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

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We and others have recently cloned a non-receptor, calcium-dependent tyrosine kinase (CADTK; also known as PYK2, CARKβ, and RAFTK) that shares both overall domain structure and 45% amino acid identity with p125FAK. We have studied the signaling, activation, and potential function of these related enzymes in GN4 rat liver epithelial cells that express CADTK and p125FAK at roughly similar levels. p125FAK 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 p70S6K pathways but not with the stimulation of mitogen-activated protein kinase or p90RSK. In this report we assessed the contribution of CADTK and p125FAK to tyrosine phosphorylation of focal contact proteins. In adherent GN4 cells, the constitutive activity of p125FAK was correlated with basal paxillin, tensin, and p130CAS 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 paxillin complexes that persisted in untreated cells revealed CADTK reduced. Immunoprecipitation of CADTK from GN4 cell line agonist-dependent paxillin tyrosine phosphorylation is similarly reduced. In transfected 293(T) cells, complexes were largely independent of the tyrosine phosphorylation state of either protein. Surprisingly, we did not detect p125FAK:paxillin complexes in immunoprecipitates using either of two p125FAK antibodies. When CADTK and p125FAK 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, p130CAS, 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 p125FAK 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 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 after treatment with 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), [Arg8]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 CARKβ (19). CADTK and p125FAK 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. p125FAK, 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, p125FAK sits at a conceptual and physical juncture where it is capable of integrating cell structure and signaling.

p125FAK does not contain SH2 or SH3 domains but can

1 The abbreviations used are: EGF, epidermal growth factor; CADTK, calcium-dependent tyrosine kinase; p125FAK, p125 focal adhesion tyrosine kinase; p130CAS, p130 Crk-associated substrate; p70S6K, p70 ribosomal S6 kinase; p90RSK, 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; Me2SO, 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, p125FAK creates binding sites for other SH2 domain-containing proteins including Src tyrosine kinase family members (28–30). p125FAK also exhibits a proline-rich region that can interact with SH3 domain-containing proteins including p130CAS (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 p125FAK to focal adhesions (36). However, others have reported that paxillin association may not be required for p125FAK focal adhesions’ localization (37). As noted, tyrosine-phosphorylated p125FAK binds (and may activate) Src family tyrosine kinases, concentrating these enzymes and other proteins within focal adhesions, in effect assembling a family tyrosine kinases, concentrating these enzymes and other proteins within focal adhesions, in effect assembling a large potential signaling complex (5, 6, 24).

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 MeSO. 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-p125FAK polyclonal antibody, A17, was purchased from Santa Cruz Biotechnology. Anti-p130CAS 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-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 293T cells) were kindly provided by Dr. Brian Varum (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% (w/v) glycerol); (ii) high salt buffer (500 mM NaCl, 20 mM HEPES (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM NaF, and 10% (w/v) glycerol); or (iii) RIPA lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1% Triton X-100, 2 mM EDTA, 10% (w/v) glycerol, 0.1% SDS, and 0.5% deoxycholate). For all cell lysis buffers the following reagents were added before use: 1 mM Na3VO4, 20 mM β-mercaptoethanol, 10 μg/ml leupeptin, and 100 μg/ml aprotinin.

Cell lysates were clarified by centrifugation at 14,000 × g for 20 min at 4 °C. Lysate cell content was determined prior to immunoprecipitation.

Anti-Tyr(P), p125FAK, 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 at 14,000 × g for 20 min, 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.). 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 p125FAK in Human 293(T) Cells—The full-length CADTK cDNA was isolated as described (16) and was subcloned into a pcDNA3 vector (Invitrogen). Human p125FAK cDNA, subcloned into the pcDNA vector, was kindly provided by Dr. William Cance (University of North Carolina). The control vector (pcDNA3), pcDNA3-CADTK, and pcDNA3-p125FAK 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, p125FAK, paxillin, or p130CAS.
Ang II Increases Paxillin, Tensin, and p130CAS 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 p125FAK and paxillin, the 115-kDa tyrosine-phosphorylated proteins was paxillin. Immunoprecipitation and anti-Tyr(P) immunoblot revealed that Ang II treatment of GN4 cells increased 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. 1A). 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. 1C) and p130CAS (Fig. 1E) tyrosine phosphorylation. In contrast to paxillin, both tensin and p130CAS 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. 1G) but may also suggest that tensin and p130CAS are more susceptible to tyrosine phosphatases than paxillin. Tensin but not p130CAS also exhibited a significant gel mobility shift with Ang II treatment (Fig. 1C). 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 acid analysis may resolve this issue.

Agonist-dependent CADTK and Paxillin Tyrosine Phosphorylation—Ang II and other agonists known to stimulate CADTK followed by comparison of the Tyr(P) content of paxillin, CADTK, and p125FAK. Agonists that significantly increased CADTK tyrosine phosphorylation (Fig. 2A) were the most effective at acutely increasing paxillin tyrosine phosphorylation (Fig. 2C). 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. 2A, lane 3) and slightly stimulated paxillin tyrosine phosphorylation (Fig. 2C, lane 3), whereas Ang II and sorbitol maximally stimulated CADTK and paxillin Tyr(P) content. In contrast, p125FAK tyrosine phosphorylation was only minimally altered by agonist treatment (Fig. 2B).
Therefore, basal paxillin tyrosine phosphorylation may be determined by constitutive p125<sup>FAK</sup> activity. The correlation between CADTK and paxillin tyrosine phosphorylation suggests that G<sub>a</sub> 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<sup>FAK</sup> 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. 3A). p125<sup>FAK</sup> expression and tyrosine autophosphorylation in response to Ang II stimulation was similar in the GN4 and WB cell lines (Fig. 3C). Thus the similar basal level of paxillin tyrosine phosphorylation in WB and GN4 (Fig. 3A) cells correlated with the equivalent p125<sup>FAK</sup> levels (Fig. 3C). 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. 4A, lane 6), almost totally inhibited thapsigargin-dependent paxillin tyrosine phosphorylation (Fig. 4C, lane 6). Preincubation with BAPTA-AM alone decreased both basal p125<sup>FAK</sup> and paxillin tyrosine phosphorylation (Fig. 4B). 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<sup>FAK</sup> tyrosine autophosphorylation and prevents paxillin tyrosine phosphorylation in Swiss 3T3 cells (22, 39, 41, 48). To test whether cytochalasin D inhibited CADTK, p125<sup>FAK</sup>, 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<sup>FAK</sup> tyrosine phosphorylation (Fig. 5B). 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,
tyrosine phosphorylation. (37), we tested whether tyrosine-phosphorylated paxillin bound to 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-p125FAK (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).

i.e. that p125FAK 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 p125FAK. Since paxillin associates with p125FAK in chicken embryo fibroblasts (37), we tested whether tyrosine-phosphorylated paxillin bound to CADTK or p125FAK 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 antiseraum to CADTK or with one of two antibodies to p125FAK (2A7 or A17). CADTK tyrosine phosphorylation was stimulated in Ang II-treated cells, and p125FAK, 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 p125FAK 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 Tyr402 and demonstrated that this mutation drastically decreases CADTK autophosphorylation in the same manner that Tyr397 mutation reduces p125FAK autophosphorylation.3 Thus, the equivalent level of maximal tyrosine phosphorylation in GN4 cell CADTK and p125FAK provides a reasonable indication that p125FAK 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 p125FAK (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 p125FAK (see Refs. 36 and 37 and Fig. 9) in adherent cells, little or no tyrosine-phosphorylated paxillin can be found in precipitable p125FAK-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 antiseraum; 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, Arg II or thapsigargin treatment did not alter CADTK-paxillin association; the complex exists prior to agonist treatment (Fig. 7A). The results were the same after lysis in a high salt buffer (Fig. 7B), 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. 7C). 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 p125FAK—We next tested whether disrupting the cytoskeleton structure with cytochalasin D could disrupt the CADTK-paxillin or p125FAK association. As shown in Fig. 8A, 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-p125FAK complexes were not detected under these conditions (with or without cytochalasin D) (Fig. 8B). The antibody, 2A7, used to immunoprecipitate p125FAK is the same antibody that detects the p125FAK-paxillin association in chicken embryo fibroblasts (37). Paxillin immunoblotting after immunoprecipitaiting with p125FAK antibody, A17, also failed to detect paxillin-p125FAK complexes in GN4 cells (data not shown).

3 X. Li and H. S. Earp, unpublished data.
CADTK and Paxillin Tyrosine Phosphorylation

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 (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 p125FAK have distinct effects on the MAPK pathway.

To broaden the comparison between CADTK and p125FAK, 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 p125FAK 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 p60CSK and Csk, both of which are potentially regulated and/or localized through p125FAK (30). In this report, we show that Ang II rapidly increased tyrosine phosphorylation of paxillin, tensin, and p130CAS 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 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 p125FAK have distinct effects on the MAPK pathway.

To examine another potential CADTK substrate complex, we tested CADTK immunoprecipitates from CADTK-transfected 293(T) cells for the presence of p130CAS. CADTK overexpression in human 293(T) cells allows detection of CADTK-p130CAS association (Fig. 10) as defined by co-immunoprecipitation. In contrast to paxillin, the CADTK-p130CAS 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 (p130CAS), 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 p70S6K but not MAPK or p90RSK (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 p125FAK have distinct effects on the MAPK pathway.

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p125FAK (Fig. 3). Ang II treatment only minimally altered p125 FAK (49, 50), and both are close to the consensus sequence (YEEI) recognized by the SH2 domain of p60src (51). Our preliminary data show that CADTK and its homologous domains are present in CADTK: (875 RTDDLVYH-4X. Li, M. D. Schaller, and H. S. Earp, unpublished results. 4 but this does not preclude the involvement of another tyrosine kinase. In this latter scenario, activated CADTK could recruit and activate a tyrosine kinase with an affinity for CADTK Tyr^397 greater than its affinity for p125FAK 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 p125FAK 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 (PBS1) and 2 (PBS2). Both PBS1 (919 RSNDKRYENVTGLVKAVIEMR35) and PBS2 (1025 VDAKNLDDVQARL34) homologous domains are present in CADTK: (577 RTDDL VYHNMTLVEAVLEI384) and (686 VDAKNLDDAVQAKY399) 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, p125FAK-paxillin association was not detected in GN4 cells even though protein expression levels of p125FAK and CADTK appear to be similar. Further experiments will be necessary to determine the mechanism (direct or indirect) and domains involved in CADTK and p125FAK 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 p125FAK.

Stratton et al.4 have shown that the proliferative effect of CADTK on paxillin in vitro, but this does not preclude the action of a CADTK-associated tyrosine kinase. Fig. 6 demonstrates a high level of p125FAK 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^397 greater than its affinity for p125FAK 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.

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Since they are expressed in the same cells, p125FAK and CADTK are not likely to be cell type-specific functional equivalents. For example, CADTK cannot fully replace p125FAK as p125FAK-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 p125FAK appear to have different abilities to complex with paxillin suggests subtle differences in function, even with re-
pect to the cytoskeleton. Further experiments will help us define the relationship between these two protein tyrosine kinases.

Ang II treatment also increases p130CAS and tensin tyrosine phosphorylation, and overexpression of CADTK in 293(T) cells demonstrated CADTKp130CAS complexes (Fig. 10). Transfected cells with Rous sarcoma virus or spreading of cells on extracellular matrix induces both p130CAS and tensin tyrosine phosphorylation (24, 57). Indeed, both p125FAK and Src can phosphorylate these two cytoskeletal proteins in vitro (58, 59). p125FAK can associate with p125FAK in vivo through its SH3 domain (32, 34). Interestingly, the proline-rich region of CADTK, and this may be the basis of CADTK-p130 CAS association detected in transfected 293(T) cells (Fig. 10). Whether CADTK directly phosphorylates p130CAS and tensin remains to be determined.

The role of tyrosine phosphorylation of paxillin, p130CAS, 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, p130CAS, and tensin may also provide binding sites for the SH2 containing proteins and initiate unique signaling pathways.

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REFERENCES

1. Hall, A. (1994) *Annu. Rev. Cell Biol.* 10, 31–54
2. Clark, A. E., and Brugge, J. S. (1995) *Science* 269, 233–239
3. Richardson, A., and Parsons, J. T. (1995) *BioEssays* 17, 220–236
4. Schaller, M. D., and Parsons, J. T. (1994) *Curr. Opin. Cell Biol.* 6, 705–710
5. Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Stanke, G., and Winkler, J. (1995) *Annu. Rev. Cell Dev. Biol.* 11, 379–416
6. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* 11, 549–589
7. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J. Biol. Chem.* 267, 23439–23442
8. Kornberg, L., Earp, H. S., Turner, C., Prokop, C., and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 8392–8396
9. Guinebault, C. P., Payrastre, B., Raccoud-Sultan, C., Mazarguil, H., Breton, M., Masco, G., Plantsavid, M., and Chap, H. (1995) *J. Cell Biol.* 129, 831–842
10. Farkas, M. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996) *J. Biol. Chem.* 271, 13649–13655
11. Schaller, J. D., Schaller, M. D., and Parsons, J. T. (1993) *J. Cell Biol.* 123, 993–1005
12. Tachibana, K., Sato, T., D’Avirro, N., and Morimoto, C. (1995) *J. Exp. Med.* 182, 1089–1100
13. Zohn, I., Yu, H., Li, X., Cox, A. D., and Earp, H. S. (1995) *Mol. Cell. Biol.* 15, 6160–6165
14. Graves, L. M., He, Y., Lambert, J., Hunter, D., Li, X., and Earp, H. S. (1997) *J. Biol. Chem.* 272, 1920–1928
15. Turner, C. E., Pietras, K. M., Taylor, D. S., and Molloy, C. J. (1995) *Cell Sci.* 108, 333–342
16. Leduc, I., and Meloche, I. (1995) *J. Biol. Chem.* 270, 4401–4404
17. Turner, C. E., Glenney, J. R., and Burridge, K. (1990) *J. Cell Biol.* 111, 1059–1068
18. Turner, C. E. (1994) *BioEssays* 16, 47–52
19. Rankin, S., and Rozenburg, E. (1994) *J. Biol. Chem.* 269, 704–710
20. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, V. W., Rines, R. L., and Parsons, J. T. (1994) *Mol. Cell. Biol.* 14, 1680–1688
21. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* 72, 767–778
22. Illic, D., Furuta, Y., Kanazawa, S., Takede, N., Sobue, K., Nakatani, S., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995) *Nature* 377, 539–544
23. Li, J., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) *Blood* 88, 417–428
24. Kanner, S. B., Reynolds, A. B., Vines, R. L., and Parsons, J. T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3328–3332
25. Petch, A., Bockholt, S. M., Bouton, A., Parsons, J. T., and Burridge, K. (1995) *J. Cell Biol.* 130, 1371–1379
26. Bockholt, S. M., and Burridge, K. (1994) *J. Biol. Chem.* 268, 14565–14567
27. Vuori, K., and Rooslahti, E. (1996) *J. Biol. Chem.* 272, 22559–22562
28. Lo, S. H., Weisberg, E., and Chen, L. B. (1994) *Trends Biochem. Sci.* 19, 817–823
29. Vuori, K., Hirai, H., Aizawa, S., and Rooslahti, E. (1996) *Mol. Cell. Biol.* 16, 2696–2613
30. Birge, R. B., Fajardo, J. E., Reichman, C., Shoelson, S. E., Songyang, Z., Cantley, L. C., and Hanafusa, H. (1993) *Mol. Cell. Biol.* 13, 4458–4456
31. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., and Sasaki, T. (1995) *J. Biol. Chem.* 270, 21206–21219
32. Schaller, M. D., Boggman, C. A., Cobb, B. C., Reynolds, A. B., and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 5192–5196
33. Kanner, S. B., Reynolds, A. B., Vines, R. L., and Parsons, J. T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 8328–8332
34. Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S., and Hattori, S. (1994) *Mol. Cell. Biol.* 14, 5485–5500
35. Feller, S. M., Ben R., Hanafusa, H., and Baltimore, D. (1994) *Trends Biochem. Sci.* 19, 453–458