Angiotensin II Activates at Least Two Tyrosine Kinases in Rat Liver Epithelial Cells

SEPARATION OF THE MAJOR CALCIUM-REGULATED TYROSINE KINASE FROM p125FAK∗

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In rat liver epithelial cell lines (WB or GN4), angiotensin II (Ang II) stimulates cytosolic tyrosine kinase activity, in part, through a calcium-dependent mechanism. In other cell types, selected hormones that activate Gi- or Gs-coupled receptors stimulate the soluble tyrosine kinase, p125FAK. Immunoprecipitation of p125FAK from Ang II-activated GN4 cells demonstrated a doubling of p125FAK kinase activity. However, an additional Ang II-activated tyrosine kinase (or kinases) representing the majority of the total activity was detected when the remaining cell lysate, immunodepleted of p125FAK, was reimmunoprecipitated with an anti-phosphotyrosine antibody. Cytochalasin D pretreatment blocks G-protein receptor-dependent tyrosine phosphorylation in Swiss 3T3 cells. While cytochalasin D decreased the Tyr(P) content of 65–75-kDa substrates in Ang II-treated GN4 cells, it did not diminish tyrosine phosphorylation of 115–130-kDa substrates, again suggesting activation of at least two tyrosine kinase pathways in GN4 cells. To search for additional Ang II-activated enzymes, we used molecular techniques to identify 20 tyrosine kinase sequences in these cell lines. None was the major cytosolic enzyme activated by Ang II. Specifically, JAK2, which had been shown by others to be stimulated by Ang II in smooth muscle cells, was not activated by Ang II in GN4 cells. Finally, we purified Tyr(P)-containing tyrosine kinases from Ang II-treated cells, using anti-Tyr(P) and ATP affinity resins; 80% of the tyrosine kinase activity migrated as a single 115–120-kDa tyrosine-phosphorylated protein immunologically distinct from p125FAK. In summary, Ang II activates at least two separate tyrosine kinases in rat liver epithelial cells; p125FAK and a presumably novel, cytosolic 115–120-kDa protein referred to as the calcium-dependent tyrosine kinase.

In analyzing EGF1-dependent tyrosine phosphorylation in rat liver epithelial cell, two waves of Tyr(P) substrate phosphorylation occurring at 5 and 60 s were noted (1). The latter group appeared to be phosphorylated in part due to activation of a second process (e.g. a downstream tyrosine kinase) (2). Subsequently, we identified several of the same substrates in Ang II, vasopressin, or epinephrine-treated cells, and determined that these G-protein-coupled receptors stimulated tyrosine phosphorylation in a calcium-dependent, protein kinase C-independent manner (2–4). Since virtually all tyrosine kinases autophosphorylate on tyrosine residues, immune complex tyrosine kinase activity could be assessed in anti-Tyr(P) immunoprecipitates from control and Ang II-treated cells. Ang II increased tyrosine kinase activity within 15–30 s; maximal activation was seen within 1 to 2 min. Activation was abrogated by intracellular chelators that blunt the Ang II-induced calcium signal (3). The mechanism by which calcium increases tyrosine kinase activity remains unclear, but the schema is presumably indirect because tyrosine kinase activity can not be stimulated by adding calcium to cell-free extracts of these cells. Thus, our previous work showed that G-protein-coupled receptors generating a calcium signal rapidly, but indirectly, activate one or more tyrosine kinase(s) (2–4).

Other hormones activating G-protein-coupled receptors also increase tyrosine phosphorylation (e.g. bombesin (5, 6), bradykinin (7), thrombin (8–10), carbachol (11), endothelin (5, 6), cholecystokinin (12), lysophosphatidic acid (13), and fMet-Leu-Phe (14)), and our demonstration of Ang II- and vasopressin-dependent tyrosine phosphorylation has been confirmed by several groups (15–20). While many of these hormone receptors (like Ang II) activate Gt-proteins increasing phospholipase C activity and intracellular calcium (21), several activate Gt-coupled receptors, which would not be expected to raise intracellular calcium (22). Whatever the mechanism, several investigators established that stimulation of Gt- or Gt-coupled receptors activated a 125-kDa tyrosine kinase. This kinase, p125FAK, localizes to focal adhesions (23–25) and had been cloned as a substrate of p60src (26, 27). In concurrent work, several laboratories, including our own, showed that signaling via integrins also stimulated p125FAK phosphorylation and activity (28–31). This analogy between G-protein stimulation and cell surface perturbation is extended by the fact that both integrin (32) and hormone stimulation (see below) activate MAP kinase.

Many of the hormones noted above that activate tyrosine phosphorylation also stimulate cell proliferation and the immediate-early gene expression that accompanies growth factor action. The latter is likely to occur by activation of the MAP kinase (ERK 1 and ERK 2) pathway (33, 34), or the newly characterized c-Jun N-terminal kinase (JNK) pathway (35). In fact, both Gt-coupled receptors (in some instances via a calcium-dependent mechanism) and Gt-coupled receptors have been shown to activate MAP kinase (15, 16, 20, 36–39). In addition, our laboratory has shown recently that Ang II treatment of rat

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† The abbreviations used are: EGF, epidermal growth factor; Ang II, angiotensin II; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis.
liver epithelial cells produces a 200-fold activation of JNK through a calcium- and tyrosine kinase-dependent process. In summary, data from several cell types suggest that the selective Gq- and Gs-coupled receptors as well as other agonists (e.g., integrin stimulation) can activate p125FAK MAPK, and JNK. The potential stimulation of several pathways thought to be controlled by tyrosine phosphorylation led us to ask whether Ang II stimulated p125FAK or another tyrosine kinases(s) or both.

We report that Ang II stimulates at least two cytosolic tyrosine kinases. The first, p125FAK, is tyrosine-phosphorylated and activated in these cells in an Ang II-dependent manner; however, greater than 80% of the tyrosine kinase activity resides in another molecule that can be distinguished from p125FAK. Molecular means were used to identify 20 tyrosine kinases in the cells, but none appear to be the calcium-dependent cytosolic tyrosine kinase. In contrast, we have partially purified an autophosphorylating kinase from Ang II-treated cells by sequential affinity chromatography. This activity migrates as a 115–120-kDa Tyr(P) protein that can be separated electrophoretically and distinguished from pp125FAK. The possibility is raised that distinct Ang II-dependent intracellular signaling pathways are activated through separate tyrosine kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—EGF was purified from mouse salivary glands as described previously (40). Human Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was obtained from Sigma. Ang II was prepared in 50 mM acetic acid. EGF was prepared as 100-fold concentrated solutions in 10 mM NaPi (pH 7.4), 150 mM NaCl containing 0.1% bovine serum albumin. Cytochalasin D (Calbiochem) was prepared as stock solutions in dimethyl sulfoxide (final concentration ≤ 0.1%, v/v). Anti-p125FAK, monoclonal antibody 2A7, and polyclonal antibody BC-2 were generously provided by Dr. James Ihle, St. Jude’s. Monoclonal anti-Tyr(P) antibody, PT66, and PT66-agarose were purchased from Sigma.

**Cell Cultures**—WB and GN4 cell were maintained at 37°C in Richter’s improved minimal essential medium containing 10% fetal bovine serum and 0.1 μM insulin in a humidified 5% CO2 atmosphere as described previously (3). Seven to 10 days before each experiment, WB cells of passages 19–26 or GN4 cell passages 5–15 were seeded onto plastic culture dishes (Costar) and used when they reached confluence.

**Anti-Tyr(P) Immunoblotting**—Cell treatments and anti-Tyr(P) immunoblotting were performed essentially as described previously (2). Cell lysates of WB or GN4 cells grown in 150-mm diameter culture dishes, subcellular fractions, PT66 immunoprecipitates, or affinity resin fractions were probed in a similar fashion. Briefly, in some experiments treatment incubations were terminated by rapid aspiration of the medium and addition of 250 μl of ice-cold RIPA buffer. Cell extracts were scraped from culture dishes and solubilized by addition of 125 μl of 3-fold concentrated sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and heating to 100°C for 3 min. For experiments in which homogenate, cytosol, and membrane fractions were separated, four 60-mm plates preincubated with 200 μM vanadate for 15 min and were then treated with vehicle or Ang II for 1 min. Cells were scraped into vanadate-containing immunoprecipitation buffer (see below) and homogenized in a 2-ml Teflon glass homogenizer with a motor driven pestle on ice (30 strokes). One-mL aliquots were ultracentrifuged in a Beckman TL100 centrifuge at 105,000 × g for 45 min. Proteins from cell lysates, subcellular fraction, or affinity procedures were separated by electrophoresis on 7, 8, or 10% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed by sequential incubation with anti-Tyr(P) antibodies and 125I-protein A (2–10 μg/ml) or rabbit anti-mouse IgG (10 μg/ml) or using ECL reagent. Molecular weights were estimated by using prestained standards (Sigma).

**Anti-Tyr(P), p125FAK, JAK, and EGF Receptor Immunoprecipitation**—Confluent cultures of WB or GN4 cells in 60-mm dishes, subcellular fractions or affinity column fractions, were immunoprecipitated in ice-cold lysis buffer, referred to as NLB (20 mM HEPES (pH 7.3) containing 500 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 10% (v/v) glycerol, and 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 kallikrein inhibitor units of aprotinin per ml). Lysates were clarified prior to immunoprecipitation by centrifugation at 14,000 × g for 10 min at 4°C. Tyrosine-phosphorylated proteins were precipitated by incubation with monoclonal anti-Tyr(P) antibody PT66, 5 μl of protein A/G-agarose (Santa Cruz), or 25 μl of protein A/G-agarose (Santa Cruz), for 2 h at 4°C. Immune complexes were collected by centrifugation at 14,000 × g for 3 min at 4°C, were washed twice with this lysis buffer, and if used for immune complex tyrosine kinase activity, the precipitates were washed twice with 2-fold concentrated tyrosine kinase assay buffer (50 mM sodium HEPES (pH 7.6), 60 mM Na3VO4, 2 mM MgCl2, 0.2 mM Na3VO4, 0.2% Nonidet P-40), and then were resuspended with 70 μl of this buffer. In some experiments, an aliquot (10–20 μl) of the washed immune complex was removed for anti-Tyr(P) immunoprecipitation after SDS-PAGE on 8% gels as described. Efficient anti-Tyr(P) recovery of tyrosine phosphoproteins and kinase activity required high NaCl concentration (500 mM) in the cell lysate buffer. We postulate that Tyr(P) residues are dissociated from endogenous binding domains (e.g., Src homology domains) at high ionic strength and thereby are made accessible for immunoprecipitation. In cell lysates or affinity fractions, p125FAK was precipitated using 2–10 μl of monoclonal 2A7; EGF receptor was precipitated with anti-rat polyclonal EGF receptor antibody 1392 prepared in this laboratory. JAKs 1, 2, and 3 were immunoprecipitated after lysis in NLB with specific antisera provided by Dr. James Ihle.

**Immune Complex Tyrosine Kinase Assay and In Vitro Autophosphorylation Reactions**—Routine, 10–20-μl aliquots of immune complex suspension were precipitated for 5 min at 4°C with 160 μg of the synthetic tyrosine kinase substrate poly(Glu97-Tyr20) or the control substrate poly(Glu) (Sigma). Reactions (90 μl total reaction volume) were initiated by addition of 5 μl of [γ-32P]ATP (2–10 μCi/reaction) for cell lysate experiments. Affinity fractions were assayed with 60 μM ATP, 5–20 μCi of [γ-32P]ATP to equalize the ATP concentration in various fractions. After 4–10 min at 25°C, 50 μl of the reaction mix was spotted onto Whatman No. 3MM paper. The papers were washed with acidic ethanolic acid, air-dried, and assayed by liquid scintillation for acid-insoluble 32P. Tyrosine kinase activity was defined as 32P incorporation in counts/min occurring in the presence of poly(Glu97-Tyr20) minus that occurring in the presence of poly(Glu80-Tyr20). This defined phosphorylation above the background of endogenous protein phosphorylation. The rates of tyrosine phosphorylation so measured were linear for at least 15 min and were proportional to the amount of cell lysate used for immunoprecipitation. In experiments in which endogenous substrate autophosphorylation was assessed, the poly(Glu97-Tyr20) was not added to the reaction mixture. The reactions were terminated at 4–10 min by boiling in SDS-sample buffer. Samples were subjected to 7–8% SDS-PAGE; gels were stained, destained, dried, and subjected to autoradiography on Kodak AR film. In some instances gels were transferred to Immobilon and subjected to two dimensional phosphoamo acid analysis as described previously (2).

**Degenerate PCR Cloning of Tyrosine Kinase Domains**—Unknown tyrosine kinase domains were amplified with degenerate oligonucleotides designated TK forward (ccg gtt ctc gat cat cgn gtn gtm cgn eg) and TK reverse (ctg can acc tgg tgg ctn ta cag cta tgg acc), encoding the conserved sequences HIRDLAA and DWSFGVY, respectively, as described (41). The first strand cDNA template was prepared from poly(A) mRNA made by cesium chloride isolation of total RNA from the WB cell line followed by oligo(dT) selection. The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase in RT buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, and 3 mM MgCl2). 15 units of RNA guard (Pharmacia Biotech Inc.), 100 μg of random hexamers, and 1.25 μM each dNTP in a 20-μl reaction. The volume of the cDNA was adjusted to 25–60 μl with TE following first strand synthesis. PCR amplifications were carried out with 5 μl of random-primed first strand cDNA in a 50-μl reaction volume of PCR buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.1% Triton X-100, 0.2 μg/ml of each dNTP, 10 μg/ml of each of 2.5 units of Taq DNA polymerase (Life Technologies, Inc.) under the following conditions: 5 min at 94°C, 1 min at 55°C, and 2 min at 72°C (1 cycle), 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C (22 cycles), and 1 min at 94°C, 1 min at 55°C, and 10 min at 72°C (1 cycle). The PCR product was then gel-purified, cut with XhoI and CiaI at sites included in the PCR primers, and subcloned into pBluescript SK+ (Stratagene). In later experiments, the PCR product was TA-cloned (Invitrogen) as per manufacturer’s instructions. The 190 cloned PCR products were sequenced by dyeoxy termination sequencing (Sequenase...
nase 2.0, U. S. Biochemical Corp.), grouped into families (40 of 190 were Abl), and the sequences compared to the GenBank data base with the Blast algorithm.

Expression Cloning of WB Cell Tyrosine Kinases—A cDNA library was constructed in Agt11 using oligo(dT)-primed poly(A)-containing RNA purified from confluent cultures of WB cells. Recombinants were screened for cDNAs encoding active tyrosine kinases by a modification of the method of Letwin et al. (42). A total of 480,000 plaque-forming units from the unamplified phage library was used to infect Escherichia coli strain Y1090. Infected cells were plated in LB top agarose on 10–150-mm LB agar plates and incubated for 3 h at 42°C. When plaques had become visible, expression of insert sequences was induced by applying nitrocellulose filters, prewet with 10 mM IPTG, to the surface of the agarose and continuing incubation at 37°C. After 4 h, filters were rinsed briefly with 50 mM Tris-Cl, 150 mM NaCl (pH 7.5), plus 0.05% sodium azide (“RB”) and blocked overnight in RB containing 3% bovine serum albumin (Boehringer Mannheim). Plaques containing proteins phosphorylated on tyrosine were identified by immunoblottting the filters with purified polyclonal anti-phosphotyrosine antibodies followed by 125I-protein A as described. Twenty positive plaques were detected. Phage from the 12 plaques giving rise to the strongest isopropyl-1-thio-
β-D-galactopyranoside induction and anti-phosphotyrosine immunoblotting, yielding 8 purified clones with Lambdaorb reagent (Promega). Insert DNAs were excised by restriction and endonuclease digestions with EcoRI and ligated into EcoRI-cut pBluescript II KS+ (Stratagene). Partial nucleotide sequences of insert cDNAs, obtained using the Sequenase 2.0 kit (U. S. Biochemical Corp.), were compared to known sequences using the GCG analysis package.

Affinity Purification—In a typical preparation, 50–150-mm tissue culture plates of confluent GN4 cells were treated with 1 μM Ang II for 1 min and scraped into 1 ml of NBL/plate. After centrifugation, the lysate was tumbled with 1 ml of PT66-agarose, washed five times, and eluted with 10 ml of NBL with 10 mM phenylphosphate. The Tyr(P) elute was brought to 50 mM MgCl2 in 150 mM NaCl and incubated with 0.5 ml of ATP-Sepharose synthesized by one of us (L. Graves). Briefly, 4-aminomino-lido-ATP was synthesized by reacting ATP with N-ethyl-N’- (3-dimethylaminopropyl)carbodiimide and phenylendiamine. The product, isolated by high performance liquid chromatography, was analyzed by mass spectrometry to confirm the identity. The 4-aminomino-lido-ATP derivative was coupled by carbodiimide chemistry to Sepharose resin containing a carboxyl group attached to a 6-carbon linker (Pharmacia ECH Sepharose). After transfer to a Bio-Rad 1–ml column, the resin was washed with 150 mM and 500 mM NaCl containing buffers and the purified ATP-binding proteins were eluted with 1 mM ATP in six to eight 1-ml fractions. Fractions were combined and used for immunoprecipitation (2 h at 0°C, as described) with p125FAK or EGF receptor antibodies or were concentrated by incubation with PT66-agarose overnight at 0°C. Some aliquots were autophosphorylated in immune complex kinase assays, as described, and subjected to gel electrophoresis and autoradiography. Alternatively, aliquots were prepared for gel-electrophoresis, followed by silver staining, or after transfer to nitrocellulose, for Tyr(P) immunoblotting. For some gel electrophoresis analyses, the bisacrylamide concentration was reduced from 1 to 0.5% to increase the separation in the 120-kDa region.

RESULTS

The Ang II-dependent Tyr(P) Substrates and Tyrosine Kinase(s) Are Cytosolic Proteins—The subcellular localization of Ang II-dependent Tyr(P) substrates and tyrosine kinase activity was investigated in WB and rat liver epithelial cells. Previous experiments had assessed the time course of substrate phosphorylation and kinase activity in extracts of cells lysed in detergent-containing buffers with 500 mM NaCl, protease, and phosphatase inhibitors (2). To determine subcellular localization, cells preincubated with 200 μM vanadate to preinhibit cellular Tyr(P) phosphatases were incubated with Ang II for 1 min, the time of maximal tyrosine kinase activation. Preincubation with vanadate prolonged the Ang II-dependent tyrosine phosphorylation response, but did not detectably alter the substrates phosphorylated. Cells were scraped, homogenized on ice, and centrifuged at 105,000 × g for 45 min. After resuspension of the pellet membranes, Triton X-100 was added to a portion of the original homogenate (kept on ice during subcellular fractionation), as well as to the cytosol and membrane fractions. Following electrophoresis of the samples and transfer to nitrocellulose, an anti-Tyr(P) immunoblot was performed (Fig. 1). The majority of the Tyr(P) substrates in Ang II-treated WB cells were localized to the cytosolic fraction. In contrast, EGF-stimulated tyrosine phosphorylation of substrates both in the cytosolic and membrane fractions; the major substrate in the membrane fraction was the 170-kDa EGF receptor.

To determine the localization of the tyrosine kinase(s) activated in Ang II-treated cells, the experiment was repeated using GN4 cells, a chemically transformed line derived from WB, that expresses −3-fold more Ang II-stimulated tyrosine kinase activity. After separating homogenate, cytosol, and membrane fractions, detergent was added, followed by immunoprecipitation with anti-phosphotyrosine monoclonal antibody PT66. Immune complex tyrosine kinase activity was performed as described under “Experimental Procedures” assessing the transfer of 32P from γ-32P]ATP to the exogenous substrate poly(Glu80-Tyr20). In immunoprecipitates from the homogenate fraction, Ang II treatment increased tyrosine kinase activity by about 2.5-fold (2,637 cpm for control to 6,190 cpm for Ang II-treated). At least 75–80% of this activity was found in the immunoprecipitates of cytosolic Tyr(P) proteins (740 cpm for control, 4,596 cpm for Ang II-treated). In contrast, little activity was found in the immunoprecipitates from the membrane fraction (699 cpm for control, 313 cpm for Ang II-treated). Thus, the majority of the Tyr(P) substrates and virtually all of the increased tyrosine kinase activity were found in the cytosolic fraction.

p125FAK Is Activated in Ang II-treated Cells, but Is Not the Major Ang II-dependent Tyrosine Kinase—The fact that the Ang II-dependent tyrosine kinase activity was soluble and that there were Tyr(P) substrates in the p115–130-kDa region led us to test whether p125FAK was the major kinase activated. GN4 cells treated with vehicle or Ang II for 1 min were subjected to single or sequential immunoprecipitation protocols using control mouse Ig (mlg), the anti-Tyr(P) monoclonal antibody PT66, or the FAK monoclonal antibody 2A7. Immunoprecipitates from treated cells were subjected to electrophoresis, transfer to nitrocellulose, and Tyr(P) immunoblotting with PT66 as described under “Experimental Procedures” (Fig. 2). Immunoprecipitation with irrelevant mlg did not precipitate Tyr(P)-containing proteins from either control or Ang II-
treated cells. Immunoprecipitation of p125FAK (Fig. 2, lanes 3 and 6) demonstrated tyrosine phosphorylated p125FAK in control GN4 cells; Ang II slightly increased p125FAK Tyr(P) content at 1 min, the time of maximal Ang II-dependent tyrosine phosphorylation. However, FAK was not the major component in Ang II-treated cells. Immunoprecipitation with anti-Tyr(P) PT66 showed the previously described large increase in Tyr(P) substrates after Ang II treatment (Fig. 2, lanes 2 and 5). To determine more precisely the portion of Tyr(P) phosphoprotein in the p120–125 region that was pp125FAK, a sequential immunoprecipitation was performed. The first immunoprecipitation with either irrelevant mlg or 2A7 anti-FAK was performed at 0 °C in vanadate-containing buffers. The supernatants remaining from the first immunoprecipitation were then immunoprecipitated with PT66. Preclearance of nearly all immunoreactive FAK by 2A7 had little effect on the amount of PT66 immunoprecipitable Tyr(P) substrate from Ang II-treated cells (Fig. 2, lanes 10 and 11).

While the above indicated that p125FAK is only a small fraction of the Tyr(P) substrate, it did not determine whether p125FAK was the major autophosphorylating kinase in immune complexes. Tyrosine kinase activity assays were performed on 2A7 (p125FAK monoclonal) and PT66 (anti-Tyr(P) antibody) immunoprecipitates. In five experiments, the immune complex p125FAK activity measured by the ability of p125FAK immunoprecipitates to phosphorylate poly(Glu80-Tyr20) nearly doubled when isolated from cells after Ang II treatment (1 min), from an average of 6,747 cpm for control to 11,730 cpm for Ang II. The immune complex tyrosine kinase activity of the PT66 immunoprecipitate paired with a representative p125FAK experiment rose from a control of 15,656 cpm to 66,342 cpm after Ang II treatment (1 min). Autophosphorylation activity was also assessed by repeating the single and sequential immunoprecipitation protocol in control or Ang II-treated GN4 cells and assessing incorporation from [γ-32P]ATP into precipitated protein in vitro, followed by gel electrophoresis and autoradiography of dried gels as described under “Experimental Procedures.” Fig. 3 demonstrates that p125FAK autokinase activity in 2A7 immunoprecipitates is slightly elevated (lanes 3 and 6) in Ang II-treated cells (1 min), corresponding to the increase in immune complex poly(Glu80-Tyr20) activity noted above. Ang II-dependent PT66 immune complex auto kinase activity was much greater (Fig. 3, lanes 2 and 5). In the sequential protocol, immunoprecipitation removing nearly all immunoreactive p125FAK from the supernatant did little to diminish the subsequent PT66-immunoprecipitable autokinase activity seen in the 115–120-kDa region (Fig. 3, lanes 10 and 11). Examination of lanes 5, 10, and 11 in Fig. 3 revealed a diffuse band above 120 kDa of phosphorylated protein and one sharp band of in vitro phosphorylated protein at ~115–120 kDa. On shorter exposures of the autoradiograph, this single band is even more prominent. This is observed either before (lane 10) or after (lane 11) the removal of p125FAK by immunoprecipitation, suggesting that a major autophosphorylation protein was not removed by p125FAK antibody. 32P-Phosphoamino acid analysis by two dimensional thin layer chromatography has shown that all [32P]phosphate added in vitro was on tyrosine residues (data not shown). This analysis suggested that an autophosphorylating kinase at 115–120 kDa may be the most prominent in vitro labeled protein, a supposition substantiated by the purification discussed below.

In studies of p125FAK activation via G-protein-coupled receptors, others tested whether the cytoskeletal was involved in the signal transduction pathway (19, 24). Preincubation of cells with cytochalasin D, an agent that disrupts actin microfilaments and cytoskeletal movement, prevented the tyrosine phosphorylation by G-protein-coupled receptor agonists (e.g., bombesin) in Swiss 3T3 cells (19, 24). GN4 cells were preincubated with 2 μM cytochalasin D for 2 h prior to stimulation with Ang II (1 min). Fig. 4 demonstrates that cytochalasin D had little or no effect on Ang II-dependent tyrosine phosphorylation of substrates in the 115–130-kDa region. We did not assess p125FAK tyrosine phosphorylation in this experiment, but Fig. 2 demonstrated that depletion of Tyr(P) p125FAK from the 115–130-kDa substrates region would not alter the Tyr(P) immunoblottting pattern in Ang II-treated cells. Cytochalasin D
Other than p125 FAK—because Ang II activates at least two tyrosine kinase pathways.

In other cell types, Tyr(P) substrates in this region have proved to be the cytoskeletal protein paxillin, which is tyrosine-phosphorylated in cells stimulated by G-protein-coupled receptors (24). In conclusion, Ang II stimulates rapid tyrosine phosphorylation of two groups of substrates: one sensitive to cytochalasin D (90–120-kDa region). The tyrosine phosphorylation in the 65–75-kDa region stimulated by Ang II is inhibited by cytochalasin.

Pretreatment did, however, distinctly inhibit tyrosine phosphorylation of 65–75-kDa substrates in Ang II-treated GN4 cells. In other cell types, Tyr(P) substrates in this region have proved to be the cytoskeletal protein paxillin, which is tyrosine-phosphorylated in cells stimulated by hormones binding to G-protein-coupled receptors (24). In conclusion, Ang II stimulates rapid tyrosine phosphorylation of two groups of substrates: one sensitive and one insensitive to cytochalasin D. This may occur because Ang II activates at least two tyrosine kinase pathways.

Attempts to Identify an Ang II-regulated Tyrosine Kinase Other than p125 FAK—Molecular technologies and available antibodies were used to identify the range of tyrosine kinases present in WB rat liver epithelial cells. First, a cDNA library that had been constructed using WB cell polyA mRNA was used for cDNA expression cloning to identify novel tyrosine kinases that might not have canonical kinase domain sequences but would be detected if expressed in E. coli, which have neither tyrosine kinases nor tyrosine phosphatases. Anti-Tyr(P) cDNA expression cloning identified three tyrosine kinases (Fer, Fyn, Bek). Failing to identify a novel cytosolic tyrosine kinase, degenerative PCR cloning using the tyrosine kinase domain primers was performed (41, 43). A total of 20 tyrosine kinases were identified in WB cells, including the EGF receptor and Src (identified using antibodies), the three kinases noted above detected by expression cloning, and 15 more identified by PCR. The latter group included three that were novel when detected (JAK3 (44), Tyro 3 (45), and an Elk-like kinase). Ten of the kinases were membrane-bound receptors (EGF receptor, Bek, Axl, Tyro 3, Tyro 10, Eck, Elk-like, Flk, Flt, and IGF-I receptor), and two were Src family members (Fyn and Src); neither group would likely be the Ang II-regulated kinase whose activity is >80% soluble. Of the eight other intracellular tyrosine kinases (Fer, Abl, Abl-related, Csk-1, FAK, JAK1, JAK2, and JAK3), all but Csk-1 and Abl-related were ruled out as the major calcium-dependent kinase in double immunoprecipitation experiments similar to those done in Figs. 2 and 3 (data not shown). Csk-1 is not autophosphorylated, and thus would not be a kinase activity immunoprecipitated with anti-Tyr(P) antibodies (46). This left the abl-related sequence (47) (which we have not formally ruled out, but which is not in the correct molecular weight range). Since a recent report demonstrated Ang II-dependent JAK2 activation in rat smooth muscle cells (48), we specifically tested for the ability of Ang II and a known JAK2 stimulus, growth hormone (49), to stimulate JAK2 autophosphorylation in GN4 cells. Fig. 5 shows that Ang II failed to increase JAK2 tyrosine phosphorylation at 1, 15, or 30 min, while human growth hormone clearly increases JAK2 tyrosine phosphorylation.

Purification—GN4 cells grown to confluence were treated with Ang II for 60 s, and cell lysates were harvested by detergent lysis. Lysates were incubated with anti-phosphotyrosine PT66-agarose, washed, and eluted with phenylphosphate, resulting in a substantial purification with respect to protein. Multiple Tyr(P) substrates were isolated. This fraction (termed Tyr(P) elute in Fig. 6) was passed over an ATP-Sepharose column (ATP linked with a spacer through the γ-phosphate), allowing efficient isolation of ATP-binding Tyr(P) proteins. Greater than 90% of the silver-staining protein and Tyr(P) substrate was not adsorbed and was found in the flow-through fractions of the ATP column.

Fig. 6 (panel A) shows an analysis at the end of a typical purification using a standard gel polyacrylamide gel. At least five Tyr(P)-containing proteins adsorbed to the ATP column (p170, p140, p125, p115–120, and p75) were eluted with 1 mM ATP and were concentrated by overnight reincubation with PT66-agarose. This last step was followed by washing and extraction of the purified proteins in SDS sample buffer (final Tyr(P) step). The lower band in the 115–120-kDa region corresponded to the most heavily autophosphorylated protein seen in Fig. 3. A second purification was examined using a gel with a low bisacrylamide concentration to enhance separation of phosphoproteins in the 120-kDa region (Fig. 6B). The gel allows visualization of the 115–120-kDa Tyr(P) protein below the 120-kDa prestained marker separated from the preponderance of Tyr(P) substrates above the 120-kDa marker. Most of these did not adsorb to or elute from the ATP column. This can be seen even more clearly in Fig. 6C, which shows the results from a third purification in which the ATP eluate was concentrated ~20-fold using a final Tyr(P) step. Again the low bisacrylamide gel was used. This third purification was also used to confirm the specificity of the ATP affinity column by identifying two of the trace Tyr(P) proteins in final ATP eluate as the EGF receptor and p125 FAK. The eluate from the ATP affinity resin was aliquoted into three fractions, which were concentrated by precipitation with either PT66-agarose (labeled the final Tyr(P) step), rat EGF receptor polyclonal antisera, 1382, or the p125 FAK monoclonal 2A7. The PT66 lane was loaded with only 1/25 the sample used in the EGF receptor or p125 FAK lanes.
Downstream signaling from G-protein-coupled receptors is extraordinarily diverse as these receptors are involved in some manner in most physiologic processes. In general, stimulation of G-protein-coupled receptors results in activation of Ser/Thr kinases (21, 22). However, in certain cell types, hormones or agonist lipids that bind to G-protein-coupled receptors stimulate tyrosine phosphorylation, and sometimes even cell proliferation. In addition, this subset of hormone G-protein-coupled receptors can activate the MAP kinase pathway (15, 16, 20, 36–39) and can lead to immediate early gene expression (50).
In rat liver epithelial cells, Ang II alone not only stimulates proliferation, it also activates the MAP kinase and JNK kinase pathways and modifies transcription factor activity as adju­dged by increased AP-1 binding. The Ang II actions on MAP kinase and JNK are protein kinase C-dependent, as well as calcium/Calmodulin-independent, yet both actions are inhibited by genistein, implicating the involvement of a tyrosine kinase. Activation of JNK in a calcium-dependent, tyrosine kinase-de­pendent manner has not been observed in other cells (51). Thus, binding to the Ang II receptor and the resultant βγ and calcium signals may have differential consequences depending upon the downstream signaling elements expressed in the cell types under study. The expression of a novel tyrosine kinase activated in a G-protein- and/or calcium-dependent manner may allow additional Ang II-dependent actions in some cells, i.e. Ang II may stimulate multiple pathways in cells in which it activates more than one tyrosine kinase.

Subcellular fractionation demonstrated that many of the Tyr(P) substrates and virtually all of the activated tyrosine kinase in Ang II-treated cells were soluble. Our catalogue of rat liver epithelial cell tyrosine kinases yielded several intracellular candidates (p125FAK and the JAKs) as potential calcium-regulated tyrosine kinases in the 115–130-kDa range. p125FAK is both tyrosine phosphorylated and minimally activated in Ang II-treated cells, but it is a minor component of the Ang II-dependent response (Figs. 2 and 3). The JAK kinases, while often found in the particulate fraction, presumably non-covalently bound to their associated cytokine receptor (52), could be released from the membrane and activated by a calcium-de­pendent step. In addition, another group reported that Ang II activated JAK2 in smooth muscle cells (48), and a second group showed Ang II stimulation of serum-inducible element binding (53), often regarded as evidence of JAK pathway activation (52). We have not seen evidence of increased serum-inducible element binding in Ang II-treated GN4 cells, nor do we detect significant JAK2 tyrosine phosphorylation in an Ang II-treated rat liver epithelial cells (Fig. 7). Evidence for JAK2 activation in smooth muscle cells and its absence in rat liver epithelial cells confirms the cell type complexity of intracellular tyrosine kinase signalling.

The p125FAK immunoprecipitation experiments (Figs. 2 and 3) suggested that another tyrosine kinase was activated in Ang II-treated cells; the kinase purification supports this conclusion. The sharp band of activity seen in immune complexes (Fig. 3) appears to be the major autophosphorylating kinase (Fig. 3) appears to be the major autophosphorylating kinase activity isolated by large scale Tyr(P) and ATP affinity chro­matography. We thank Nancy Kaiser for manuscript preparation.

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