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 C, 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 p125FAK and both enzymes are expressed in WB and GN4 cells. Angiotensin II, which only slightly increases p125FAK 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 activity 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 (Ca²⁺), which in turn regulates myriad cellular processes, including gene expression and cell growth (1–3). The Ca²⁺ signal acts, in part, by controlling serine and threonine protein phosphorylation through multiple mechanisms, for example, by modulating Ca²⁺/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 Gq-coupled receptors (e.g. angiotensin II, or Ang II) 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 Ca²⁺ (e.g. thapsigargin) increase tyrosine phosphorylation, in part, by activating a soluble, Ca²⁺-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, p125FAK (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 Ca²⁺ signal activates the c-Jun N-terminal kinase (JNK) through a Ca²⁺/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 p125FAK. However, CADTK/PYK2 and p125FAK 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, p125FAK 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 p125FAK, 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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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. 1A). 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 898–998) 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. 1B. 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 Sina Lev and Joseph Schlessinger, we determined that purified rat CADTK was recognized on immunoblots by antiserum generated against the catalytic domain of human PYK2. 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, p125FAK (22, 23). CADTK/PYK2 cDNA clones were recently isolated independently by two other groups using strategies to detect p125FAK 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 p125FAK. CADTK and p125FAK exhibit approximately 45% amino acid identity (rat CADTK versus mouse p125FAK) 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 p125FAK and CADTK, and it will be of interest to study the...
function of these two homologous enzymes when they are expressed in the same cell.

Agonist-dependent CADTK Activation—CADTK was rapidly tyrosine-phosphorylated in GN4 cells treated with Ang II (Fig. 2A) 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 Ca²⁺ and significantly attenuated CADTK autophosphorylation in response to Ang II (Fig. 2A). As shown in Fig. 2B, Ang II and another agonist that stimulates JNK in GN4 cells (e.g., TNF-α, UV radiation, and anisomycin) failed to stimulate CADTK. 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-32P]ATP, and poly(Glu₄-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
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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. 4A). 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 PLC\(\gamma\)-dependent Ca\(^{2+}\) signal in these cells) stimulated JNK activity 5–6-fold more effectively in GN4 than in WB cells (Fig. 5A). 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. 5A). Furthermore, immunoblot analysis of immunoprecipitated JNK protein showed nearly equal levels of 46- and 54-kDa JNK isoforms (lower and upper bands, respectively) in GN4 and WB cells. An additional experiment (Fig. 5B) 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-dependent 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...
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 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.

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. 5A), and (ii) the substantial activation of CADTK by LPA (Fig. 2B) 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. 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 bradykinin-dependent MAP kinase activation (17). Our data may suggest that key components coupling CADTK to MAP kinase

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Footnotes:

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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