Colocalization of chaperone Cpn60, proinsulin and convertase PC1 within immature secretory granules of insulin-secreting cells suggests a role for Cpn60 in insulin processing

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Accepted 20 March; published on WWW 10 May 2000

SUMMARY

Many of the mechanisms that control insulin processing and packaging by interaction with different elements along the secretory pathway remain poorly understood. We have investigated the possibility that Cpn60, a member of the heat shock protein family, may be present in rat insulin-secreting cells, participating in the proinsulin-insulin maturation process. Immunofluorescence and high resolution immunocytochemical studies revealed the presence of the Cpn60 protein all along the insulin secretory pathway, being particularly abundant over the proinsulin-containing immature secretory granules. Double-labeling experiments showed associations between Cpn60 and proinsulin, as well as between Cpn60 and PC1 convertase, with a preferential binding to proinsulin. These findings paralleled those of coimmunoprecipitation studies showing the Cpn60 chaperone and the mature form of the PC1 convertase in proinsulin immunoprecipitates, as well as the PC1 in Cpn60 immunoprecipitates from total islet cell extracts. In vitro binding of Cpn60 to proinsulin, insulin and glucagon was also documented. Cpn60, significantly abundant in proinsulin-containing secretory granules where conversion of proinsulin to insulin takes place, and the colocalization of the chaperone with proinsulin and PC1 convertase suggest that the Cpn60 protein may play a role directing precise molecular interactions during insulin processing and/or packaging.

Key words: Chaperonin, Pancreas, Insulin, Proinsulin, Secretion, Immunocytochemistry

INTRODUCTION

Secretion of insulin involves synthesis on ribosomes of the precursor form of the hormone, preproinsulin, followed by segregation in the rough endoplasmic reticulum and subsequent removal of the signal peptide. Resulting proinsulin chains are then transferred from the rough endoplasmic reticulum to the Golgi apparatus, where packaging into secretory granules takes place (Steiner et al., 1984). Conversion of proinsulin into insulin and C-peptide, by the combined action of two convertases PC1 and PC2, occurs in acidifying clathrin-coated secretory granules at the trans-side of the Golgi apparatus (Orci et al., 1986, 1987; Malide et al., 1995). Finally, after appropriate stimulation, mature secretory granules are transported toward the plasma membrane, to which they fuse to discharge their content into the extracellular space. Although most of the cellular and molecular mechanisms that control synthesis and secretion of pancreatic endocrine products are being identified, the processing, packaging and organization of secretory products along the secretory pathway still remain poorly understood.

The presence of molecular chaperones, specialized proteins that play essential roles in enabling polypeptides to reach biologically active forms in a variety of cellular compartments, has been well demonstrated in both eukaryotic and prokaryotic cells (Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). Components of the most-studied family of chaperone proteins, the chaperonins, are known to be involved in successful folding, sorting, transmembrane transport and assembly of oligomeric polypeptide complexes (Hemmingsson et al., 1988; Ellis and Van der Vies, 1991; Gatenby, 1992; Hendrick and Ulrich-Hartl, 1993). Two chaperonins, Cpn60 and Cpn10 (also known as GroEL and GroES), have been found in bacteria (Chandrasekhar et al., 1986; Terlesky and Tabita, 1991; Torres-Ruiz and McFadden, 1992), higher plants (Hemmingsson et al., 1988; Bertsch et al., 1992; Hartman et al., 1992), and mitochondria and peroxisomes of mammalian cells (McMullin and Hallberg, 1988; Lubben et al., 1990; Velez-Granell et al., 1995). Pieces of evidence suggest that chaperonins may play decisive roles in cell secretion (Agard, 1993; Craig, 1993), and we have previously shown that chaperonins are involved in the proper packaging and aggregation of secretory proteins in pancreatic acinar cells, and proposed that they could prevent early enzyme activation.
Cpn60 immunolabelings were conducted using rabbit polyclonal antibodies following standard techniques. In order to minimize nonspecific interactions, primary antibodies were incubated overnight at 4°C with Protein A-Sepharose prior to performing the immunoprecipitation of the lysates. Material bound to the Protein A-Sepharose beads after immunoprecipitation was further separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose filters using the Mini-PROTEAN II and Mini Trans-Blot cell system, as recommended by the manufacturer (BioRad Laboratories Ltd., Canada). Western blotting immunodetections of Cpn60 and PC1 proteins on nitrocellulose filters were conducted following well-described methods (Towbin and Gordon, 1984), using the commercial anti-Cpn60 antibody or the N-terminal-directed anti-PC1 antibody, diluted 1:2000. Either the BM chemiluminescence western-blotting kit (Boehringer-Mannheim, Germany) or the alkaline phosphatase technique revealed the presence of antigen-antibody complexes on filter membranes. Staining of total proteins in the immunoprecipitate, bound to the filter membranes, was performed using 0.1% Coomassie Blue in 50% methanol for 15 minutes followed by several destaining washes with 10% acetic acid in 50% methanol.

The affinity of Cpn60 for pancreatic hormones was further defined in vitro by a dot immunoblotting assay. 1 μg each of insulin, proinsulin and glucagon (all from Sigma Co., St Louis, MO, USA) were applied onto a nitrocellulose membrane using the BRL slot filtration manifold apparatus (Bethesda Research Laboratories, Bethesda, MD, USA). After blocking for 2 hours with 0.5% BSA in TBS (10.5 mM Tris-HCl and 257 mM NaCl, pH 7.5), the membrane was incubated with 1 μg/ml of purified Cpn60 protein with or without 1 mM ATP in TBS for 3 hours at room temperature. Further steps to detect hormone-Cpn60 complexes were performed using the polyclonal anti-Cpn60 antibody diluted 1:200 and the alkaline phosphatase technique, as previously described (Velez-Granell et al., 1994). Cpn60 protein immobilized onto the nitrocellulose membrane was used as a reference. Omission of the Cpn60 protein incubation after immobilization of hormones was used as an internal control.

RESULTS

Cpn60 in pancreatic endocrine cells

Immunofluorescence labeling studies on pancreas tissue sections clearly demonstrated the presence of the Cpn60 chaperone in pancreatic endocrine cells. A strong positive signal was readily seen in the cytoplasm of islet cells (Fig. 1a). Signal was virtually absent or significantly decreased in control experiments (Fig. 1b). Immunolabeling for Cpn60 at the electron microscope level confirmed the immunofluorescence findings and demonstrated the presence of Cpn60 over both pancreatic insulin- and glucagon-secreting cells. Gold particles revealing Cpn60 antigenic sites were located over the rough endoplasmic reticulum, the Golgi apparatus and the secretory granules of the insulin-secreting cells (Fig. 2). Although both immature and mature insulin secretory granules were labeled, gold particles were more abundant over the immature ones (Fig. 2). As expected, some labeling was also evident over mitochondria. Gold particles revealing Cpn60 antigenic sites

within zymogen granules (Velez-Granell et al., 1994; Arias et al., 1994; Bruneau et al., 2000).

We demonstrate in the present study that Cpn60 chaperonin in pancreas is not restricted to the acinar portion of the tissue. It was also detected in insulin and glucagon-secreting endocrine cells using high-resolution immunocytochemistry and western immunoblotting analysis. Cpn60 protein was revealed all along the secretory pathway of the rat pancreatic insulin-secreting cells. The fact that Cpn60 appeared to be preferentially concentrated within proinsulin-containing secretory granules and was found to be associated to proinsulin and PC1 convertase prompted us to postulate its implication in the proinsulin-insulin maturation process.

MATERIALS AND METHODS

Antibodies

Cpn60 immunolabelings were conducted using rabbit polyclonal antibodies raised against the Cpn60 protein isolated either from E. coli (Epiconcentre Technologies, Madison, WI, USA) or from C. vinosum (Torres-Ruiz and McFadden, 1992). Other specific antibodies employed for immunodetections included a rabbit polyclonal antibody directed against the N-terminal end of mPC1 (Seidah et al., 1991), a guinea pig anti-bovine insulin antibody and a monoclonal antibody against proinsulin (ICN immunobiologics, Lisle, IL, USA).

Immunocytochemistry

Pancreatic tissue from normal Sprague-Dawley rats (100-200 g body mass) was fixed in situ with 1% glutaraldehyde and small fragments were processed for embedding in paraffin, Lowicryl K4M or Unicryl, as previously described (Bendayan, 1995). Cpn60 in rat pancreas was detected by immunofluorescence using the previously characterized polyclonal anti-Cpn60 antibodies on 5 μm thick paraffin sections, following standard techniques. Working conditions have been already described in detail (Velez-Granell et al., 1994).

For studies at the electron microscope level the high-resolution protein A-gold immunolabeling technique was applied (Bendayan, 1995). Thin sections from Lowicryl- or Unicryl-embedded rat pancreas, and primary antibodies against Cpn60, PC1, insulin and proinsulin, were used. Labeling conditions have already been reported (Velez-Granell et al., 1994; Malide et al., 1995; Bendayan, 1989). Sections were stained with uranyl acetate and examined with a Philips 410 electron microscope. Control experiments included preadsorption of each antibody with its corresponding antigen prior to labeling, and omission of the primary antibody with incubation with the protein A-gold complex alone (Bendayan, 1995). Double labelings for Cpn60 and insulin, proinsulin or PC1 were also performed following reported techniques (Bendayan, 1995). The presence of the Cpn60 chaperonin in double-labeling experiments was always revealed by 10 nm gold particles while insulin, proinsulin or PC1 were revealed by 15 nm gold particles, although occasionally 5 nm gold particles were also used. Quantitative evaluations of single (over 100 micrographs) and double (at least 25 micrographs at 42,250 magnification) labelings were conducted as previously described (Bendayan, 1995). The percentage of labeling association was further obtained from the double-labeling experiments (Fig. 1b). Immunolabeling for Cpn60 at the electron microscope level revealed all along the secretory pathway of the rat pancreatic insulin-secreting cell. The fact that Cpn60 appeared to be preferentially concentrated within proinsulin-containing secretory granules and was found to be associated to proinsulin and PC1 convertase prompted us to postulate its implication in the proinsulin-insulin maturation process.

Immunoprecipitation and immunodetection of Cpn60 on nitrocellulose filters

Islets from adult rat pancreas were isolated as previously reported in order to obtain total islet cell extracts (Arias and Bendayan, 1993). After isolation, the islets were rinsed with phosphate-buffered saline and lysed in a modified RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% Nonidet P40, 0.25% sodium deoxycholate, 200 μg/mL PMSF and 1× protease inhibitor cocktail (Sigma Co., St Louis, MO, USA). Complete cell disruption was accomplished by repeatedly forcing the lysate through a syringe with a 25-gauge needle followed by a cycle of freezing and thawing. Cell lysate was then clarified by centrifugation at 500 rpm for 5 minutes and kept at −20°C until use. Immunoprecipitation studies of islet cell extracts were conducted using Protein-A-Sepharose and the anti-proinsulin or the anti-Cpn60 antibodies following standard techniques. In order to minimize nonspecific interactions, primary antibodies were incubated overnight at 4°C with Protein A-Sepharose prior to performing the immunoprecipitation of the lysates. Material bound to the Protein A-Sepharose beads after immunoprecipitation was further separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose filters using the Mini-PROTEAN II and Mini Trans-Blot cell system, as recommended by the manufacturer (BioRad Laboratories Ltd., Canada). Western blotting immunodetections of Cpn60 and PC1 proteins on nitrocellulose filters were conducted following well-described methods (Towbin and Gordon, 1984), using the commercial anti-Cpn60 antibody or the N-terminal-directed anti-PC1 antibody, diluted 1:2000. Either the BM chemiluminescence western-blotting kit (Boehringer-Mannheim, Germany) or the alkaline phosphatase technique revealed the presence of antigen-antibody complexes on filter membranes. Staining of total proteins in the immunoprecipitate, bound to the filter membranes, was performed using 0.1% Coomassie Blue in 50% methanol for 15 minutes followed by several destaining washes with 10% acetic acid in 50% methanol.
Colocalization of Cpn60 with proinsulin and insulin

Immature secretory granules are known to be the site of proinsulin conversion (Orci et al., 1986, 1987; Malide et al., 1995). The particular augmentation of Cpn60 labeling seen on immature granules thus suggests a distinct biological role for this chaperone protein during the processing of insulin. Double-labeling experiments for Cpn60 and insulin, proinsulin and PC1 convertase were performed. Gold particles revealing Cpn60, proinsulin and PC1 were particularly abundant on immature granules after proinsulin/Cpn60 and PC1/Cpn60 double-labeling experiments (Fig. 5). Although the number of gold particles revealing insulin antigenic sites was particularly abundant over mature secretory granules after insulin/Cpn60 double labeling, those revealing Cpn60 were few in number (Fig. 5b). These findings led us to postulate a hypothetical situation where Cpn60 colocalizes and binds to proinsulin within immature secretory granules. More stringent quantitative assessment of the labelings was thus conducted to determine the levels of association after double-labeling experiments. While 59% of the gold particles revealing Cpn60 were found to be associated with those revealing proinsulin on the immature granules, only 12.5% of association was found in the Golgi apparatus. Equivalent association values for mature granules could not be obtained since these were virtually devoid of proinsulin labeling (Fig. 5). Association of Cpn60 with insulin at the level of the mature granules was about 34%. On the other hand, due to the low level of Cpn60 labeling and the high labeling of insulin in the mature granules, only 3% of the gold particles revealing insulin were found to be associated with gold particles revealing Cpn60. Counterpart association values for immature granules or Golgi apparatus were not, however, performed due to the well-documented cross-reaction of the anti-insulin antibody with proinsulin and the absence of mature insulin in these compartments.

Additional evidence to confirm the particular proinsulin-Cpn60 interaction came from co-immunoprecipitation studies using the anti-proinsulin monoclonal antibody. In fact, a single 57 kDa band corresponding to the Cpn60 protein was clearly found after western blot analysis of material previously immunoprecipitated by the anti-proinsulin antibody from total islet extracts (Fig. 6).

Colocalization of Cpn60 with PC1/3 endoprotease

The involvement of convertases in the processing of proinsulin has been well documented (Baillyes et al., 1992; Benjannet et al., 1992; Lipkind and Steiner, 1999; Malide et al., 1995). To test whether there is a concerted action of convertases and Cpn60 in the processing of proinsulin inside immature secretory granules, double-labeling experiments for Cpn60 and PC1 were performed. As expected, gold particles revealing Cpn60 or PC1 were particularly abundant over the immature secretory granules. About 30% of gold particles revealing Cpn60 were found associated to those revealing PC1 (Fig. 5c). As for Cpn60 protein, the mature 66 kDa form of the PC1/3 endoprotease was also detected in the proinsulin immunoprecipitate (Fig. 6). For PC1-Cpn60 interaction, an immunoprecipitation of the islet extracts was further carried out with the anti-Cpn60 antibody. A band of 66 kDa

Table 1. Densities of labeling* obtained with the anti-Cpn60 over different cellular compartments of the rat pancreatic insulin-secreting cells, under specific and control conditions

<table>
<thead>
<tr>
<th>Cellular compartment</th>
<th>Anti-Cpn60</th>
<th>Preadsorbed anti-Cpn60</th>
</tr>
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<tbody>
<tr>
<td>Endoplasmic reticulum</td>
<td>10.04±1.44</td>
<td>1.88±0.47</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>12.89±2.21</td>
<td>0.43±0.43</td>
</tr>
<tr>
<td>Immature granules</td>
<td>89.92±5.57</td>
<td>2.97±0.42</td>
</tr>
<tr>
<td>Mature granules</td>
<td>27.03±3.21</td>
<td>5.53±0.68</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>9.36±1.58</td>
<td>1.85±0.51</td>
</tr>
<tr>
<td>Nucleus</td>
<td>3.75±1.40</td>
<td>0.15±0.06</td>
</tr>
</tbody>
</table>

*Gold particles/μm². Values are means ± s.e.m.
Corresponding to the mature form of PC1 was also revealed after western analysis of the Cpn60 immunoprecipitate with the anti-PC1 antibody (Fig. 6).

Coomassie Blue staining of total proteins in the immunoprecipitate reveals only a few bands, corresponding mainly to those revealed by the anti-Cpn60 and the anti-PC1 (Fig. 6). When the anti-Cpn60 polyclonal antibody was used for immunoprecipitation, an additional band was clearly present at about 50 kDa molecular mass, corresponding most probably to unrelated contaminating immunoglobulins present in the polyclonal serum (Harlow and Lane, 1988).

Finally, double labeling with the anti-proinsulin and the anti-PC1 antibodies demonstrated a strong colocalization of these two proteins (Fig. 5d).

**In vitro binding of Cpn60**

The binding capacity of Cpn60 protein to various pancreatic peptides bound to nitrocellulose filters was determined. The addition of Cpn60 protein in the presence of ATP to insulin, proinsulin and glucagon bound to nitrocellulose filters in vitro resulted in hormone-Cpn60 association, as detected by the anti-Cpn60 antibody (Fig. 7). No hormone-Cpn60 association could be detected in the absence of ATP, or in the absence of chaperone protein in the control experiment (Fig. 7). Pure
Cpn60 protein bound to the nitrocellulose filter was used as internal positive control.

**DISCUSSION**

We have provided immunocytochemical and biochemical evidence for the presence of Cpn60 chaperone along the secretory pathway of insulin-secreting cells of pancreatic islets. Cpn60 was found to be preferentially associated with proinsulin within immature granules of the insulin-secreting cells. While a satisfactory explanation for the biological significance of this finding is not yet available, the presence of Hsp60 chaperones outside the well-documented mitochondrial location, particularly along the secretory pathway compartment of exocrine acinar cells, has been already reported (Arias et al., 1994; Velez-Granell et al., 1994, 1995; Soltys and Gupta, 1999; Bruneau et al., 2000). Concentration of Cpn60 within immature secretory granules in insulin-secreting cells is a novel finding that contrasts with its distribution in exocrine acinar cells. In the acinar cells, Cpn60 chaperone, like other molecular chaperones (Bruneau et al., 2000), follows the well-characterized secretory pathway, being sequentially concentrated along the ER-Golgi-condensing vacuole-zymogen granule, to be finally discharged into the pancreatic juice (Velez-Granell et al., 1994).

Further, significant association of Cpn60 with amylase and lipase suggested a role in their proper folding and assembly that may prevent early autoactivation (LeGall and Bendayan, 1996; Bruneau et al., 2000). The intracellular distribution of Cpn60 chaperone in insulin-secreting cells that we obtained using similar analytical methodologies was remarkably different, strongly suggesting that it has precise biological functions. The present results accord with those of a previous report showing the presence of Hsp60 protein in insulin secretory granules from mouse pancreas (Brudzynski et al., 1992). In contrast to our results, however, Hsp60 was reported to be restricted to mitochondria and mature secretory granules, and a mechanism of extramitochondrial protein export has recently been proposed (Brudzynski et al., 1992; Soltys and Gupta, 1999). Although differences in animal tissue properties, such as the site of proinsulin-insulin conversion, could well explain such disparities between the findings, the properties of the various antibodies used could also explain the results. In fact, the same authors recently described different labeling patterns when two other monoclonal antibodies raised against yeast and human Hsp60 proteins were used (Brudzynski et al., 1995). Moreover, it is now clear that many of the Hsps initially considered to be singular actually represent entire families of proteins (Feder and Hofmann, 1999). Thus, it is possible that closely related proteins share highly antigenic domains or, alternatively, that a single protein with accurate temporal and/or spatial postranslational modifications can mask precise recognition epitopes from specific antibodies (Brudzynski et al., 1995; Khan et al., 1998).
In any case, the specific labelings reported here were found to be identical for at least two different polyclonal antibodies raised against the Cpn60 protein, isolated either from *C. vinosum* (Torres-Ruiz and McFadden, 1992) or from *E. coli* (Epicentre Technologies, Madison, WI, USA). In both cases, the findings contrast with those obtained for acinar cells found in sections from the same tissue, which were used as internal controls for the experiments. Close agreement between morphological and biochemical evidence of Cpn60 location supports the theory that this chaperone has a still undefined role during the biogenesis of the secretory granule in the insulin-secreting cells.

There is substantial evidence that for optimal functional properties, Cpn60 interacts with Cpn10, a 10.5 kDa protein. A. E. Arias and others

**Fig. 5.** Double-labeling experiments. (a) Double labeling of Cpn60 and proinsulin. Cpn60 was revealed by 10 nm gold-Protein A while proinsulin was revealed by 15 nm gold-Protein A. Both labels are present over the Golgi cisternae (G) and the immature secretory granules (ig). Several colocalizations of the large and small particles are visible (encircled), particularly in immature granules. The mature secretory granules (mg) appear to be unlabeled. (b) Double labeling of Cpn60 and insulin. Very few small particles (10 nm) revealing Cpn60 are found over the mature secretory granules (mg) which, on the other hand, are well labeled for insulin (15 nm gold particles). (c) Double labeling of PC1 and Cpn60. Colocalisations of PC1 (10 nm gold particles) and Cpn60 (5 nm gold particles) in immature secretory granules (ig) are encircled. Mature secretory granules (mg) are not labeled. Mitochondria (M) show labeling for Cpn60. (d) Double labeling of PC1 and proinsulin. Labeling for proinsulin (15 nm gold particles) and PC1 (5 nm gold particles) is mainly located over the immature secretory granules (ig). Mature granules (mg) show little labeling. Colocalizations of proinsulin and PC1 are encircled. Bars, 5 μm.
Involvement of Cpn60 in insulin processing

Martin et al. (1993) have shown the functional stoichiometry to be consistent with one 7-subunit Cpn10 heptamer per 14-subunit Cpn60 oligomer. Along with this, we have morphological evidence that, as in the exocrine pancreatic acinar cells (Velez-Granell et al., 1994), Cpn10 is also present in immature insulin-secreting granules (our unpublished data).

Although abundant information on the molecular and biochemical mechanisms of insulin synthesis is already available, understanding of hormone processing and packaging inside the secretory granule is still lacking (Bailyes et al., 1992). It is generally accepted that the conversion of precursor proinsulin molecule into insulin and C-peptide in rat pancreas chaperonine (Xu et al., 1997). Martin et al. (1993) have shown the functional stoichiometry to be consistent with one 7-subunit Cpn10 heptamer per 14-subunit Cpn60 oligomer. Along with this, we have morphological evidence that, as in the exocrine pancreatic acinar cells (Velez-Granell et al., 1994), Cpn10 is also present in immature insulin-secreting granules (our unpublished data).

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occurs in acidifying clathrin-coated secretory vesicles at the trans-side of the Golgi apparatus (Orci et al., 1986, 1987). Whether changes in pH are solely responsible for activation of the processing machinery leading to precise assembly of proteins inside the secretory granule is still unclear. How processing machinery works, and how the proteins or protein complexes are sorted out into the secretory pathway to accurately form secretory granules, are the subjects of intensive research (Dannies, 1999). Cpn60 protein was detected after immunoprecipitation of total islet cell extracts with a monoclonal antibody against proinsulin. Corresponding and non-exclusive binding of the chaperone to proinsulin was also observed in hormone association studies in vitro. The ATP-dependency of chaperone-hormone association fits with the firmly established concept that Hsp60 chaperones require ATP for full expression of their biological function (Landry and Gierasch, 1994; Sigler et al., 1998). Although circumstantial, these results clearly support our immunocytochemical findings of the preferential association of Cpn60 chaperone with proinsulin inside immature secretory granules. Biochemical and immunocytochemical evidence for the presence of the Cpn60 along the secretory pathway of pancreatic endocrine cells, with particular enrichment inside immature secretory granules, was obtained.

Table 2. Comparison of immunogold labeling densities* expressed as percentage values, for endocrine and exocrine pancreatic proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>RER</th>
<th>GA</th>
<th>IG</th>
<th>MG</th>
<th>References</th>
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<tbody>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Proinsulin</td>
<td>0.34</td>
<td>21.98</td>
<td>77.36</td>
<td>0.32</td>
<td>Unpublished results</td>
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<tr>
<td>Insulin</td>
<td>3.96</td>
<td>5.82</td>
<td>30.02</td>
<td>60.20</td>
<td>Bendayan, 1989</td>
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<tr>
<td>C-peptide</td>
<td>6.93</td>
<td>10.65</td>
<td>27.11</td>
<td>55.56</td>
<td>Bendayan, 1989</td>
</tr>
<tr>
<td>PCI</td>
<td>9.14</td>
<td>11.69</td>
<td>54.91</td>
<td>24.27</td>
<td>Malide et al., 1995</td>
</tr>
<tr>
<td>Cnp60</td>
<td>4.47</td>
<td>6.46</td>
<td>68.20</td>
<td>20.84</td>
<td>Present results</td>
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<td>Acinar cell</td>
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<tr>
<td>Amylase</td>
<td>6.78</td>
<td>15.10</td>
<td>26.60</td>
<td>51.52</td>
<td>Bendayan, 1984</td>
</tr>
<tr>
<td>Cnp60</td>
<td>8.14</td>
<td>18.88</td>
<td>28.17</td>
<td>44.52</td>
<td>Velez-Granell et al., 1994</td>
</tr>
</tbody>
</table>

*Results were calculated from data reported in the references, and are expressed as a percentage of total labeling density over that seen in the secretory pathway compartments. RER, rough ER; GA, Golgi apparatus; IG, immature granules; MG, mature granules.

‡Values for the condensing vacuole compartment.
granules, leads us to predict a role for the Cpn60 chaperone during processing and/or packaging of mature secretory proteins.

Along the maturation process of proinsulin, two convertases, PC1 and PC2, have thus far been recognized (Bailey et al., 1992; Benjamett et al., 1992; Lipkind and Steiner, 1999). We have recently provided evidence for the involvement of both convertases in the processing of proinsulin (Malide et al., 1995; Bendayan et al., 1995). Thus, we decided to test the hypothesis of a concerted action between Cpn60 and convertases in the processing of proinsulin. Finding the mature form of PC1 in proinsulin immunoprecipitates from total islet cell extracts supported this view. Although higher amounts of Cpn60 and PC1 proteins within immature secretory granules of insulin-secreting cells have already been reported in the literature (Table 2), we could only demonstrate a lower level of Cpn60 and PC1 association (30%) after quantitative analysis of double labelings. This lower level of association, as compared to that of Cpn60-proinsulin (59%), found by immunocytochemistry, may be explained by lower direct Cpn60-PC1 interaction or by a masking of epitopes necessary for recognition by the anti-PC1 antibody. Nevertheless, differences in the percentage associations between Cpn60 and proinsulin or PC1 emphasise the value of the immunocytochemical methodology that reveals the colocalization of two or more molecules in the same cellular compartment. As for the biological significance of the reported findings, we propose as a working hypothesis that the Cpn60 chaperone assists the adequate conformational folding of both proinsulin and PC1/PC3 endoprotease along the secretory pathway to permit accurate interaction between them during secretory granule formation. Several examples of molecular chaperones working along the insulin secretory pathway have been reported. Bip, the most abundant and best characterized molecular chaperone of the ER, which belongs to the heat shock protein 70 family, has already been demonstrated to assist the assembly of insulin intermediates (Schmitz et al., 1995). CSP-L1, a novel synaptic vesicle-associated protein that contains the signature J-domain of the DnaJA molecular chaperone/Hsp family of proteins, has also been found closely associated with membranes of insulin secretory granules (Brown et al., 1998). Cpn60 would thus be another molecular chaperone ensuring the precise molecular conformation of insulin in the secretory pathway. Interestingly, there are also reports of significant roles for Hsp60 proteins in the maturation of precursor pro-caspase-3 by upstream activator proteases during apoptosis (Xanthoudakis et al., 1999), and in interactions with extracellular molecules once they have been secreted (Bassan et al., 1998).

In summary, we have shown the presence of Cpn60 protein along the secretory pathway of pancreatic endocrine cells. The significant abundance of Cpn60 within immature secretory granules, together with its particular colocalization with proinsulin and PC1 convertase, suggest that Cpn60 protein may direct accurate molecular interactions during insulin processing and/or packaging.

We would like to thank Drs. J. A. Torres-Ruiz and N. Seidah for providing us with the anti Cpn60 and the anti-PC1 antibodies, respectively. We also acknowledge Diane Gingras for her technical assistance and Jean Lévéillé for photographic work. This work was supported by grants from the Medical Research Council of Canada.

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