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Adapter Protein CRKII Signaling Is Involved in the Rat Pancreatic Acini Response to Reactive Oxygen Species

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Abstract Recent studies demonstrate that reactive oxygen species (ROS) are important mediators of acute pancreatitis, whether induced experimentally or in necrotizing pancreatitis in humans; however, the cellular processes involved remain unclear. Adapter protein CrkII, plays a central role for convergence of cellular signals from different stimuli. Cholecystokinin (CCK), which induces pancreatitis, stimulates CrkII tyrosine phosphorylation and CrkII protein complexes, raising the possibility it can be important in the acinar cell responses to ROS. Therefore, our aim was to investigate whether CrkII signaling is involved in the biological response of rat pancreatic acini to H_2O_2 and the intracellular mediators implicated. Treatment of isolated rat pancreatic acini with H_2O_2 rapidly stimulates CrkII phosphorylation, measured as electrophoretic mobility shift and by using a phosphospecific antibody (pTyr221). Tyrosine kinase blocker B44 inhibits the higher phosphorylation state, demonstrating that it occurs mainly in tyrosine residues. H_2O_2-induced CrkII phosphorylation is time- and concentration-dependent, showing maximal effect with 3 mM H_2O_2 at 5 min. The intracellular pathways induced by H_2O_2 leading to CrkII tyrosine phosphorylation do not involve PKC, intracellular calcium, PI3-K or the actin cytoskeleton integrity. ROS generation clearly promotes the formation of protein complex CrkII–PYK2. In conclusion, ROS clearly affect the key adapter protein CrkII signaling by two ways: stimulation of CkII phosphorylation and a functional consequence: formation of CrkII–protein complexes. Because of its central role in activating more distal pathways, CrkII might likely play an important role in the ability of ROS to induce pancreatic cellular injury and pancreatitis. J. Cell. Biochem. 97: 359–367, 2006.

Key words: reactive oxygen species; CrkII; signaling; phosphorylation; pancreatic acini

Recent studies indicate that extracellular stimuli generate or require reactive oxygen species (ROS) to transmit their intracellular signals successfully [Lander, 1997; Kamata and Hirata, 1999]. In epithelial cells such as pancreatic acini it has been shown that treatment with ROS donor H_2O_2 induced several intracellular effects including the release of calcium from intracellular stores [Pariente et al., 2001], activation of ERK, JNK, and $p38^{MAPK}$ transduction pathways [Dabrowski et al., 2000], cytokine production [Seo et al., 2002], and actin cytoskeletal reorganization [Rosado et al., 2002]. Moreover, ROS are known to be pathogenic factors and it has been suggested an important role of these species in the early pathogenesis of the acute pancreatitis induced by cholecystokinin (CCK) receptor activation [Steer, 1998; Dabrowski et al., 1999].

A new oncogene identified from a chicken tumor that activated a cellular adapter-type SH2-SH3-containing protein led to the name of *crk* (chicken tumor virus regulator of kinase) [Feller, 2001]. The product of the oncogene in human and mouse, c-Crk, is expressed as two distinct proteins of 28 and 40 kDa, respectively. c-CrkI contains one SH2 and one SH3 domain, while c-CrkII has an additional SH3 domain [Matsuda et al., 1992].

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The Crk family seems to be involved in different signaling systems including JNK pathway activation, actin cytoskeleton regulation, receptor tyrosine kinases signaling pathways, and pathogenesis of different leukemias related with the Bcr/Abl tyrosine kinases [Buday, 1999; Feller, 2001]. A peculiar feature of CrkII is that, unlike other adapter proteins, CrkII itself is phosphorylated on tyrosine. Tyrosine kinases such as the cytoplasmic c-Abl and the epidermal growth factor (EGF) receptor, which bind to SH3 and SH2 domains of CrkII, respectively, phosphorylate CrkII in tyrosine [Feller et al., 1994; Ren et al., 1994; Hashimoto et al., 1998]. The SH2 domain of CrkII binds to phosphotyrosine-containing proteins such as $p130^{Cas}$, paxillin, or Cbl [Buday, 1999; Feller, 2001]. The SH3 domain of CrkII binds to guanine nucleotide exchange factors, such as C3G, which in turn activates transduction cascades related with small GTP binding proteins, such as Rap1, that leads to activation of MAPK family members and subsequent activation of transcription factors [Buday, 1999; Feller, 2001].

Because CrkII tyrosine phosphorylation and protein complex formation are stimulated by growth factors and as well by sphingosine 1-phosphate, angiotensin II, bombesin, engagement of T-cell receptor, or B-cell antigen receptor [Buday, 1999; Feller, 2001], the adapter CrkII represents a convergence pathway of the actions of numerous mitogenic stimuli.

Recent studies show that CCK, which induces experimental pancreatitis, increases tyrosine phosphorylation of CrkII [Andreolotti et al., 2003] and stimulates CrkII–protein complexes [Tapia et al., 1999; Andreolotti et al., 2003] raising the possibility it can be relevant in the pancreatic acini response to ROS. Therefore, the aim of this work was to investigate whether CrkII signaling is involved in the biological response of rat pancreatic acini to ROS donor H_2O_2 and the intracellular mediators implicated. Our results indicate that ROS are potent activators of the CrkII phosphorylation cascade by a mechanism(s), that is independent of the intracellular calcium mobilization, PKC activation, phosphoinositide synthesis, or actin cytoskeleton organization. ROS generation clearly has a functional consequence: the formation of CrkII protein complex with PYK2. We postulate that CrkII signaling could serve then as a possible mediator of the activation of signaling cascades induced by ROS donor in pancreatic acini

MATERIALS AND METHODS

Materials

Male Wistar rats (150–200 g) were obtained from the Animal Section, Veterinary Resources Branch, NIH, Bethesda, MD, or from the animal farm, Faculty of Veterinary, UEX, Spain; purified collagenase (CLSPA) from Worthington Biochemicals, Freehold, NJ; COOH-terminal octapeptide of cholecystokinin (CCK-8) from Peninsula Laboratories, Belmont, CA, thapsigargin, tyrphostin B44, and GF109203X from Calbiochem, La Jolla, CA; BAPTA-AM, from Bachem AG, Switzerland; anti-Crk, anti-PYK2, and anti-phosphotyrosine mAb (PY20) from Transduction Laboratories, Lexington, KY; phospho specific anti-pY221Crk pAb from Cell Signaling, Beverley, MA.

Methods

Pancreatic acini isolation. Dispersed rat pancreatic acini were prepared according to modifications [Garcia and Jensen, 1998] of the procedure previously published [Peikin et al., 1978]. Unless otherwise stated, the standard incubation solution contained (mM): HEPES (25.5) [pH 7.4]; NaCl (98); KCl (6); NaH_2PO_4 (2.5); sodium pyruvate (5); sodium fumarate (5); sodium glutamate (5); glucose (11.5); $CaCl_2$ (0.5); $MgCl_2$ (1); glutamine (2); albumin 1% (w/v); trypsin inhibitor 1% (w/v); vitamin mixture 1% (v/v); and amino acid mixture 1% (w/v) performed with 100% O_2 as the gas phase.

Immunoprecipitation. Dispersed pancreatic acini from one rat were preincubated with standard incubation solution for 3 h at 37°C. Acini were then incubated with agonists at concentrations and times indicated, washed with phosphate buffered saline (PBS) with 0.2 mM Na_3VO_4, and sonicated 5 s at 4°C in lysis buffer. Lysates were centrifuged at 10,000 g for 15 min. Protein concentration in supernatant was standardized to 500 μg/ml. For tyrosine phosphorylation determination lysates (500 μg) were incubated overnight at 4°C with anti-phosphotyrosine (PY20) mAb (4 μg) and 25 μl of protein A-agarose. For co-immunoprecipitation studies, lysates (400 μg) were incubated with 4 μg of anti-Crk mAb for 2 h at 4°C. Then the immune complexes were incubated with 4 μg of rabbit anti-mouse IgG and 25 μl of protein A-

agarose for 1 h at 4°C. Immunoprecipitates were washed three times with PBS and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Western blotting. Proteins in total cellular lysates or immunoprecipitates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Western blotting was realized as previously described [Ferris et al., 1999; Tapia et al., 1999] using as the primary antibody concentration: 0.25 μg/ml anti-Crk and anti-PYK2 or a dilution of 1:1000 of anti-pY221 CrkII pAb.

RESULTS

Identification of CrkII Protein in Rat Pancreatic Acini and Regulation by H_2O_2

Proteins from whole cell acinar lysates were separated by SDS–PAGE and analyzed by Western blotting. Results using anti-Crk specific antibody (Fig. 1A) showed two bands at the suitable molecular weight of CrkII (40/42 kDa). It is well established that the electrophoretic mobility shift of CrkII is due to the phosphorylation state of the protein; thus the upper band corresponds to the more phosphorylated CrkII and the lower band corresponds to the less phosphorylated state of the CrkII protein [Beitner-Johnson and LeRoith, 1995]. In lysates from non-treated acinar cells the majority of CrkII was present in the lower band (lane 1). In whole lysates from acinar cells treated with 1 mM H_2O_2 the majority of CrkII is shifted to the upper band, indicating an increase on the phosphorylation state of the protein (lane 3). Similar results were obtained when pancreatic acini were treated with 10 nM CCK-8 (lane 4), which is one of the best established physiological regulators of the pancreatic acinar cells [Williams, 2001], or after addition of 300 mM sorbitol to the incubation medium to induce an osmotic stress (lane 2).

In order to investigate whether CrkII tyrosine phosphorylation was involved in its electrophoretic mobility shift, we pretreated pancreatic acini with B44, a general inhibitor of protein tyrosine kinases. Previously, we showed that pretreatment of rat pancreatic acini with 300 μM B44 for 2 h caused almost a complete inhibition of general tyrosine phosphorylation either in basal or stimulated conditions [Tapia et al., 1999]. Pretreatment of these cells with

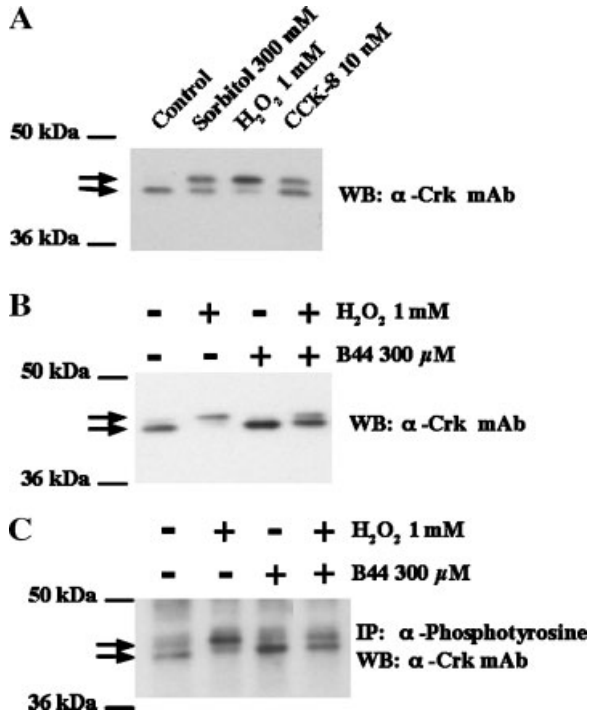

Fig. 1. H_2O_2 treatment leads to CrkII tyrosine phosphorylation in pancreatic acini. **A**: Rat pancreatic acini were treated for 5 min with 300 mM sorbitol or 1 mM H_2O_2 or 10 nM CCK-8 and then lysed. **B** and **C**: Rat pancreatic acini were preincubated for 2 h with the tyrosine kinase inhibitor B44 (300 μM) followed by treatment for 5 min with vehicle (**lanes 1**, **3**) or with 1 mM H_2O_2 (**lanes 2**, **4**) and then lysed. Whole cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine mAb (α-phosphotyrosine). Cell lysates or immunoprecipitates were analyzed by Western blotting (WB) using anti-Crk mAb. CrkII positions are indicated as arrows on the **left**. Results shown are representative of three other independent experiments, each one performed in duplicate.

B44 showed that the majority of CrkII protein after H_2O_2 treatment remained in the lower band, indicating that the electrophoretic mobility shift induced by H_2O_2 was prevented by this tyrosine kinase inhibitor (Fig. 1B, lane 4).

To further confirm whether CrkII was tyrosine phosphorylated, we first immunoprecipitated phosphotyrosine-containing proteins from acinar lysates using an anti-phosphotyrosine monoclonal specific antibody. Immunopurified proteins were then analyzed by Western blotting using a specific anti-Crk antibody (Fig. 1C). Two phosphotyrosine-containing bands appeared in untreated acini (lane 1) while treatment with H_2O_2 resulted in an increase of the intensity in the upper band (lane 2), which corresponds to the more phosphotyrosine-containing band of CrkII. Pretreatment with B44 inhibited the H_2O_2-induced shift to the upper

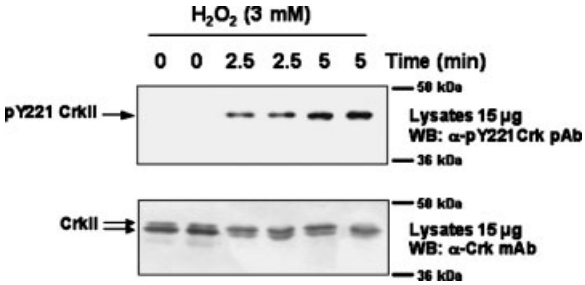

Fig. 2. H_2O_2 treatment leads to site-specific tyrosine phosphorylation of CrkII in rat pancreatic acini. Rat pancreatic acinar cells were treated with H_2O_2 (3 mM) for the indicated times and then lysed. Whole cell lysates (15 µg) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting using the phospho specific anti-pY221Crk pAb (**upper panel**). Membranes were stripping and reprobing with anti-Crk mAb (**lower panel**). pY221 CrkII and CrkII positions are indicated as arrows on the **left**. Results shown are representative of three other independent experiments, each one performed in duplicate.

band in pancreatic acini (lane 4). These results were further confirmed by using antibodies in the reverse order, immunoprecipitation with an anti-Crk antibody, and subsequent Western blotting analysis using an anti-phosphotyrosine antibody (data not shown).

In addition, our results show that CrkII is phosphorylated in the tyrosine Y221 after H_2O_2 treatment (Fig. 2, upper panel) where it can be observed that this phosphorylation correlates with the mobility shift of upper band of CrkII (lower panel).

Phosphorylation of CrkII Induced by H_2O_2 Is Time- and Dose-Dependent

In pancreatic acini, H_2O_2 caused a marked and rapid increase on the CrkII phosphorylation level, as evidenced by an electrophoretic mobility shift. CrkII phosphorylation induced by 1 mM H_2O_2 was time-dependent (Fig. 3A) presenting a marked increase detected within 1 min after addition of 1 mM H_2O_2 and a maximum reached within 5 min (60% of total CrkII was shifted to the upper band). Phosphorylation degree returns to basal values at 20 min. The higher CrkII phosphorylation state induced by H_2O_2 was dose-dependent with a maximal increase detected at 3 mM H_2O_2 (Fig. 3B).

Effect of Intracellular Calcium on CrkII Phosphorylation in Pancreatic Acini

Intracellular calcium plays a central role in the main function of pancreatic acinar cells through its involvement in different intracellular pathways. Therefore, our next aim was to investigate the involvement of calcium in H_2O_2-induced CrkII phosphorylation. We preincubated pancreatic acini with different

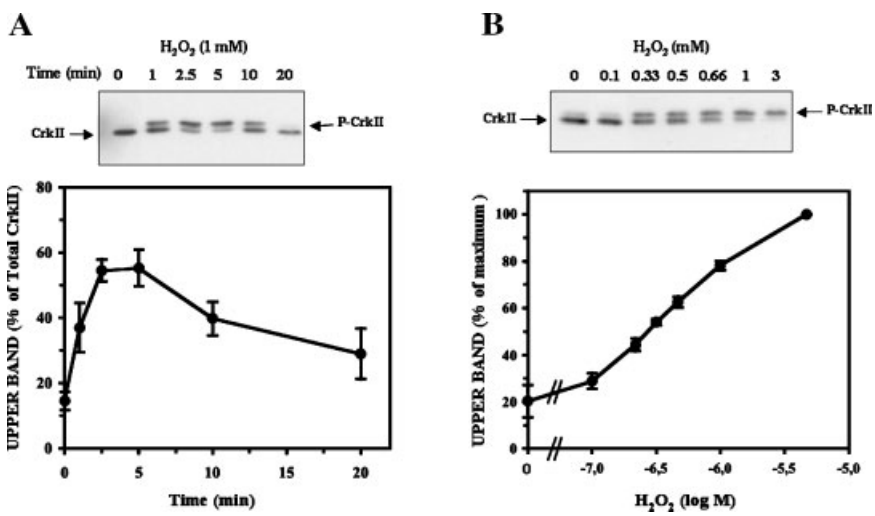

Fig. 3. Time-course (**A**) and dose-dependence (**B**) of H_2O_2 induction of CrkII tyrosine phosphorylation. Rat pancreatic acini were treated with H_2O_2 at times (A) and concentrations (B) indicated and then lysed. Cell lysates (10 µg) were resolved by SDS–PAGE and analyzed by Western blotting using anti-Crk mAb. Quantification of bands was performed by scanning densitometry and is represented in the graphs. Results shown are representative of four independent experiments, each one performed in duplicate. A: **Upper panel** shows a representative experiment with H_2O_2 (1 mM) at the indicated times. Values shown in the graph are mean ± SEM, expressed as the percentage of CrkII upper band with respect to total CrkII (upper and lower band). B: **Upper panel** shows a representative experiment where acini were treated for 5 min with indicated H_2O_2 concentrations. Values are mean ± SEM expressed as the percentage of maximal increase caused by 3 mM H_2O_2 above control unstimulated values.

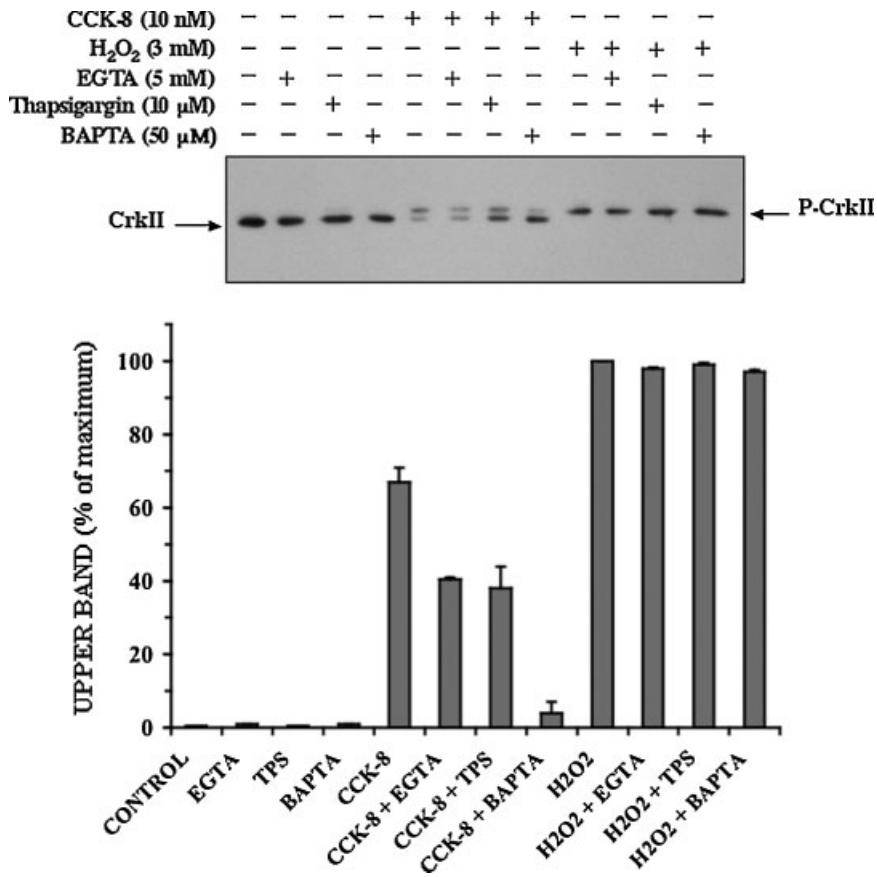

Fig. 4. Calcium independence of H_2O_2 induction of CrkII electrophoretic mobility shift in pancreatic acini. Acini were pretreated 30 min at 37°C in a calcium-free medium (with EGTA 5 mM) either in absence or presence of thapsigargin (10 μM) or BAPTA/AM (50 μM). Acini were further incubated for 5 min with vehicle, H_2O_2 (3 mM) or CCK-8 (10 nM). CrkII electrophoretic mobility shift was analyzed by Western blotting as described. **Upper panel** results shown are representative of three others. **Lower panel** results are mean ± SEM expressed as a percentage of CrkII maximal electrophoretic motility shift (obtained with H_2O_2 treatment in a medium with normal calcium concentration).

compounds in a calcium-free medium (with ethylene glycol-bis (β aminoethyl ether) tetra-acetic acid, EGTA, 5 mM) before addition of 3 mM H_2O_2. The calcium-free medium, which decreases calcium influx in response to CCK-8 in pancreatic acini, as previously shown [Tapia et al., 1999], or 30 min preincubation with thapsigargin (10 μM), a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, which totally abolishes the CCK-8-induced increase of intracellular calcium in pancreatic acini [Metz et al., 1992], or depletion of intracellular calcium by 30 min preincubation with BAPTA-AM (50 μM), markedly decreased the CCK-induced CrkII phosphorylation (Fig. 4, lane 5) by 41.5 ± 2.9%, 35.5 ± 4.0%, and 84.0 ± 11.6%, respectively (Fig. 4, lanes 6–8). However, contrasting with these effects in response to CCK, none of these pretreatments was able to reduce the CrkII phosphorylation induced by H_2O_2 (Fig. 4, lanes 10–12).

Effects of Protein Kinase C (PKC), Phosphoinositide 3-Kinase (PI3-kinase), and the Disruption of Actin Cytosqueleton on CrkII Phosphorylation

At present little is known about the intracellular pathways related to CrkII tyrosine phosphorylation. To determine whether PKC pathway activation is involved in H_2O_2-increased CrkII phosphorylation we pretreated pancreatic acini for 2 h with 5 μM of GF109203X, a PKC inhibitor [Garcia et al., 1997]. PKC inhibition alone or in combination with intracellular calcium stores depletion (using thapsigargin) had not effect on H_2O_2 stimulation of CrkII phosphorylation in acinar cells (Fig. 5A, lanes 4 and 6, respectively). In order to elucidate the intracellular pathways by which H_2O_2 leads to CrkII phosphorylation, we next study whether phosphoinositide metabolism is mediating H_2O_2-induced CrkII

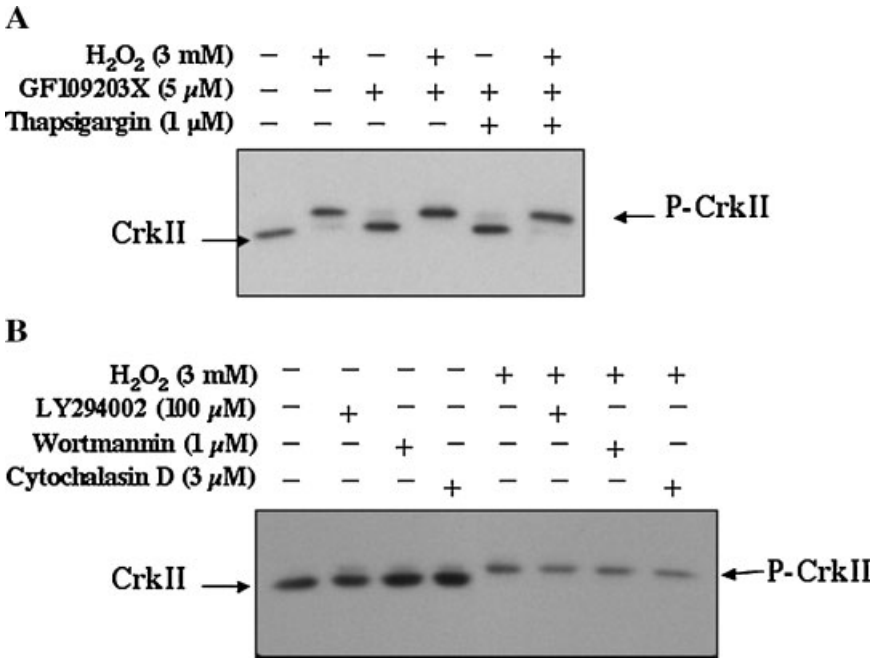

Fig. 5. Effect of different inhibitors on CrkII electrophoretic mobility shift in rat pancreatic acini. **A**: Pancreatic acini were pretreated with 1 μM thapsigargin (30 min) in a calcium-free medium (with EGTA 5 mM) or with 5 μM GF109203X (2 h) either alone or in combination. Acini were then incubated for further 5 min with vehicle or with H_2O_2 (3 mM). CrkII electrophoretic mobility shift was evaluated by Western blotting as described in Figure 1. Results shown are representative of three others. **B**: Pancreatic acini were pretreated for 2 h either in the absence or presence of 100 μM LY294002, 1 μM Wortmannin, or 3 μM Cytochalasin D. Acini were then incubated for further 5 min with vehicle or H_2O_2 (3 mM) and then lysed. CrkII electrophoretic mobility shift was analyzed by Western blotting as described. Results shown are representative of three others.

phosphorylation. Acinar cells were then pretreated with 100 μM LY294002 or 1 μM Wortmannin, two unrelated PI3-kinase inhibitors [Rankin et al., 1996]. Inhibition of the PI3-K pathway had no effect on the induction of CrkII phosphorylation by H_2O_2 (Fig. 5B, lanes 6 and 7). Because of the known effect of ROS on actin filament polymerization in pancreatic acinar cells [Rosado et al., 2002], we evaluated whether the integrity of the actin cytoskeleton is mediating the effects of H_2O_2 on CrkII phosphorylation by incubating cells with 3 μM cytochalasin D, a selective disrupter of the actin filament network [Salmon et al., 1984; Tapia et al., 1999]. Disruption of the actin cytoskeleton did not affect H_2O_2 induced CrkII phosphorylation (Fig. 5B, lane 8).

H_2O_2 Treatment Induces the Rapid Formation of CrkII Complex With Protein PYK2

H_2O_2 caused a marked and rapid increase on the formation of CrkII complex with protein PYK2 (Fig. 6). CrkII complex formation induced by 3 mM H_2O_2 was time-dependent, presenting a marked increase detected within 2.5 min after addition of H_2O_2 (fold increase of 3.2 ± 0.7).

DISCUSSION

In this study, we have demonstrated in vivo in rat pancreatic acini that treatment with the ROS donor, H_2O_2, leads to tyrosine phosphorylation of the adapter protein CrkII, which has been shown to be present as two bands with different electrophoretic mobility in these cells [Andreolotti et al., 2003] or other cell types [Beitner-Johnson and LeRoith, 1995], corresponding to two phosphorylation states of the protein. It is well known that CrkII tyrosine phosphorylation occurs after growth factor stimulation [Buday, 1999; Feller, 2001]. However, we demonstrated here for the first time that a different type of stimuli, such as the generated after H_2O_2 treatment, leads to CrkII phosphorylation in tyrosine residues in vivo in a concentration dependent manner. Treatment of rat pancreatic acini with H_2O_2 evokes a CrkII functional consequence, the rapid formation of CrkII protein complex with PYK2. Concentrations of H_2O_2 that we have used (mM range) have been previously used in rat pancreatic acini, failing to show any visible deleterious effect in these cells [Pariente et al., 2001; Rosado

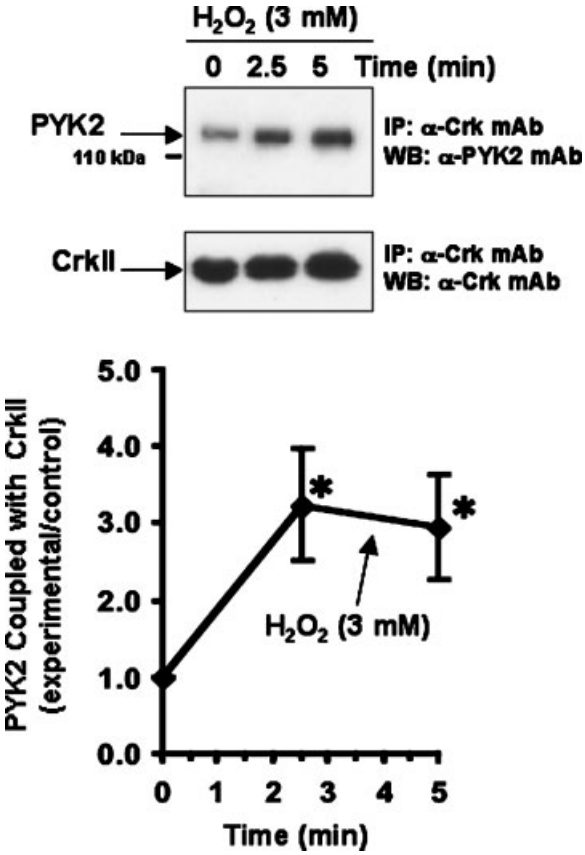

Fig. 6. Ability of H_2O_2 to stimulate the association of endogenous CrkII with PYK2 in rat pancreatic acini. Rat pancreatic acini were incubated with 3 mM H_2O_2 for the indicated times and then lysed. Proteins (400 μg) were immunoprecipitated (IP) with anti-Crk mAb (4 μg) for 2 h at 4°C, and the immunoprecipitates were fractionated using SDS–PAGE. Western blotting (WB) was performed with anti-PYK2 mAb (**upper panels**) or with anti-Crk mAb (**middle panel**). The upper/middle panels show results from a typical experiment representative of two others in duplicate. The **lower panel** is the mean ± SEM of three experiments expressed as the ratio of the PYK2 immunoprecipitated with CrkII versus the control. $^*P < 0.05$.

et al., 2002]. It is interesting to mention that in rat pancreatic acini H_2O_2 is a more potent stimulus of CrkII phosphorylation than the gastrointestinal hormone CCK or hyperosmotic medium inducer sorbitol at concentration that is able to increase the stress pathway of $p38^{MAPK}$ in these cells [Schafer et al., 1998].

We have confirmed that this electrophoretic mobility change induced by H_2O_2, sorbitol, and CCK is due to phosphorylation on tyrosine residues by mean of two different approaches using B44, which we have previously used as an effective inhibitor of tyrosine kinase in these cells [Tapia et al., 1999]. In the present study, B44 pretreatment greatly inhibits both, the H_2O_2-induced electrophoretic shift in acinar lysates and the increase induced by H_2O_2 in the upper phosphotyrosine-immunopurified CrkII band. Both experimental approaches strongly support the conclusion that the upper band of the protein contains CrkII is phosphorylated on tyrosine residues after H_2O_2 treatment. We have further confirmed that tyrosine 221 is rapid and potently phosphorylated after H_2O_2 treatment and that this phosphorylation correlates with the mobility shift to the upper band of CrkII. This direct relationship observed between the upper band and the increase on tyrosine phosphorylation of CrkII after H_2O_2 treatment is in total agreement with previous studies using other stimuli in rat pancreatic acini [Andreolotti et al., 2003] or other cell types [Beitner-Johnson and LeRoith, 1995].

At present, nothing is known about the intracellular pathways leading to CrkII tyrosine phosphorylation induced by the ROS donor H_2O_2. We have recently reported in pancreatic acini that CrkII tyrosine phosphorylation caused by CCK-8 treatment is dependent on the presence of intracellular calcium, which plays a permissive role [Andreolotti et al., 2003]. Moreover, it has been recently shown that H_2O_2 treatment in these cells causes calcium mobilization from CCK-8- and thapsigargin-sensitive stores [Pariente et al., 2001; Williams, 2001]. We have demonstrated in this study that (a) treatment with H_2O_2 in a calcium-free medium, or (b) abolishing the H_2O_2-induced cytosolic calcium mobilization with thapsigargin or (c) depleting intracellular calcium with BAPTA-AM does not modify CrkII electrophoretic mobility shift induced by H_2O_2. These different approaches support the conclusion that the mobilization of a relevant intracellular messenger in pancreatic acini, intracellular calcium, is not involved on CrkII tyrosine phosphorylation caused by H_2O_2 treatment.

PKC pathway plays an important role in the physiology of the pancreatic acinar cells [Williams, 2001]. Our results suggest that PKC activation is not an intracellular mediator involved in H_2O_2-stimulated CrkII phosphorylation in pancreatic acini. Moreover, simultaneous PKC and intracellular calcium stimulation fails to affect CrkII mobility shift. Thus, intracellular pathways that mediate CrkII phosphorylation in response to H_2O_2 appear to involve PKC-independent mechan-

isms. Previously, we have shown in these cells that CCK-stimulated tyrosine phosphorylation of different proteins was mediated by PKC-dependent and -independent mechanisms [Ferris et al., 1999]. Recent results in pancreatic acini indicated that activation of PKC was not essential for actin polymerization after H_2O_2 treatment [Rosado et al., 2002].

Our group previously showed that tyrosine phosphorylation of different proteins in pancreatic acini was dependent on both the ability of these cells to synthesize phosphoinositides [Rosado et al., 2000] and the integrity of the actin cytoskeleton [Ferris et al., 1999]. Interestingly, our results demonstrate that CrkII phosphorylation induced by H_2O_2 is independent of phosophoinositide metabolism and the disruption of the actin cytoskeleton. These results agree with a previous study where we have demonstrated that CrkII tyrosine phosphorylation pathway does not involve either PI3-K cascade or actin cystoskeleton integrity after stimulation of pancreatic acini with CCK [Andreolotti et al., 2003].

Recently, it was demonstrated that ROS are involved in the pathogenesis of acute pancreatitis [Dabrowski et al., 1999] and strongly activate MAPK intracellular cascades in pancreatic acinar cells [Dabrowski et al., 2000]. Several studies searching for a functional role of CrkII have recently suggested that Crk is an alternative pathway to activate MAPK signaling cascades, mainly the JNK [Matsuda et al., 1992; Tanaka et al., 1994; Blakesley et al., 1997]. The present work demonstrates that CrkII phosphorylation and formation of a complex with protein PYK2 is a very rapid mediator of the H_2O_2 effects in vivo in pancreatic acini and in the intact animal (data not shown), suggesting that CrkII would act very upstream in the intracellular cascade(s) activated by H_2O_2. Taken all above-mentioned data, we could speculate that ROS donor H_2O_2, by rapidly modulating CrkII phosphorylation and formation of a complex with PYK2 could signal to JNK pathway, which activation is increased in the acute pancreatitis [Dabrowski et al., 1996]. This idea is reinforced with our results showing that CrkII phosphorylation occurs rapidly in vivo in the intact animal in response to supramaximal doses of CCK [Andreolotti et al., 2003], which is one of the best established model of experimental acute pancreatitis [Steer, 1998; Dabrowski et al., 1999].

In summary, results in this study clearly support the conclusion that tyrosine phosphorylation of the adapter protein CrkII and formation of CrkII protein complexes in rat pancreatic acini is induced by different stimuli in vivo other than growth factors or CCK, such as H_2O_2. Tyrosine phosphorylation of CrkII induced by H_2O_2 in acinar cells occurs by an intracellular pathway(s) that is independent of PKC activation, increase in intracellular calcium concentration, PI3-K pathway or the integrity of the actin cytoskeleton. The tyrosine phosphorylation of CrkII correlates well with the induction of CrkII complex with PYK2 after H_2O_2 treatment. Our data suggest that CrkII tyrosine phosphorylation might likely be an important very upstream mediator of some of the described H_2O_2 cellular actions in vivo in rat pancreatic acini.

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