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Identification and characterization of a novel protein histidine kinase in the islet β cell: evidence for its regulation by mastoparan, an activator of G-proteins and insulin secretion

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Abstract

Using insulin-secreting cells, we previously demonstrated that specific proteins associated with the cytosolic, secretory granule, and mitochondrial fractions undergo a novel type of phosphorylation on their histidine residues. Subsequently, we identified these proteins as the nucleoside diphosphate kinase (NDPK) [Kowluru and Metz, Biochemistry 1994;33:12495–503], the β subunit of trimeric GTP-binding proteins [Kowluru *et al*., Biochem J 1996;313:97–107], and the α subunit of succinyl-CoA synthetase [Kowluru, Diabetologia 2001;44:89–94], respectively. Since several other enzymes of intermediary metabolism (e.g. ATP-citrate lyase and glucose-6-phosphatase) also undergo histidine phosphorylation, these initial findings may have a more generalized significance to β cells. Herein, we characterized a novel protein histidine kinase in pancreatic β cells, and determined it to be acid- and heat-labile as well as alkali-resistant in its phosphorylation of histone 4. Such an activity was detected in normal rat islets, human islets, and clonal β (HIT-T15 and INS-1) cells, and could utilize either ATP or GTP as a phosphoryl donor (with K_m values in the range of 60–100 μM). On a size-exclusion column, its molecular mass was estimated to be in the range of 60–70 kDa. It was stimulated by divalent cations ($Mg^{2+} > Mn^{2+} >$ control $= Ca^{2+} = Zn^{2+} = Co^{2+}$), but was resistant to polyamines. It was inactivated by known *in vitro* inhibitors of protein histidine phosphorylation (e.g. UDP or cromoglycate). Mastoparan, a global activator of G-proteins and insulin secretion from isolated β cells, but not mastoparan-17, its inactive analog, stimulated histidine kinase activity and histidine phosphorylation of G_β subunit and insulin secretion from isolated rat islets. These studies identify, for the first time, a protein kinase activity in the pancreatic β cell that does not act on traditional -Ser, -Tyr, or -Thr residues. They also establish a possible link between histidine kinase activity and G_β phosphorylation in isolated β cells.

Keywords: Pancreatic β cell; Histidine phosphorylation; G-proteins; Mastoparan; Insulin secretion; Nucleoside diphosphate kinase

1. Introduction

In most cells, including the islet β cell, transduction of extracellular signals involves ligand binding to a receptor, often followed by the activation of one or more GTP-binding proteins (G-proteins) and their effector systems [1–3]. The pancreatic β cell is unusual in that glucose, the major physiological agonist, lacks an extracellular receptor. Instead, events consequent to glucose metabolism promote insulin secretion via the generation and/or altered distribution of diffusible second messengers, such as ions, cyclic nucleotides, and biologically active lipids [4–6]. Changes in calcium concentration not only initiate insulin secretion, but also regulate various enzymes such as protein kinases, phosphodiesterases, adenylyl cyclases, and phospholipases, thereby facilitating insulin secretion [4–6]. In addition to calcium-dependent protein kinase(s), several other kinases, including calmodulin-, cyclic nucleotide-, phospholipid-dependent protein kinases, tyrosine kinases, and mitogen-activated protein kinases have been described in β cells (see Ref. [7] for a review). The majority of these kinases mediate phosphorylation of endogenous β cell proteins using ATP as the phosphoryl donor. Previously,

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Abbreviations: CRG, cromoglycate; DTT, dithiothreitol; G-proteins, guanine nucleotide-binding regulatory proteins; Mas, mastoparan; Mas-7, mastoparan-7; Mas-17, mastoparan-17; NDPK, nucleoside diphosphate kinase; pHMB, *p*-hydroxymercuribenzoate; P-Ser, phosphoserine; P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; and P-His, phosphohistidine.

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we reported [8] preliminary evidence for the localization of a novel protein kinase in β cells that selectively uses GTP as a phosphoryl donor and uniquely phosphorylates specific proteins (e.g. β subunit of trimeric G-proteins) at histidine residues. We further demonstrated that this phosphate, in turn, is transferred to free GDP (or GDP liganded to G-proteins) to yield free GTP (or GTP bound to G-proteins).

To date, the most phosphorylated amino acids identified include serine [P-Ser], threonine [P-Thr], and tyrosine [P-Tyr]. Phosphoamino acids exhibit differential sensitivities to acidic and alkaline pH conditions [9,10]. P-Ser and P-Thr, which form *O*-p(alcoholic *O*-monoester) linkages, are stable at acidic pH and fairly unstable under alkaline conditions. P-Tyr, which forms *O*-p(phenolic *O*-monoester), is stable under acidic and alkaline conditions. Therefore, due to their stability under acidic conditions, P-Ser, P-Thr, and P-Tyr are readily identified after acid hydrolysis of phosphorylated proteins. However, acid-labile phosphoramidate linkage has been reported [10] in histidine [P-His], arginine [P-Arg], and lysine [P-Lys]. It is not surprising that very little information is available on the number of proteins with P-His, since its phosphate is rapidly lost during identification of phosphoamino acids under standard acid hydrolysis conditions or under conditions used for SDS–PAGE (see below). It is estimated that P-His may account for 6% of total protein phosphorylation in eukaryotes [10]. In this context, it has also been shown that P-His undergoes rapid dephosphorylation in crude cellular extracts [11] including pancreatic islet cell lysates, as we reported in Ref. [8].

Several studies have appeared on the localization, characterization, and regulation of protein histidine kinases in multiple cell types [12–16]. Wei and Matthews [16] first reported a filter paper-based protein kinase assay that selectively quantitated acid-labile and alkali-stable phosphorylation reactions. Employing this assay, these investigators reported the purification and characterization of a protein histidine kinase from *Saccharomyces cerevisiae*, using histone 4 as the substrate [17]. They demonstrated that the yeast histidine kinase activity selectively phosphorylated the histidine residue at the 75th position, but not the 18th residue on histone 4 [17]. These studies were the first to describe not only a convenient and reliable method for the quantitation of protein histidine phosphorylation, but also the characterization of a novel protein histidine kinase in cellular preparations. Using two different assay methods (i.e. SDS–PAGE and the Nytran filter paper assay), we report, herein, the localization and characterization of a protein histidine kinase in the lysates of insulin-secreting cells, namely normal rat islets, human islets, and pure β (HIT-T15 and INS-1) cells. We also demonstrate a potential regulation of this activity by mastoparan, an activator of G-proteins and insulin secretion from normal rat islets. Our studies also establish a link between activation of histidine kinase and the phosphorylation of the G_{β} subunit in isolated islet β cells. To our knowledge, this is the first study of localization of protein histidine kinase in an endocrine cell, in general, and the pancreatic β cell, in particular. This study is the seventh [8,18–22] in a series of our studies that addresses the putative roles of protein histidine phosphorylation in the pancreatic β cell with relevance to insulin secretion.

2. Materials and methods

2.1. Materials

[γ-^{32}P]GTP (30 Ci/mmol) and [γ-^{32}P]ATP (600 Ci/mmol) were purchased from NEN-DuPont. Nucleoside diphosphates, triphosphates, and histone 4 were purchased from Boehringer Mannheim. Spermine, spermidine, UDP, and CRG were purchased from the Sigma Chemical Co. Nytran filter papers were purchased from Schleicher & Schuell. Mastoparan was purchased from Sigma. Mastoparan-17 and mastoparan-7 were purchased from Peninsula Laboratories, and Biomol, respectively. Purified α and βγ subunits of transducin were provided by Dr. Akio Yamazaki, Kresge Eye Institute. HIT-T15 and INS-1 cells were provided by Dr. Paul Robertson (Pacific Northwest Research Institute) and Dr. Claes Wollheim (University of Geneva), respectively. Human islets were provided by the Islet Isolation Core Facility, Washington University School of Medicine. P-His standards were provided by Professor Harry Matthews (University of California).

2.2. Isolation and fractionation of pancreatic islets

Pancreatic islets were isolated from Sprague–Dawley rats (300–400 g body weight) by a collagenase digestion method, as described previously [8,18–22]. Homogenates of isolated β cells were prepared using an isotonic medium, and membrane and soluble fractions from normal rat islets, human islets, and HIT-T15 and INS-1 cells were isolated by a differential centrifugation method, using procedures that we described earlier [8,18–22].

2.3. Phosphorylation of the β subunit and its quantification

The phosphorylation reaction was carried out in a total volume of 100 μl consisting of 50 mM Tris–HCl, pH 7.4, 2 mM DTT, 3 mM $MgCl_2$, and membrane protein (15 μg); [γ-^{32}P]GTP or [γ-^{32}P]ATP was added at 37° for 2 min. Purified α and/or βγ subunits of transducin (5 μg) were also included in the assay medium, in different combinations, as indicated in the text. The phosphorylation reaction was terminated by the addition of Laemmli stop solution. Since the P-His of the β subunit is heat-labile [8], samples were incubated in the sample buffer at room temperature for 30 min prior to SDS–PAGE. Following separation of

proteins by SDS–PAGE, gels were routinely fixed in methanol:acetic acid:water (4:1:5; by vol.) for 1.5 hr and dried at room temperature. Short fixation times were chosen in order to prevent the loss of label from P-His under acidic conditions [8,18]. Labeled proteins were identified by autoradiography, and their labeling intensities were quantified by scanning individual lanes using a Zeineh Video Laser Densitometer (Biomed Instruments, Inc.) that was interfaced to an IBM computer equipped with software to calculate the individual peak areas.

2.4. Identification of P-His

Besides employing stringent conditions for the identification of the phosphorylated amino acid, i.e. acid-lability, alkali-stability, and heat lability as well as the sensitivity to known inhibitors of protein histidine phosphorylation (see below), we confirmed the phosphorylated amino acid as histidine by amino acid analyses using authentic P-His standards (provided by Professor Harry Matthews). We previously reported [8] the sensitivity of P-His residues of G_β subunit to various histidine modifying reagents such as hydroxylamine and diethylpyrocarbonate. Data from these experimental approaches aided us to prove conclusively that the phospho amino acid residue was, indeed, histidine.

2.5. Quantitation of histidine kinase activity by the Nytran paper assay

This assay was done according to Wei and Matthews [16] without further modifications. In brief, the assay mixture consisted, in a total volume of 100 µl, of 0.6 mg/mL of histone 4, 0.2 mM [^{32}P]ATP or [^{32}P]GTP, 15 mM magnesium chloride, 50 mM Tris–HCl, pH 7.5, and β cell lysates as indicated in the text. The reaction was carried out at 37° for 5 min, and was terminated by incubating the mixture in 0.5 N NaOH at 60° for 30 min. The base-treated reaction mixture was transferred directly to the Nytran papers, previously soaked overnight in 1 mM ATP, pH 9.0, at room temperature and air dried. Then the papers were transferred to a beaker with 200 mL of 10 mM sodium pyrophosphate at pH 9.0 and gently stirred at room temperature for 30 min to remove both unreacted ATP and phosphate released in the hydrolysis step. The filters were air-dried under an infrared lamp, and the radioactivity associated with filters was quantitated by scintillation spectrometry.

2.6. Partial purification of histidine kinase

Histidine kinase was partially purified on a Sephacryl S-100 column as described previously [16,17]. In brief, HIT-T15 cell cytosol (corresponding to about 1 mg protein) was loaded onto a Sephacryl S-100 column (13 × 0.7 cm) that was equilibrated with 0.15 M NaCl, 50 mM $NaPO_4$, pH 7.5, 6 mM $MgCl_2$, 1 mM DTT, and 0.2 mM EDTA. Fractions (corresponding to 125 µl each) were collected, and histidine kinase activity was assayed in each fraction using histone 4 as the substrate as described above. The molecular mass of the histidine kinase was calculated based on the elution profiles of proteins of known molecular sizes, including bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotonin (6.5 kDa).

2.7. Other methods

The protein concentration in the samples was assayed according to Bradford using bovine serum albumin as a standard [8,18]. The statistical significance of the differences between experimental conditions was determined by Student's *t*-test. P values <0.05 were considered significant.

3. Results

3.1. Histidine phosphorylated proteins in the pancreatic β cell

Previous studies from our laboratory have identified at least three proteins that undergo phosphorylation at a histidine residue; these include NDPK, succinyl-CoA synthetase, and the β subunit of trimeric G-proteins [8,18–22]. During the course of these studies, we also obtained evidence [8] to indicate that unlike NDPK, which undergoes autophosphorylation at a histidine residue, the phosphorylation of the β subunit is catalyzed by a histidine kinase endogenous to the islet β cell [8]. Data in Fig. 1 confirm our earlier observations. Incubation of the islet membrane fraction with [^{32}P]GTP (and ATP; unpublished observation) resulted in the phosphorylation of the β subunit of trimeric G-proteins (data being represented as densitometric scans of the phosphorylated protein). Purified β subunit alone, or in combination with the purified α subunit of trimeric G-proteins, did not undergo phosphorylation (Fig. 1). Addition of purified β subunit to the membrane fraction, however, significantly increased the phosphorylation of the β subunit (Fig. 1). These data suggest that a membrane-associated factor or kinase may be required for the phosphorylation of G_β subunit, compatible with previous reports [8,23]. Based on these data we undertook the following studies to examine whether a histidine kinase activity is localized in insulin-secreting cells. In these studies, we quantified histidine kinase activity using histone 4 as the substrate, which has been shown by several investigators to be an ideal substrate for the quantitation of histidine kinase activity in cell lysates [13,16,17].

3.2. Quantitation of protein histidine phosphorylation

At least two methods have been utilized for the assay of histidine kinase activity in broken cell preparations

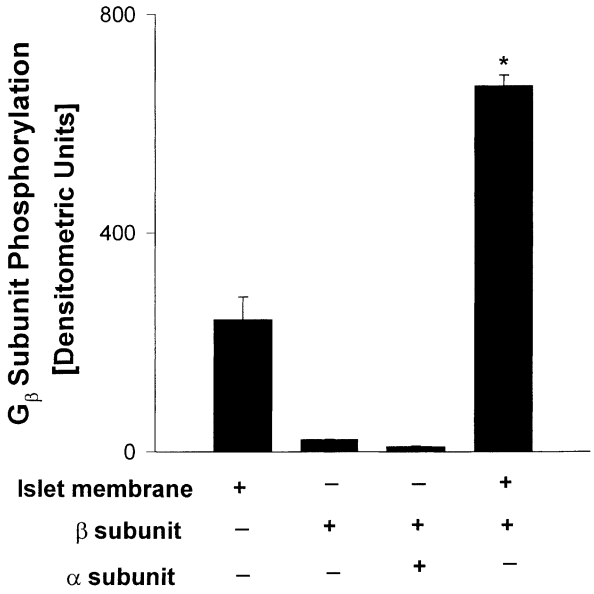

Fig. 1. Requirement for a membrane-associated factor for the phosphorylation of the β subunit of heterotrimeric G-proteins. Total particulate fraction from normal rat islets (15 μg protein) was incubated with [γ-^{32}P]GTP in the absence or presence (5 μg) of purified α and β subunits of transducin (see Section 2 for additional details). G_β subunits were provided as a βγ complex. Labeled proteins were separated by SDS–PAGE and identified by autoradiography. The intensity of the phosphorylated β subunit (identified immunologically as before; [8]) was quantified by densitometry. Data are expressed as arbitrary densitometric units and are means ± SEM or the variance from 2–3 individual determinations. Key: (*) $P < 0.001$ vs the degree of phosphorylation demonstrable using islet membranes alone.

[13,16,17]. The first one involves phosphorylation of histone 4 (with an apparent molecular mass of 11–12 kDa) in the presence of cell lysates using [γ-^{32}P]ATP or [γ-^{32}P]GTP as phosphoryl donors, followed by separation of the phosphorylated proteins on SDS gels and identification of the phosphorylated histone by autoradiography. The second method involves phosphorylation of histone 4 by cell lysates using [γ-^{32}P]ATP or [γ-^{32}P]GTP as phosphoryl donors followed by trapping the phosphorylated histone on Nytran filters under conditions where only histidine phosphorylated proteins are retained (see Section 2). Advantages of the Nytran filter paper assay over the SDS–PAGE assay include the ability to measure a large number of samples in relatively short time periods. Also, the data are much more readily quantifiable with a significant degree of accuracy over the conventional SDS–PAGE, autoradiography, and densitometry [16,17]. In the current study, we determined histidine kinase activity in insulin-secreting cells using these two methods.

3.2.1. SDS–PAGE

Data in Fig. 2A indicate a time-dependent phosphorylation of histone 4 catalyzed by rat islet lysates. Data in Fig. 2B demonstrate a concentration-dependent phosphorylation of histone 4 also catalyzed by rat islet lysates. Additionally, we observed that histone-phosphorylating histidine kinase is sensitive to divalent cations. Data in Fig. 3 suggest that maximal phosphorylation of histone 4 was demonstrable in the presence of magnesium, albeit significant phosphorylation of histone was also demonstrable in the presence of manganese (Fig. 3). Other divalent cations such as calcium, cobalt, and zinc were ineffective (Fig. 3). We also observed that polyamines such as spermine and spermidine (up to 3 mM) had no demonstrable effects on the phosphorylation of histone (additional data not shown). Together, these data indicate a magnesium requirement for the optimal activation of histidine kinase activity in the pancreatic β cell lysates.

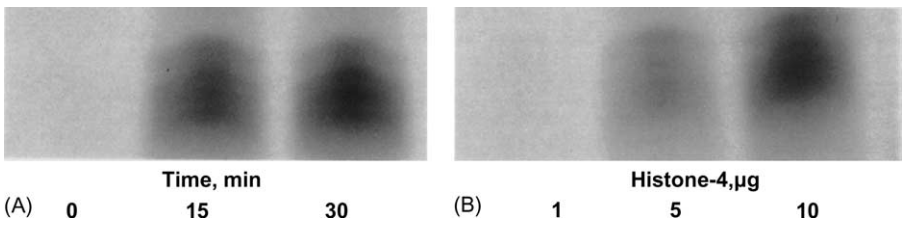

Fig. 2. Histidine kinase-mediated histidine phosphorylation of histone 4 in normal rat islet lysates: time-course and histone 4 concentration-dependence. (A) Lysates from normal rat islets were incubated with 10 μg of purified histone 4 and [γ-^{32}P]ATP (see Section 2) for different time intervals (0–30 min) indicated in the figure. Labeled proteins were separated by SDS–PAGE and identified by autoradiography. Data are representative of two experiments with identical results. (B) Lysates from normal rat islets were incubated for 15 min with 1–10 μg of purified histone 4 and [γ-^{32}P]ATP (see Section 2) as indicated in the figure. Labeled proteins were separated by SDS–PAGE and identified by autoradiography. Data are representative of two experiments with identical results.

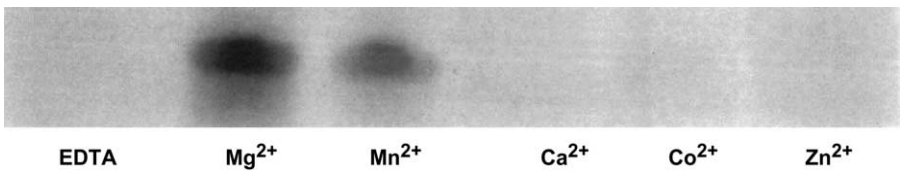

Fig. 3. Metal ion-specificity of histidine kinase in normal rat islet lysates. Lysates from normal rat islets were incubated with 10 μg of purified histone 4 and [γ-^{32}P]ATP (see Section 2) for 15 min in the presence of EDTA (10 mM) or divalent cations (3 mM each) as indicated in the figure. Labeled proteins were separated by SDS–PAGE and identified by autoradiography. Data are representative of two experiments with identical results.

3.2.2. Nytran filter paper assay

The histone-phosphorylating histidine kinase activity was then quantified by Nytran filter paper assay in four types of insulin-secreting cells, namely normal rat islets, human islets, HIT-T15 cells, and INS-1 cells. Interestingly, the specific activity of this kinase (expressed as picomoles of ^{32}P incorporated into histone 4 per milligram of protein per minute) was much higher in human islets and INS-1 cells than in rat islets and HIT-T15 cells. Such an activity represented 1.75 ± 0.14 and 1.79 ± 0.77 pmol/min/mg protein in rat islet and HIT-T15 cell lysates, respectively. The histone-phosphorylating histidine kinase amounted to 3.55 ± 0.86 and 3.65 ± 0.29 pmol/min/mg protein in human islet and INS-1 cell lysates, respectively (additional data not shown).

3.3. Subcellular distribution of the histidine kinase activity

Rat islet homogenates were subjected to a single-step centrifugation step at 105,000 *g* for 90 min at 4° to obtain total particulate (pellet) and total soluble (supernatant) fractions. Histone phosphorylating activity was then quantified in these two fractions using the Nytran filter paper assay using [^{32}P]ATP or [^{32}P]GTP as phosphoryl donors. These data indicated that approximately 55 and 45% of the total ATP-sensitive histidine kinase activity was associated with the total particulate and soluble fractions, respectively. Comparable distribution of GTP-sensitive histidine kinase activities (i.e. 45% in the membrane and 55% in the soluble fractions) was demonstrable in rat islets (N = 3 experiments; additional data not shown). All further experiments on the characterization of the histidine kinase (below) were carried out in lysates to reflect the combined activities of both membrane-associated and soluble forms of this enzyme.

3.4. Substrate specificity of the β-cell histidine kinase

In the next series of studies, we examined substrate (ATP or GTP) specificity for the histidine kinase in insulin-secreting cells. These studies are important, since we have reported ([8]; also in Fig. 1) a GTP-specific phosphorylation of the β subunit in rat islet membranes and secretory granule fractions. For this purpose, [γ-^{32}P]ATP or [γ-^{32}P]GTP was used as a phosphoryl donor to quantitate the phosphorylation of histone 4 catalyzed by islet lysates. Data in Fig. 4 indicate that the β cell histidine kinase utilizes both ATP and GTP as phosphoryl donors, albeit the degree of histone 4 phosphorylation was much smaller in the presence of GTP, compatible with data reported in the case of histidine kinase from yeast [16,17]. In subsequent experiments, we determined the K_m values for ATP and GTP by measuring the catalytic activity of this enzyme in the presence of various concentrations (0–500 μM) of either ATP or GTP. Data from these studies indicated comparable K_m values for both of these nucleotide triphosphates that were in the range

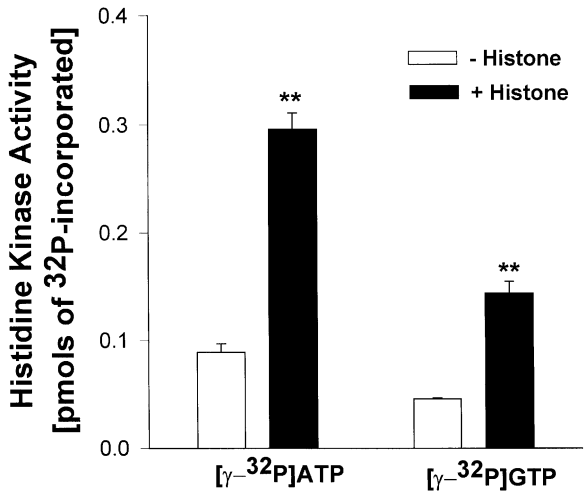

Fig. 4. ATP- and GTP-sensitive histidine kinase activity in rat islet cell lysates. Lysates from normal rat islets were incubated in the absence or presence of purified histone 4 and [γ-^{32}P]ATP or [γ-^{32}P]GTP (see Section 2) as indicated in the figure. The degree of histone 4 phosphorylation was quantified by the Nytran filter paper assay (see Section 2). Data are means ± SEM from at least three individual preparations carried out in triplicate. Key: (**) $P < 0.001$ vs no histone controls.

of 60–100 μM (additional data not shown). These values are comparable to those reported for the yeast enzyme [16,17].

3.5. pH Optima, stability characteristics, and sensitivity to inhibitors

The pH optimum of the ATP-sensitive enzyme was found to be around 7.0, whereas the GTP-sensitive enzyme exhibited a relatively broader pH optimum (i.e. 7.0 to 8.0; additional data not shown). We have reported previously that the histidine phosphorylation of the islet cytosolic NDPK was sensitive to -SH modifying reagents such as pHMB [19]. Data in Fig. 5A show that the β cell histidine kinase activity was inhibited by pHMB in a concentration-dependent manner. We also observed that other known inhibitors of histidine phosphorylation, namely UDP and CRG, significantly inhibited the endogenous protein histidine phosphorylation as well as exogenously added histone 4 phosphorylation (Fig. 5B). Furthermore, phosphorylation of histone 4 was heat-labile, consistent with known characteristics of P-His phosphorylation (Fig. 5B). In a limited number of studies, we confirmed the identity of the phosphorylated amino acid as histidine, using an authentic P-His standard (see Section 2; additional data not shown). These data clearly suggest that the histidine kinase we report in this study has many properties comparable to those of other histidine kinases, including NDPK.

3.6. Partial purification and molecular weight determination

HIT-T15 cell lysates were subjected to molecular sieving chromatography on a Sephacryl-100 column.

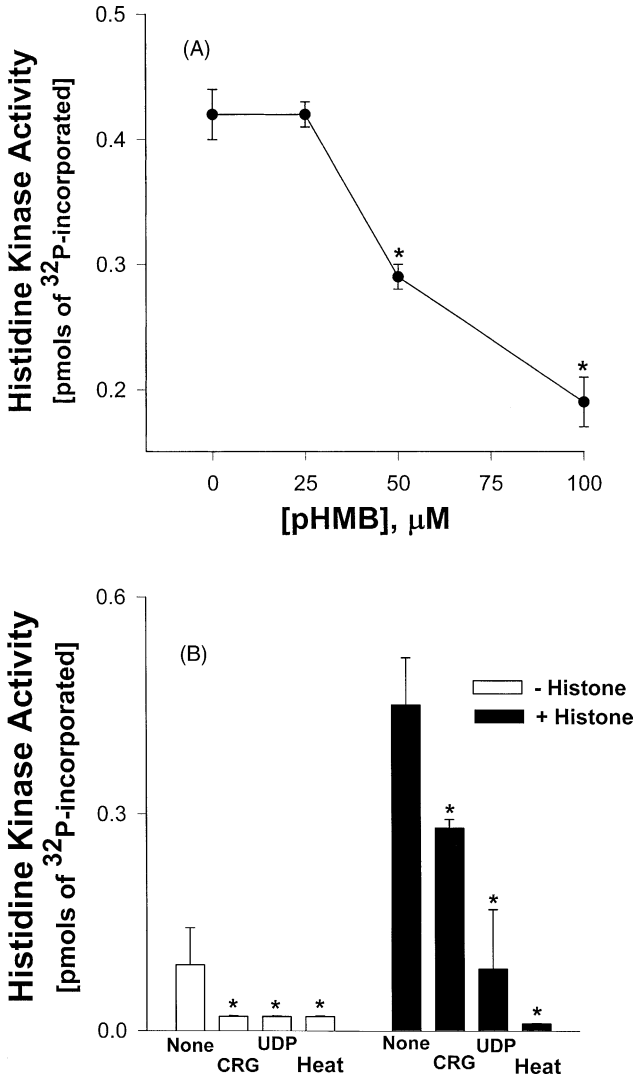

Fig. 5. Sensitivity of the β cell histidine kinase to known inhibitors of protein histidine phosphorylation. (A) Histone 4 phosphorylation was quantitated in lysates from HIT-T15 cells in the presence of different concentrations of pHMB, as indicated in the figure. Data are means ± SEM from two individual experiments carried out in triplicate. Key: (*) $P < 0.001$ vs no pHMB control. (B) Endogenous (no added histone) or histone 4 phosphorylation was quantitated in HIT-T15 cell lysates in the presence of CRG (5 mM) or UDP (10 mM) as indicated in the figure. In thermal stability experiments, the lysate was heated at 95° for 10 min, following which the proteins were brought back to 37° by cooling, and histidine kinase activity was then assayed in those preparations. Data are means ± SEM from two individual experiments carried out in triplicate. Key: (*) $P < 0.001$ vs untreated controls.

Experimental details, including fractionation conditions, are described under Section 2. Histidine kinase activity was measured in the column fractions and is plotted in Fig. 6. These data indicate the emergence of a single peak with histone-phosphorylating histidine kinase activity. Based on the elution profiles of proteins with known molecular weights (see Section 2), we estimate the molecular mass of the histidine kinase to be in the range of 60–70 kDa.

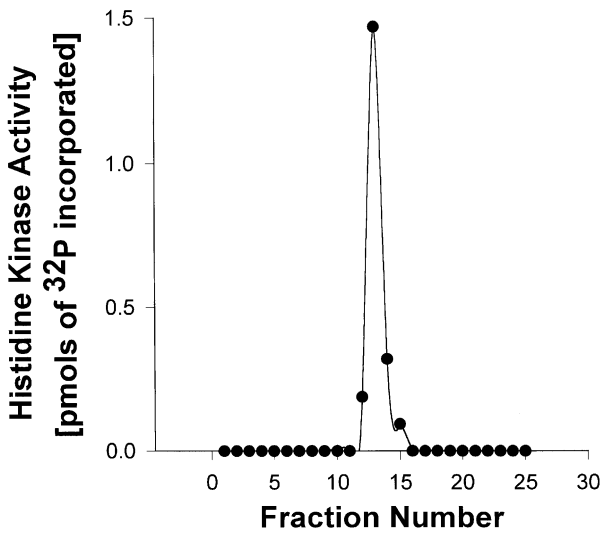

Fig. 6. Molecular weight determination of histidine kinase from HIT-T15 cell lysates by Sephacryl S-100 column chromatography. Histidine kinase was partially purified on a Sephacryl column as described under Section 2. In brief, HIT-T15 cell lysate (corresponding to about 1 mg protein) was loaded onto a Sephacryl S-100 column (13 × 0.7 cm), which was equilibrated with 0.15 M NaCl, 50 mM $NaPO_4$, pH 7.5, 6 mM $MgCl_2$, 1 mM DTT, and 0.2 mM EDTA. Fractions (corresponding to 125 μL each) were collected, and histidine kinase activity was assayed in each fraction using histone 4 as the substrate as described above. The molecular weight of the histidine kinase was calculated based on the elution profiles of proteins of known molecular sizes, including bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotonin (6.5 kDa).

3.7. Regulation of histidine phosphorylation by mastoparan, a global activator of G-proteins

The next series of studies were aimed at examining the effects of mastoparan, a tetradecapeptide from wasp venom, on histidine kinase activity and phosphorylation of the β subunit of trimeric G-proteins from isolated normal rat islets. The choice of mastoparan was based on the fact that it is a global activator of G-proteins, and it has been shown in several studies, including our own [18,19,24–26], that mastoparan stimulates exocytosis of insulin from isolated islets and clonal β cell preparations, involving G-proteins endogenous to β cells. Therefore, we examined the regulation by mastoparan of G_β subunit phosphorylation and histidine kinase activity in rat islets. Further, in these studies, as a negative control, we studied effects of mastoparan-17, a structurally similar, but functionally inactive analog of mastoparan, on these three parameters.

Data in Fig. 7A indicate a structure-specific activation of the phosphorylation of β subunit by mastoparan analogs with the following rank order: mastoparan-7 > mastoparan > mastoparan-17. Mastoparan-7 is a more potent analog of mastoparan in the activation of G-proteins [19]. We and others (see Ref. [1] for a review) have reported a similar rank order for the activation of β

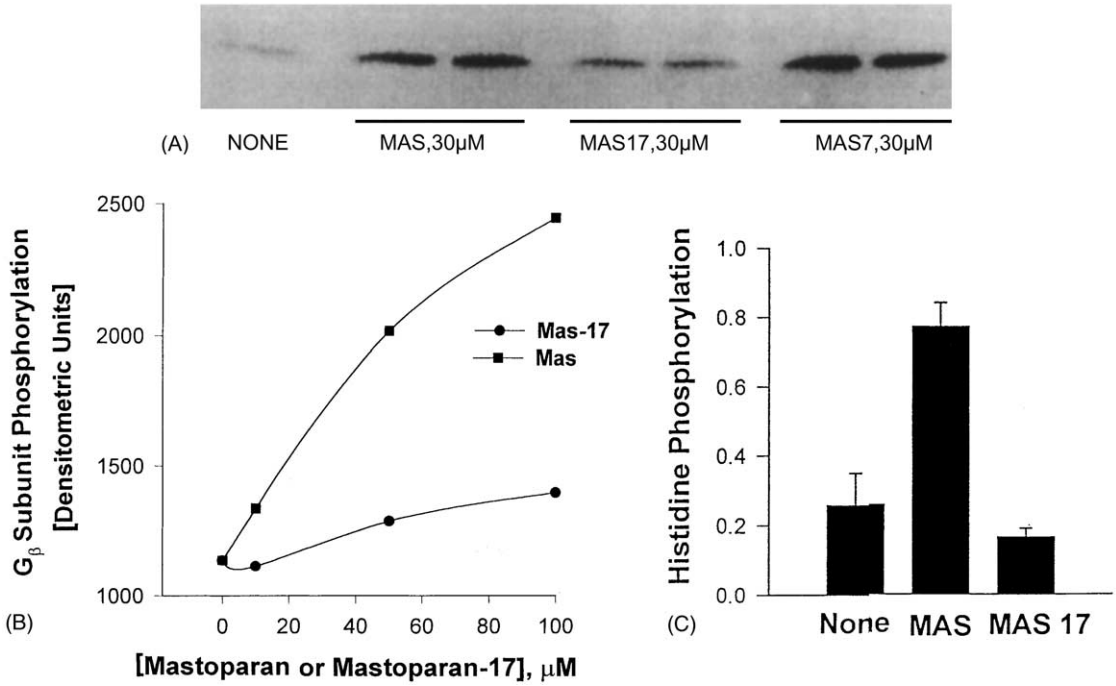

Fig. 7. Stimulation by mastoparan, but not its inactive analog, mastoparan-17, of the phosphorylation of G_β subunit and histidine kinase activity in normal rat islets. G_β subunit phosphorylation (panels A and B) and histidine kinase activity (panel C) were quantified as described in Section 2. The data in panels A and B were derived using rat islet membrane fraction, and the data in panel C were derived using rat islet homogenates. (A) G_β subunit phosphorylation studies were carried out in the presence of 30 μM each of mastoparan (Mas), mastoparan-7 (Mas-7; a more potent analog of mastoparan), or mastoparan-17 (Mas-17; an inactive analog of mastoparan), as indicated in the figure. Data are representative of three experiments with identical results. (B) G_β subunit phosphorylation studies were carried out in the presence of increasing concentrations (0–100 μM) of mastoparan or mastoparan-17, as indicated in the figure. These data are representative of two experiments and are provided as densitometric scans of the phosphorylated β subunit. (C) Histidine kinase assays were carried out in the presence of either mastoparan or mastoparan-17 (30 μM each). Data are expressed as picomoles of [^{32}P] incorporated into histone 4, and are the means ± SEM from three experiments carried out in duplicate.

cell G-proteins by mastoparan analogs. We also observed that the stimulatory effects of mastoparan on G_β subunit phosphorylation were concentration-dependent (Fig. 7B). It may be mentioned that the modest effects of stimulation of G_β subunit phosphorylation by mastoparan-17, an inactive analog of mastoparan (Fig. 7, A and B), may not be due to its effects on histidine phosphorylation, since we found very little effect of mastoparan-17 on histidine kinase even at 30 μM (Fig. 7C). Data in Fig. 7C show a similar stimulatory effect of mastoparan on the histone-phosphorylating histidine kinase activity. As indicated above, mastoparan markedly stimulated this activity, in contrast to mastoparan-17 which failed to activate histidine kinase activity. The data in Fig. 7 establish a relationship between histidine kinase activation and β subunit phosphorylation lysates derived from rat islets. Furthermore, they identify additional regulatory loci for mastoparan, which has been shown to activate G-proteins, specifically via the activation of GTP/GDP exchange processes.

4. Discussion

The main objective of this study was to further examine the roles of protein histidine phosphorylation in G-protein activation in the pancreatic β cell, which we have shown previously to be important in the stimulus–secretion coupling in the pancreatic β cell leading to insulin secretion [8,18–22]. We reported earlier that the β subunit of trimeric G-proteins undergoes phosphorylation at a histidine residue, which, in turn, is transferred to the GDP-bound to the α-subunit of the trimeric G-protein (inactive) to yield the GTP-bound, active conformation of the G-protein. In those studies, we also described that, unlike the nucleoside diphosphate kinase, which undergoes autophosphorylation at a histidine residue [19], the G_β subunit phosphorylation might require endogenous histidine kinase activity [8], compatible with data from other laboratories in other cell types [23]. In the present study, we describe localization of a novel histidine kinase activity in all four types of insulin-secreting cells, namely normal rat islets, human islets, and clonal β (HIT-T15 and INS-1) cells. These data on the relative abundance of this novel protein kinase activity in all the insulin-secreting cells suggest a critical regulatory role for this enzyme in stimulus–secretion coupling of the β cell, especially in light of the fact that we have demonstrated P-His phosphorylation of G_β subunit in all these cell types [8]. Further, we demonstrated regulation of such an activity by mastoparan, a global activator of G-proteins and insulin secretion from isolated islets, thus establishing a

potential relationship between activation of histidine kinase and G_β subunit phosphorylation in isolated β cells. Our data thus provide a basis for future studies to directly link activation of G-proteins (via transphosphorylation mediated by this novel histidine kinase) to insulin secretion elicited by nutrient secretagogues and G-protein receptor coupled agonists.

Interestingly, the putative involvement of histidine phosphorylation in signal transduction in response to extracellular stimuli was first described in bacterial systems [27–29]. Despite numerous technical difficulties to accurately quantify protein histidine phosphorylation, primarily due to its extreme instability, several recent studies have studied protein histidine phosphorylation in multiple cell types [12–17]. For example, Matthews and coworkers [16,17] reported purification of a monomeric histidine kinase from *S. cerevisiae* with an apparent molecular mass of 32 kDa, and specifically used ATP (also GTP, but with minimal affinity) to phosphorylate histone 4. The K_m for ATP was 60 μM, and required divalent cations for optimal activity (Mg^{2+} and Mn^{2+}, but not Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}). Spermine and spermidine were ineffective. In another study, Motojima and Goto reported [12] histidine phosphorylation of a 36 kDa protein by a histidine kinase in liver extracts. They also demonstrated localization of an okadaic acid-resistant phosphatase activity (with an apparent molecular mass of 45 kDa). Using an HPLC method, they demonstrated copurification of the kinase and P36 substrate at a 70–75 kDa size. These data indicate that the liver histidine kinase is different from the yeast enzyme originally described by Matthews and coworkers [16,17]. Urushidani and Nagao [14] also reported autophosphorylation, at a histidine residue, of a 40 kDa protein localized in the membrane fraction derived from rabbit gastric mucosa. Data from sequence analyses indicated that this protein might represent the α subunit of extramitochondrial succinyl-CoA synthetase or its homologue. Autophosphorylation of this protein was stimulated by GDP, Ras (a small molecular mass G-protein), and myelin basic protein, and the protein was rapidly dephosphorylated in the presence of ATP, succinate, and CoA. Furthermore, Hegde and Das [15] have shown that Ras stimulated the phosphorylation of a 36 kDa protein at a histidine residue in liver membranes. More recently Besant and Attwood [13] purified and characterized a histone 4 phosphorylating histidine kinase activity from porcine thymus. This enzyme appears to have certain similarities with the yeast enzyme, including molecular mass, which was estimated to be approximately 34–41 kDa. Based on the elution profile on a size-exclusion column, we estimated the β cell histidine kinase to be approximately 60–70 kDa. However, our studies do not rule out the possibility that it might be comprised of more than one subunit. Additional studies are underway to purify this protein and determine its structure and subunit composition in the islet β cell.

Based on the above-described data, there appear to be noticeable similarities between the β cell and the yeast histidine kinases. For example, both use ATP as well as GTP as phosphoryl donors (ATP > GTP in both cases; appear to have comparable K_m values 60 μM), both exhibit similar metal ion requirements, and both were resistant to polyamines. The principal difference appears to be the size of the enzyme. The *β* cell enzyme is approximately 60–70 kDa in size in contrast to the yeast enzyme, which has been shown to be about 32 kDa.

In support of our hypothesis that cellular activation leads to rapid protein histidine phosphorylation, Crovello *et al.* [30] provided the first direct evidence for the induction of rapid and reversible histidine phosphorylation in mammalian cells upon activation. Using human platelets, they demonstrated transient phosphorylation of P-selectin at a histidine residue by thrombin or collagen. Our current studies do support the formulation that P-His phosphorylation is important in insulin exocytosis from the β cell as well. We demonstrated that the β cell enzyme is activated in a structure-specific manner by mastoparan. Mastoparan and mastoparan-7 are potent activators of insulin secretion, in contrast to mastoparan-17, which is inactive [18,19]. We observed similar specificities for the activation by mastoparan analogs of histidine kinase activity as well as the β subunit phosphorylation in rat islet homogenates. Mastoparan and mastoparan-7, but not mastoparan-17, have been shown to activate both trimeric as well as low molecular mass G-proteins, specifically by increasing the GTP/GDP exchange and GTPase activity [1,31,32]. While several previous studies, including our own [18,19,24–26] have demonstrated insulinotropic effects of mastoparan, the current study reports for the first time that mastoparan-mediated signaling events could include activation of protein histidine phosphorylation in the pancreatic β cell.

In summary, we report localization of a novel histidine kinase activity in the pancreatic β cell. To our knowledge, this is the first report of localization of histidine kinase activity in any endocrine cell. Accumulating evidence suggests that several enzymes/proteins of intermediary metabolism undergo phosphorylation at critical histidine residues. Some of these include: nucleoside diphosphate kinase [19,33], ATP-citrate lyase [34,35], succinyl thiokinase [22], glucose-6-phosphatase [36], and the β subunit of trimeric G-proteins [8]. Therefore, the histidine kinase that we report herein could subserve the function of phosphorylating some of these proteins. Clearly, our data establish a biochemical link between the activation of histidine kinase and the activation of G-protein β subunit phosphorylation through the use of mastoparan, a global G-protein activator. Additional studies are needed to understand precisely the regulation of this enzyme by nutrient insulin secretagogues and G-protein coupled receptor agonists to conclusively establish a link between activation of G-proteins (via activation of this kinase) and insulin secretion from isolated β cells. Advancement along these lines will,

indeed, depend upon identification of specific inhibitors of this novel protein kinase in intact β cells.

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