecific staining (Figs. 7 and 8, *Mock*). FAK was clearly localized to focal adhesions, whereas CAKβ localization was largely cytoplasmic (Figs. 7 and 8, FAK and CAKβ). In a small percentage of cells, CAKβ was present in focal adhesions and/or smaller focal adhesion-like structures. The control chimeras FFF (Fig. 7) and CCC (Fig. 8) were localized as their wild type counterparts, demonstrating that the amino acid substitutions in the chimeric kinases did not alter subcellular localization. As predicted, the FAK C terminus directed focal adhesion localization of FCF, CCF, and CFF (Figs. 7 and 8). These results demonstrate that the C terminus of FAK contains a potent focal adhesion targeting sequence, since it can target the CAKβ N terminus and catalytic domain (CCF) to focal adhesions. Like CAKβ, CFC localization was diffuse (Fig. 8). However, quite interestingly, the C terminus of CAKβ directed focal adhesion localization when the N terminus of FAK was present (Fig. 7, FFC and FCC). This suggested that the C terminus of CAKβ is also sufficient to direct focal adhesion localization. Furthermore, substitution of the FAK N terminus may be permissive for the focal adhesion targeting activity of the CAKβ C terminus.

The morphology changes observed in cells expressing certain chimeric kinases raised questions regarding the integrity of their focal adhesions. To visualize focal adhesions, cells were co-stained for paxillin (Fig. 8). Chimeric proteins that contained the N terminus of FAK were well spread and exhibited characteristic staining of paxillin in focal adhesions.[2] Surprisingly, even in apparently well spread cells expressing CAKβ, CCC, CCF, CFC, and CFF, paxillin localization was altered to varying degrees, suggesting that expression of these kinases altered focal adhesion structure. The Rho family of GTPases plays a central role in regulating the actin cytoskeleton (52). In particular, Rho activity is important for generating tension
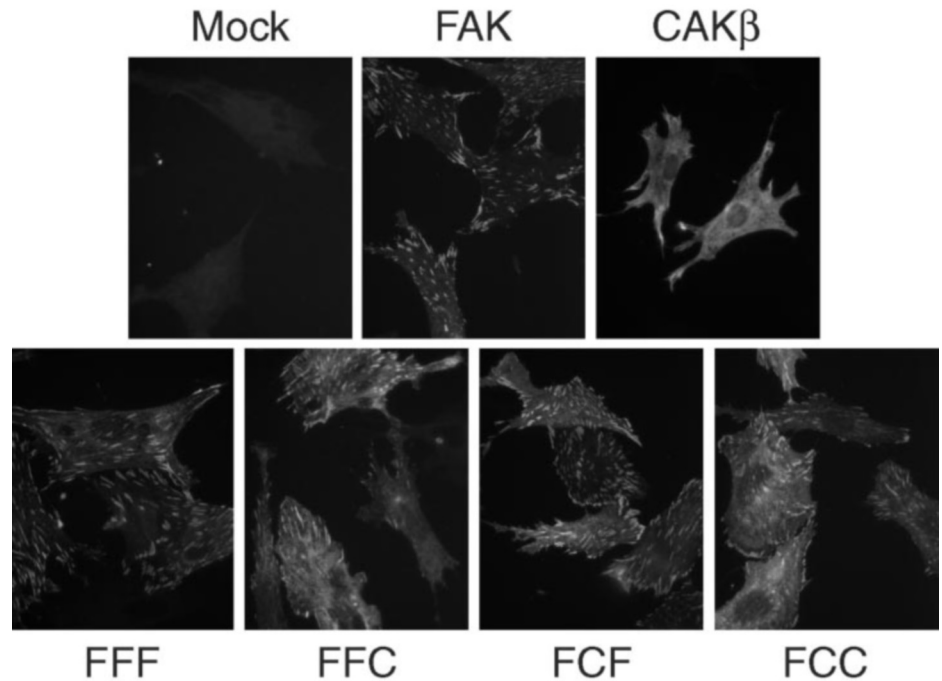
FIG. 7. **Subcellular localization of chimeras containing the N terminus of FAK.** Mock-transfected CE cells and CE cells expressing FAK, CAKβ, FFF, FFC, FCF, and FCC were plated onto fibronectin-coated coverslips and cultured in complete media for 16 h. Cells were then processed and labeled with the KT3 epitope tag antibody to reveal the localization of the kinases as described under "Experimental Procedures."

which is associated with the formation of actin stress fibers and focal adhesions (53). Therefore, the changes in cell shape and focal adhesion structure observed in certain chimera-expressing cells might be mediated by down-regulation of Rho activity. Since CCF cells exhibited the most dramatic morphological changes, the level of activated, endogenous RhoA in mock and CCF cells was assessed using the GST-RBD pull-down assay (Fig. 9). The amount of active RhoA recovered from mock and CCF cells was equal, suggesting that there was no difference in the activation state of RhoA between these cells (Fig. 9, *Rho blot*). As controls for this assay, RhoA Q63L (constitutively active) and RhoA T19N (dominant negative) were analyzed. The GST-RBD fusion protein bound to RhoA Q63L, but not RhoA T19N (Fig. 9, *Myc blot*). Therefore, it appears that alteration of cell morphology is not mediated by changes in RhoA activity.

DISCUSSION

A comparative analysis of FAK and CAKβ revealed both similarities and intriguing differences in their function (7). The goal of this study was to define the sequences within FAK and CAKβ that mediate their unique properties. Our approach was to construct and comprehensively characterize a full set of chimeric FAK/CAKβ proteins. Chimeric proteins have been used previously to study the activities of FAK and CAKβ (14, 32, 35, 54). These studies have provided insight into selected activities of FAK and CAKβ. However, additional valuable information was obtained through the biochemical and biological analysis of novel chimeric molecules. The results, which are summarized in Table I, revealed an unexpected role for the N-terminal domain in regulating substrate phosphorylation, focal adhesion localization and cell morphology, and provided an explanation for enhanced phosphorylation of substrates by CAKβ relative to FAK.

Tyrosine phosphorylation of substrates *in vivo* correlated perfectly with the origin of the catalytic domain, whereby the catalytic domain of CAKβ induced greater levels of phosphotyrosine than the catalytic domain of FAK. Unexpectedly, the N terminus had a striking impact on catalytic activity. Chimeras with the N-terminal domain of FAK and catalytic domain of CAKβ (FCF and FCC) elicited even higher levels of substrate

phosphorylation than CAKβ itself. The degree to which chimeric kinases elevated substrate phosphorylation *in vivo* was proportional to their level of catalytic activity in an *in vitro* kinase assay.

The mechanism by which the FAK and CAKβ catalytic domains support different levels of catalytic activity is unclear. One obvious possibility was that elevated catalytic activity was the result of enhanced coupling to Src family kinases. Two lines of evidence suggest that this was not the case. First, chimeras with the FAK catalytic domain bound more c-Src, yet exhibited lower activity *in vitro*. Second, the inclusion of PP2, a Src-specific inhibitor (55) in the *in vitro* kinase assays had no effect upon the autophosphorylation activity of the chimeras.[2] Thus, the presence of Src kinases in these immune complexes had a negligible contribution to the observed differences in catalytic activity *in vitro*. Rather, the intrinsic activity of the kinase itself determines the baseline level of catalytic activity.

Interestingly, exchanging the CAKβ N terminus for the FAK N terminus in the context of the CAKβ catalytic domain (*i.e.* CCC, CCF to FCC, FCF) resulted in elevated c-Src binding. Increased c-Src binding correlated with higher levels of catalytic activity of these chimeras *in vitro* and *in vivo*. Further, FCF and FCC exhibited higher levels of autophosphorylation on Tyr^{402} and increased phosphorylation on activation loop regulatory tyrosines Tyr$^{579/580}$, which when phosphorylated, promote maximal catalytic activity (50, 51). Therefore, modulation of Src binding by the N terminus in the context of the CAKβ catalytic domain was associated with elevated phosphorylation of key regulatory tyrosines, providing a potential mechanism by which the N terminus may regulate catalytic activity.

It was previously suggested that the N-terminal domain of FAK kinases might impinge upon and negatively regulate catalytic activity, since deletion of the N terminus of FAK led to elevated catalytic activity in some cases (56, 57). However, deletion of the N terminus in other cases has little effect upon catalytic activity (18). A recent study of JAK3 also suggests a mechanism by which the N-terminal FERM domain of FAK kinases could regulate catalytic activity through an intramolecular interaction. The N-terminal FERM domain of JAK3
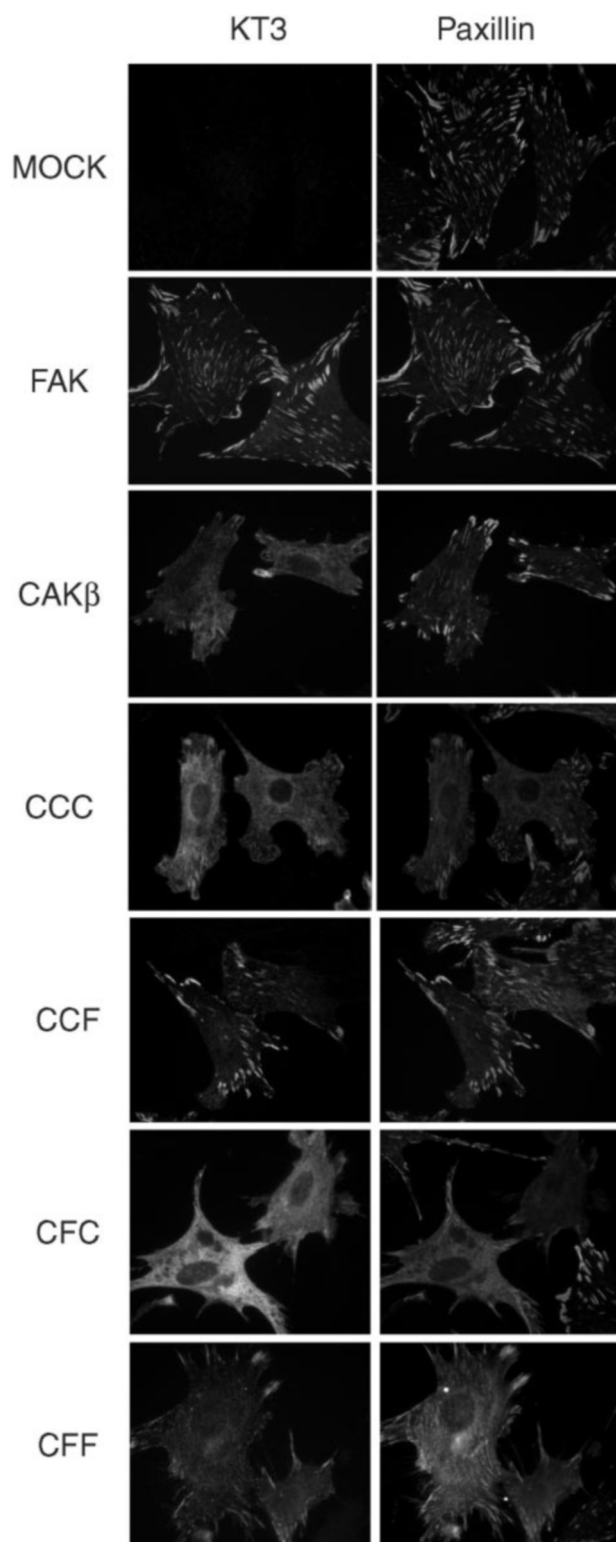
KT3 Paxillin

MOCK

FAK

CAKβ

CCC

CCF

CFC

CFF

FIG. 8. **Subcellular localization of chimeras containing the N terminus of CAKβ.** Mock-transfected CE cells and CE cells expressing FAK, CAKβ, CCC, CCF, CFC, and CFF were plated onto fibronectin-coated coverslips and cultured in complete media for 16 h. Cells were co-stained with the KT3 epitope tag antibody to reveal kinase localization and the anti-paxillin antibody 8605 to visualize focal adhesions.

physically associates with the C-terminal kinase domain, and this interaction is important for catalytic activity (58). Similar intramolecular interactions within CAKβ or the chimera could have inhibitory or stimulatory effects on catalytic activity. Al-
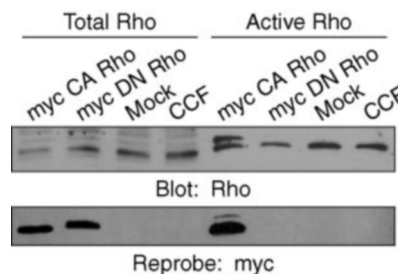
Total Rho | Active Rho

FIG. 9. **RhoA activity is similar in mock and CCF cells.** The activity of RhoA in confluent mock-transfected CE cells and cells expressing CCF, as well as cells expressing Myc-RhoA Q63L (constitutively active, positive control) and Myc-RhoA T19N (dominant negative, negative control) was assessed as described under "Experimental Procedures." Membranes were Western blotted for Rho (*upper panel*) and stripped and reprobed with anti-Myc antibody to reveal exogenous Rho proteins (*lower panel*).

ternatively, the N terminus of CAKβ could recruit negative regulators, *e.g.* phosphatases, or the N terminus of FAK may recruit positive regulators into complex with the chimeras. While the molecular details remain to be established, it is clear that the presence of the N terminus of FAK with the catalytic domain of CAKβ resulted in increased Src binding, which was associated with pronounced effects on the tyrosine phosphorylation of these kinases, as well as their catalytic activity.

Tyrosine phosphorylation on the chimeric kinases correlated, albeit not perfectly, with two domains: the C termini and catalytic domains. Chimeric kinases containing the C-terminal domain of FAK tended to be more highly phosphorylated. All chimeras containing this domain efficiently targeted to focal adhesions and thus enhanced tyrosine phosphorylation may be due to subcellular localization. These results are consistent with the conclusion drawn previously from a study using FAK/CAKβ chimeric proteins (14). It has been suggested that localization and complex formation in focal adhesions may be sufficient for the activation and phosphorylation of FAK. This may explain why CAKβ phosphorylation is low in cells in which it is diffusely localized (14, 59). Interestingly, further analysis revealed that localization to focal adhesions cannot be the sole determinant of a high level of tyrosine phosphorylation, since the FCC chimera targeted to focal adhesions, yet is poorly tyrosine phosphorylated. Thus the C-terminal domain of FAK may play a role in addition to targeting to promote tyrosine phosphorylation.

The identity of the catalytic domain of the chimera also contributed to levels of phosphotyrosine on chimeric kinases *in vivo*. Chimeras with the FAK catalytic domain tended to be more highly phosphorylated than chimeras with the CAKβ catalytic domain, despite the fact that CAKβ catalytic activity *in vitro* and *in vivo* is greater than that of FAK. Therefore, relative tyrosine phosphorylation *in vivo* is not an indication of relative catalytic activity. In support of this observation, a previous report demonstrated that FAK catalytic activity was low when NIH3T3 cells in culture were serum-starved, and maximal after replating on fibronectin, although FAK was highly phosphorylated in both cases (60). These data suggest that the FAK catalytic domain could promote kinase phosphorylation via another mechanism. Since more c-Src was found in complex with FAK than CAKβ, it is possible that FAK binds Src kinases with a higher affinity than CAKβ, resulting in elevated phosphorylation of Src-dependent sites on FAK/CAKβ. FAK contains a consensus Src SH3 domain binding site ([368]RA**LPSIPKL**[376]) in the proximity of the Src SH2 domain binding site which is not highly conserved in CAKβ ([374]NS**LP-QIPTL**[382]). Alternatively, enhanced Src binding might impair

TABLE I
Summary of Results

	Substrate pY[a] *in vivo*	Catalytic activity *in vitro*	Kinase pY *in vivo*	Focal adhesion targeting	Morphological changes
FFF	+	+	+++	+++	−[b]
FFC	+	+	++	++	−
FCF	++++	++++	+++	+++	−
FCC	++++	++++	+	++	−
CCC	+++	+++	+	−	+
CCF	+++	+++	++	+++	++
CFC	+	+	++	−	++
CFF	+	++	+++	++	++

[a] pY, tyrosine phosphorylation.
[b] None detected.

dephosphorylation of the major autophosphorylation site *in vivo*.

FAK and FRNK localize prominently to focal adhesions in adherent cells (1). Conversely, there are conflicting reports regarding the subcellular localization of CAKβ. The autonomously expressed C-terminal domain of CAKβ (CRNK/PRNK) localizes to focal adhesions (7, 19). However, in CE cells, only a subset (~10%) of CAKβ expressing cells exhibit focal adhesion staining. These data suggest that the FAT sequence within the CAKβ C terminus is functional, but may be masked in the context of the full length protein (7). Several groups have employed FAK/CAKβ chimeric proteins to explore differences in FAK and CAKβ localization. The FAK C terminus was universally capable of directing chimeric kinases to focal adhesions. Like CFF and CCF, chimeric proteins PFhy1 and Pyk2/FAK-CT (which resemble CCF) were targeted to focal adhesions (14, 32, 54). These results demonstrate that the FAK FAT sequence was dominant over a potential opposing localization signal in the CAKβ N terminus or catalytic domain.

While there is consensus that the FAT sequence of FAK can target CAKβ to focal adhesions, there is conflicting data regarding the ability of the C terminus of CAKβ to target chimeric proteins to focal adhesions. Clearly, FFC and FCC localized to focal adhesions in CE cells. The CAKβ FAT sequence might be slightly less efficient than that of FAK since FFF and FCF exhibited slightly more discrete focal adhesion localization than FFC and FCC. That the C terminus of CAKβ can direct focal adhesion targeting contrasts with previous reports of chimeric proteins FPhy2 (similar to FFC) and FAK/Pyk2-CT (similar to FCC), which were diffusely localized in NIH3T3 cells and FAK null cells, respectively (32, 54). Discrepant results regarding the localization of similar chimeric proteins may reflect slight differences in domain structure or the use of different cell systems. Since CE cells are primary cells that form well-defined focal adhesions, we believe that it is a system well suited for these analyses.

The C terminus of CAKβ was capable of directing chimeric kinases containing the N terminus of FAK to focal adhesions (FFC and FCC), but not those that contained the N terminus of CAKβ (CCC and CFC). A potential explanation for this phenomenon is that the N terminus of FAK houses a second focal adhesion targeting activity that compensates for the absence of a strong C-terminal CAKβ FAT sequence. In support of this notion, the N terminus of FAK contains a β_1 integrin binding site (61). Additionally, the N termini of both FAK and CAKβ contain a putative FERM domain, which has been implicated in linkage to transmembrane proteins (3, 62, 63). However, an ancillary targeting sequence in the N terminus of FAK must be weak, since it cannot independently target FAK to focal adhesions (18). Alternatively, the N terminus of CAKβ may oppose the focal adhesion targeting activity of the C terminus of CAKβ. The CAKβ N terminus could fold back onto the molecule

and block the FAT sequence. It may also direct the protein to or anchor the protein in another location in the cell.

It was shown previously that treatment of cells expressing CAKβ, but not FAK, with the tyrosine phosphatase inhibitor vanadate induced dramatic changes in cell morphology (7). In an effort to determine which domain of CAKβ mediated changes in cell morphology, the morphology of cells expressing chimeras was observed. Expression of kinases containing the N terminus of CAKβ resulted in a profound loss of the typical fibroblastic morphology. Strikingly, these morphological changes occurred in the absence of vanadate. One explanation for the morphology change is that expression of certain chimeras induced apoptosis. CAKβ is activated by a variety of stress signals and has been directly implicated in signaling pathways downstream of known apoptotic agents (25, 26, 36, 64). Further, Xiong and Parsons (1997) showed that expression of CAKβ/Pyk2 and chimeric proteins Pyk2/FAK1 and Pyk2/FAK2 (similar to CFF and CCF, respectively) in Rat-1 fibroblasts resulted in apoptosis in a high percentage of cells. These data suggested that the N terminus of CAKβ and catalytic activity were required for maximum induction of cell death. Although morphological changes that occur in CE cells are also dependent upon the N terminus of CAKβ, cell rounding was not associated with an increase in apoptotic cell death. Therefore, it seems more likely that these kinases induce cell rounding by eliciting changes in cytoskeletal structure.

The notion that FAK family kinases impinge upon the actin cytoskeleton is not a new one. Both associate physically and biochemically with actin-based structures and actin cytoskeleton regulatory proteins (1, 65). The importance of FAK family kinases in regulation of the cytoskeleton is underscored by the fact that FAK-null cells have increased stress fiber formation and a transient migration defect. This affect has been attributed to a deregulation of Rho signaling (29, 66). Attenuation of CAKβ expression in osteoclast-like cells causes cell retraction and a dramatic decrease in the area of the cell (67). Similar to CE cells, it was recently reported that CAKβ expression induced cell rounding in Swiss 3T3 cells, and that this effect was dependent upon the N terminus of the kinase. This morphology was attributed to changes in actin cytoskeletal structure (68).

The mechanism by which chimeras containing the N terminus of CAKβ mediate dramatic cell shape changes in CE cells is unclear. The primary effect is not decreased adhesion or an inability to spread, since there were no detectable difference in the ability of these cells to initially attach or spread onto fibronectin, and the kinetics of spreading were not altered.[2] However, these cells may have a defect in maintenance of focal adhesions or cell shape. This may be due to direct affects on the structure of focal adhesions and/or the actin cytoskeleton. In support of this hypothesis, paxillin staining was altered even in well spread cells, suggesting that changes in focal adhesion structure may precede gross morphological changes. It is inter-

esting to note that RhoA activity was unaltered in CCF cells, suggesting that an alternate signaling pathway may be involved in regulating cell morphology. The results in CE cells are clearly phenomenological since CAKβ is not expressed in this cell type. However, these finding may provide insight into the physiological function of CAKβ in cells with dynamic cytoskeletons, such as macrophages and osteoclasts. The challenge for the future is to determine how FAK-like kinases regulate changes in cell structure through elucidation of the signaling pathways linking FAK kinases and cytoskeletal dynamics.

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