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Paxillin Is Tyrosine-phosphorylated by and Preferentially Associates with the Calcium-dependent Tyrosine Kinase in Rat Liver Epithelial Cells*

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Xiong Li and H. Shelton Earp‡

From the Department of Pharmacology and Medicine and the Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7295

We and others have recently cloned a non-receptor, calcium-dependent tyrosine kinase (CADTK; also known as PYK2, CAKβ, and RAFTK) that shares both overall domain structure and 45% amino acid identity with p125^{FAK}. We have studied the signaling, activation, and potential function of these related enzymes in GN4 rat liver epithelial cells that express CADTK and p125^{FAK} at roughly similar levels. p125^{FAK} is nearly fully tyrosine-phosphorylated in resting GN4 cells. In contrast, while CADTK is not tyrosine-autophosphorylated in untreated cells, angiotensin II increases CADTK Tyr(P) by 5–10-fold. With regard to signaling, CADTK activation is correlated with stimulation of c-Jun N-terminal kinase and p70^{S6K} pathways but not with the stimulation of mitogen-activated protein kinase or p90^{RSK}. In this report we assessed the contribution of CADTK and p125^{FAK} to tyrosine phosphorylation of focal contact proteins. In adherent GN4 cells, the constitutive activity of p125^{FAK} was correlated with basal paxillin, tensin, and p130^{CAS} tyrosine phosphorylation. A rapid increase in the tyrosine phosphorylation of each protein was detected after treatment with angiotensin II or other agonists that stimulate CADTK; the prolonged 3–4-fold increase in paxillin tyrosine phosphorylation was the most substantial change. In the WB cell line that expresses 3-fold less CADTK than GN4 cell line agonist-dependent paxillin tyrosine phosphorylation is similarly reduced. Immunoprecipitation of CADTK from GN4 cells revealed CADTK·paxillin complexes that persisted in 500 mM NaCl but not in 0.1% SDS cell lysis buffer. The complexes were largely independent of the tyrosine phosphorylation state of either protein. Surprisingly, we did not detect p125^{FAK}·paxillin complexes in immunoprecipitates using either of two p125^{FAK} antibodies. When CADTK and p125^{FAK} were transiently overexpressed in 293(T) cells, both enzymes associated with paxillin, but the avidity of CADTK appeared to be greater. In addition, in transfected 293(T) cells, complexes between CADTK and another potential substrate, p130^{CAS}, were detected. In summary, in GN4 rat liver epithelial cells stimulation of CADTK was highly correlated with paxillin tyrosine phosphorylation; in addition, CADTK but not p125^{FAK} was complexed to paxillin at detectable levels. This suggests that agonist-dependent cytoskeletal changes in epithelial cells might proceed, in part, by CADTK-dependent mechanisms.

The once separate fields of cell structure and signal transduction have converged due to a number of recent observations. Growth factors and hormones are now known to regulate cell shape and mobility; conversely, proteins involved in cell attachment and cytoskeleton formation can clearly be induced to generate intracellular signals (1–6). For example, integrins engagement stimulates tyrosine phosphorylation (7–10) and alters gene expression (6, 11).

Several years ago our laboratory noted that EGF[1] treatment of rat liver epithelial cells produced several waves of protein tyrosine phosphorylation, the second occurring ~30 s after the initial accumulation of EGF-dependent Tyr(P) substrates (12). At least some of the secondary substrates, those in the p115–130-kDa region, corresponded in molecular size to Tyr(P) substrates observed following integrin cross-linking. The delayed EGF-dependent activity was, in part, due to stimulation of a soluble protein tyrosine kinase that appeared to be activated by the small EGF-dependent calcium signal (13). Kinase activation was even greater with the higher intracellular calcium levels produced by angiotensin II (Ang II), [Arg^8]vasopressin, or epinephrine (13, 14). Thapsigargin which increases intracellular calcium without stimulating protein kinase C also stimulated tyrosine phosphorylation, an effect greatly diminished by pretreatment of cells with BAPTA-AM, an intracellular calcium chelator (13, 14).

We have now purified the calcium-dependent tyrosine kinase (CADTK) from rat liver epithelial cells (15) and obtained a complete cDNA (16). Rat CADTK encodes the same protein as human PYK2 (17), RAFTK (18), and rat CAKβ (19). CADTK and p125^{FAK} are highly related, and thus comparison is instructive. The 45% overall amino acid identity is particularly striking within the tyrosine kinase and C-terminal domains. p125^{FAK}, originally identified as a v-*src* substrate is localized to focal adhesions (20) where a network of proteins connects the extracellular matrix to the actin cytoskeleton (4) and is activated in response to cellular interaction with the extracellular matrix (7, 10, 21, 22). This latter effect is often correlated with but may or may not be causally related to signals that activate the MAPK pathway (23–27). In summary, p125^{FAK} sits at a conceptual and physical juncture where it is capable of integrating cell structure and signaling.

p125^{FAK} does not contain SH2 or SH3 domains but can

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‡ To whom correspondence should be addressed. Tel.: 919-966-2335; Fax: 919-966-3015.

[1] The abbreviations used are: EGF, epidermal growth factor; CADTK, calcium-dependent tyrosine kinase; p125^{FAK}, p125 focal adhesion tyrosine kinase; p130^{CAS}, p130 Crk-associated substrate; p70^{S6K}, p70 ribosomal S6 kinase; p90^{RSK}, p90 ribosomal S6 kinase; MAPK, mitogen-activated protein kinase; Tyr(P), tyrosine phosphorylation; Ang II, angiotensin II; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester; Me_2SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

interact with proteins containing SH2 and SH3 binding domains. When autophosphorylated on tyrosines, $p125^{FAK}$ creates binding sites for other SH2 domain-containing proteins including Src tyrosine kinase family members (28–30). $p125^{FAK}$ also exhibits a proline-rich region that can interact with SH3 domain-containing proteins including $p130^{CAS}$ (31–34). Additionally, the C terminus encompasses the focal adhesion targeting domain that forms direct or indirect complexes with several focal adhesion proteins including paxillin (35). The association with paxillin presumably occurs through two vinculin homology domains within the C-terminal focal adhesion targeting sequence; in turn, paxillin binding has been suggested to localize $p125^{FAK}$ to focal adhesions (36). However, others have reported that paxillin association may not be required for $p125^{FAK}$ focal adhesions' localization (37). As noted, tyrosine-phosphorylated $p125^{FAK}$ binds (and may activate) Src family tyrosine kinases, concentrating these enzymes and other proteins within focal adhesions, in effect assembling a large potential signaling complex (5, 6, 24).

$p125^{FAK}$ has been most extensively studied in fibroblasts, a cell type that does not express CADTK (19) (data not shown). The latter kinase is abundant in neural tissue and is expressed in epithelial cells and hematopoietic cells (16–18). However, CADTK is not excluded from cells of mesenchymal origin as we have found it to be activated by multiple agonists in rat smooth muscle cells.[2] While some cells express only one or the other enzyme, at least several cell types including rat liver epithelial cells express CADTK and $p125^{FAK}$. CADTK is rapidly activated by a G_q protein-coupled signal using either the calcium- or protein kinase C-dependent branches of phospholipase C-activated pathways (15–17). Neither the effect of calcium nor protein kinase C appear to be direct (calcium does not directly activate the purified enzyme and protein kinase C activation is slower). In Swiss 3T3 cells, $p125^{FAK}$ is rapidly stimulated by bombesin and other neuropeptides (38–41). However, in cells in which both enzymes are expressed the stimulation of CADTK activity is more rapid, of greater magnitude, and is far more dependent upon hormonal and growth factor stimulation than changes in $p125^{FAK}$ (16, 17).[2] For example, in adherent WB and GN4 cells, $p125^{FAK}$ exhibits a high basal level of tyrosine phosphorylation and is only minimally increased by Ang II treatment, whereas CADTK is stimulated by 5–10-fold (16). In many instances $p125^{FAK}$ activation is correlated with stimulation of the MAPK pathway although a direct causal relationship is not universally agreed upon. The calcium-dependent activation of CADTK in GN4 liver epithelial cells does not activate MAPK (16) but is correlated with substantial increases in c-Jun N-terminal kinase and the $p70^{S6}$ kinase ($p70^{S6K}$) activities (16, 42, 43).

But what of CADTK's interaction with and phosphorylation of cytoskeletal proteins, a dominant feature of $p125^{FAK}$ biology? In this article we report that Ang II treatment stimulates the tyrosine phosphorylation of paxillin, tensin, and $p130^{CAS}$. The effect on paxillin phosphorylation is the most dramatic and prolonged. While $p125^{FAK}$ probably regulates basal paxillin tyrosine phosphorylation in adherent GN4 cells (since CADTK activity is minimal), the rapid and extensive paxillin tyrosine phosphorylation observed after agonist stimulation correlates with both the activation and expression of CADTK. Moreover, even though the levels of tyrosine-phosphorylated $p125^{FAK}$ and CADTK are similar in GN4 cells, paxillin forms a noncovalent complex with CADTK, whereas paxillin·$p125^{FAK}$ complexes could not be detected. Overexpression of CADTK and $p125^{FAK}$ by transient transfection again revealed the increased affinity of paxillin for CADTK and also demonstrated CADTK·$p130^{CAS}$ complexes. Thus, in GN4 cells, hormonal activation of CADTK significantly increases paxillin tyrosine phosphorylation, acute changes that are played out upon a background level of extracellular matrix dictated protein tyrosine phosphorylation mediated, in part, by $p125^{FAK}$.

[2] A. Brinson, X. Li, Y. He, H. S. Earp, and L. M. Graves, unpublished results.

EXPERIMENTAL PROCEDURES

Materials—Ang II was purchased from Sigma and prepared in 50 mM acetic acid prior to use. Thapsigargin and BAPTA-AM were purchased from Sigma and Biomol, respectively, and prepared in Me_2SO. Anti-paxillin monoclonal antibody and anti-Tyr(P) monoclonal antibody (RC20H) were purchased from Transduction Laboratories. Anti-Tyr(P) monoclonal antibody (PT66) was purchased from Sigma. Anti-$p125^{FAK}$, monoclonal antibody 2A7, and polyclonal antibody BC3 were generously provided by Drs. Tom Parsons (University of Virginia) and Michael Schaller (University of North Carolina). Anti-$p125^{FAK}$ polyclonal antibody, A17, was purchased from Santa Cruz Biotechnology. Anti-$p130^{CAS}$ monoclonal and polyclonal antibodies were purchased from Transduction Laboratories and Santa Cruz Biotechnology, respectively. Anti-tensin monoclonal antibody was generously provided by Dr. Keith Burridge (University of North Carolina). Rabbit anti-rat CADTK polyclonal antisera were raised to two glutathione *S*-transferase fusion proteins (amino acids 1–80 and 680–860 of rat CADTK, respectively) as described previously (16).

Cell Cultures—Rat liver epithelial cells (GN4 or WB) were cultured in Richter's minimal essential medium supplemented with 10% fetal bovine serum and 0.1 μM insulin as described earlier (15). Human 293 embryonic kidney cells with SV40 T antigen (human 293(T) cells) were kindly provided by Dr. Brian Varnum (Amgen) and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Cell Lysate Preparation—Cell lysis was performed essentially as described previously (15). Briefly, cells treated with agonists were scraped into ice-cold cell lysis buffer. One of three lysis buffers was used and referred to as follows: (i) low salt lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM NaF, and 10% (v/v) glycerol); (ii) high salt buffer (500 mM NaCl, 20 mM HEPES (pH 7.3), 1% Triton X-100, 5 mM EDTA, 50 mM NaF, and 10% (v/v) glycerol); or (iii) RIPA lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 2 mM EDTA, 10% (v/v) glycerol, 0.1% SDS, and 0.5% deoxycholate). To all cell lysis buffers the following reagents were added before use: 1 mM Na_3VO_4, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml. Cell lysates were clarified by centrifugation at 14,000 $\times$ *g* for 10 min at 4 °C and the protein content determined prior to immunoprecipitation.

Anti-Tyr(P), $p125^{FAK}$, CADTK, and Paxillin 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, followed by addition of 20 μl of protein A or protein A/G-agarose beads (Santa Cruz) for 1 h. Immune complexes were collected by centrifugation (in some cases the supernatant was used for a second antibody immunoprecipitation). Immune complexes were then washed three times with the lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE, transferred to Immobilon (Millipore), and detected by incubating the blots with the specified antibody as described (15). Immunoblots were incubated with either goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies and developed using enhanced chemiluminescence according to the manufacturer's procedure (Amersham Corp.). When indicated, immunoblots were stripped in the buffer (62.5 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol) at 50 °C for 30 min, and reprobed with another specific antibody.

Transient Expression of CADTK and $p125^{FAK}$ in Human 293(T) Cells—The full-length CADTK cDNA was isolated as described (16) and was subcloned into a pcDNA3 vector (Invitrogen). Human $p125^{FAK}$ cDNA, subcloned into the pcDNA3 vector, was kindly provided by Dr. William Cance (University of North Carolina). The control vector (pcDNA3), pcDNA3-CADTK, and pcDNA3-$p125^{FAK}$ were transfected into subconfluent human 293(T) cells with LipofectAMINE according to the manufacturer's procedure (Life Technologies, Inc.). After 48 h, transfected cells were harvested and lysed on ice with low salt buffer, and the lysates were analyzed by immunoprecipitation, followed by immunoblotting with anti-Tyr(P), CADTK, $p125^{FAK}$, paxillin, or $p130^{CAS}$.

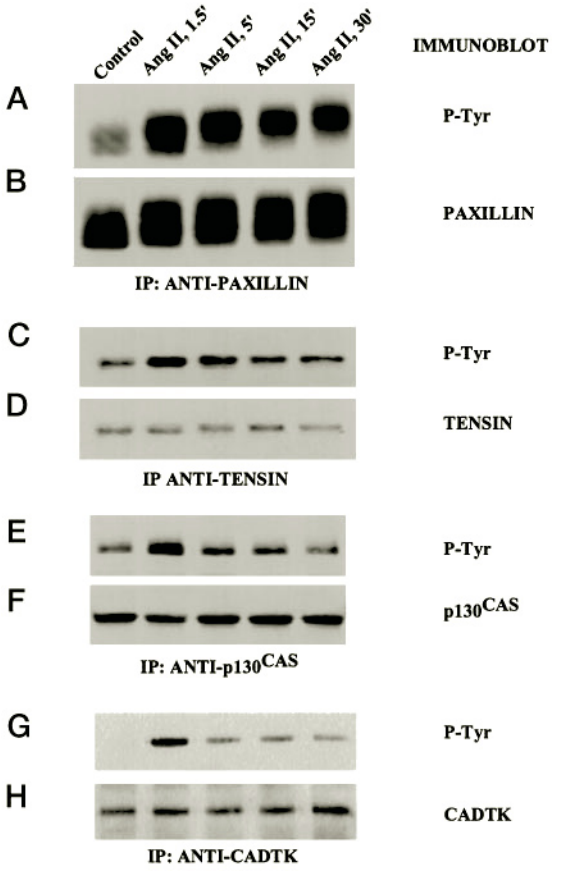

FIG. 1. **Ang II stimulates paxillin, p130^{CAS}, tensin, and CADTK tyrosine phosphorylation in GN4 cells.** Rat liver epithelial cells (GN4) were treated with Ang II (1 μM) for the times indicated. Cells were then lysed, and immunoprecipitates (*IP*) were prepared using anti-paxillin, tensin, p130^{CAS}, and CADTK antibodies. Immunoprecipitates were analyzed by SDS-PAGE, followed by transfer of proteins to Immobilon and immunoblotting with the anti-Tyr(P) antibody (RC20H). Blots were stripped and reprobed with anti-paxillin (*B*), and anti-tensin (*D*), anti-p130^{CAS} (*F*), and anti-CADTK (*H*) antibodies, respectively.

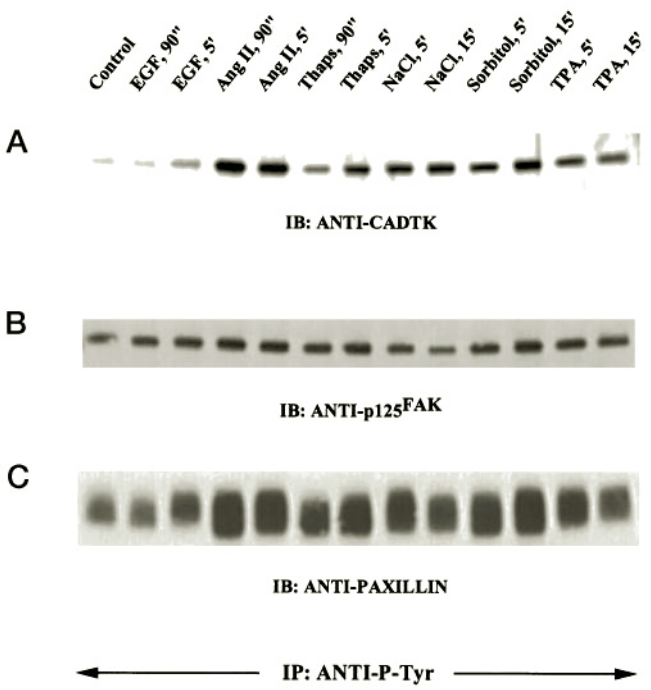

FIG. 2. **CADTK agonists increase paxillin tyrosine phosphorylation in GN4 cells.** GN4 cells were treated with EGF (100 ng/ml), Ang II (1 μM), thapsigargin (*Thaps*) (2 μM), NaCl (0.7 M), sorbitol (0.4 M), and phorbol esters (*TPA*) (100 nM) for the times indicated. Equal amounts of cell lysate were immunoprecipitated (*IP*) with anti-Tyr(P) antibody (pT66). The immunoprecipitates were then analyzed by SDS-PAGE, followed by immunoblotting (*IB*) with anti-CADTK (*A*), anti-p125^{FAK} (*B*), and anti-paxillin (*C*) antibodies, respectively.

RESULTS

Ang II Increases Paxillin, Tensin, and p130^{CAS} Tyrosine Phosphorylation—As previously reported, Ang II and thapsigargin increase the tyrosine phosphorylation of proteins with a molecular weight of p115–130, p72/75, and p66 (13–15). One of the 115-kDa tyrosine-phosphorylated proteins was identified as the major calcium-dependent tyrosine kinase (CADTK). Since integrin engagement stimulates p125^{FAK} and paxillin Tyr(P) and Ang II has been shown to increase paxillin tyrosine phosphorylation in smooth muscle cells (44, 45), we tested whether one of the GN4 cell p72/75-kDa tyrosine phosphoproteins was paxillin. Immunoprecipitation and anti-Tyr(P) immunoblot revealed that Ang II treatment of GN4 cells increased paxillin tyrosine phosphorylation by 90 s, an effect that persisted for at least 30 min (Fig. 1*A*). Paxillin tyrosine phosphorylation could be detected at 15–30 s coincident with the tyrosine phosphorylation of CADTK (data not shown). Ang II stimulation also altered paxillin electrophoretic mobility, suggesting sequential, multi-site phosphorylation on Tyr, Ser, or Thr residues. The mobility shift, while detected at early time points, became more pronounced at 5 min.

Ang II treatment also rapidly increased tensin (Fig. 1*C*) and p130^{CAS} (Fig. 1*E*) tyrosine phosphorylation. In contrast to paxillin, both tensin and p130^{CAS} reached their maximum level of tyrosine phosphorylation by 90 s and gradually decreased their Tyr(P) content. This more closely parallels the time course of CADTK tyrosine phosphorylation (Fig. 1*G*) but may also suggest that tensin and p130^{CAS} are more susceptible to tyrosine phosphatases than paxillin. Tensin but not p130^{CAS} also exhibited a significant gel mobility shift with Ang II treatment (Fig. 1*C*). The gel mobility shift persists even though total Tyr(P) as assessed by immunoblot recedes. It is likely that the gel mobility shift of both paxillin and tensin progress as due to Ser/Thr phosphorylations or by tyrosine phosphorylation at new sites. Tryptic phosphopeptide mapping followed by phosphoamino analysis may resolve this issue.

Agonist-dependent CADTK and Paxillin Tyrosine Phosphorylation Are Correlated—Rat liver epithelial cells express both p125^{FAK} and CADTK. Stimulation with Ang II leads to a variable alteration in p125^{FAK} tyrosine phosphorylation and autokinase activity ranging from barely detectable to 50–100% increases (15). This is presumably due to the fact that p125^{FAK} is near maximally phosphorylated in adherent, confluent GN4 cells. In contrast, CADTK tyrosine phosphorylation is barely detectable in control cells but is rapidly induced (5–10-fold) by Ang II or other agonists. To test whether CADTK or p125^{FAK} stimulation correlated with acute changes in paxillin tyrosine phosphorylation, GN4 cells were treated with a series of agonists known to stimulate CADTK followed by comparison of the Tyr(P) content of paxillin, CADTK, and p125^{FAK}. Agonists that significantly increased CADTK tyrosine phosphorylation (Fig. 2*A*) were the most effective at acutely increasing paxillin tyrosine phosphorylation (Fig. 2*C*). In general, the extent of CADTK and paxillin Tyr(P) was correlated. For example, prolonged EGF treatment (5 min) slightly increased CADTK tyrosine phosphorylation (Fig. 2*A, lane 3*) and slightly stimulated paxillin tyrosine phosphorylation (Fig. 2*C, lane 3*), whereas Ang II and sorbitol maximally stimulated CADTK and paxillin Tyr(P) content. In contrast, p125^{FAK} tyrosine phosphorylation was only minimally altered by agonist treatment (Fig. 2*B*).

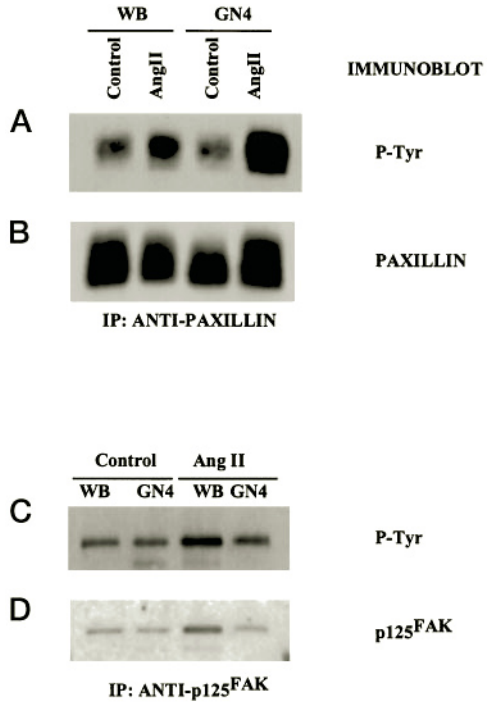

FIG. 3. **Comparison of paxillin tyrosine phosphorylation in GN4 and WB cell lines in response to Ang II stimulation.** Rat liver epithelial cells (GN4 and WB cells) were treated with Ang II (1 μM, 90 s). Cells were then lysed, and immunoprecipitates (*IP*) were prepared with anti-paxillin antibody (*A* and *B*) or anti-p125^{FAK} antibody (*C* and *D*). Immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting with anti-Tyr(P) antibody (RC20H) (*A* and *C*). Blot was stripped and reprobed with anti-paxillin antibody (*B*) or anti-p125^{FAK} antibody (*D*).

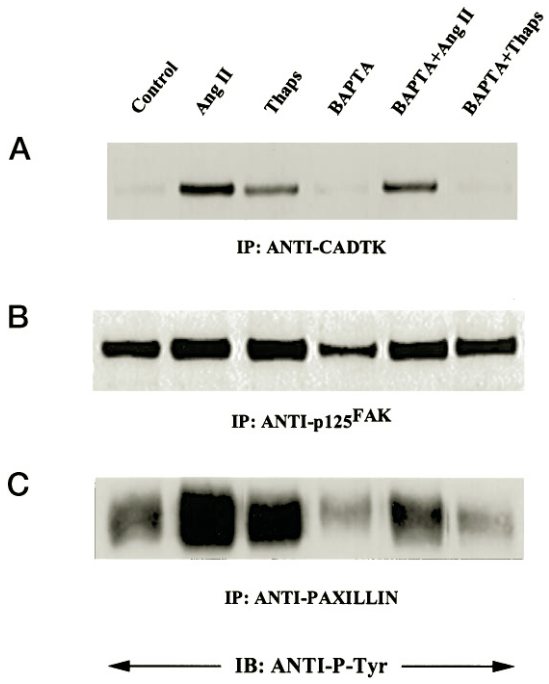

FIG. 4. **The intracellular calcium chelator, BAPTA-AM, inhibits CADTK tyrosine autophosphorylation and paxillin tyrosine phosphorylation to a greater degree than it inhibits p125^{FAK} tyrosine phosphorylation.** GN4 cells were pretreated with 50 μM BAPTA-AM for 20 min, followed by Ang II (1 μM) and thapsigargin (*Thaps*) (2 μM) stimulation (90 s). Cells were then lysed, and equal amounts of cell lysates were immunoprecipitated (*IP*) with anti-CADTK (*A*), anti-p125^{FAK} (*B*), and anti-paxillin (*C*) antibodies. Immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting (*IB*) with anti-Tyr(P) antibody (RC20H), respectively.

Therefore, basal paxillin tyrosine phosphorylation may be determined by constitutive p125^{FAK} activity. The correlation between CADTK and paxillin tyrosine phosphorylation suggests that G_q protein-coupled receptor and other agonists stimulate additional paxillin tyrosine phosphorylation via CADTK.

Agonist-stimulated Paxillin Tyrosine Phosphorylation Correlates with CADTK Expression and Extent of Activation—To further examine the role of the two enzymes, Ang II-dependent paxillin tyrosine phosphorylation was investigated in cells known to express similar levels of p125^{FAK} but different levels of CADTK. We had previously shown that confluent GN4 cells (derived by chemical transformation of a normal rat liver epithelial cell line, WB) have ~3–4 times more Ang II-dependent tyrosine phosphorylation than its parent WB cell (13). Immunoprecipitation with specific CADTK antiserum demonstrated that GN4 cells exhibit ~4–5-fold higher maximal Ang II-dependent CADTK tyrosine phosphorylation (90 s) than similarly treated WB cells (16). Likewise, Ang II treatment resulted in significantly more paxillin tyrosine phosphorylation in GN4 cells than in WB cells (Fig. 3*A*). p125^{FAK} expression and tyrosine autophosphorylation in response to Ang II stimulation was similar in the GN4 and WB cell lines (Fig. 3*C*). Thus the similar basal level of paxillin tyrosine phosphorylation in WB and GN4 (Fig. 3*A*) cells correlated with the equivalent p125^{FAK} levels (Fig. 3*C*). The much greater Ang II-dependent paxillin tyrosine phosphorylation in GN4 cells was observed consistently. It supports the hypothesis that increased CADTK activation acutely regulates paxillin Tyr(P) content.

Intracellular Calcium Chelation and Cytochalasin D Block Ang II-dependent Paxillin Tyrosine Phosphorylation—CADTK activation by Ang II or thapsigargin is, in part, a calcium-dependent process. To test the calcium dependence of Ang II and thapsigargin-stimulated paxillin tyrosine phosphorylation, GN4 cells were preincubated with the cell-permeable, calcium chelator BAPTA-AM that abolishes the calcium signal in these cells (13, 14). BAPTA attenuated the acute increase in both CADTK and paxillin tyrosine phosphorylation in response to Ang II (Fig. 4, *A* and *C*). The effect of BAPTA was not complete since Ang II also regulates CADTK activity via protein kinase C. However, BAPTA, which completely blocked thapsigargin-dependent CADTK activation (Fig. 4*A, lane 6*), almost totally inhibited thapsigargin-dependent paxillin tyrosine phosphorylation (Fig. 4*C, lane 6*). Preincubation with BAPTA-AM alone decreased both basal p125^{FAK} and paxillin tyrosine phosphorylation (Fig. 4*B*). These results suggest that calcium-dependent activation of CADTK is in part responsible for initiating paxillin tyrosine phosphorylation in response to Ang II and thapsigargin.

Paxillin, a member of the complex of cytoskeletal proteins, is concentrated at focal contacts, the cytoplasmic face of regions attaching the cell to the extracellular matrix (46, 47). Cytochalasin D, an agent that disrupts actin microfilaments and cytoskeletal movement, inhibits p125^{FAK} tyrosine autophosphorylation and prevents paxillin tyrosine phosphorylation in Swiss 3T3 cells (22, 39, 41, 48). To test whether cytochalasin D inhibited CADTK, p125^{FAK}, or paxillin tyrosine phosphorylation, GN4 cells were preincubated with cytochalasin D and subsequently treated with Ang II or NaCl, another agonist that stimulates CADTK activity (see Ref. 16 and Fig. 2). Cytochalasin D pretreatment dramatically changed cell morphology (data not shown) and partially inhibited Ang II and NaCl-stimulated CADTK (Fig. 5, *A* and *C*) and basal p125^{FAK} tyrosine phosphorylation (Fig. 5*B*). The effect of cytochalasin D on paxillin tyrosine phosphorylation was even more dramatic; both basal and stimulated paxillin Tyr(P) were nearly eliminated by cytochalasin D pretreatment. If our thesis is correct,

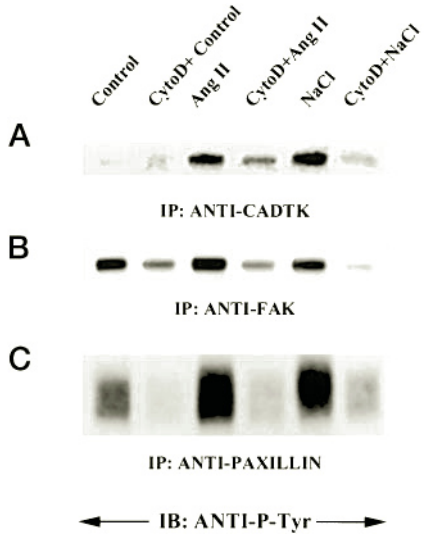

FIG. 5. **Cytochalasin D inhibits CADTK, p125^{FAK}, and paxillin tyrosine phosphorylation.** GN4 cells were pretreated with 2 μM cytochalasin D (*CytoD*) for 2 h and then treated with Ang II (1 μM) for 90 s or NaCl (0.7 M) for 5 min. Equal amounts of cell lysates were then immunoprecipitated with anti-CADTK (*A*), anti-p125^{FAK} (*B*), and anti-paxillin (*C*) antibodies, respectively. Immunoprecipitates (*IP*) were then analyzed by SDS-PAGE, followed by immunoblotting (*IB*) with anti-Tyr(P) antibody (RC20H).

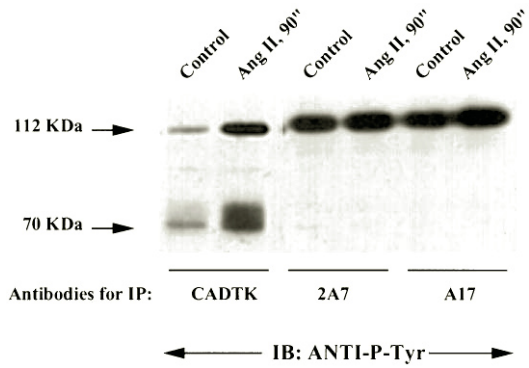

FIG. 6. **Tyrosine-phosphorylated 68–75-kDa proteins complex with CADTK but not with p125^{FAK}.** GN4 cells were treated with Ang II (1 μM) for 90 s. Cells were then lysed and immunoprecipitated with anti-CADTK or one of two anti-p125^{FAK} (*2A7*, *A17*) antibodies. Immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting (*IB*) with the anti-Tyr(P) antibody (RC20H).

i.e. that p125^{FAK} is responsible for basal paxillin tyrosine phosphorylation in adherent cells and CADTK is responsible for the hormonally induced increment, this experiment suggests that both processes require an intact cytoskeleton.

Tyrosine-phosphorylated Paxillin Associates with CADTK in Ang II-treated Cells—The results in Figs. 1–3 suggested that Ang II stimulates paxillin tyrosine phosphorylation and that this agonist-dependent process is best correlated with activation and expression of CADTK rather than p125^{FAK}. Since paxillin associates with p125^{FAK} in chicken embryo fibroblasts (37), we tested whether tyrosine-phosphorylated paxillin bound to CADTK or p125^{FAK} in control and Ang II-treated GN4 cells. Confluent GN4 cells were treated with vehicle or Ang II for 90 s, lysed, and immunoprecipitated with antiserum to CADTK or with one of two antibodies to p125^{FAK}, 2A7 or A17. CADTK tyrosine phosphorylation was stimulated in Ang II-treated cells, and p125^{FAK}, which runs slightly above the 115-kDa CADTK, was tyrosine-phosphorylated in both control and Ang II-stimulated GN4 cells (Fig. 6). Equal amounts of cell lysates were used for all immunoprecipitations and were immunoblotted with the same anti-Tyr(P) antibody; thus, the result depicted in Fig. 6 suggests that maximal level of tyrosine-phosphorylated p125^{FAK} is at least equivalent and in fact is probably slightly greater than that of CADTK. Because we do not have an antibody that recognizes both enzymes, we have compared total Tyr(P) in the immunoprecipitated kinases. The sequence and autophosphorylation sites in both kinases are highly conserved. Moreover, we have mutated the predicted major tyrosine autophosphorylation site of CADTK Tyr^{402} and demonstrated that this mutation drastically decreases CADTK autophosphorylation in the same manner that Tyr^{397} mutation reduces p125^{FAK} autophosphorylation.[3] Thus, the equivalent level of maximal tyrosine phosphorylation in GN4 cell CADTK and p125^{FAK} provides a reasonable indication that p125^{FAK} and CADTK expression are similar in these cells.

Surprisingly, 68–75-kDa range Tyr(P) proteins were associated with CADTK, but there was little or no 68–75-kDa Tyr(P) protein associated with p125^{FAK} (Fig. 6). Analysis of the CADTK immunoprecipitation with paxillin antibody showed that at least some of the 68–75-kDa Tyr(P) protein was paxillin (see below). The result suggests that while paxillin can associate with p125^{FAK} (see Refs. 36 and 37 and Fig. 9) in adherent cells, little or no tyrosine-phosphorylated paxillin can be found in precipitable p125^{FAK}·paxillin complexes.

Paxillin and CADTK Associate in Intact Cells—Having observed CADTK association with tyrosine-phosphorylated paxillin in GN4 cells, we further investigated the nature of the CADTK·paxillin complex. GN4 cells were treated with vehicle, Ang II, or thapsigargin and lysed in three different lysis buffers, low salt, high salt, and 0.1% SDS RIPA buffer. Cell lysates were first immunoprecipitated using anti-CADTK antiserum; subsequently the CADTK-cleared lysate was reimmunoprecipitated with anti-paxillin antibody. Following SDS-PAGE and transfer, a paxillin immunoblot demonstrated nearly equal amounts of paxillin present in CADTK immunocomplexes precipitated in low salt buffer from either control or agonist-treated cells. Thus, Ang II or thapsigargin treatment did not alter CADTK-paxillin association; the complex exists prior to agonist treatment (Fig. 7*A*). The results were the same after lysis in a high salt buffer (Fig. 7*B*), a condition that would disrupt protein-protein association based on Tyr(P)-SH2 domain interaction. CADTK-paxillin association was eliminated in 0.1% SDS suggesting that native conformation of one or both proteins was required (Fig. 7*C*). The above results suggested that CADTK-paxillin association *in vivo* is an intrinsic property of the proteins and does not require CADTK or paxillin tyrosine phosphorylation.

CADTK Appears to Bind More Avidly to Paxillin Than p125^{FAK}—We next tested whether disrupting the cytoskeleton structure with cytochalasin D could disrupt the CADTK-paxillin or p125^{FAK} association. As shown in Fig. 8*A*, CADTK associated with paxillin regardless of whether the GN4 cells were treated with cytochalasin D or not. Although, cytochalasin D pretreatment inhibited CADTK tyrosine phosphorylation and prevented paxillin tyrosine phosphorylation in Ang II-treated cells (Fig. 5), it did not disrupt the CADTK-paxillin association. Paxillin·p125^{FAK} complexes were not detected under these conditions (with or without cytochalasin D) (Fig. 8*B*). The antibody, 2A7, used to immunoprecipitate p125^{FAK} is the same antibody that detects the p125^{FAK}-paxillin association in chicken embryo fibroblasts (37). Paxillin immunoblotting after immunoprecipitating with p125^{FAK} antibody, A17, also failed to detect paxillin·p125^{FAK} complexes in GN4 cells (data not shown).

[3] X. Li and H. S. Earp, unpublished data.

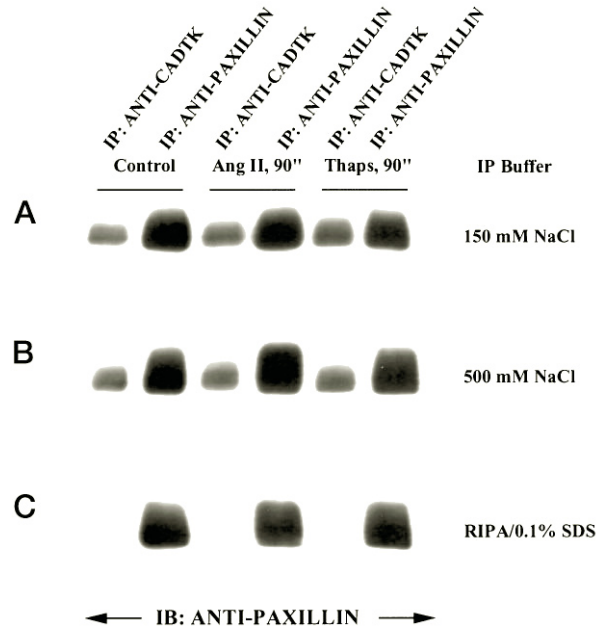

FIG. 7. **CADTK and paxillin form a non-covalent complex.** GN4 cells were treated with Ang II (1 μM) or thapsigargin (2 μM) for 90 s. Cells were then lysed in three different lysis buffers, low salt buffer (*A*), high salt buffer (*B*), and RIPA buffer, containing 0.1% SDS (*C*), respectively. Equal amounts of cell lysates were immunoprecipitated (*IP*) with anti-CADTK antiserum. The supernatant of the first immunoprecipitation was then immunoprecipitated with anti-paxillin antibody. Both first and second immunoprecipitates were then analyzed by SDS-PAGE, followed by immunoblot (*IB*) with anti-paxillin antibody.

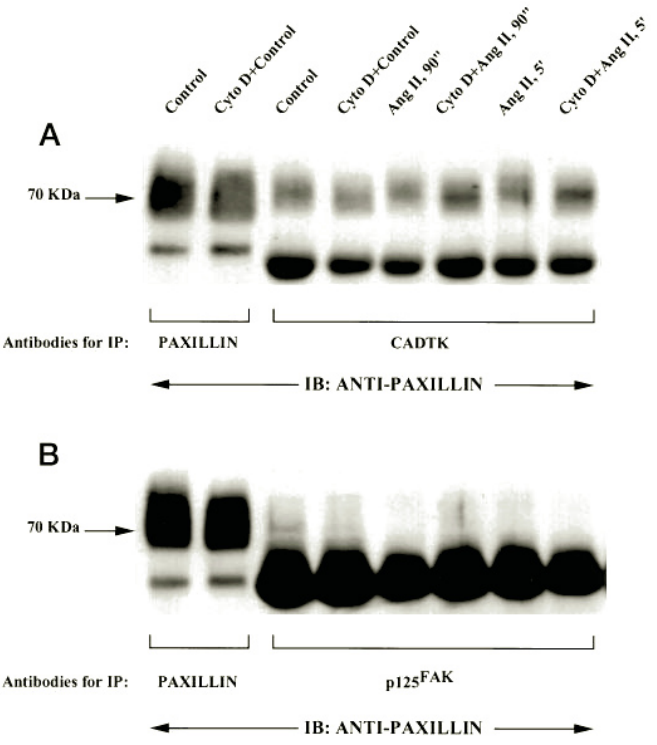

FIG. 8. **CADTK-paxillin association does not require the integrity of the cytoskeleton.** GN4 cells were pretreated with or without cytochalasin D (2 μM) for 2 h, followed by Ang II (1 μM) stimulation for the indicated times. Equal amounts of cell lysates were then immunoprecipitated (*IP*) with anti-CADTK, anti-$p125^{FAK}$, or anti-paxillin antibodies, respectively. Immunoprecipitates were analyzed by SDS-PAGE, followed by immunoblotting (*IB*) with anti-paxillin antibody.

To further examine the paxillin complexes, $p125^{FAK}$ and CADTK were overexpressed by transient transfection cDNAs driven by cytomegalovirus promotor in human 293(T) cells. 293(T) cells exhibit minimal $p125^{FAK}$ expression (as seen on an overexposed immunoblot), but we could not detect any endogenous CADTK. As shown in Fig. 9, after transfection, $p125^{FAK}$ tyrosine phosphorylation level was much higher than that of CADTK (Fig. 9*A*). Immunoprecipitation demonstrated $p125^{FAK}$·paxillin complex under this condition (Fig. 9*C*), ruling out an experimental artifact as the reason for our failure to detect $p125^{FAK}$·paxillin complex in GN4 cells.

To examine another potential CADTK substrate complex, we tested CADTK immunoprecipitates from CADTK-transfected 293(T) cells for the presence of $p130^{CAS}$. CADTK overexpression in human 293(T) cells allows detection of CADTK-$p130^{CAS}$ association (Fig. 10) as defined by co-immunoprecipitation. In contrast to paxillin, the CADTK·$p130^{CAS}$ complex was not detected in GN4 cells (data not shown), but the experiment in Fig. 10 does demonstrate a direct interaction of this potential substrate ($p130^{CAS}$), kinase (CADTK) pair.

DISCUSSION

Ang II initiates its signal via G_q protein-coupled receptor-activating phospholipase C, serine/threonine kinases, and at least one tyrosine kinase that we have recently purified and cloned and referred to as CADTK (16). Although CADTK is stimulated by a calcium signal, the calcium effect is indirect as neither calcium nor calcium/calmodulin directly activate the enzyme. Treatment of GN4 cells with phorbol esters also activates CADTK, but the activation is slower than that of Ang II or thapsigargin and is therefore also probably indirect. Thus, the mechanism by which calcium and other signals regulate CADTK is currently unknown. In GN4 cells, thapsigargin, which produces a calcium signal and activates CADTK, also stimulates both c-Jun N-terminal kinase and $p70^{S6K}$ but not MAPK or $p90^{RSK}$ (16, 42, 43). However, in PC12 cells, agonist-dependent CADTK/PYK2 stimulation increased Shc tyrosine phosphorylation and MAPK activity. We are currently trying to understand the basis of this cell type difference in CADTK signaling and must, in addition, determine whether CADTK and $p125^{FAK}$ have distinct effects on the MAPK pathway.

To broaden the comparison between CADTK and $p125^{FAK}$, we investigated the Ang II-dependent tyrosine phosphorylation of paxillin, a protein tyrosine phosphorylated upon engagement of cell surface integrins (37). In Swiss 3T3 cells (which do not express CADTK), paxillin and $p125^{FAK}$ become coordinately and substantially tyrosine-phosphorylated in response to a variety of stimulators, including bombesin, lysophosphatidic acid, platelet-derived growth factor, and GTPγS (38–41, 48). Paxillin can also be phosphorylated *in vitro* by $p60^{src}$ and Csk, both of which are potentially regulated and/or localized through $p125^{FAK}$ (30). In this report, we show that Ang II rapidly increased tyrosine phosphorylation of paxillin, tensin, and $p130^{CAS}$ in adherent GN4 rat liver epithelial cells. The effect on paxillin is the greatest. The increment in paxillin tyrosine phosphorylation produced by CADTK agonists (i) is rapid (Fig. 1), (ii) correlates with CADTK activation for a range of agonists (Figs. 2 and 5), (iii) correlates with the CADTK expression and activation in GN4 and WB cells (Fig. 3), and (iv) is largely calcium-dependent (Fig. 4). A key element of this argument is depicted in Fig. 6. Tyrosine-phosphorylated $p125^{FAK}$ is expressed at levels that are similar or slightly greater than the maximal level of tyrosine-phosphorylated CADTK. Since it is known that the major autophosphorylation site in $p125^{FAK}$ is Tyr^{397} (30, 49) and we have demonstrated the equivalent site Tyr^{402} is the major autophosphorylation site in CADTK,[3] the comparison of maximal tyrosine phosphorylation of these two enzymes provides a reasonable estimate of their relative expression. $p125^{FAK}$ is near maximally tyrosine-phosphorylated

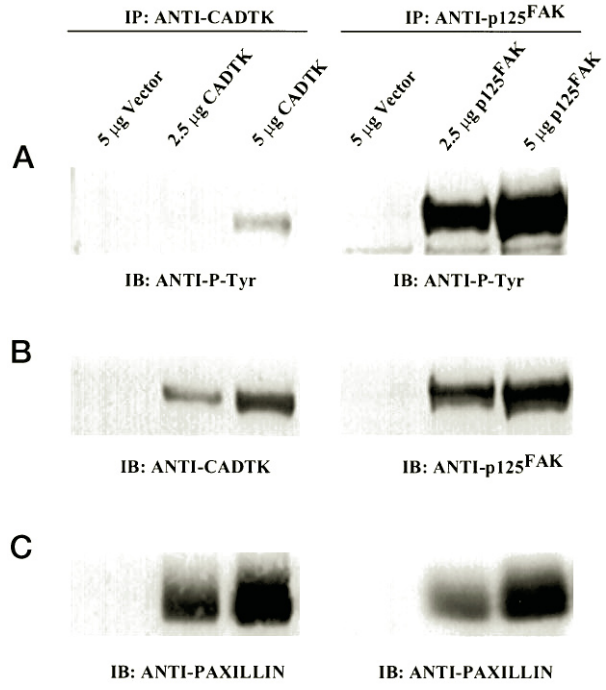

FIG. 9. **CADTK-paxillin and p125^{FAK}-paxillin associations are present in CADTK and p125^{FAK}-transfected human 293(T) cells.** Human 293(T) cells were transiently transfected with CADTK and p125^{FAK} expression constructs. Equal amounts of cell lysates were immunoprecipitated (*IP*) with either anti-CADTK or anti-p125^{FAK} antibodies. Immunoprecipitates were then analyzed by SDS-PAGE, followed by immunoblot (*IB*) with anti-Tyr(P) (*A*), anti-CADTK or anti-p125^{FAK} (*B*), and anti-paxillin (*C*) antibodies. This experiment is representative of three separate transfections.

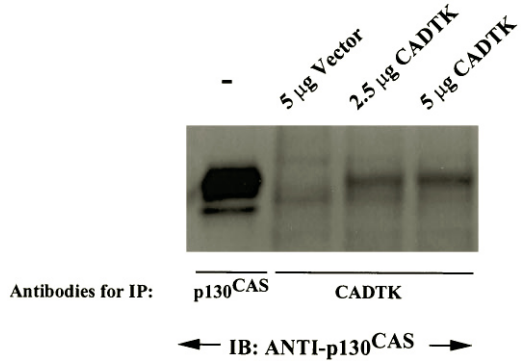

FIG. 10. **CADTK·p130^{CAS} complex is present in human 293(T) cells transfected with CADTK.** Human 293(T) cells were transiently transfected with either 2.5 or 5 μg of pcDNA3-CADTK expression constructs or with 5 μg of pcDNA3 vector. Equal amounts of cell lysates were immunoprecipitated (*IP*) with either anti-p130^{CAS} or anti-CADTK (1–80) antibodies. Immunoprecipitates were then analyzed by SDS-PAGE, followed by immunoblot (*IB*) with an anti-p130^{CAS} antibody.

in confluent, untreated cells. Since p125^{FAK} activity is thought to be primarily controlled by extracellular factors, the near maximal level of p125^{FAK} activation may well dictate the basal level of paxillin tyrosine phosphorylation. This is concordant with the fact that basal level of paxillin Tyr(P) is equivalent in WB and GN4 cell lines expressing similar amounts of p125^{FAK} (Fig. 3). Ang II treatment only minimally altered p125^{FAK} tyrosine phosphorylation (Fig. 6) and autokinase activity (15), but Ang II increases CADTK tyrosine phosphorylation (Fig. 2) and activity (16). This activation correlates with a substantial increase in paxillin and smaller but definable increases in tensin and p130^{CAS} tyrosine phosphorylation.

The intracellular calcium chelator, BAPTA, significantly inhibits thapsigargin-dependent CADTK and paxillin tyrosine phosphorylation. In agonist-treated cells, BAPTA inhibition has less effect on p125^{FAK} tyrosine phosphorylation than on CADTK (Fig. 4), suggesting that CADTK is the tyrosine kinase responsible for the G_q protein-coupled receptor-mediated increase in paxillin tyrosine phosphorylation. However, we cannot rule out other possibilities. First, CADTK tyrosine autophosphorylation like that of p125^{FAK} may provide SH2 domain binding sites to recruit tyrosine kinases, such as Src family tyrosine kinases (2, 28, 29, 49, 50). These enzymes in turn may phosphorylate paxillin. We have shown a major autophosphorylation site (Tyr^{402} AEI) on CADTK is identical to Tyr^{397} AEI in p125^{FAK} (49, 50), and both are close to the consensus sequence (YEEI) recognized by the SH2 domain of p60^{src} and its family members (51). Our preliminary data show that CADTK immune complexes can directly phosphorylate the N-terminal portion of paxillin *in vitro,*[4] but this does not preclude the action of a CADTK-associated tyrosine kinase. Fig. 6 demonstrates a high level of p125^{FAK} Tyr(P) in resting cells, presumably phosphorylated at Tyr^{397}. The rapid, agonist-induced increases in both CADTK and paxillin tyrosine phosphorylation suggest direct CADTK phosphorylation of paxillin but again do not rule out CADTK autophosphorylation followed by recruitment of another tyrosine kinase. In this latter scenario, activated CADTK could recruit and activate a tyrosine kinase with an affinity for CADTK Tyr^{402} greater than its affinity for p125^{FAK} Tyr^{397}. Alternatively, other secondary mechanisms of activation might occur via SH3-proline-rich region interactions. It seems more likely that agonist-activated CADTK directly phosphorylates paxillin.

The CADTK and p125^{FAK} similarity is higher within the focal adhesion targeting domain that has two vinculin homologous domains (36). These two domains, which are essential for paxillin binding, have been named paxillin binding sequence 1 and 2 (PBS1 and PBS2). Both PBS1 (^{919}RSNDKVYENVTGLVKAVIEM^{938}) and PBS2 (^{1029}VDAKNLLDVIDQARL^{1043}) homologous domains are present in CADTK: (^{875}RTDDLVYHNVMTLVEAVLEL^{894}) and (^{986}VDAKNLLDAVDQAKV^{999}) with 55 and 73% identity, respectively. Treatment with cytochalasin D shows that CADTK·paxillin complexes do not require either tyrosine phosphorylation or an intact cytoskeleton (Figs. 7 and 8) suggesting that CADTK may directly associate with paxillin through primary protein structures, *e.g.* the two PBS1- and PBS2-like domains. In fact, we have demonstrated that a glutathione *S*-transferase N terminus of paxillin binds to CADTK synthesized by *in vitro* translation of the rat CADTK cDNA using rabbit reticulocytes.[3] Surprisingly, p125^{FAK}-paxillin association was not detected in GN4 cells even though protein expression levels of p125^{FAK} and CADTK appear to be similar. Further experiments will be necessary to determine the mechanism (direct or indirect) and domains involved in CADTK and p125^{FAK} interactions with cytoskeletal proteins. This information could then be used to determine, in epithelial cells, whether the affinity of CADTK for paxillin is higher than that of p125^{FAK}.

Since they are expressed in the same cells, p125^{FAK} and CADTK are not likely to be cell type-specific functional equivalents. For example, CADTK cannot fully replace p125^{FAK} as p125^{FAK}-deficient mice, produced by gene targeting, result in mutant embryos (52). However, the two enzymes have similarities; for example, cell adherence to extracellular matrix leads to CADTK/RAFTK activation (53). The fact that CADTK and p125^{FAK} appear to have different abilities to complex with paxillin suggests subtle differences in function, even with re-

[4] X. Li, M. D. Schaller, and H. S. Earp, unpublished results.

spect to the cytoskeleton. Further experiments will help us define the relationship between these two protein tyrosine kinases.

Ang II treatment also increases $p130^{CAS}$ and tensin tyrosine phosphorylation, and overexpression of CADTK in 293(T) cells demonstrated CADTK·$p130^{CAS}$ complexes (Fig. 10). Transfecting cells with Rous sarcoma virus or spreading of cells on extracellular matrix induces both $p130^{CAS}$ and tensin tyrosine phosphorylation (24, 54–57). Indeed, both $p125^{FAK}$ and Src can phosphorylate these two cytoskeletal proteins *in vitro* (58, 59). $p130^{CAS}$ can associate with $p125^{FAK}$ *in vivo* through its SH3 domain (32, 34). Interestingly, the proline-rich region of $p125^{FAK}$ (^{711}APPKPSR^{717}), which interacts with the $p130^{CAS}$ SH3 domain, is almost identical to (^{703}PPPKPSR^{709}) in CADTK, and this may be the basis of CADTK-$p130^{CAS}$ association detected in transfected 293(T) cells (Fig. 10). Whether CADTK directly phosphorylates $p130^{CAS}$ and tensin remains to be determined.

The role of tyrosine phosphorylation of paxillin, $p130^{CAS}$, and tensin in G protein-coupled receptor-directed signaling is currently unknown. Although no enzymatic activity has been described, paxillin and tensin do form complexes with other cytoskeletal proteins such as vinculin and talin (47, 58). Tyrosine-phosphorylated paxillin, $p130^{CAS}$, and tensin may also provide binding sites for the SH2 containing proteins such as Crk family members (30, 34, 60, 61). In turn, Crk can associate with a number of proteins through its SH3 domains, such as two guanine nucleotide exchange proteins, C3G and SOS, and a non-receptor tyrosine kinase, Abl (62–64). It will be interesting to see whether CADTK induces paxillin, $p130^{CAS}$, and tensin tyrosine phosphorylation at sites different than those phosphorylated by $p125^{FAK}$; if so, CADTK may bring in different SH2 containing proteins and initiate unique signaling pathways.

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