Selective Degradation of Insulin within Rat Liver Endosomes

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Abstract. To characterize the role of the endosome in the degradation of insulin in liver, we employed a cell-free system in which the degradation of internalized 125I-insulin within isolated intact endosomes was evaluated. Incubation of endosomes containing internalized 125I-insulin in the cell-free system resulted in a rapid generation of TCA soluble radiolabeled products (t1/2, 6 min). Sephadex G-50 chromatography of radioactivity extracted from endosomes during the incubation showed a time dependent increase in material eluting as radioiodotyrosine. The apparent Vmax of the insulin degrading activity was 4 ng insulin degraded-min-1·mg cell fraction protein-1 and the apparent Km was 60 ng insulin-mg cell fraction protein-1. The endosomal protease(s) was insulin-specific since neither internalized 125I-epidermal growth factor (EGF) nor 125I-prolactin was degraded within isolated endosomes as assessed by TCA precipitation and Sephadex G-50 chromatography. Significant inhibition of degradation was observed after inclusion of p-chloromercuribenzoic acid (PCMB), 1,10-phenanthroline, bacitracin, or 0.1% Triton X-100 into the system. Maximal insulin degradation required the addition of ATP to the cell-free system that resulted in acidification as measured by acridine orange accumulation. Endosomal insulin degradation was inhibited markedly in the presence of pH dissipating agents such as nigericin, monensin, and chloroquine or the proton translocase inhibitors N-ethylmaleimide (NEM) and dicyclohexylcarbocyanine (DCCD). Polyethylene glycol (PEG) precipitation of insulin-receptor complexes revealed that endosomal degradation augmented the dissociation of insulin from its receptor and that dissociated insulin was serving as substrate to the endosomal protease(s). The results suggest that as insulin is internalized it rapidly but incompletely dissociates from its receptor. Dissociated insulin is then degraded by an insulin specific protease(s) leading to further dissociation and degradation.

Materials and Methods

Ligand Radioiodination, Protein Determination, and Materials

Porcine insulin (26.8 IU·mg-1, gift of Eli Lilly, Indianapolis, IN), mouse EGF (Collaborative Research, Inc., Waltham, MA) and ovine prolactin (30.5 IU·mg-1, gift of National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD) were radioiodinated by the chloramine T method (43) with modifications as described previously (55) to specific activities of 102-174 μCi·µg-1, 191-251 μCi·µg-1, and 100-148 μCi·µg-1, respectively. Protein in subcellular fractions was determined by the method of Bradford (17). All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Canada (Montreal, Canada), or Anachemia Inc. (Montreal, Canada).

Preparation of the Golgi-Endosome (GE) Fraction Containing Internalized 125I-Insulin, 125I-EGF, and 125I-Prolactin

A GE fraction containing internalized ligands was prepared as described previously (9, 46) with minor modifications. Female Sprague Dawley rats (140-180 g) that had been fasted 14-16 h were injected with 140-180 ng of either 125I-insulin, 125I-EGF, or 125I-prolactin via the hepatic portal vein. 5 min after injection animals were sacrificed by decapitation, allowed to exsanguinate via the cervical wound, and the livers were removed rapidly and minced in ice cold 0.25 M sucrose in HKM buffer (20 mM Hepes, pH 7.4, 25 mM KCl, 10 mM MgCl2). Livers were homogenized with 4-5 strokes of a motor driven Teflon pestle (Thomas Scientific, Swedesboro, NJ) (2,300...
Cell-Free Assay for Degradation of Insulin within Isolated Endosomes

Endosomes containing 5-20 ng $^{125}$I-insulin/mg cell fraction protein$^{-1}$ were suspended in a cell-free system after recovery from the density gradient (final concentrations: 40-50 $\mu$g cell fraction protein/ml$^{-1}$ in 20 mM Hepes, pH 7.4, 250 mM sucrose, 150 mM KCl, 5 mM NaCl, 15 mM MgCl$_2$, 1 mM DTT, 10 mM ATP-K$^+$). Samples were incubated at 37°C for varying lengths of time after which the integrity of internalized $^{125}$I-insulin was assessed by precipitation in ice cold 10% TCA.

To determine the pH optimum of endosomal degradation, isolated endosomes were suspended in the cell-free system (minus ATP) buffered with 25 mM potassium acetate, Hepes, or imidazole to pH 3-8. Nigerecin (10 $\mu$M) and valinomycin (10 $\mu$M) were included to equilibrate endosomal pH with that of the buffers and samples were incubated for 30 min after which degradation of $^{125}$I-insulin was assessed by acid solubility of radiolabel.

Extraction of Radioactivity from Isolated Endosomes

Rats were injected via the portal vein with 2 $\mu$g $^{125}$I-insulin/100 g (body weight) and 5 min later, animals were sacrificed, and the GE fraction isolated. Isolated endosomes were suspended in the cell-free system for either 0 min (4°C) or 15 min (37°C) after which, 1 M acetic acid and 0.1% Triton X-100 were added to samples on ice. Samples were centrifuged at 110,000 $g_{av}$ (TL 100.2, Beckman Instruments) for 30 min, and the supernatants decanted carefully and frozen. By this method, > 96% of the radioactivity was extractable. Thawed samples were chromatographed on Sephadex G-50 columns (1.2 x 55-cm) and eluted with 1 M acetic acid. Radioactivity within endosomes containing internalized $^{125}$I-EGF was acid extracted as above and chromatographed on similar columns. Samples which eluted at the positions of intact $^{125}$I-insulin or $^{125}$I-EGF (the peak of radioactivity and one sample on either side of the peak) were pooled, neutralized, and assessed for specific binding to liver plasma membranes at 4°C for 14 h as described previously (56).

Cell-Free Acidification Assay

ATP-dependent acidification was assayed as described by Glickman et al., (38) with minor modifications. The GE fraction harvested from the density gradient was slowly diluted in assay buffer (final concentration, 10 $\mu$g cell fraction protein/ml$^{-1}$, 2 mM Hepes, 2 mM Tris, pH 7.0, 150 mM KCl, 6 mM MgCl$_2$, 1 mM DTT) and pelleted at 16,000 $g_{av}$, for 1 h (60Ti rotor, Beckman Instruments). The pellet was then gently resuspended in the same buffer with three strokes of a Dounce homogenizer (Wheaton Scientific, Millville, NJ) using a type B pestle and equilibrated in buffer for 3-6 h at 4°C. Acridine orange was then added to the membranes to a final concentration of 6 $\mu$g ATP was added (final concentration 0.4 mM) and acidification monitored by recording the change in the absorption spectrum of acridine orange as it concentrated within acidic compartments. The absorption difference at 492 and 540 nm was recorded with a dual wavelength spectrophotometer (Aminco DW-2; American Instrument Co., Silver Spring, MD), kindly provided by Dr. J. G. Joly (Hôpital Saint-Luc, Montreal, Canada).

Polyethylene Glycol Precipitation (PEG) of Insulin-Receptor Complexes

GE fractions were suspended in the cell-free degradation assay system for 0 and 15 min $\pm$ ATP in the presence or absence of 1 mM $\beta$-chloromercuribenzenoic acid (PCMBA). Subsequently, samples containing <50 $\mu$g cell fraction protein were suspended in HMT buffer (20 mM Hepes, pH 7.4, 10 mM MgCl$_2$, 0.1% Triton X-100) for 10 min after which PEG 8000 and rabbit gamma globulins were added to final concentrations of 10% and 2 mg/ml$^{-1}$, respectively. The precipitate was pelleted (20,000 $g_{av}$, 45 min, IEC, DPR 6000), the supernatant decanted carefully, and both the pellet and supernatant counted for radioactivity in a Compugamma counter (1285; LKB Instruments Inc., Gaithersburg, MD; 65% efficiency).

Electron Microscopy of GE Fractions

GE fractions were suspended in the cell-free system for either 0 min (4°C) or 30 min (37°C) and subsequently were fixed on ice with 2.5% glutaraldehyde, 100 mM sodium cacodylate, pH 7.4. Aliquots containing ~30 $\mu$g cell fraction protein were filtered onto nitrocellulose filters (Millipore Corporation, MA) using a filtration apparatus (5). Samples were then postfixed in 2% O$_2$Cl in 100 mM sodium cacodylate, pH 7.4 for 2 h at 4°C, and stained en bloc with 1% tannic acid in 100 mM sodium cacodylate (pH 7.0) (66), and 8% uranyl acetate in 100 mM maleic buffer (pH 5.0) (44), and subsequently processed for routine microscopy.

Results

Internalization and Cell-Free Degradation of Insulin in Intact Endosomes

At 5 min after the injection of low levels of insulin (100 ng/100 g bw) hormone was concentrated maximally within endosomes of the GE fraction isolated from liver homogenates. $^{125}$I-Insulin was 40 ± 4 (mean ± SD, n = 9) fold enriched in the fraction as compared to the parent homogenate. This 5-min internalization period has been shown previously to result in the entry of insulin into endosomes but not lysosomes (12, 57).

The cell-free system described by Diment and Stuhl (26) was used to evaluate the degradation of internalized $^{125}$I-insulin within endosomes of the GE fraction (Fig. 1). Degradation of $^{125}$I-insulin was rapid between 0 and 15 min of incubation at 37°C (t$_1/2$ of initial velocity, 6 min) and reached a plateau by 60 min of incubation with 71 ± 5% of the internalized hormone degraded. Inclusion of an ATP regenerating system into the cell-free system (20 mM creatine phosphate, 5 IU creatine phosphokinase) or further addition of Mg$^{2+}$-ATP at 15 and 30 min of incubation resulted in no further increase in the concentration of insulin degraded over time (not shown). Sephadex G-50 chromatography (Fig. 2) of radioactivity extracted from endosomes of the GE fraction isolated from rats given a higher insulin dose (2 $\mu$g $^{125}$I-insulin/100 g bw) showed a threefold increase in the size of the peak eluting at the position of radioiodotyrosine (peak III) after 15 min of incubation in the cell-free system. Radioactivity eluting at the position of intact $^{125}$I-insulin (peak II) was evaluated for specific binding to liver plasma membranes. At 0 min and 15 min of incubation, specific binding of extracted radioactivity eluting in peak II was 28 and 31% /50 $\mu$g cell fraction protein, respectively. Negligible binding to liver plasma membranes was observed for material eluting in peaks I or III (specific binding, 0.6 and 0.5%, respectively).

The selectivity of endosomal degradation was assessed by incubating endosomes of the GE fraction containing internalized $^{125}$I-EGF or $^{125}$I-prolactin in the cell-free system. Degradation of $^{125}$I-EGF within isolated endosomes was not detectable by acid precipitation (Fig. 1) or Sephadex G-50 chromatography (Fig. 2). Radioactivity extracted from endosomes at 0 min and 15 min of incubation that eluted as intact $^{125}$I-EGF (peak IV) showed specific binding to liver plasma membranes of 11 and 10%/50 $\mu$g cell fraction protein, respectively (not shown). No degradation of $^{125}$I-prolactin within isolated endosomes was observed as evaluated by acid precipitation (Fig. 1). Hence, only insulin was degraded to acid soluble constituents in the cell-free system. Endosomal degradation of internalized $^{125}$I-insulin followed apparent
Figure 1. Selective degradation of internalized \( ^{125}\)I-insulin within isolated intact endosomes. Endosomes containing internalized \( ^{125}\)I-insulin (●), \( ^{125}\)I-EGF (△) or \( ^{125}\)I-prolactin (■) were suspended and maintained in the cell-free system for the indicated times. The integrity of each ligand was assessed by precipitability of radiolabel in cold 10% TCA (mean ± SD, n = 3).

Michaelis-Menten kinetics with an apparent \( V_{\text{max}} \) of 4 ng insulin degraded-min\(^{-1}\)-mg cell fraction protein\(^{-1}\) and apparent \( K_m \) of 60 ng insulin-mg cell fraction protein\(^{-1}\) (Fig. 3).

Structural integrity of endosomes during incubation in the cell-free system was evaluated by electron microscopy. The freshly prepared GE fraction (Fig. 4 A) consisted of a heterogeneous mix of elements of the Golgi apparatus and endosomes filled with their characteristic content of lipoprotein-like particles. Little difference in morphology was observed after incubation for 30 min at 37°C in the cell-free system (Fig. 4 B).

Figure 2. Chromatography of radioactivity extracted from GE fraction. Radiolabel was acid extracted from GE fractions containing internalized insulin (A and B) or \( ^{125}\)I-EGF (C and D) at 0 min (A and C) or 15 min (B and D) of incubation in the cell-free system and analyzed by Sephadex G-50 chromatography. The void volume of the column (peak I) and the elution positions of intact \( ^{125}\)I-insulin (peak II), \( ^{125}\)I-tyrosine (peak III), and intact \( ^{125}\)I-EGF (peak IV) are indicated.

Figure 3. In vivo dose-response of endosomal insulin degradation. Rats were injected via the portal vein with increasing doses of \( ^{125}\)I-insulin (0.05–20 μg/100 g bw) to internalize increasing concentrations of insulin within endosomes. Isolated GE fractions were suspended in the cell-free system and the reciprocal of the initial velocity of insulin degradation (1/\( V_0 \), ng degraded-min\(^{-1}\)-mg cell fraction protein\(^{-1}\)) was assessed as a function of the reciprocal of the concentration of insulin within endosomes (1/\( S \), ng·mg cell fraction protein\(^{-1}\)).

Effect of Protease Inhibitors and Detergent on Endosomal Degradation of Insulin

To characterize the endosomal insulin-specific degrading activity, we included various protease inhibitors in the cell-free system and assessed their effect on \( ^{125}\)I-insulin degradation (Table I). Addition of leupeptin, pepstatin, aprotinin, antipain, PMSF, or EDTA had no significant effect on \( ^{125}\)I-insulin degradation. However, both PCMB and 1,10-phenanthroline inhibited degradation by 100 and 91%, respectively. Inclusion of bacitracin into the cell-free system resulted in a 40% inhibition of degradation. Significant inhibition of degradation was also observed after disruption of isolated endosomes with 0.1% Triton X-100.

ATP-dependent Acidification of Endosomes Is Required for Maximal Degradation of Insulin

Diment and Stahl (26) reported that degradation of mannose-BSA within isolated macrophage endosomes was dependent on the presence of ATP during in vitro incubation. Therefore, we examined the nucleotide requirements for the insulin-degrading activity present within isolated endosomes. Maximal degrading activity was found when ATP was included in the cell-free system (Table II). Significant but lower activity was found in the absence of ATP. This was not likely because of endogenous ATP since inclusion of an ATP-depleting system (glucose + hexokinase) produced no significant reduction in \( ^{125}\)I-insulin degradation. Substitution of ATP with AMP-PNP, ADP, GTP, or a mixture of ADP and GTP to promote ATP formation by a possible nucleoside diphosphokinase did not result in a restoration of maximal insulin degrading activity observed with ATP.

To test whether the effect of ATP on endosomal insulin degradation was mediated through activation of the endosomal proton translocase and consequent endosomal acidification, we included various pH dissipating agents and proton translocase inhibitors into the cell-free system and as-
Table I. Effect of Protease Inhibitors and Detergent on \(^{125}\text{I}-\text{insulin Degradation in Endosomes}\)

| Addition                   | Concentration | \(^{125}\text{I}-\text{insulin degraded}\) (percentage of control) |
|----------------------------|---------------|------------------------------------------------------------------|
| None                       |               | 100                                                              |
| Leupeptin                  | 10 \(\mu g \cdot ml^{-1}\) | 99.1 \(\pm 4.2^*\)                                               |
| Pepstatin                  | 10 \(\mu g \cdot ml^{-1}\) | 98.9 \(\pm 5.7^*\)                                               |
| Aprotinin                  | 10 \(\mu g \cdot ml^{-1}\) | 99.1 \(\pm 2.9^*\)                                               |
| Antipain                   | 10 \(\mu g \cdot ml^{-1}\) | 83.1 \(\pm 19.1^*\)                                              |
| PMSF                       | 1 mM          | 89.2 \(\pm 13.5^*\)                                              |
| PCMB                       | 1 mM          | 0 \(\pm 0.9^*\)                                                  |
| EDTA                       | 1 mM          | 99.6 \(\pm 1.9^*\)                                               |
| 1,10-Phenanthroline        | 1 mM          | 8.8 \(\pm 0.4^*\)                                                |
| Bacitracin                 | 5 mg \cdot ml^{-1}\ (each) | 60.5 \(\pm 3.9^*\)                                                |

Detergent

| Triton-X-100               | 0.1%          | 22.9 \(\pm 4.8^*\)                                               |

Degradation of endocytosed \(^{125}\text{I}-\text{insulin} was assessed in isolated GE fractions incubated in the cell-free system in the presence of the indicated compounds. Degradation was determined by acid solubility of radiolabel at 30 min of in vitro incubation. Results are expressed as a percent of \(^{125}\text{I}-\text{insulin} degradation observed in the standard cell-free system as described in Materials and Methods. All observations are means of three separate experiments \(\pm SD\) with significance evaluated by \(t\) test.

* Not significant.

\(P \approx 0.0001.\)

Figure 4. Electron microscopy of endosome fractions. (A) At 0 min of incubation in the cell-free system, both stacked sacules of the Golgi apparatus (G) and intact endosomes (En) with their characteristic content of lipoprotein-like particles (lp) are observed. Contamination of the fraction by other organelles is negligible. (B) After a 30-min incubation at 37°C in the cell-free system, the structural integrity of both endosomes and Golgi apparatus was maintained with no evidence of organelle aggregation or fusion. Bar, 0.5 \(\mu m\).

Table II. ATP-dependent Acidification of Isolated Intact Endosomes Is Required for Maximal Insulin Degradation

| Addition                  | Concentration | \(^{125}\text{I}-\text{insulin degraded}\) (percentage of control) |
|---------------------------|---------------|------------------------------------------------------------------|
| Nucleotides               |               |                                                                  |
| ATP                       | 10 mM         | 100                                                              |
| None                      |               | 35.4 \(\pm 4.2^*\)                                               |
| None + glucose + hexokinase | 5 mM + 10 U \(\cdot ml^{-1}\) | 31.9 \(\pm 0.2^*\)                                               |
| AMP-PNP                   | 10 mM         | 24.5 \(\pm 6.2^*\)                                               |
| ADP                       | 10 mM         | 40.0 \(\pm 2.9^*\)                                               |
| GTP                       | 10 mM         | 52.8 \(\pm 4.2^*\)                                               |
| ADP + GTP                 | 10 mM (each)  | 26.2 \(\pm 1.6^*\)                                               |
| Ionophores                |               |                                                                  |
| Nigerin                   | 50 \(\mu M\)  | 17.4 \(\pm 0.9^*\)                                               |
| Monensin                  | 50 \(\mu M\)  | 20.6 \(\pm 4.1^*\)                                               |
| Valinomycin               | 50 \(\mu M\)  | 83.3 \(\pm 2.8^*\)                                               |
| Weak base                 |               |                                                                  |
| Chloroquine               | 2 \(\mu M\)   | 16.3 \(\pm 1.1^*\)                                               |
| Proton pump inhibitors    |               |                                                                  |
| NEM                       | 10 \(\mu M\)  | 23.6 \(\pm 4.5^*\)                                               |
|                           | 1 \(\mu M\)   | 26.8 \(\pm 2.6^*\)                                               |
| DCCCD                     | 10 \(\mu M\)  | 50.4 \(\pm 14.6^*\)                                              |
|                           | 1 \(\mu M\)   | 38.7 \(\pm 2.2^*\)                                               |
| Oligomycin                | 5 \(\mu g \cdot ml^{-1}\) | 90.2 \(\pm 4.8^*\)                                               |
| Vanadate                  | 100 \(\mu M\) | 99.9 \(\pm 2.9^*\)                                               |
| Amiloride                 | 2 \(\mu M\)   | 96.6 \(\pm 3.7^*\)                                               |

Endosomal degradation of \(^{125}\text{I}-\text{insulin} observed between 0 and 30 min of in vitro incubation after the addition of the indicated compounds is expressed as a percent of the degradation observed in the standard cell-free system (mean \(\pm SD, n = 3\)). Significance was evaluated by \(t\) test. NEM, N-ethylmaleimide; DCCCD, dicyclohexylcarbodiimide.

* Not significant.

\(P \approx 0.0001.\)

\(P \approx 0.001.\)

\(P \approx 0.005.\)
activity of the GE fraction using the method of Glickman et al. (38) (Fig. 5). After addition of ATP, components of the GE fraction acidified with an initial rate that was dependent on the concentration of cell fraction protein (Fig. 5 and inset). The pH gradients generated through proton translocase activity were dissipated rapidly after addition of nigericin to the cell-free system. Acridine orange absorption levels were restored above initial baseline levels after addition of nigericin (Fig. 5, inset).

The effect of pH on endosomal degradation of insulin was tested by incubating endosomes containing internalized 125I-insulin in the cell-free system buffered to pH 3-8 minus ATP. Before assay, both nigericin and valinomycin (10 #M) were included to equilibrate endosomal pH with that of the buffer. The pH gradients generated through proton translocase activity were dissipated rapidly after addition of nigericin to the cell-free system. Acridine orange absorption levels were restored above initial baseline levels after addition of nigericin (Fig. 5, inset).

The pH dependency of insulin degradation in GE fractions. GE fractions containing 6 ng insulin-mg cell fraction protein−1 were suspended in the cell-free system (minus ATP) buffered with potassium acetate, Hepes, or imidazole (25 mM) at various pHs ranging from 3 to 8. Nigericin (10 #M) and valinomycin (10 #M) were included to equilibrate endosomal pH with that of the buffer. Degradation of 125I-insulin was assessed after 30 min of incubation by acid solubility of radiolabel (mean ± SD, n = 3). Maximal degradation was observed at pH 5.5 and normalized to 100%.

These results suggested that lowering of the endosomal pH through proton translocase activity was required for maximal activity of the insulin protease(s).

We tested directly the ATP-dependent proton translocase activity of the GE fraction using the method of Glickman et al. (38) (Fig. 5). After addition of ATP, components of the GE fraction acidified with an initial rate that was dependent on the concentration of cell fraction protein (Fig. 5 and inset). Before assay, both nigericin and valinomycin (10 #M) were included to equilibrate endosomal pH with that of the buffer. The pH gradients generated through proton translocase activity were dissipated rapidly after addition of nigericin to the cell-free system. Acridine orange absorption levels were restored above initial baseline levels after addition of nigericin (Fig. 5, inset).

The pH of endosomal degradation of insulin was tested by incubating endosomes containing internalized 125I-insulin in the cell-free system buffered to pH 3–8 minus ATP. Before assay, both nigericin and valinomycin (10 #M) were included to equilibrate endosomal pH with that of the buffer. The pH gradients generated through proton translocase activity were dissipated rapidly after addition of nigericin to the cell-free system. Acridine orange absorption levels were restored above initial baseline levels after addition of nigericin (Fig. 5, inset).

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The effect of pH on endosomal degradation of insulin was tested by incubating endosomes containing internalized 125I-insulin in the cell-free system buffered to pH 3–8 minus ATP. Before assay, both nigericin and valinomycin (10 #M) were included to equilibrate endosomal pH with that of the buffer. The pH gradients generated through proton translocase activity were dissipated rapidly after addition of nigericin to the cell-free system. Acridine orange absorption levels were restored above initial baseline levels after addition of nigericin (Fig. 5, inset).
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30 min of incubation or inclusion of an ATP regenerating system had no effect on ligand degradation then the limited extent of degradation observed likely reflects the lability of the endosomal insulin proteolytic activity in vitro.

Based on the assays employed (i.e., acid precipitability and Sephadex G-50 chromatography), we were unable to detect degradation of EGF or prolactin. The possibility that these ligands may be in endosomal components distinct to those internalizing insulin was considered unlikely. Prior studies have shown that at 5-min postinjection, insulin (45), EGF (34), and prolactin were localized within the same endosomes as those harboring internalized asialoglycoprotein.

A rapid and subtle processing of EGF occurring most probably in endosomes has been documented in fibroblasts by several groups (50, 54, 63, 69). These processing steps have been attributed to disparities between hepatic and fibroblast endosomes, or possibly, the absence of endosomal components containing this activity in the GE fraction.

Diment and Stahl (26) have previously demonstrated endosomal degradation of mannose-BSA internalized via the mannose receptor of macrophages. With an identical cell-free system to that employed here, Diment and Stahl (26) observed inhibition of degradation by leupeptin, pepstatin, antipain, and PMSF and have identified a membrane bound cathepsin D in macrophage endosomes as responsible for proteolysis of internalized mannose-BSA (27). By contrast, we found no effect of leupeptin, pepstatin, antipain, PMSF, aprotinin, or EDTA on insulin degradation in liver endosomes. The sensitivity of endosomal insulin degradation to PMSF is similar to the characteristics of a cytosolic insulin degrading enzyme recently cloned (1). The cytosolic localization of this insulin degrading enzyme (64, 65) and its near neutral pH optimum (29, 30) suggest that a distinct but related enzyme may be located within hepatic endosomes.

Electron microscopy demonstrated that endosomes remained intact during the cell-free incubations. Indeed, inclusion of 0.1% Triton X-100 in the cell-free system resulted in significant inhibition of degradation. This was also observed by Diment and Stahl (26) for degradation of mannose-BSA within macrophage endosomes. The observation likely is explained by a dissipation of the endosomal pH gradient (vide infra) and also serves to rule out any contaminating lysosomes as responsible for the insulin degrading activity observed in vitro.

The observation that ATP was required for maximal insulin degradation prompted us to examine whether endosomal acidification through proton translocase activity was required for optimal degrading activity. The GE fraction was highly active in ATP dependent acridine orange uptake (Fig. 5). The fraction, however, is heterogeneous consisting of Golgi sacules and lipoprotein-filled endosomes. Although acidotropic agents have been shown to accumulate in trans Golgi components (for review, see reference 4), we suggest that the ATP-dependent acridine orange uptake of the GE fraction was mainly because of endosomes. Both the pH optima of Golgi apparatus sugar transferase activities (11, 18) and the lack of fusion activity of the influenza hemagglutinin precursor HAO (16) during biosynthetic traverse of the Golgi apparatus indicate that Golgi compartments are not very acidic (16). In this context, the contribution of contaminating endosomes in the Golgi fraction of Glickman et al. (38) for proton translocase activity had not previously been evaluated.

The inhibition of insulin degradation observed after inclusion of proton ionophores, the weak base chloroquine, and proton translocase inhibitors in the cell-free system supported our conclusion that degradation was regulated by the proton translocating activity of endosomes. A small but significant inhibitory effect of the potassium ionophore valinomycin was observed; this effect was likely because of an increase in endosomal [K+] that may inhibit the maximal extent of proton translocation.

The demonstration of optimal insulin degrading activity at pH 5.5 (Fig. 6) also supported the contention that degradation was regulated by endosomal acidification. However, even at pH 7.4, 22% of maximal degradation was observed.
This value was consistent with results obtained after the inclusion of nigericin, monensin, or chloroquine into the cell-free system. However, under the standard incubation conditions, when endosomal pH was not equilibrated with that of the cell-free system, insulin degradation in the absence of ATP was higher than the expected 22%; i.e., 35% of control (Table II). We suggest that this 13% difference may be because of degradation occurring within a population of endosomes that were acidified in vivo and retained their protons during isolation and in vitro incubation.

The PEG precipitation studies demonstrated that in freshly prepared endosomes, the majority of internalized insulin (59%) was dissociated from receptor. That this represented maximal insulin dissociation was shown by the inclusion of PCMB into the cell-free system (Fig. 7, D); no further dissociation was observed in the presence of ATP. Only when degradation proceeded maximally (+ATP) was net insulin dissociation observed (Fig. 7, B). Hence, despite the decrease in pH expected after adding ATP or by incubating endosomes at pH 5.5 (see Fig. 7 legend) no further effect on $K_0$ was observed. As explained elsewhere (6, 10), the extent of ligand dissociation is expected to be limited by the small volume of endosomes ($\sim 10^{-17}$ liters) and by the high intraluminal concentration of free insulin that would quickly counteract pH induced changes in $K_0$. By coupling degradation and dissociation in the endosome, rapid and complete removal of internalized receptor-bound insulin may therefore be expected. The observations on incubations carried out -ATP (Fig. 7, C) were noteworthy. Here, insulin degradation occurred with no loss of receptor-bound insulin observed. Thus, free insulin was serving as the preferred substrate for the insulin degrading activity.

A number of the reported sites of proteolytic cleavage of insulin (31–33) occur in the receptor binding domain of insulin (24). Dissociation of insulin from its receptor would facilitate availability of these sites to the endosomal protease(s). However, selective degradation of internalized ligands within the isolated endosomes was not a consequence of selective dissociation as 60% of prolactin and 43% of EGF were dissociated from receptor in freshly isolated endosomes. Analysis of the primary sequences of porcine insulin (15), ovine prolactin (36), and mouse EGF (20, 60) and knowledge of the sites of cleavage of insulin in liver (31–33) show seven potential sites for prolactin processing but no analogous sites for EGF. Selectivity of endosomal processing may be a consequence of secondary and tertiary structural differences between insulin and prolactin that enable internalized insulin, but not prolactin, to serve as substrate for the endosomal protease(s).

The observations presented here extend our past studies (12, 14, 40, 56) and those of Pease et al. (53) and resolve several previously puzzling observations. These include (a) the extraordinarily rapid kinetics of insulin clearance from liver as opposed to prolactin and EGF (61); (b) the selective effect of chloroquine in accumulating internalized insulin but not prolactin in subcellular fractions enriched in endosomes (12, 47, 57); (c) the identification of insulin fragments in endosomes corresponding to the in vivo sites of insulin cleavage (40); and (d) the inability to document extensive entry of insulin into acid phosphatase enriched lysosomes (7, 12, 57).

For liver, insulin degrading activity has been reported at the cell surface (70) in lysosomes (22) and in the cytosol (64, 65). These studies may indicate sites of proteolytic activity that are unavailable for interacting with the majority of cell-associated insulin under physiological conditions.

The demonstration of insulin proteolytic activity in endosomes extends the work of several groups who have documented proteolytic activity and specific proteases in components of the endosomal apparatus (2, 26, 27, 37, 40, 53, 59, 63, 69). The significance of endosomal processing of internalized ligands remains speculative. Several interpretations propose a model of lysosomal maturation involving a biogenetic pathway of newly synthesized lysosomal proteases through endosomal components (19, 37, 59). However, the targeting of insulin specific proteases to endosomes may be of physiological significance. Insulin is rapidly degraded in vivo by two processes. A nonselective, nonreceptor mediated high capacity pathway via the proximal convoluted tubule of the kidney (8) that ensures rapid clearance of insulin from the circulation (35) and a selective receptor mediated pathway in the liver. The liver parenchymal cell is the first major target organ to interact with insulin from the pancreas and is responsible for degrading the majority of incoming insulin (29, 30). After initial binding to hepatic surface receptors, insulin activates receptor tyrosine kinase activity and the receptor is rapidly internalized (39, 48). As isolated liver endosomes harbor activated insulin receptor kinase after internalization (48, 49), insulin dissociation and degradation consequent to endosomal acidification may limit the extent and duration of insulin receptor kinase activity and thereby ensure rapid termination of the insulin response in liver.

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