A Kex2-related endopeptidase activity present in rat liver specifically processes the insulin proreceptor

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The insulin proreceptor is cleaved by limited proteolysis post-translationally at an Arg-Lys-Arg-Arg site to generate its mature α - and β -subunit form. An ^{35}S -labelled insulin proreceptor substrate preparation and a 15-mer peptide substrate that mimics the amino acid sequence around and including the insulin proreceptor processing site (IRP-peptide) has revealed an endopeptidase activity that catalyses insulin proreceptor cleavage in a rat liver subcellular fraction. Under optimal conditions, normal ^{35}S -labelled insulin proreceptor substrate processing by this fraction was quantitative. This fraction was not able to process an ^{35}S -labelled insulin proreceptor variant substrate (where the Arg⁻¹ of the tetrabasic cleavage site had been replaced by Ala⁻¹), similarly to previous *in vivo* observations, suggesting that this endopeptidase activity has physiological relevance. Biochemical characterization of the insulin proreceptor/IRP-peptide processing revealed this rat liver endopeptidase activity to have a broad pH range (> 70% maximal activity between pH 5.5 and 10.0) and a pH optimum of pH 8–10. It was Ca²⁺-dependent activity, maximally active between 0.5 and 5 mM Ca²⁺ and half-

maximally activated between 50 and 90 μM Ca²⁺. Endoproteolytic activity was not inhibited by group-specific inhibitors of serine-, cysteinyl or aspartyl proteinases or by 1,10-phenanthroline; however, EDTA and 1,2-cyclohexanediaminetetraacetic acid did inhibit the activity, but this was accounted for by Ca²⁺ chelation. The IRP-peptide substrate assay enabled measurement of an apparent K_m of 22 μM and a $V_{\max}$ of 18.6 pmol/min for this endopeptidase activity. These biochemical characteristics suggest the insulin proreceptor processing endopeptidase activity to be a legitimate member of the Kex2-related proprotein convertase family. Immunoblotting detected furin and PACE4 proteins (both members of this family) to be present in the rat liver subcellular fraction containing insulin proreceptor processing activity. Since the biochemical characteristics of the insulin proreceptor processing endopeptidase activity mostly resembled those of furin activity, it is likely that insulin proreceptor proteolytic maturation can be catalysed by furin in the liver.

INTRODUCTION

Many proteins that traverse the secretory pathways of mammalian cells are synthesized as larger inactive precursors and processed post-translationally by limited proteolysis, usually at sites containing basic amino acids [1]. This applies not only to proproteins that follow the regulated or constitutive secretory pathways [2] but also to certain plasma membrane proproteins, such as the insulin receptor.

The insulin receptor is initially synthesized as a high-molecular-mass precursor 'proreceptor', and is then processed by limited proteolysis at a tetrabasic -Arg-Lys-Arg-Arg- sequence [3,4] to give the mature α - and β -subunits [5,6]. Proteolytic conversion of the insulin proreceptor is of absolute importance to the insulin-responsive cell. Inhibition of this processing (as instigated by mutagenesis of Arg⁻¹ or Arg⁻⁴ of the tetrabasic processing site [7,8] does result in correct glycosylation, dimerization and delivery of the insulin proreceptor to the plasma membrane. However, these unprocessed insulin proreceptor variants are essentially biologically inactive, showing altered insulin binding, defective ligand-receptor internalization, down-regulation, autophosphorylation and insulin-resistance [7–9]. The subcellular localization of insulin proreceptor conversion processing has not yet been identified but, analogous to that for other proproteins

that follow the constitutive secretory pathway (such as proalbumin [10–12]), it is thought to be initiated intracellularly in the *trans*-Golgi network and continue in a post-Golgi transport vesicle compartment, before the final destination of the plasma membrane is reached [6].

There is now an emerging family of eukaryotic proprotein processing endopeptidases that are related to bacterial subtilisin and the yeast Kex2 protease (for reviews see [13–15]). A series of mammalian Kex2 homologues have been discovered [15], named PC3 [16] (also known as PC1 [17]), PC2 [18,19], furin [20,21] (also known as PACE), PACE4 [22], PC4 [23] and PC6 [24]. PC2 and PC3 are located only in neuroendocrine cells [16,19], PC4 is specific to the testis [23] and PC6 appears to be gut-specific [24]. In contrast, furin and PACE4 are essentially ubiquitously expressed [22,25,26] and, since the insulin receptor is found on most cells [27], furin and PACE4 appear to be the prime candidates for the insulin proreceptor processing endopeptidase. Indeed, it has been demonstrated in co-transfection experiments that furin is capable of correctly processing the liver secretory proteins complement pro-C3 and proalbumin [28]. While it is tempting to suggest that furin and/or PACE4 could be the insulin proreceptor processing endopeptidase activity, this has yet to be demonstrated. In this study, however, we have identified a Ca²⁺-dependent endopeptidase activity in rat liver that is

Abbreviations used: Aha, hexanoic acid; CDTA, 1,2-cyclohexanediaminetetra-acetic acid; E-64, [N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amido-(4-guanido)butane; IRP-peptide, insulin receptor precursor peptide; IRP-substrate, biotinylated peptide based on IRP-peptide; PMSF, phenylmethanesulphonyl fluoride; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK').

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capable of specifically processing the insulin proreceptor. Biochemical characterization of this activity suggested it to be a typical member of the mammalian Kex2-homologue family. Furthermore, it appeared that these characteristics are very similar to those of furin [29,30].

EXPERIMENTAL

Materials

L-[³⁵S]Methionine (1.2 mCi/mmol) was from New England Nuclear Research Products (Cambridge, MA, U.S.A.) Avidin-agarose and biotin were from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other analytical-grade biochemicals were from Sigma or Fisher. The NIH 3T3-IR cells [31] that overexpress the normal human insulin receptor were generously provided by Dr. Jonathan Whittaker (State University of New York, Stony Brook, NY, U.S.A.). CHO-HIR P1-A cells [8], which overexpress a variant of the human insulin receptor that is not processed *in vivo* due to Arg¹ at the processing site being replaced by Ala, were a gift from Dr. Donald Steiner (University of Chicago, Chicago, IL, U.S.A.). Polyclonal rabbit antisera to the C-terminal tail of PC2 and the N-terminal region of PACE4 were raised in this laboratory [32]. Antiserum to the N-terminal of PC3 (mature) was a gift from Dr. Iris Lindberg (Louisiana State University Medical Center, New Orleans, LA, U.S.A.), and anti-furin serum [33] was generously provided by Dr. Al Rehemtulla (Genetics Institute, Cambridge, MA, U.S.A.).

Isolation of a rat liver subcellular fraction

Livers were obtained from 200–250 g Sprague–Dawley rats. Preparation of a rat liver low-density vesicle fraction was as previously described using discontinuous sucrose density and sucrose-flotation gradients [34]. The low-density vesicular fraction at a 0.85 M/0.25 M sucrose interface of the second sucrose-flotation gradient (arbitrarily named the GV-1 fraction) was collected to assess for insulin proreceptor processing activity. A similar rat liver subcellular fraction has previously been shown to contain proalbumin processing activity [34,35]. Marker enzyme analysis was performed, using the enzyme assays previously described [36,37], to characterize the organellar composition of the GV-1 fraction compared with that of the starting homogenate.

Pulse-chase radiolabelling and insulin (pro)receptor immunoprecipitation

Approximately 75% confluent NIH 3T3-IR cells in 2.5 cm diameter round plates were pre-incubated in methionine-free Dulbecco's minimal essential media for 2 h at 37 °C as described [31]. The cells were then pulse-radiolabelled for 30 min at 37 °C in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 0.1% (w/v) BSA, 16.7 mM glucose and 167 µCi/ml [³⁵S]methionine (specific radioactivity 1000 Ci/mmol). After this pulse incubation, the buffer was aspirated away and the cells washed four to five times with Krebs–Ringer/BSA buffer containing 100 mM methionine. The cells were then chased at 37 °C for increasing time periods in the absence of [³⁵S]methionine and the presence of 100 mM methionine. After the chase incubation, the cells were placed on ice and scraped into 1 ml of lysis buffer, consisting of 50 mM Hepes (pH 7.4), 1% (v/v) Triton X-100, 10 µM phenylmethanesulphonyl fluoride (PMSF), 10 µM E-64, 10 µM pepstatin A, 10 µM Tos-Lys-CH₂Cl (TLCK), 0.1 mM leupeptin

and 0.1% (w/v) sodium azide. The extract was transferred to 1.5 ml Microfuge tubes, sonicated (25 W, 10 s), and then centrifuged (13000 g; 1 min) to remove any cellular debris. The cell lysate was pre-cleared by incubation with 60 µl/ml Pansorbin (Calbiochem, La Jolla, CA, U.S.A.) for 1 h at 4 °C. The sample was then centrifuged (13000 g; 1 min), the pellet discarded and 0.5 µg of protein of semi-purified CT1 monoclonal antibody (raised against a synthetic peptide that mimics the amino acid sequence of the C-terminal cytoplasmic tail of the insulin receptor [38,39]) and 3 µg of affinity-purified rabbit anti-mouse IgG were added to the supernatant and incubated at 4 °C for 18 h with rotary mixing. The insulin (pro)receptor/CT1 antibody/rabbit anti-mouse complex was then precipitated by addition of 60 µl of 10% (w/v) Pansorbin, and rotary-mixed for 1–2 h at 4 °C. The tubes were then centrifuged (10000 g; 2 min) and the supernatant discarded. The pellets were washed three times with 1 ml of 50 mM Hepes (pH 8.0) containing 0.1% Triton X-100, 0.1% SDS and 150 mM NaCl as previously described [32]. The pellets were then heated (5 min at 95 °C) in 40 µl of Laemmli sample buffer [40] in the presence or absence of 1 mM dithiothreitol, and the immunoprecipitated [³⁵S]insulin (pro)receptor was analysed on 5% (w/v) acrylamide non-reducing or 7.5% (w/v) acrylamide reducing SDS/PAGE and fluorography as described [32].

Radiolabelled insulin proreceptor preparation

Normal human ³⁵S-labelled insulin proreceptor was prepared by biosynthetically radiolabelling NIH 3T3-IR cells [31] with [³⁵S]methionine essentially as described above. The NIH 3T3-IR cells were pulse-radiolabelled for 30 min at 37 °C as described above. A 30 min pulse-radiolabelling period was mostly used to ensure that only ³⁵S-labelled insulin proreceptor, rather than mature ³⁵S-labelled insulin receptor, was produced [31]. Alternatively, NIH 3T3-IR cells were pulsed for 30 min with [³⁵S]methionine and then chased for 60 min, to prepare ³⁵S-labelled insulin proreceptor that was in a dimeric as well as a monomeric form (see Figure 2). After the pulse-radiolabelling incubation, the buffer was aspirated away and the cells washed four to five times with Krebs–Ringer/BSA buffer containing 100 mM methionine. The cells were then scraped off the well on ice and sonicated in 1 ml of the lysis buffer. The cell lysate was pre-cleared, then centrifuged (13000 g; 1 min), and 100 µl of a 50% (w/v) suspension of CT1 antibody coupled to Sepharose-4B in PBS (pH 7.5) was added to the supernatant [39]. This suspension was incubated at 4 °C for 18 h with rotary mixing to immunoprecipitate ³⁵S-labelled insulin proreceptor. This sample was centrifuged (13000 g; 1 min) and the pellet washed three times in 1 ml lysis buffer. The immunoprecipitated ³⁵S-labelled insulin proreceptor was then eluted from this pellet by adding 500 µl of CT1 synthetic peptide (that mimics the C-terminus of the insulin receptor and to which the CT1 antibody was raised [39]) in lysis buffer (20 µg/ml), and incubating at 4 °C for 8 h with rotary mixing. The peptide–CT1 antibody–Sepharose was re-pelleted by centrifugation (13000 g; 1 min) and the supernatant removed. The pellet was washed twice with 250 µl of lysis buffer, and the resultant wash supernatants were pooled with the elution supernatant. This eluant was then lyophilized and the dried material was resuspended in 0.1% (v/v) Triton X-100. Such an elution avoided harsh conditions of high salt or low pH and ensured that the immunopurified insulin (pro)receptor was in a relatively renatured state for use as an *in vitro* substrate in order to analyse insulin proreceptor processing endopeptidase activity. This preparation was used as the ³⁵S-labelled insulin proreceptor substrate, containing 5000–10000 c.p.m./µl obtained per well of 3T3-IR cells. A preparation of ³⁵S-labelled human insulin pro-

IRP-substrate: Biotin-CONH-(CH₂)₅-CONH-Val-Pro-Arg Pro-Ser-**Arg-Lys-Arg-Arg**-Ser-Leu-Gly-Asp-Val-[¹²⁵I]Tyr

IRP-peptide: Val-Pro-Arg-Pro-Ser-**Arg-Lys-Arg-Arg**-Ser-Leu-Gly-Asp-Val-Tyr

Figure 1 Amino acid sequence of the peptide-substrate mimicking the human insulin proreceptor processing site

For further details, see the Experimental section.

receptor variant containing Ala at position -1 was prepared by the same procedure, except that CHO-HIR P1-A cells [8] were biosynthetically radiolabelled with [³⁵S]methionine for 60 min at 37 °C.

Insulin proreceptor processing assay

The processing of ³⁵S-labelled insulin proreceptor or ³⁵S-labelled insulin proreceptor containing Ala⁻¹ was assessed by incubating the GV-1 fraction (20–30 µg of protein) for between 0 and 180 min at 30 °C in 20 µl (final volume) of 20 mM sodium acetate buffer (pH 5.5), 0.1 % (w/v) Brij-35, 5 mM CaCl₂, 10 µM pepstatin A, 100 µM Tos-Phe-CH₂Cl (TPCK), 10 µM E-64 and 5000–10000 c.p.m. of an ³⁵S-labelled insulin proreceptor preparation. The incubation was stopped by the addition of 3 × Laemmli sample buffer (pH 8.6) [40]. These samples were then subjected to SDS/7.5 % (w/v) PAGE under reducing conditions. The electrophoresis gel was fixed in 75 % (v/v) 10 M acetic acid/23 % (v/v) propanol for 10 min, and incubated for 2 h in 20 % (w/v) diphenyloxazole (PPO) in the acetic acid/propanol fixative. The gel was then washed with cold running water for 20 min, dried and subjected to fluorography.

Insulin proreceptor processing site peptide-substrate (IRP-substrate) assay

A biotinylated peptide that mimics the amino acid sequence around and including the tetrabasic processing site (Arg⁷²⁰-Lys⁷²¹-Arg⁷²²-Arg⁷²³) of the human insulin proreceptor sequence Val⁷¹⁵-Val⁷²⁸ [3] was synthesized and used as a peptide substrate for a rapid and sensitive analysis of insulin proreceptor processing activity (Figure 1). The IRP-substrate was biotinylated at the N-terminal tail, which permitted its binding and precipitation with avidin-agarose [41]. A hexanoic acid (Aha) linker between the N-terminal biotin and the IRP-peptide was added to ensure that the biotin was readily available for binding to avidin. The IRP-substrate contains a tyrosine residue at the C-terminal end for iodination. Solid-phase synthesis of the IRP-peptide was performed on an Applied Biosystems model 430A synthesizer with D-(+)-biotin and Aha coupled manually, as previously described [41]. IRP-peptide iodination was as previously described using a lactoperoxidase method [41]. The specific radioactivity of purified [¹²⁵I]IRP-substrate was 1.5 mCi/µg.

Unless otherwise stated, the biotinylated [¹²⁵I]IRP-substrate peptide processing was analysed by incubating aliquots of the GV-1 fraction (0.5 µg of protein) for 45 min at 30 °C in 25 µl of 80 mM Tris/HCl (pH 7.0), 0.08 % (w/v) Brij-35, 5 mM CaCl₂, 1 µM pepstatin A, 10 µM Tos-Lys-CH₂Cl, 1 µM E-64, 2 mM PMSF and 10000–20000 c.p.m. of [¹²⁵I]IRP-substrate. The reaction was stopped by heating at 95 °C for 5 min. A 20 µl sample of a 50 % (w/v) suspension of avidin-agarose in 0.5 ml of 0.2 M sodium phosphate buffer (pH 8.0)/0.2 M NaCl was added in order to bind biotinylated peptides, as previously described [41]. IRP-substrate assay tubes were then centrifuged (10000 g; 5 min) to pellet the avidin-agarose-biotinylated peptide complex, and the supernatant was separated from the pellet. The radio-

activity in the pellet and supernatant were then counted in a γ-radiation counter, and the ratio between these values indicated the amount of peptide substrate cleavage, so that the percentage of peptide processing could be readily calculated. Radioactivity in the pellet was representative of intact biotinylated ¹²⁵I-labelled substrate, whereas radioactivity in the supernatant was that of a non-biotinylated ¹²⁵I-labelled product generated from cleavage at the tetrabasic sequence.

For *K_m* and *V_{max}* determinations, IRP-substrate (2 × 10⁴ c.p.m.) processing was monitored as a marker in the presence of increasing non-biotinylated, non-radiolabelled IRP-peptide concentrations. No difference in rat liver GV-1 fraction endopeptidase activity-mediated cleavage of non-biotinylated IRP-peptide compared with that of biotinylated IRP-substrate was observed.

Reversed-phase C₁₈ h.p.l.c. separation of the products produced from cleavage of 1 µM of IRP-peptide by the rat liver GV-1 fraction (5 µg of total protein), and identification of these products by automated amino acid analysis, was performed as previously described [41].

Immunoblot analysis

Immunoblot analysis of the rat liver GV-1 fraction was performed as described (Bio-Rad alkaline phosphatase immunoblot kit [32]) using at least a 1:500 dilution of antisera against PC2 and PC3 [32], furin [33] and PACE 4. Polyclonal anti-PACE4 serum was raised in rabbits (on a customer-based service by Hazelton Research Products Inc., Denver, PA, USA) against peptides (coupled to keyhole limpet haemocyanin) that mimicked amino acids Gln¹⁴¹-Asp¹⁵⁵ (designated pro-PACE) and Gln¹⁵⁰-Asp¹⁶² (designated mat-PACE) of PACE4 [22], as previously described [32].

Other procedures

Protein concentration was determined by the BCA assay method (Pierce) using BSA as a standard. Free Ca²⁺ concentrations were calculated from reference stability constants as previously described [42]. *K_m* and *V_{max}* were determined by Lineweaver-Burk analysis [43]. Results are presented as means ± S.E.M. and any statistical significant differences between groups were analysed by Student's *t* test.

RESULTS

Post-translational processing of human insulin proreceptor *in vivo*

A pulse-chase experiment assessed the biosynthesis and post-translational processing of human insulin proreceptor in NIH 3T3-IR cells. After a 30 min pulse-radiolabelling period, cells were chased for periods of up to 12 h and the insulin (pro)receptor was immunoprecipitated with CT1 antibody (see the Experimental section). The ³⁵S-labelled insulin receptor was then analysed by 5 % (w/v) acrylamide non-reducing and 7.5 % (w/v) acrylamide reducing SDS/PAGE followed by fluorography (Figure 2). The insulin receptor was initially synthesized as a

Figure 2 Post-translational processing of the insulin proreceptor

NIH 3T3-IR cells were pulse-radiolabelled for 30 min with [^{35}S]methionine and then chase-incubated for periods of up to 12 h, and insulin receptor forms were specifically immunoprecipitated from cell lysates, as described in the Experimental section. Half of each sample was then analysed by 7.5% reducing (a) and the other half by 5% non-reducing (b) SDS/PAGE followed by fluorography. The migration of molecular size standards is indicated on the left, and of various molecular mass forms of the insulin (pro)receptor on the right.

187 kDa single-chain monomeric precursor (210 kDa after glycosylation 1 h after the pulse radiolabelling period; Figure 2a [5,6]). At 1 h into the chase period, the monomeric form of the insulin receptor underwent dimerization (210 kDa to 400 kDa; Figure 2b), in parallel to proteolytic processing of the insulin proreceptor to its α -subunit (126 kDa) and β -subunit (93 kDa)-containing mature form (Figure 2a). Full maturation of the insulin proreceptor to its tetrameric α - and β -subunit form was complete by 6 h (Figures 2a and 2b).

Specific processing of the insulin proreceptor by the rat liver subcellular fraction

A subcellular fraction from rat liver was prepared, and arbitrarily named the GV-1 fraction. Rat liver subcellular preparations similar to the GV-1 fraction used in this study have previously been shown to contain a specific Ca^{2+} -dependent proalbumin processing endopeptidase activity [34,35,44]. By marker enzyme analysis (Table 1), the GV-1 fraction was 6-fold enriched in the Golgi apparatus compared with the starting homogenate. Plasma membrane micelles were also enriched in the GV-1 fraction, although to a lesser extent (2.8-fold; Table 1), but no other organelle was significantly improved. Although the GV-1 preparation was relatively heterogeneous in organellar content, > 99% of rat liver lysosomes removed from the GV-1 fraction (Table 1), which was a particularly important consideration when investigating a non-lysosomal proprotein processing proteinase activity such as a proprotein convertase [1,14].

As substrates for *in vitro* processing experiments, we mostly used immunoaffinity-purified ^{35}S -labelled insulin proreceptors from 30 min-pulse-radiolabelled NIH 3T3-IR cells. As indicated in Figure 2, this preparation consisted mostly of the monomeric 187 kDa proreceptor. However, in a few experiments we also

Table 1 Marker enzyme analysis of an isolated rat liver subcellular fraction containing insulin proreceptor processing activity

A low density subcellular fraction (named the GV-1 fraction) was isolated from rat liver using discontinuous sucrose density followed by sucrose-flotation gradient centrifugation as previously described, and marker enzyme analyses were performed in order to characterize the organellar composition of the initial homogenate and the final GV-1 fraction as stated (see the Experimental section). The fold enrichment in specific activity of a marker in GV-1 fraction versus the starting homogenate was determined. The means of results from two subcellular fractionations are shown. ND, not detectable.

Protein marker	Homogenate	GV-1 fraction	Enrichment (fold)
Lactate dehydrogenase (cytosol) ($\Delta A_{340}/\text{min}$ per mg of protein)	10.3	2.6	0.25
Citrate synthase (mitochondria) ($\Delta A_{415}/\text{min}$ per mg of protein)	46.0×10^{-3}	ND	—
NADPH:cytochrome <i>c</i> reductase (microsomes) ($\Delta A_{550}/\text{min}$ per mg of protein)	0.60	1.0	1.67
Aryl sulphatase (lysosomes) (nmol/min per mg of protein)	214.0	2.1	0.01
5'-Nucleotidase (plasma membrane) (nmol/min per mg of protein)	47.3	132.8	2.8
Galactosyltransferase (Golgi) ($\mu\text{mol}/\text{min}$ per mg of protein)	0.5	3.0	6.0

used an ^{35}S -labelled proreceptor preparation from 30 min-pulse-radiolabelled/60 min-chased NIH 3T3-IR cells, which contained a heterogeneous mixture of 187 kDa monomeric, 210 kDa glycosylated monomeric and 400 kDa dimeric forms of the proreceptor, as well as a small amount of the mature α - and β -subunit form (Figure 2).

We have demonstrated that the rat liver GV-1 fraction prepared (Table 1) has the ability to correctly process a normal human ^{35}S -labelled insulin proreceptor substrate preparation *in vitro* to its mature α - and β -subunit form (Figure 3a). This processing appeared to be specific in that no other processing products (other than the 126 kDa α -subunit and 93 kDa β -subunit; Figure 3a) were observed, even with prolonged incubation periods of 18 h. In addition, the ratio of [^{35}S]methionine-labelled α - to β -subunit by densitometric scanning was 1:2.4, which corresponds to the 8:20 ratio of methionines in the α - and β -subunits of the insulin receptor [3]. When the ^{35}S -labelled insulin proreceptor substrate obtained from 60 min-chased cells was used as a substrate, it was found that this insulin proreceptor processing activity was also able to process the 400 kDa dimeric form of the insulin proreceptor to the mature α - and β -subunit-containing insulin receptor form (results not shown). The specific nature of this insulin proreceptor processing endopeptidase activity was reaffirmed in that under identical *in vitro* conditions, the ^{35}S -labelled human insulin proreceptor variant substrate containing Ala $^{-1}$ was not processed (Figure 3b). This particular insulin proreceptor variant has been previously shown not to be processed either in transfection experiments [7] or *in vivo* [8].

Specific processing of the IRP-peptide by the rat liver subcellular fraction

The IRP-peptide (Figure 1), which mimics the amino acid sequence surrounding the tetrabasic cleavage site in the insulin proreceptor molecule [3], was synthesized as an additional

Figure 3 Insulin proreceptor processing by a rat liver subcellular fraction *in vitro*

Normal human ³⁵S-labelled insulin proreceptor and a ³⁵S-labelled human insulin proreceptor variant with Ala at position -1 were prepared as described in the Experimental section. Processing of these substrates by a rat liver subcellular GV-1 fraction (25 µg of total protein) *in vitro* at 30 °C, pH 7.0 and 5 mM Ca²⁺ for periods of up to 3 h was also as described in the Experimental section. A typical analysis by reducing SDS/7.5% PAGE and fluorography is shown of a time course for processing of normal ³⁵S-labelled insulin proreceptor (a) and of ³⁵S-labelled human insulin proreceptor variant containing Ala⁻¹ (b) in parallel. Left-hand arrows indicate the migration of molecular mass standards, and right arrows indicate various molecular mass forms of the insulin (pro)receptor.

substrate to monitor insulin proreceptor processing activity. The IRP-peptide was processed by the rat liver GV-1 fraction (5 µg of total protein), and h.p.l.c. analysis of this proteolytic cleavage revealed only a single product (eluting at fraction 13; Figure 4b). This cleavage product had an amino acid composition equivalent to a peptide of the sequence Ser-Leu-Gly-Asp-Val-Tyr (Figure 4). These data are consistent with a single cleavage C-terminal to the tetrabasic sequence of the IRP-peptide (see Figure 1). Modification of the IRP-peptide by attaching an N-terminal biotin and an Aha linker sequence (IRP-substrate; Fig. 1) has enabled a rapid and sensitive peptide substrate assay method to be developed, similar to that previously described for proinsulin processing [41].

Characterization of the rat liver insulin proreceptor processing activity

We have further characterized the insulin proreceptor processing endopeptidase activity contained in the rat liver GV-1 subcellular fraction *in vitro*, using both ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate assays. In initial experiments, optimal condi-

Figure 4 Amino acid analysis of products generated from the processing of the IRP-peptide by a rat liver subcellular fraction

The processing of the IRP-peptide (1 µM) by the isolated rat liver GV-1 fraction (5 µg of total protein) for 3 h at 30 °C was measured as described in the Experimental section. Generated products of IRP-peptide proteolysis were detected by C₁₈ reversed-phase h.p.l.c. monitoring for tyrosine absorbance at 280 nm. The only peptide product detected (fractions 14–15) was subjected to amino acid analysis (see the Experimental section). (a) H.p.l.c. profile for GV-1 fraction only; (b) h.p.l.c. profile for GV-1 fraction + 1 µM IRP-peptide. Amino acid analysis of the major h.p.l.c. product from IRP-peptide processing gave the following results (relative molar ratios): Asp, 1.2; Ser, 1.2; Gly, 1.3; Tyr, 1.0; Val, 1.1; Leu, 1.2.

tions for these assays were set. Processing of the ³⁵S-labelled insulin proreceptor substrate at pH 7.0 and 5 mM Ca²⁺ by the GV-1 fraction was linear up to 120 min when using 20 µg of total protein lysate. Processing of the [¹²⁵I]IRP-substrate at pH 7.0 and 5 mM Ca²⁺ by the GV-1 fraction was linear up to 60 min when using 0.5 µg of total protein lysate. For optimal sensitivity and quantification in further analysis, standard conditions of a 60–90 min incubation with ~20 µg of total protein lysate were used for ³⁵S-labelled insulin proreceptor substrate processing, and a 45 min incubation period with 0.5 µg of protein were used for [¹²⁵I]IRP-substrate processing.

Insulin proreceptor processing activity was both Ca²⁺ and pH-dependent (Figure 5), as determined by both ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate processing assays. At 5 mM Ca²⁺, insulin proreceptor processing activity was >70% maximally active between pH 5.5 and 10.0 (Figure 5b), indicating a broad pH range similar to those of Kex-2 protease [45] and furin [30]. This was in subtle contrast to proalbumin processing endopeptidase activity in similar rat liver subcellular fractions, where a pH optimum of pH 6.0 has been determined, although proalbumin processing at alkaline pH values was not extensively characterized [35,44]. The pH profile of insulin proreceptor processing activity had a 'shoulder' at pH 6.0 (Figure 5b); this

Figure 5 Ca²⁺- and pH-dependence of the rat liver insulin proreceptor processing endopeptidase activity

Analysis of ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate processing by the rat liver GV-1 fraction was carried out as described in the Experimental section, at various Ca²⁺ concentrations (buffered with 2 mM EDTA) at a constant pH of 7.0 (a), or at various pH values at a constant 5 mM Ca²⁺ concentration (b). The buffers used to obtain the required pH were: 50 mM sodium citrate (pH 3.0–4.5), 50 mM Na-Mes (pH 5.0–6.5), 50 mM Tris/acetate (pH 7.0–8.5), 50 mM glycine/HCl (pH 9–10) and 50 mM NaOH/phosphate (pH 11–12). Results (means ± S.E.M. for at least four independent observations) are expressed as percentages of the maximum processing in the experiment (75–85% for ³⁵S-labelled insulin proreceptor in 90 min; and 60–70% for [¹²⁵I]IRP-substrate in 45 min).

might be suggestive of there being two endopeptidase activities capable of processing the insulin proreceptor present in the rat liver GV-1 fraction, with different pH optima at pH 6 and pH 8–10. However, this was unlikely, since *in vitro* IRP-substrate processing by purified furin has been determined to have a very similar broad pH profile to that observed in the present study for the rat liver insulin proreceptor processing endopeptidase activity (C. J. Rhodes and A. Rehmtulla, unpublished work). It should be noted that the pH-dependence of a Kex2-related endopeptidase activity, such as furin, can vary depending on the buffer used in *in vitro* studies [30]. In addition, it is possible that a given Kex2-related endopeptidase activity might process different proprotein substrates with differing pH requirements. Nevertheless, although the pH optimum of the insulin proreceptor processing activity was between pH 8 and 10, a standard assay pH of 7.0 was used in most assays since this was considered to be a physiologically relevant pH for insulin proreceptor processing in the Golgi vesicle compartment of hepatocytes [6].

Table 2 Effect on rat liver insulin proreceptor processing endopeptidase activity of replacement of Ca²⁺ with other bivalent cations

Analysis of ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate processing by the isolated liver GV-1 fraction was assessed at 30 °C over 60 min (³⁵S-labelled insulin proreceptor) or 45 min ([¹²⁵I]IRP-substrate) at pH 7.0, in the presence or absence of 10 mM EDTA and a bivalent cation (15 mM) as described in the Experimental section. Results are means ± S.E.M. (four independent observations for [¹²⁵I]IRP-substrate processing and at least two independent observations for ³⁵S-labelled insulin proreceptor processing) and are expressed as a percentage of the control processing at 5 mM CaCl₂ in the absence of EDTA [control values for ³⁵S-labelled insulin proreceptor, 72.8 ± 8.7%/60 min per 25 µg of protein (*n* = 3), and for the [¹²⁵I]IRP-substrate, 60.7 ± 3.8% per 45 min per µg of protein (*n* = 4)].

Addition	Processing activity (% of control)	
	[¹²⁵ I]IRP-substrate	³⁵ S-labelled insulin proreceptor
5 mM CaCl ₂ (control)	100	100
10 mM EDTA	2.2 ± 0.8	2.0
10 mM EDTA + 15 mM NiCl ₂	6.9 ± 2.0	7.2
10 mM EDTA + 15 mM BaCl ₂	10.2 ± 3.4	11.1
10 mM EDTA + 15 mM MgCl ₂	8.4 ± 2.7	12.2
10 mM EDTA + 15 mM CdCl ₂	7.6 ± 2.1	0
10 mM EDTA + 15 mM CaCl ₂	112.9 ± 10.8	121.2
10 mM EDTA + 15 mM CoCl ₂	12.7 ± 2.8	5.6
10 mM EDTA + 15 mM ZnCl ₂	8.3 ± 2.1	1.1
10 mM EDTA + 15 mM CuCl ₂	3.6 ± 1.2	0
10 mM EDTA + 15 mM SrCl ₂	14.2 ± 2.0	31.3
10 mM EDTA + 15 mM MnCl ₂	11.0 ± 1.9	8.2

At pH 7.0, insulin proreceptor processing activity was negligible below 1 µM Ca²⁺, but fully active between 0.5 and 5 mM Ca²⁺ (Figure 5a) for both ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate. The concentration for half-maximal activation (*K*_{0.5}) of insulin proreceptor processing activity by Ca²⁺ was similar for both substrates used: *K*_{0.5} = 66.2 ± 8.9 µM Ca²⁺ (*n* = 3) for ³⁵S-labelled insulin proreceptor substrate and *K*_{0.5} = 73.8 ± 9.3 µM Ca²⁺ (*n* = 3) for [¹²⁵I]IRP-substrate. Ca²⁺ chelation by 10 mM EDTA in the ³⁵S-labelled insulin proreceptor or [¹²⁵I]IRP-substrate assays resulted in total inhibition of insulin proreceptor processing activity (Table 2). However, full insulin proreceptor processing activity could be recovered by restoration of a 5 mM Ca²⁺ concentration, but not by any other cation tested (Table 2). This indicated the distinct Ca²⁺-dependence of the rat liver insulin proreceptor processing activity.

The effects of group-specific proteinase inhibitors on the processing of both ³⁵S-labelled insulin proreceptor and [¹²⁵I]IRP-substrate by the GV-1 fraction endopeptidase activity are outlined in Table 3. Insulin proreceptor processing activity was not inhibited by serine, cysteinyl, or aspartyl proteinase inhibitors. The cysteinyl proteinase inhibitor *p*-chloromercuribenzoate did inhibit insulin proreceptor processing activity; however, this was probably due to the mercuric moiety of this compound, since Hg²⁺ similarly inhibited the processing (Table 3). The metal ion chelators EDTA and 1,2-cyclohexanediaminetetra-acetic acid (CDTA) completely inhibited processing activity, but the effect of EDTA could be overcome by the addition of an excess of Ca²⁺ (Table 2), reaffirming the Ca²⁺-dependence of this activity. The specific Ca²⁺ requirement of insulin proreceptor processing activity was also reaffirmed in that the specific heavy metal cation 1,10-phenanthroline did not significantly inhibit this activity (Table 3).

The IRP-peptide substrate assay was used to determine the apparent kinetic parameters, *V*_{max}, and *K*_m, of the GV-1 fraction insulin proreceptor processing activity. Human insulin pro-

Table 3 Effect of various proteinase inhibitors on rat liver insulin proreceptor processing endopeptidase activity

Analysis of ^{35}S -labelled insulin proreceptor and [^{125}I]IRP-substrate processing by the isolated liver GV-1 fraction was assessed at 30 °C over 60 min (for ^{35}S -labelled insulin proreceptor) or 45 min ([^{125}I]IRP-substrate) at pH 7.0, 5 mM CaCl_2 , in the presence of various proteinase inhibitors as described in the Experimental section. Results (means of at least two independent observations) are expressed as percentage inhibition compared with the appropriate control. The control values for ^{35}S -labelled insulin proreceptor processing were 65–75%/60 min per 25 μg of protein, and for [^{125}I]IRP-substrate processing they were 60–70%/45 min per μg of protein. Abbreviation: GEMSA, guanidinoethylmercaptosuccinic acid. NA, not assessed.

Inhibitor (mM)	Processing activity (% of control)	
	Insulin proreceptor	IRP-peptide
PMSF (10)	84.7	73.0
PMSF (1)	102.2	93.5
Iodoacetic acid (1)	91.3	81.9
Iodoacetamide (1)	106.5	99.8
N-Ethylmaleimide (1)	98.5	105.0
Pepstatin A (1)	100.7	75.8
E-64 (1)	96.2	101.0
Tos-Lys- CH_2Cl (1)	88.9	92.0
Tos-Phe- CH_2Cl (1)	96.4	94.0
EDTA (10)	5.3	0.8
CDTA (10)	2.0	0
1,10-Phenanthroline (10)	83.2	97.7
GEMSA (0.1)	98.6	NA
Leupeptin (1)	78.1	93.2
Antipain (1)	74.2	59.4
p-Chloromercuribenzoate (0.1)	7.6	0
HgCl ₂ (0.1)	0	0

receptor substrate could not be used to determine these parameters, since not enough protein substrate could be obtained. Nevertheless, Lineweaver–Burk analysis of IRP-peptide substrate processing indicated an apparent K_m of $22.2 \pm 2.1 \mu\text{M}$ ($n = 4$) and a $V_{\max}$ of $18.6 \pm 2.6 \text{ pmol/min}$ ($n = 4$) (Figure 6). Based on a steady-state concentration of insulin receptor in liver of 12 pmol/mg of membrane protein and a half-life of $\sim 12 \text{ h}$, this processing activity is quite likely sufficient to completely process the proreceptor to the mature receptor protein *in vivo*.

Immunoblot analysis of mammalian Kex2-related proteins in the rat liver subcellular fraction

Immunoblot analysis of the rat liver GV-1 fraction was carried out in order to determine which members of the mammalian Kex2-related endopeptidases were present, and thus might be inferred to be a candidate for an insulin proreceptor processing endopeptidase. Antisera raised against PC1/3 and PC2 [32] were unable to detect these proteins in the liver Golgi vesicle fraction (results not shown). However, both furin and PACE4 were found to be present in the isolated rat liver Golgi vesicle fraction (Figure 7). Anti-furin serum specifically recognized an apparent 90 kDa form of furin [46], presumably mature furin [47]. The antiserum against pro-PACE4 specifically recognized a 122 kDa form of PACE4, and that against mat-PACE4 detected a 106 kDa form of PACE4 (Figure 7). Although further experiments will be necessary in order to examine the biosynthesis and post-translational modification of PACE4, it is likely that the 122 kDa form of PACE4 is a precursor form, since the peptide that this proPACE antiserum recognizes mimics the amino acid sequence around and including the putative processing site of PACE4 [22]. Likewise, the 105 kDa mature form of PACE4 was

Figure 6 Apparent K_m and $V_{\max}$ values for rat liver insulin proreceptor processing endopeptidase activity with the IRP-peptide substrate

Processing of biotinylated [^{125}I]IRP-substrate by the rat liver GV-1 fraction lysate (25 μg of total protein) was assessed between 2 and 60 min at various concentrations (20-fold, range centred on the apparent K_m) of non-biotinylated, non-radiolabelled IRP-peptide at 5 mM Ca^{2+} , pH 7.0, and 30 °C as described (see the Experimental section), and the initial rate (V_0) of peptide-substrate processing was calculated. A typical V_0 versus substrate concentration ($[S]$) plot is shown in (a), and the derived Lineweaver–Burk plot is shown in (b). The calculated apparent K_m and $V_{\max}$ values are the means $\pm$ S.E.M. of four independent Lineweaver–Burk plots.

detected by the mat-PACE4 antiserum, which was raised against a peptide that mimics the N-terminus of a proteolytically cleaved mature form of PACE4. The primary amino acid sequence of PACE4 predicts (after signal peptide removal) a pro-form of PACE4 of about 118 kDa and a mature PACE4 form of approx. 107 kDa [22].

DISCUSSION

It has been suggested that insulin proreceptor processing is most likely initiated in the *trans*-Golgi [6,48], analogous to the situation for other proproteins that traverse the constitutive secretory pathway [2]. In this study we have characterized a Ca^{2+} -dependent endopeptidase activity present in a rat liver subcellular fraction that is capable of correctly processing the insulin proreceptor to its mature α - and β -subunit form. This insulin proreceptor processing endopeptidase activity was apparently specific, since (a) no other proteolytic products of insulin proreceptor processing were observed other than the mature insulin receptor 126 kDa α -subunit and 93 kDa β -subunit (Figure 3a), as observed *in vivo* (Figure 2); (b) the proteolytic cleavage of the insulin

Figure 7 Immunoblot analysis of mammalian Kex2-related endopeptidases in an isolated rat liver subcellular fraction containing insulin proreceptor processing activity

The rat liver GV-1 fraction was subjected to immunoblot analysis as described in the Experimental section using specific antisera against furin (lane 2) precursor PACE4 (pro-PACE4; lane 4) and mature PACE4 (mat-PACE4; lane 6). Control immunoblot analyses using non-immune serum for furin (lane 1) and pre-immune serum for PACE4 (pro-PACE4, lane 3; mat-PACE4, lane 5) are also shown. Left arrows indicate the migration of molecular mass standards. The molecular masses of forms of furin and PACE4 recognized by the specific antisera and not by the non- or pre-immune sera is also indicated.

proreceptor occurred only C-terminal to the tetrabasic Arg-Lys-Arg-Arg processing site, yielding a correct ratio of [^{35}S]-methionine in the α - versus the β -subunit of the mature insulin receptor; (c) in relation to previous *in vivo* observations [7,8], the endopeptidase activity failed to process the human insulin proreceptor variant containing Ala at position-1 in parallel experiments where normal insulin proreceptor was processed; and (d) the rat liver subcellular GV-1 fraction used, although heterogeneous, was relatively enriched in Golgi vesicle marker enzyme activity over that of other organelles, and this is the predicted intracellular compartment where insulin proreceptor processing takes place *in vivo* [5,6,48]. In addition, this fraction was relatively (> 99%) free of lysosomal contamination, thus reducing the possibility of non-specific proteolysis. All these points meet the previously defined criteria for identification of a specific proprotein converting endopeptidase activity [1].

Determination of the rat liver insulin proreceptor processing activity using ^{35}S -labelled insulin proreceptor as a substrate was technically limiting, because it was slow and a second analysis (SDS/PAGE) was required to determine the specific processing. Therefore a convenient IRP-peptide substrate assay method (based on a peptide that mimicked the amino acid sequence of the human insulin proreceptor processing site) was used, as previously developed for characterizing proinsulin processing endopeptidases [41]. The IRP-peptide substrate was processed in the same manner as the insulin proreceptor substrate (Figure 5; Tables 2 and 3). The IRP-substrate assay was more sensitive, in that a 20-fold less amount of the endopeptidase preparation was necessary to give a comparable degree of processing to that observed with the [^{35}S]-labelled insulin proreceptor substrate. The IRP-substrate method was also quicker and more convenient, in that no second SDS/PAGE analysis was necessary. Furthermore, combined use of the IRP-peptide and [^{125}I]IRP-substrate was able to determine an apparent K_m , the low micromolar range for the insulin proreceptor processing activity (Figure 6), comparable with those previously observed for Kex2 protease [45] and furin [29,30] processing of synthetic fluorogenic tetrapeptide substrates.

In general, the biochemical characteristics revealed for the

insulin proreceptor processing activity using both insulin proreceptor and the IRP-peptide as substrates suggested this endopeptidase activity to be a legitimate member of the mammalian Kex2-related proprotein convertase family [14,15]. It was apparent that this endopeptidase activity was Ca^{2+} -dependent, was not inhibited by the metalloproteinase inhibitor 1,10-phenanthroline, was susceptible to inhibition by Hg^{2+} and was relatively resistant to serine-, aspartyl or cysteinyl proteinase inhibitors. In comparison with the known characteristics of other members of the Kex2-related convertase family, the biochemical characteristics of the liver Golgi vesicle insulin proreceptor processing activity are generally most like those of furin. The insulin proreceptor processing endopeptidase has a relatively broad pH range and alkaline pH optimum that are reminiscent of Kex2 [45] and furin activities [29,30]. PC1/3 and PC2, in contrast, both have narrow pH ranges with an acidic pH optimum of pH 5.5 [41]. Ca^{2+} requirements for furin activity [30] and the insulin proreceptor processing activity are also comparable, in that these activities are fully activated between 0.5 and 5 mM Ca^{2+} and show half-maximal activation between 50 and 250 μM Ca^{2+} . In addition, the insulin proreceptor processing endopeptidase activity appeared to be specific for a tetrabasic processing site, in that the human insulin proreceptor variant containing Ala at position-1 was not processed by this endopeptidase activity. An Arg⁻¹ residue been suggested to be essential for proprotein processing in the constitutive secretory pathway [49]. This is unlike Kex2 [45], PC1/3 and PC2 [41,50], which are able to process dibasic substrates, but more like furin activity which has been demonstrated to have a much greater specificity for tetrabasic processing sites [29,30,51–53]. The tetrabasic preference of the insulin proreceptor processing activity was further demonstrated in that it only hydrolysed the peptide bond C-terminal to the Arg-Lys-Arg-Arg tetrabasic processing site sequence on the IRP-peptide (corresponding to Arg⁷²³-Ser⁷²⁴ on the insulin proreceptor), and not within this sequence. Finally, the apparent K_m and $V_{\max}$ values for furin cleavage of synthetic tetrabasic peptide fluorogenic substrates [29,30] are not significantly different from these determined for the rat liver insulin proreceptor processing activity cleavage of the IRP-substrate (Figure 6).

Immunoblot analysis of the rat liver Golgi vesicle subcellular fraction containing the insulin proreceptor processing activity confirmed that PC1/3 and PC2 were not present, in line with the neuroendocrine-specific expression of these proteins [16,19]. Testis-specific PC4 and gut-specific PC6 are also not expressed in liver [23,24]. However, the immunoblot analysis showed both furin and PACE4 to be present in the rat liver Golgi vesicle fraction (Figure 7). It follows that the insulin proreceptor processing activity is analogous to either furin or PACE4 activity. The ubiquitous expression and broad specificity for cleavage of proproteins containing a tetrabasic processing site [28,54], has led to the suggestion of furin as a 'housekeeping' proprotein processing endopeptidase, present in all cells, which processes proproteins traversing the constitutive secretory pathway. A similar rat liver subcellular preparation to that used in this study has also been shown to contain a Kex2-related Ca^{2+} -dependent proalbumin processing endopeptidase activity [35], and this activity has subsequently been attributed to furin [55]. The similar *in vitro* biochemical characteristics of the insulin proreceptor processing activity and furin [29,30,53] suggest that these two activities might be one and the same. However, PACE4 is also universally expressed among mammalian cell types [22], and is additionally present in the rat liver GV-1 fraction containing furin and the insulin proreceptor processing activity. Although PACE4 has recently been demonstrated to possess

proprotein processing endopeptidase activity [56], its biochemical characteristics as a proteolytic enzyme are essentially unknown. Furin and PACE4 are both able to process certain proprotein substrates [56], and it is possible that these endopeptidase activities might both be capable of processing the insulin proreceptor. Therefore, until PACE4 proprotein processing activity is more fully characterized, insulin proreceptor processing activity cannot yet be unequivocally attributed to furin.

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REFERENCES

- Docherty, K. D. and Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625–638
- Burgess, T. L. and Kelly, R. B. (1987) *Annu. Rev. Cell. Biol.* **3**, 243–293
- Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. and Ramachandran, J. (1985) *Nature (London)* **313**, 756–760
- Ebina, Y., Ellis, L., Jarnagin, K., Ederly, M., Graf, L., Clauser, E., Ou, J.-H., Masiaz, P., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. J. (1985) *Cell* **40**, 747–758
- Kasuga, M., Hedo, J. A., Yamada, K. M. and Kahn, C. R. (1982) *J. Biol. Chem.* **257**, 10392–10399
- Olson, T. S., Bamberger, M. J. and Lane, M. D. (1988) *J. Biol. Chem.* **263**, 7342–7351
- Yoshimasa, Y., Seino, S., Whittaker, J., Kosaki, A., Kuzuya, H., Imura, H., Bell, G. I. and Steiner, D. F. (1988) *Science* **240**, 784–787
- Yoshimasa, Y., Paul, J. I., Whittaker, J. and Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 17230–17237
- Williams, J. F., McClain, D. A., Dull, T. J., Ullrich, A. and Olefsky, J. M. (1990) *J. Biol. Chem.* **265**, 8463–8469
- Edwards, K., Fleischer, B., Dryburgh, H., Fleischer, S. and Schreiber, G. (1976) *Biochem. Biophys. Res. Commun.* **72**, 310–318
- Rhodes, C. J., Brennan, S. O. and Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 14240–14245
- Orci, L., Malhotra, V., Amherdt, M., Serafini, T. and Rothman, J. E. (1989) *Cell* **56**, 357–368
- Lindberg, I. (1991) *Mol. Endocrinol.* **5**, 1361–1365
- Hutton, J. C. (1991) *Curr. Opin. Cell Biol.* **2**, 1131–1142
- Steiner, D. F., Smeekens, S. P., Ohagi, S. and Chan, S. J. (1992) *J. Biol. Chem.* **267**, 23435–23438
- Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J. and Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 340–344
- Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. and Chrétien, M. (1991) *Mol. Endocrinol.* **5**, 111–122
- Smeekens, S. P. and Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997–3000
- Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. and Chrétien, M. (1990) *DNA Cell Biol.* **9**, 415–424
- Roebroek, A. J., Schalken, J. A., Leunissen, J. A., Onnekink, C., Bloemers, H. P. and Van de Ven, W. J. (1986) *EMBO J.* **5**, 2197–2202
- Van Den Ouweland, A. M. W., van Duijnhoven, H. L. P., Keizer, G. D., Dorssers, L. C. J. and Van den Ven, W. J. M. (1990) *Nucleic Acids Res.* **18**, 664
- Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D. and Barr, P. J. (1991) *DNA Cell Biol.* **10**, 757–769
- Nakayama, K., Kim, W. S., Torii, K. S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. and Murakami, K. (1992) *J. Biol. Chem.* **267**, 5897–5900
- Feany, M. B., Lee, S., Edwards, R. H. and Buckley, K. M. (1992) *Cell* **70**, 861–867
- Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K. and Nakayama, K. (1990) *J. Biol. Chem.* **265**, 22075–22078
- Roebroek, A. J., Schalken, J. A., Bussemakers, M. J., van Heerikhuizen, H., Onnekink, C., Debruyne, F. M., Bloemers, H. P. and Van de Ven, W. J. (1986) *Mol. Biol. Rep.* **11**, 117–125
- Myers, M. G., and White, M. F. (1993) *Diabetes* **42**, 643–650
- Misumi, Y., Oda, K., Fujiwara, T., Takami, N. and Ikehara, Y. (1991) *J. Biol. Chem.* **266**, 16954–16959
- Hatsuzawa, K., Nagahama, M., Takahashi, S., Takada, K., Murakami, K. and Nakayama, K. (1992) *J. Biol. Chem.* **267**, 16094–16099
- Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 16396–16402
- Whittaker, J., Okamoto, A. K., Thys, R., Bell, G., Steiner, D. F. and Hoffman, C. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5237–5241
- Alarcón, C., Lincoln, B. and Rhodes, C. J. (1993) *J. Biol. Chem.* **268**, 4276–4280
- Rehemtulla, A., Dorner, A. and Kaufman, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8235–8239
- Quinn, P. S. and Judah, J. D. (1978) *Biochem. J.* **172**, 301–309
- Brennan, S. O. and Peach, R. J. (1988) *FEBS Lett.* **229**, 167–170
- Rhodes, C. J., Lucas, C. A., Mutkoski, R. L., Orci, L. and Halban, P. A. (1987) *J. Biol. Chem.* **262**, 10712–10717
- Davidson, H. W., Peshavaria, M. and Hutton, J. C. (1987) *Biochem. J.* **246**, 279–286
- Siddle, K. (1990) in *Peptide Hormone Secretion: A Practical Approach*. (Hutton, J. C. and Siddle, K.) pp. 97–120, IRL Press, Oxford
- Prigent, S. A., Stanley, K. K. and Siddle, K. (1990) *J. Biol. Chem.* **265**, 9970–9977
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Rhodes, C. J., Lincoln, B. and Shoelson, S. E. (1992) *J. Biol. Chem.* **267**, 22719–22727
- Storer, A. C. and Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 1–5
- Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, J. Wiley and Sons, New York
- Oda, K. and Ikehara, Y. (1988) *J. Biochem (Tokyo)* **104**, 159–161
- Brenner, C. and Fuller, R. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 922–926
- Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. and Thomas, G. (1990) *J. Cell Biol.* **111**, 2851–2859
- Ludac, R., Molloy, S. S., Thorne, B. A. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 14304–14308
- Hedo, J. A., Kahn, C. R., Hayashi, M., Yamada, K. M. and Kasuga, M. (1983) *J. Biol. Chem.* **258**, 10020–10026
- Watanabe, T., Murakami, K. and Nakayama, K. (1993) *FEBS Lett.* **320**, 215–218
- Shennan, K. I. J., Smeekens, S. P., Steiner, D. F. and Docherty, K. (1991) *FEBS Lett.* **284**, 277–280
- Hosaka, M., Nagahama, M., Kim, W.-S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. and Nakayama, K. (1991) *J. Biol. Chem.* **266**, 12127–12130
- Rehemtulla, A. and Kaufman, R. J. (1992) *Blood* **79**, 2349–2355
- Trifaró, J. M., Vitale, M. L. and Rodríguez Del Castillo, A. (1992) *Eur. J. Pharmacol.* **225**, 83–104
- Watanabe, T., Nakagawa, T., Ikemizu, J., Nagahama, M., Murakami, K. and Nakayama, K. (1992) *J. Biol. Chem.* **267**, 8270–8284
- Oda, K., Misumi, Y., Ikehara, Y., Brennan, S. O., Hatsuzawa, K. and Nakayama, K. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1353–1361
- Rehemtulla, A., Barr, P. J., Rhodes, C. J. and Kaufman, R. J. (1993) *Biochemistry* **32**, 11586–11590