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Activation of a Novel Calcium-dependent Protein-tyrosine Kinase

CORRELATION WITH c-Jun N-TERMINAL KINASE BUT NOT MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION*

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Many G protein-coupled receptors (*e.g.* that of angiotensin II) activate phospholipase Cβ, initially increasing intracellular calcium and activating protein kinase C. In the WB and GN4 rat liver epithelial cell lines, agonist-induced calcium signals also stimulate tyrosine phosphorylation and subsequently increase the activity of c-Jun N-terminal kinase (JNK). We have now purified the major calcium-dependent tyrosine kinase (CADTK), and by peptide and nucleic acid sequencing identified it as a rat homologue of human PYK2. CADTK/PYK2 is most closely related to p125^{FAK} and both enzymes are expressed in WB and GN4 cells. Angiotensin II, which only slightly increases p125^{FAK} tyrosine phosphorylation in GN4 cells, substantially increased CADTK tyrosine autophosphorylation and kinase activity. Agonists for other G protein-coupled receptors (*e.g.* LPA), or those increasing intracellular calcium (thapsigargin), also stimulated CADTK. In comparing the two rat liver cell lines, GN4 cells exhibited ~ 5-fold greater angiotensin II- and thapsigargin-dependent CADTK activation than WB cells. Although maximal JNK activation by stress-dependent pathways (*e.g.* UV and anisomycin) was equivalent in the two cell lines, calcium-dependent JNK activation was 5-fold greater in GN4, correlating with CADTK activation. In contrast to JNK, the thapsigargin-dependent calcium signal did not activate mitogen-activated protein kinase and Ang II-dependent mitogen-activated protein kinase activation was not correlated with CADTK activation. Finally, while some stress-dependent activators of the JNK pathway (NaCl and sorbitol) stimulated CADTK, others (anisomycin, UV, and TNFα) did not. In summary, cells expressing CADTK/PYK2 appear to have two alternative JNK activation pathways: one stress-activated and the other calcium-dependent.

Multiple hormones, neurotransmitters, and immune effectors rapidly raise intracellular calcium (Ca^{2+}), which in turn regulates myriad cellular processes, including gene expression and cell growth (1–3). The Ca^{2+} signal acts, in part, by controlling serine and threonine protein phosphorylation through multiple mechanisms, for example, by modulating Ca^{2+}/calmodulin dependent protein kinase (4) and protein kinase C (5, 6) activities. In the WB and GN4 rat liver epithelial cell lines (7, 8) and in some other cells (9–14), hormones that activate G_q-coupled receptors (*e.g.* angiotensin II, or Ang II)[1] stimulate tyrosine phosphorylation in addition to the expected increases in Ser/Thr phosphorylation. We have used rat liver cell lines to demonstrate that hormones and other agonists that raise intracellular Ca^{2+} (*e.g.* thapsigargin) increase tyrosine phosphorylation, in part, by activating a soluble, Ca^{2+}-dependent tyrosine kinase (CADTK) (7, 8). Purification of the major autophosphorylating tyrosine kinase from Ang II-treated rat liver cells demonstrated a 115-kDa tyrosine kinase that could be separated from another soluble tyrosine kinase present in these cells, p125^{FAK} (15).

We have also used rat liver cells to demonstrate that angiotensin II increases AP-1 binding activity in a protein kinase C-independent manner (16). In investigating this phenomenon, we showed that an intracellular Ca^{2+} signal activates the c-Jun N-terminal kinase (JNK) through a Ca^{2+}/calmodulin-independent mechanism that was blocked by the tyrosine kinase inhibitor, genistein (16). These results suggest that a novel mechanism involving CADTK may be responsible for JNK activation, in effect providing cells expressing CADTK with a new pathway of calcium-regulated gene expression. The present report extends these studies by microsequencing and cDNA cloning of the purified CADTK. These results identify CADTK as the rat homologue of the recently cloned PYK2 (17). This non-receptor tyrosine kinase is, by sequence, most closely related to p125^{FAK}. However, CADTK/PYK2 and p125^{FAK} clearly exhibit different modes of activation. For example, in rat liver epithelial cells CADTK appears to have a low activity state, even in adherent cells, and is markedly stimulated by hormonal and other agonists. In contrast, p125^{FAK} is active in adherent cells and is only minimally affected by hormonal stimulation (see "Results" and Ref.15). The downstream signaling consequences of CADTK/PYK2 also appear to diverge from those of p125^{FAK}, *i.e.* this report demonstrates that CADTK/PYK2 activation is highly correlated with the stimulation of c-Jun N-terminal kinase activity.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U69109.

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[1] The abbreviations used are: Ang II, angiotensin II; CADTK, calcium-dependent tyrosine kinase; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; MS, mass spectroscopy; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; TNF, tumor necrosis factor; EGF, epidermal growth factor; LPA, L-α-lysophosphatidic acid, oleoly; CAKβ, cell adhesion kinase β; RAFTK, related adhesion focal tyrosine kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; JNKK, JNK kinase; SEK, stress-activated extracellular kinase; PAK, p21$^{(CDC42)/(RAC)}$-activated kinase.

MATERIALS AND METHODS

Purification and Peptide Sequencing of CADTK—GN4 cells (80 150-mm tissue culture plates) were treated with 1 μM Ang II for 1 min and lysed as described (15). After centrifugation, lysate was passed over an anti-Tyr(P) monoclonal antibody (PT66) column (Sigma), eluted with 10 mM phenylphosphate, and applied to an ATP-Sepharose column in the presence of 50 mM Mg^{2+} (15). After washing, ATP-bound, tyrosine-phosphorylated proteins were eluted with 1 mM ATP, concentrated using another anti-Tyr(P) affinity step, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (15). For peptide sequencing, purified CADTK was run on a 8–16% SDS-polyacrylamide gel (Novex, San Diego, CA) and detected using Bio-Rad Copper Stain. After destaining by chelation, the 115-kDa protein was reduced and pyridylethylated, and trypsinized in the gel (24 h at 37 °C). The peptide mixture was extracted, and pooled extracts were dried, resuspended in 1% aqueous acetic acid, loaded into a 1-mm capillary containing a small amount of Poros R 2/H resin (about 100 nl of 50-μm particles), washed with 2×5 μl of 1% aqueous acetic acid, and eluted in 3×400 nl of 60:40 methanol: water, 5% acetic acid as described elsewhere (18–20). The washings were collected in a capillary and analyzed by electrospray ionization mass spectrometry and tandem mass spectrometry. An ion spray voltage of 650 V sustained a liquid flow rate of ~20 nl/min. Mass spectra were collected in either the MS or MS-MS modes, with several spectra being averaged to improve signal/noise ratio. A total of six ions were identified as representing peptides. Two of these, m/z 532 and 709, were different charge states of the same peptide. There was also an overlap of peptides with m/z 432 representing the octapeptide LGDFGLSR, and the ions at m/z 532 and 709 representing the overlapping peptide NILVASPECVKLGDFGLSR. Altogether four regions of the subsequently cloned protein were represented: residues 310–334, 554–572, 672–687, and 989–998. One additional ion, m/z 758, represented a tryptic peptide of trypsin itself.

Cloning of the CADTK cDNA from Rat Liver Epithelial Cells—Trizol® (Life Technologies, Inc.) purified GN4 cell total RNA was poly(A^+)-selected and reverse-transcribed to create a probe. This probe was used to screen a λZAP WB rat liver epithelial cell cDNA library. The largest of 10 positive clones was a 3-kilobase cDNA beginning at CADTK coding nucleotide base 1001 and extending to the 3′-untranslated region. polymerase chain reaction of reverse-transcribed GN4 poly(A^+) RNA was used to complete the 5′ coding region. The entire coding region was sequenced and has been submitted to GenBank as CADTK, accession number U69109.

CADTK Antiserum and Immunoprecipitation—The CADTK cDNA was used to create a GST fusion protein encompassing amino acids 680–860. After purification using glutathione-agarose, rabbits were injected with 500 μg of fusion protein in Freund's complete adjuvant followed by three boosts at 2-week intervals in Freund's incomplete adjuvant. The resultant antiserum recognizes CADTK and does not cross-react with $p125^{FAK}$. GN4 or WB cells (60 mm confluent dishes) were treated, lysed, incubated with CADTK-specific antisera and protein A-agarose beads (Santa Cruz Biotechnology), and washed as described (7, 8). In some experiments immunoprecipitation was performed using anti-Tyr(P) monoclonal antibody (PT66). Immunoprecipitates were subjected to 8% SDS-PAGE, transferred to Immobilon (Millipore), and immunoblotted with either CADTK antiserum or anti-Tyr(P) monoclonal antibody (PT66) as described previously (7, 8).

c-Jun N-terminal and MAP Kinase Assays—WB and GN4 cells (60-mm confluent dishes) were treated as indicated. For JNK kinase activity assays, the cells were lysed and protein content determined. 50 μg of lysed cellular protein was incubated with GST-c-Jun (1–79) on glutathione-agarose, precipitated, washed, and phosphorylated as described previously (16). The reaction mixture, boiled in SDS sample buffer, was subjected to 12% SDS-PAGE (16). Total JNK protein in WB and GN4 cells was compared by using cell lysate from 60-mm confluent dishes treated either with or without Ang II. Following immunoprecipitation with anti-JNK antibody (generously provided by Dr. John Kyriakis), the precipitates were subjected to 10% SDS-PAGE, flanked by low molecular weight prestained standards, transferred to nitrocellulose, immunoblotted using the anti-JNK antibody, and developed with protein A-conjugated horseradish peroxidase and ECL reagent. The latter decreased visualization of Ig heavy chain in the 50–55-kDa region.

MAP kinase activity state was assessed by immunoblotting of WB or GN4 cell lysates run on 15% SDS gels using anti-Erk 1 antibody (k-23) (Santa Cruz Biotechnology) (16). In other experiments, Erk-1 and Erk-2 were immunoprecipitated with anti-Erk 1 antibody (c-16) (Santa Cruz Biotechnology) and used to phosphorylate myelin basic protein in the presence of [γ-^{32}P]ATP. Phosphorylated myelin basic protein was assessed by autoradiography after 15% SDS-PAGE (21).

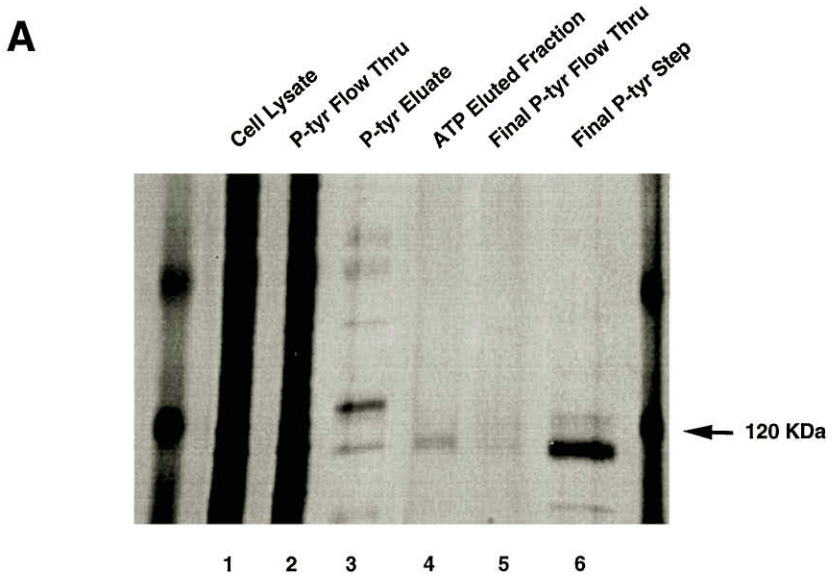
RESULTS AND DISCUSSION

Identification of CADTK—To further study the role of Ca^{2+}-dependent tyrosine phosphorylation, we purified activated CADTK from Ang II-stimulated GN4 cells, taking advantage of our previous finding that the activated enzyme was tyrosine-phosphorylated (7, 8). Following a 60-s Ang II stimulation, GN4 cells were lysed and the enzyme purified by sequential anti-phosphotyrosine antibody and ATP affinity chromatography (Fig. 1*A*). Several large scale preparations were concentrated by an additional anti-phosphotyrosine affinity step and subjected to gel electrophoresis, in-gel trypsin digestion of the purified CADTK, and analysis of the peptide mixture by Nano-Electrospray on a tandem mass spectrometer. Sequence from four peptides (amino acid residues 310–334, 554–572, 672–687, and 989–998) was identified.

We next isolated the full-length cDNA encoding rat CADTK. A WB cell cDNA library was screened, yielding a 3-kilobase cDNA encoding nucleotide 1001 to the 3′ end of the molecule. The 5′ region of the cDNA was obtained by polymerase chain reaction amplification of GN4 cell first strand cDNA. The deduced amino acid sequence from the rat CADTK cDNA clone is shown in Fig. 1*B*. Peptides sequenced from the purified protein are noted in *boxes*. Sequence comparison indicated that the cDNA was the rat homologue of a novel human non-receptor protein-tyrosine kinase, PYK2 (17). In collaboration with Sima Lev and Joseph Schlessinger, we determined that purified rat CADTK was recognized on immunoblots by antiserum generated against the catalytic domain of human PYK2.[2] The 115-kDa protein was also recognized by specific antiserum raised to a GST-CADTK fusion protein made using the rat CADTK cDNA (see "Materials and Methods"). Thus, the cDNA clone encodes the 115-kDa purified protein, as indicated by (i) comparison of sequenced peptides and predicted amino acid sequence, and (ii) recognition of the purified 115-kDa rat protein by antiserum to two regions of CADTK/PYK2. Since the 115-kDa protein was the predominant tyrosine kinase in Ang II-treated GN4 cells (15), CADTK appears to be the major calcium-dependent tyrosine kinase in rat liver epithelial cells.

This newly identified rat tyrosine kinase, which we will refer to by its functional name, CADTK, is closely related in sequence and domain structure to the focal adhesion kinase, $p125^{FAK}$ (22, 23). CADTK/PYK2 cDNA clones were recently isolated independently by two other groups using strategies to detect $p125^{FAK}$ homologues. These were reported as cell adhesion kinase β (CAKβ) (24) and related adhesion focal tyrosine kinase (RAFTK) (25). Our nucleotide sequence is identical to that of rat CAKβ with the exception of nucleotide 616 (C → A). The change predicts a Glu rather than an Ala in position 205. A Glu is found in this position in human PYK2 (21), in mouse RAFTK (25), and in mouse and human $p125^{FAK}$. CADTK and $p125^{FAK}$ exhibit approximately 45% amino acid identity (rat CADTK *versus* mouse $p125^{FAK}$) and 65% similarity. The homology is highest within the tyrosine kinase domain and the latter part of the C terminus commonly referred to as the focal adhesion targeting domain. Conversely, the identities are only in the 25% range in the N terminus and in the proline-rich domain residing between the catalytic domain and focal adhesion targeting region. We and others have not found CADTK expressed in commonly studied mouse fibroblast lines (Ref. 24 and data not shown). However, rat liver epithelial cells express both $p125^{FAK}$ and CADTK, and it will be of interest to study the

[2] S. Lev, J. Schlessinger, H. Yu, X. Li, R. Dy, L. Graves, and H. S. Earp, unpublished results.



B

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   1  MSGVSEPLSR VKVGTLRPPE GPPEPMVVVP VDVEKEDVRI LKVCFYSNSF NPGKNFKLVK CTVQTEIQEI ITSILLSGRI GPNIQLAECY GLRLKHMKSD  100
 101  EIHWLHPQMT VGEVQDKYEC LHVEAEWRYD LQIRYLPEDF MESLKEDRTT LLYFYQQLRN DYMQRYASKV SEGMALQLGC LELRRFFKDM PHNALDKKSN  200
 201  FELLEKEVGL DLFFPKQMQE NLKPKQFRKM IQQTFQQYAS LREEECVMKF FNTLAGFANI DQETYRCELI QGWNITVDLV IGPKGIRQLT SQDTKPTCLA  300
 301  EFKQIRSIRC LPLEETQAVL QLGIEGAPQS LSIKTSSLAE AENMADLIDG YCRLQGEHKG SLIIHAKKDG EKRNSLPQIP TLNLESRRSH LSESCSIESD  400
 401  IYAEIPDETL RRPGGPQYGV AREDVVLNRI LGEGFFGEVY EGVYTNHKGE KINVAVKTCK KDCTLDNKEK FMSEAVIMKN LDHPHIVKLI GIIEEEPTWI  500
 501  VMELYPYGEL GHYLERNKNS LKVPTLVLYA LQICKAMAYL ESINCVHRDI AVRNILVASP ECVKLGDFGL SRYIEDEDYY KASVTRLPIK WMSPESINFR  600
 601  RFTTASDVWM FAVCMWEILS FGKQPFFWLE NKDVIGVLEK GDRLPKPELC PPVLYTLMTR CWDYDPSDRP RFTELVCSLS DIYQMERDIA IEQERNARYR  700
 701  PPKILEPTAF QEPPPKPPRP KYKHPSQTNL LAPKLQFQVP EGLCASSPTL TSPMEYPSPV NSLHTPPLHR HNVFKRHSMR EEDFIRPSSR EEAQQLWEAE  800
 801  KIKMRQVLDR QQKQMVEDSQ WLRREERCLD PMVYMNDKSP LTPEKEAGYT EFTGPPQKPP RLGAQSIQPT ANLDRTDDLV YHNVMTLVEA VLELKNKLSQ  900
 901  LPPEEYVVVV KNVGLNLRKL IGSVDDLLPS LPASSRTEIE GIQKLLNKDL AELINKMRLA QQNAVTSLSE DCKRQMLTAS HTLAVDAKNL LDAVDQAKVV 1000
1001  ANLAHPPAF   1009
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FIG. 1. **Identification of rat calcium-dependent tyrosine kinase.** *A*, a silver-stained 8% polyacrylamide gel of fractions following CADTK purification. *Lanes 1* and *2* represent the lysate and flow through fraction from the anti-Tyr(P) agarose column (approximately 0.00001% of the total lysate and flow-through are shown). *Lane 3* shows protein eluted by 10 mM phenylphosphate from anti-Tyr(P) agarose and *lane 4* the fraction eluted from ATP-Sepharose with 1 mM ATP. The ATP eluate was concentrated with a repeat anti-Tyr(P) monoclonal purification step (*lane 6*, the flow-through of which is shown in *lane 5*). The kinase runs as a single band of 115 kDa (*lane 6* represents 4% of the purified preparation). *B*, purified CADTK was digested. Peptides were sequenced, and the full-length cDNA was cloned using rat liver epithelial cell RNA. The predicted amino acid sequence is shown with the original sequenced peptides denoted by enclosure within a *box*.

function of these two homologous enzymes when they are expressed in the same cell.

Agonist-dependent CADTK Activation—CADTK was rapidly (15 s) tyrosine-phosphorylated in GN4 cells treated with Ang II (Fig. 2*A*) or thapsigargin (data not shown). Maximal phosphorylation is observed between 60 and 90 s. Pretreatment of cells with BAPTA-AM prevented the Ang II-dependent rise in intracellular Ca^{2+} (8) and significantly attenuated CADTK autophosphorylation in response to Ang II (Fig. 2*A*). As shown in Fig. 2*B*, Ang II or another agonist that stimulates G protein-coupled receptors, LPA, were at least twice as effective as thapsigargin in activating CADTK. Thus, a calcium signal is a significant, but perhaps not the sole, determinant of CADTK activation.

We had previously shown Ca^{2+}-dependent JNK activation in GN4 cells and had postulated that the involvement of CADTK (16). Therefore, we tested whether agonists known to stimulate JNK activity also stimulated CADTK autophosphorylation. Hyperosmolarity (700 mM NaCl or 400 mM sorbitol) resulted in CADTK activation (Fig. 2*B*). NaCl gave consistently greater activation than sorbitol. The effect of hyperosmolarity on CADTK tyrosine phosphorylation was less rapid than that of Ang II and thapsigargin (data not shown), but with NaCl, the maximal activation was similar to Ang II. However, other agonists that stimulate JNK in GN4 cells (*e.g.* TNFα, UV radiation, and anisomycin) failed to stimulate CADTK (Fig. 2*B*). Ang II and thapsigargin also stimulated CADTK immune complex tyrosine kinase activity as measured by comparing CADTK immunoprecipitates from control and agonist-treated cells incubated with [γ-^{32}P]ATP, and poly(Glu_4-Tyr) as a substrate (8). Again, Ang II was approximately twice as effective as thapsigargin at elevating CADTK tyrosine kinase activity measured between 30 s and 5 min (data not shown).

Comparison of CADTK in GN4 and WB Cells—Previously we showed that GN4 cells (derived by chemical transformation of a normal rat liver epithelial line, WB; Ref. 26) exhibited 3–4 times the angiotensin II-dependent tyrosine phosphorylation of its parent WB cell line (8). Using antiserum specific for rat

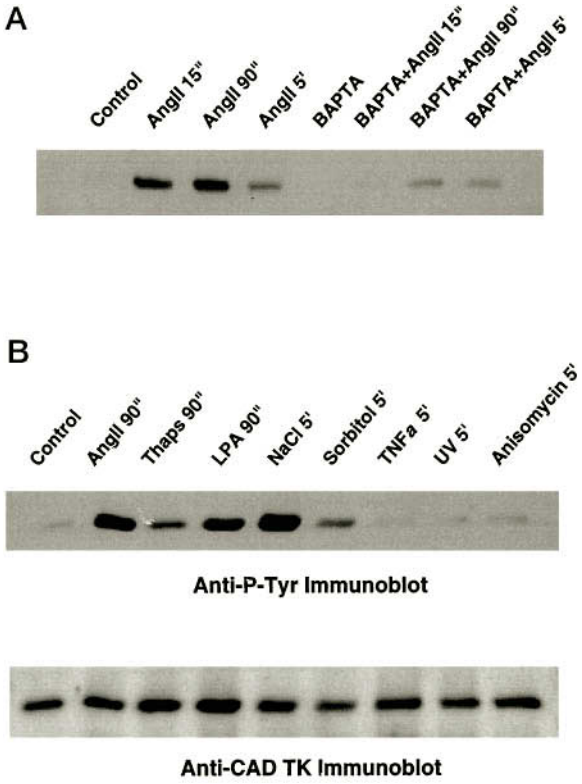

FIG. 2. **CADTK tyrosine phosphorylation in agonist-treated cells.** *A*, angiotensin II-stimulated CADTK tyrosine phosphorylation is dependent in part on a calcium signal. Cells were pretreated with or without 50 μM BAPTA-AM for 20 min prior to treatment with Ang II (1 μM) for the indicated times. Following lysis, CADTK was immunoprecipitated with anti-CADTK antiserum and subjected to SDS-PAGE, transferred to Immobilon, and immunoblotted with monoclonal anti-Tyr(P) antibody PT66. Tyrosine phosphorylation of CADTK was rapid and was greatly attenuated in cells pretreated with the cell permeant calcium chelator. *B*, agonist-induced CADTK tyrosine phosphorylation. Confluent GN4 cells were treated for 90 s with Ang II (1 μM), thapsigargin (2 μM), LPA (10 μg/ml) or for 5 min with NaCl (700 mM), sorbitol (400 mM), TNFα (60 ng/ml), UV (100 J/m^2), or anisomycin (10 μg/ml). Cells were lysed, CADTK was immunoprecipitated, and its extent of tyrosine phosphorylation was assessed by anti-Tyr(P) immunoblotting as described above. Immunoblots were stripped and reprobed with anti-CADTK antiserum to demonstrate equal CADTK immunoprecipitation from each sample.

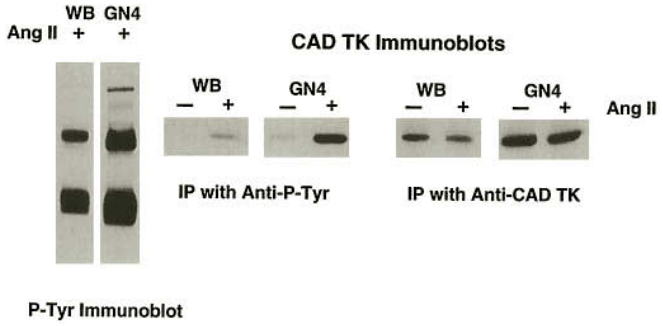

FIG. 3. **Comparison of CADTK and its Ang II-dependent activation in WB and GN4 cells.** Confluent plates (60 mM) of GN4 and WB rat liver cells were treated with vehicle or Ang II (1 μM, 90 s). Cells were lysed and equal amounts of cell protein immunoprecipitated with monoclonal anti-Tyr(P) antibody PT66 (*left panel*) or anti-CADTK antiserum (*middle* and *right panels*) as described under "Materials and Methods." *Left panel* was immunoblotted with PT66 to demonstrate the Ang II-dependent Tyr(P) protein from WB and GN4 cells. *Middle panel* was immunoblotted with PT66 to demonstrate the amount of tyrosine-phosphorylated CADTK in Ang II-treated WB and GN4 cells. *Right panel* was immunoblotted with anti-CADTK antiserum, demonstrating the difference in CADTK protein immunoprecipitated from confluent WB and GN4 cells. Confluent GN4 cells exhibited approximately 5-fold greater CADTK tyrosine autophosphorylation and 2–3-fold more CADTK protein.

CADTK, we found that Ang II-treated confluent GN4 cells consistently exhibited 4–5 fold more autophosphorylated CADTK than similarly treated WB cells (Fig. 3). This approximately 5-fold increase in CADTK tyrosine phosphorylation was consistent whether WB and GN4 cells were compared by immunoprecipitating CADTK and performing an anti-Tyr(P) immunoblot (Fig. 3) or by immunoprecipitating with an anti-Tyr(P) antibody and performing a CADTK immunoblot (data not shown). Total CADTK protein expression was approximately 2–3-fold greater in confluent GN4 cells as determined by immunoprecipitation of CADTK followed by a CADTK immunoblot (Fig. 3). Thus the increase in Ca^{2+}-dependent tyrosine phosphorylation observed in GN4 cells is secondary to both increased expression of CADTK and additional increment in the CADTK activation. Since neither Ca^{2+} nor Ca^{2+}/calmodulin appear to directly activate CADTK (8, 15, 17), exploration of CADTK regulation by Ca^{2+} may be aided by the difference in activation between WB and GN4 cells.

Activation of CADTK: Correlation with JNK but Not MAP Kinase Activation—In GN4 cells, Ang II stimulates MAP kinase and JNK, as well as increasing AP-1 binding in a protein kinase C-independent manner (16). Therefore, we examined the correlation of CADTK activation with increases in AP-1 binding activity by comparing Ang II-dependent activation of CADTK, MAP kinase, and JNK in GN4 and WB cells. Whereas Ang II-dependent CADTK activity was 4–5 fold higher in GN4 cells (Fig. 3), Ang II-dependent MAP kinase activation was similar in WB and GN4 cells (Fig. 4*A*). Furthermore, thapsigargin, which is a potent, Ca^{2+}-dependent stimulator of JNK activity (see below), only minimally activated MAP kinase in GN4 cells as determined either by gel mobility shift or myelin basic protein phosphorylation (Fig. 4, *B* and *C*, respectively). Thus, in rat liver cells, MAP kinase activation was neither correlated with CADTK activation nor substantially Ca^{2+}-dependent.

We have shown previously that Ang II and thapsigargin significantly activate JNK in GN4 cells (16). We now show that treatment with thapsigargin, Ang II, or EGF (which produces a small PLCγ-dependent Ca^{2+} signal in these cells) stimulated JNK activity 5–6-fold more effectively in GN4 than in WB cells (Fig. 5*A*). This was not due to differential JNK expression; both GN4 and WB cells exhibited similar, substantial increases in JNK activity when treated with the protein synthesis inhibitor, anisomycin (Fig. 5*A*). Furthermore, immunoblot analysis of immunoprecipitated JNK protein showed nearly equal levels of 46- and 54-kDa JNK isoforms (*lower* and *upper bands*, respectively; Fig. 5*C*) in GN4 and WB cells. An additional experiment (Fig. 5*B*) confirms the exuberant JNK activation by thapsigargin and Ang II in GN4 cells, but showed little difference in the ability of hyperosmolarity (NaCl and sorbitol), UV exposure, or anisomycin to stimulate JNK in the two cell types. Since anisomycin and UV failed to activate CADTK (Fig. 2B), it was not surprising that these two agonists activate JNK similarly in WB and GN4 cells. The UV and anisomycin results clearly indicate that CADTK-independent pathways to JNK activation exist in GN4 and WB cells. The fact that NaCl and sorbitol stimulate CADTK does not *a priori* indicate that CADTK is the only mechanism by which hyperosmolarity activates JNK. It is likely that hyperosmolarity can maximally activate JNK in WB cells through a stress pathway similar to that of UV and anisomycin, a pathway that does not vary significantly between WB and GN4 cells.

The above results demonstrate that Ca^{2+}-dependent JNK activation is well correlated with the 4–5-fold greater CADTK activation in GN4 cells (above that seen in WB cells), whereas

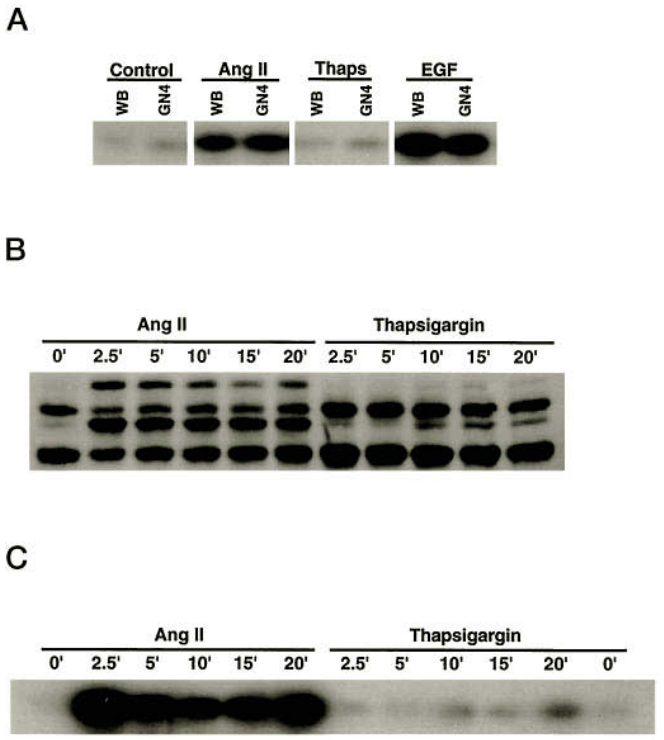

FIG. 4. **MAP kinase activation by Ang II was similar in serum-starved GN4 and WB cells and was only minimally activated by thapsigargin.** *A*, MAP kinase activity was assessed in WB and GN4 cells 5 min after stimulation with Ang II (1 μM), thapsigargin (2 μM), and EGF (100 ng/ml). Cells were lysed and immunoprecipitated with anti-Erk 1 antibody. The amount of myelin basic protein phosphorylation in treated and control cells was assessed as described under "Materials and Methods." Ang II and EGF-dependent MAP kinase activation were similar in GN4 and WB cells. *B* and *C*, time-dependent activation of MAP kinase was assessed in Ang II- and thapsigargin-treated cells by examining the gel mobility shift of MAP kinase (Erks 1 and 2) by immunoblotting (*B*) or by assessing myelin basic protein phosphorylation in the same samples (*C*). MAP kinase activation by Ang II did not correlate with the 4–5-fold higher expression of CADTK in GN4 cells.

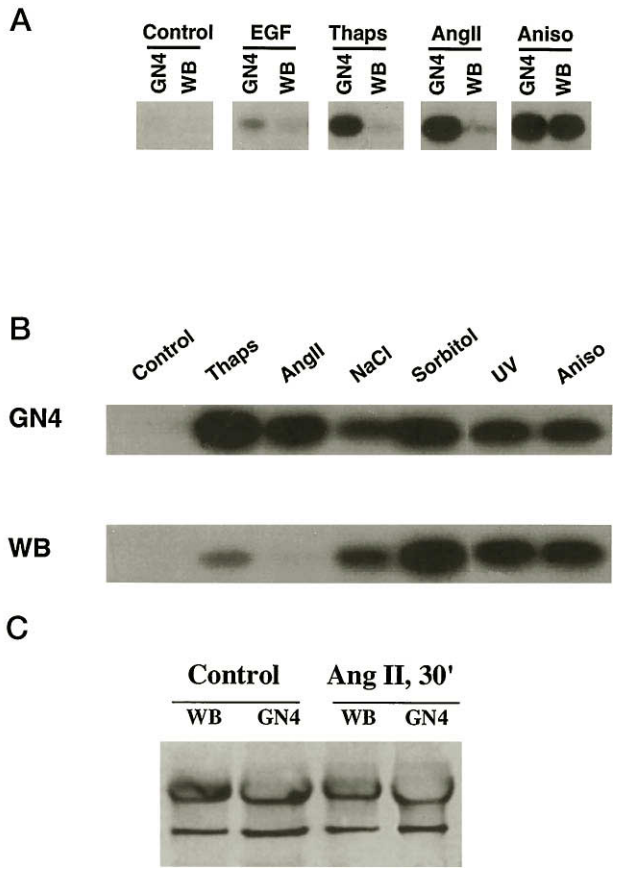

FIG. 5. **JNK activation is correlated with CADTK activation.** *A*, JNK kinase activity in GN4 and WB cells treated for 30 min with vehicle, EGF (100 ng/ml), thapsigargin (2 μM), Ang II (1 μM), or anisomycin (10 μg/ml) was assessed by measuring phosphorylation of GST c-Jun (1–79) in lysates of serum-starved cells treated for the indicated times as described under "Materials and Methods." Calcium-dependent agonists activated JNK 4–6 times more effectively in GN4 cells, which exhibit >4–5 fold activation of CADTK. Anisomycin-dependent activation of JNK was equivalent in the two cell lines. *B*, in a separate experiment, confluent GN4 and WB cells were incubated for 30 min with thapsigargin (2 μM), Ang II (1 μM), NaCl (700 mM), Sorbitol (400 mM), UV (100 J/m^2), and anisomycin (10 μg/ml). Cells were lysed and JNK activity assessed as described above. The relative JNK activation as reflected by GST c-Jun phosphorylation was at least 4–5-fold greater in GN4 cells for Ca^{2+}-dependent agonists but was equal in the two cell lines treated with agonists thought to be activated via the stress-dependent pathway. *C*, the expression of 46- and 54-kDa JNK isoforms is similar in WB and GN4 cells. Equal amounts of cell lysate were immunoprecipitated with anti-JNK antiserum from control or Ang II-treated cells, subjected to SDS-PAGE and immunoblotted as described under "Materials and Methods." The 54-kDa isoform band is distorted by the presence of the heavy chain from the immunoprecipitating antibody.

MAP kinase activation is not. Additional evidence supporting this conclusion includes: (i) the small and temporally delayed activation of CADTK caused by EGF correlates with a minimal level of JNK activation in GN4 cells, which nonetheless exceeds EGF-dependent JNK activation in WB cells (Fig. 5*A*), and (ii) the substantial activation of CADTK by LPA (Fig. 2*B*) correlates with substantial JNK activation in GN4 cells equivalent to that of thapsigargin and Ang II (data not shown).

Activation of the JNK by hormones (16) and neurotransmitters (27) demonstrates that there are hormone-dependent pathways that may well be involved in physiological control of gene expression in a cell type-specific manner; JNK is not just involved in the stress response. The pathway from membrane signal (hormone, TNFα, etc.) to JNK is not totally defined for any agonist, including those that stimulate CADTK. However, the work of others suggests that the JNK activation pathway involves the small GTP-binding proteins, such as Rac and Cdc42, PAK-like protein kinases, and MEKK (28–31). We have demonstrated at least two pathways to JNK activation in GN4 cells, those correlated with CADTK activation (Ang II and Ca^{2+}) and those not correlated (UV, TNFα, and anisomycin). Additional preliminary studies support the two pathway model in GN4 cells; cAMP and TPA significantly inhibit the Ang II and thapsigargin-dependent pathways but not the stress-related pathway.[3] Since cAMP and TPA do not inhibit anisomycin-dependent JNK activation, it is likely these agents inhibit the putative CADTK → JNK pathway prior to the JNKK/SEK step (32–36). In GN4 cells, TNFα, UV, and anisomycin pathways do not involve CADTK, and the pathway to JNK for these agonists is presumably CADTK-independent.

It is intriguing that thapsigargin-dependent activation of CADTK does not result in significant MAP kinase activation (Fig. 2) in GN4 cells. In PC12 cells, intracellular Ca^{2+} signals and PYK2 overexpression did stimulate MAP kinase. Furthermore, expression of a dominant negative PYK2 blocked bradykinindependent MAP kinase activation (17). Our data may suggest that key components coupling CADTK to MAP kinase

[3] Li, X., Yu, H., He, Q., Gravea, L., and Earp, H. S., unpublished results.

are limiting in or absent from GN4 cells, explaining the preponderance of JNK (Fig. 5) as opposed to MAP kinase (Fig. 4) activation following thapsigargin treatment. The large discrepancy between Ca^{2+}-dependent MAP kinase and JNK activation in GN4 cells serves to emphasize that the control of cell-type specific gene expression by heterotrimeric G proteins may be quite flexible. Specifically, whether a hormone activates JNK or MAP kinase in a Ca^{2+}-dependent manner may be determined by (i) expression of CADTK or like enzymes, (ii) intact pathways linking CADTK to JNK or MAP kinase, and (iii) subtle modulation of either pathway by signaling cross-talk.

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