Proinsulin Targeting to the Regulated Pathway Is Not Impaired in Carboxypeptidase E-deficient Cpefat/Cpefat Mice

(Received for publication, August 26, 1997)

Jean-Claude Irminger‡, C. Bruce Verchere§, Katharina Meyer, and Philippe A. Halban
From the Laboratoires de Recherche Louis Jeantet, University of Geneva, 1211 Geneva 4, Switzerland

Sorting of proinsulin from the trans-Golgi network to secretory granules is critical for its conversion to insulin as well as for regulated insulin secretion. The proinsulin sorting mechanism is unknown. Recently, carboxypeptidase E (CPE) was proposed as a sorting receptor for prohormones. To know whether CPE is implicated in proinsulin sorting, pancreatic islets were isolated from CPE-deficient Cpefat/Cpefat mice and Cpefat/+ controls, pulse-labeled ([3H]leucine), and then chased in basal medium (90 min) to examine constitutive secretion followed by medium with secretagogues (60 min) to stimulate regulated secretion. Secretion of labeled proinsulin via the constitutive pathway was <2% even in Cpefat/Cpefat islets. After a 150-min chase, only 13% of radioactivity remained as proinsulin in Cpefat/+ islets compared with 46% in Cpefat/Cpefat islets, reflecting slower conversion. Regulated secretion was stimulated to an equal extent from Cpefat/+ and Cpefat/Cpefat mice with 20% of the total content of labeled (pro)insulin released during the 60-min stimulatory period. It is concluded that in CPE-deficient Cpefat/Cpefat mice, proinsulin is efficiently routed to the regulated pathway and its release can be effectively stimulated by secretagogues. CPE is thus not essential for sorting proinsulin to granules.

Proinsulin is directed to the regulated secretory pathway of the pancreatic β-cell and is converted to bioactive insulin in immature secretory granules (1). At least three enzymes are needed for generating native insulin, namely the conversion endopeptidases PC1 (also known as PC3) and PC2 (2, 3) and carboxypeptidase E (also called carboxypeptidase H or CPE) for trimming residual basic C-terminal residues (4–6). Correct targeting to secretory granules is essential for the cell to produce and store bioactive insulin, which can then be released upon stimulation of the β-cell by glucose or other secretagogues. One of the crucial steps in targeting takes place in the TGN (trans-Golgi network) (7). The proprotein must somehow be recognized selectively in the TGN and then be transferred to the immature secretory granules, instead of being released through the constitutive or default pathway (3).

One hypothesis (reviewed in Ref. 3) accounting for targeting within the TGN implicates a sorting receptor with a broad specificity in the TGN membrane, which recognizes a sorting domain and thereby binds proteins to be routed to the regulated pathway. Sorting domains have been reported for several prohormones including prosomatostatin (8, 9), chromogranin (10), and the basic proline-rich protein (11). An N-terminal sorting domain has been reported for proopiomelanocortin (POMC) (12, 13), although this is controversial (14). A putative C-terminal sorting domain has also recently been proposed for the prohormone convertase PC2 (15). There is not as yet any direct experimental evidence for a sorting domain for proinsulin, although the comparison of structural features of many prohormones suggests that a region within the insulin B-chain may play such a role (16, 17).

Very recently, CPE has been proposed as the targeting receptor for POMC (18) with evidence that it interacts with the N-terminal sorting domain on this prohormone. Further, proinsulin and other proproteins, which are normally secreted through the regulated pathway but not proteins which are released through the constitutive pathway, were shown to displace POMC (or a peptide encompassing its sorting domain) from this putative sorting receptor (18). It was subsequently shown that Cpefat/Cpefat mice appear to misroute POMC, resulting in its abnormal conversion and secretion (19).

Obese Cpefat/Cpefat mice suffer from hyperproinsulinemia associated with expression of a mutated CPE, which results in destruction of the CPE protein in the rough endoplasmic reticulum (20, 21). The pancreatic β-cells of these mice are clearly granulated but the content of the granules appears to be less dense (20), suggesting storage of proinsulin instead of mature insulin. Despite this observation, it has been suggested that proinsulin fails to be targeted to the regulated pathway in β-cells of these mice and that CPE is the sorting receptor not only for POMC but for other prohormones including proinsulin itself (18, 19). Confronted by this discrepancy, we wanted to know whether CPE is indeed implicated in proinsulin sorting. To this end, we have examined whether proinsulin is efficiently targeted to the regulated secretory pathway or released in major part through the constitutive pathway in primary β-cells in islets isolated from CPE-deficient Cpefat mice.

EXPERIMENTAL PROCEDURES

Materials—C57BLKS-Cpefat/J male mice were purchased from The Jackson Laboratories, Bar Harbor, ME. Chemicals were from Fluka (Buchs, Switzerland). [3H]Leucine (120 Ci/mmol) was from ARC (St. Louis, MO). Collagenase A (catalog no. 17449) was purchased from Serva (Buchs, Switzerland).

Isolation of Intact Mouse Islets—The pancreas was removed from mice at 8 to 11 weeks of age and digested in collagenase (1 mg/ml) for 18–20 min, and the islets were hand-picked under a dissecting microscope after coloration with dithizone. For each experiment, islets from 6 mice were pooled and cultured for 24 h in Dulbecco's modified Eagle's medium (8.3 mM glucose), 10% fetal calf serum before use to recover from the isolation procedure.
RESULTS AND DISCUSSION

Intact mouse islets were isolated from Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} or Cpe\textsuperscript{fat}/+ control mice and cultured for 24 h before use. The islets were pulse-labeled (20 min, [\textsuperscript{3}H]Leu) and chased for 30-min intervals up to 90 min in basal medium containing 1.7 mM glucose. During these initial 90 min of chase, it was reasoned that under nonstimulatory conditions any newly synthesized (labeled) proinsulin in the medium would have been secreted predominantly by the constitutive pathway. At 90 min, medium containing the secretagogue mixture (see “Experimental Procedures”) was added, and the islets were incubated for an additional 60 min. Secretion of labeled proinsulin or insulin during this period reflects in major part that released via the regulated pathway. At the end of the total chase period of 150 min, the islets were extracted in acid. At this time, the majority of labeled proinsulin/insulin can be assumed to be stored in granules of the regulated secretory pathway; any proinsulin diverted to the constitutive pathway would thus have been secreted within this 150-min period.

The major products observed in the islet cell extracts of these cells after 150 min of chase (Fig. 1C) is quite in keeping with proinsulin having been directed to secretory granules in which it had been converted to insulin and stored. In Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets, by contrast, the major radioactive peak was proinsulin-I (Fig. 2C), accounting for 46.3 ± 2.4% of total proinsulin-I + II radioactivity and insulin-I+II. This is quite in keeping with proinsulin having been directed to secretory granules in which it had been converted to insulin and stored. In Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets, by contrast, the major radioactive peak was proinsulin-I (Fig. 2C), accounting for 46.3 ± 2.4% of total proinsulin-I + II radioactivity and insulin-I+II. The presence of such a secretory product in cells after 150 min is suggestive of its being stored in granules of the regulated pathway, given that no such storage compartment exists for the constitutive pathway. The peaks could correspond to arginine-extended forms of insulin or other proinsulin conversion intermediates. Given that these minor peaks each accounted for no more than 10% of the total labeled products and that the present study addressed proinsulin sorting rather than processing, they were not analyzed in more detail.

Table I

|                       | Chase medium 30–90 min | Chase medium 90–150 min | Cell extract at 150 min |
|-----------------------|------------------------|-------------------------|-------------------------|
| Cpe\textsuperscript{fat}/+ | 0.8 ± 0.4              | 8.9 ± 3.2               | 78.1 ± 5.5              |
| Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} | 1.5 ± 0.4              | 20.4 ± 5.1              | 78.1 ± 5.5              |

have been secreted within this 150-min period.

The medium and the cell extracts were analyzed on HPLC as described in Fig. 1. The radioactivity eluting from HPLC as proinsulin-insulin is expressed as a percentage of the total proinsulin-insulin radioactivity found in the chase media (basal plus stimulated) plus that found in the cell extracts. Data are mean ± S.E. for three independent experiments for Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets and one of two independent experiments for controls.

Analysis of Proinsulin Secretion by Pulse-Chase Experiments—The islets were incubated for 15 min in KRB (16.7 mM glucose) then labeled with 2 mCi/ml [\textsuperscript{3}H]leucine for 20 min in KRB (16.7 mM glucose), washed three times, and chased in KRB (+1 mM unlabeled leucine) containing 1.7 mM glucose (“basal”) for 30-min intervals up to 90 min. After each interval, the medium was collected, and fresh medium was added. From 90 to 150 min of chase, the islets were incubated in KRB containing 1.7 mM glucose and the additional secretagogues. The cells were extracted at the end of the entire 150 min of chase. A, combined basal medium from 30–90 min of chase; B, 90–150 min stimulated medium; C, cell extract. The elution times for authentic standards are as indicated by the arrows: 1, insulin I; 2, insulin II; 3, proinsulin I + II. Representative of two independent experiments.

Fig. 1. HPLC analysis of labeled (pro)insulin-like products from Cpe\textsuperscript{fat}/+ islets. The islets were labeled with [\textsuperscript{3}H]leucine for 20 min and then chased for 30-min intervals in 1.7 mM glucose up to 90 min followed by an additional 60 min of chase in medium containing 16.7 mM glucose and the additional secretagogues. The cells were extracted at the end of the entire 150 min of chase. A, combined basal medium from 30–90 min of chase; B, 90–150 min stimulated medium; C, cell extract. The elution times for authentic standards are as indicated by the arrows: 1, insulin I; 2, insulin II; 3, proinsulins I + II. Representative of two independent experiments.

Fig. 2. HPLC analysis of labeled (pro)insulin-like products from Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets. The islets were labeled and chased as in Fig. 1. A, combined basal medium from 30–90 min of chase; B, 90–150 min stimulated medium; C, cell extract. The elution times for authentic standards are as indicated by the arrows: 1, insulin I; 2, insulin II; 3, proinsulins I + II. Representative of three independent experiments.
Proinsulin Targeting in CPE-deficient Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} Mice

The reason for slow proinsulin conversion in Cpe\textsuperscript{fat} / Cpe\textsuperscript{fat} mouse islets is unknown. Although mis-sorting to the constitutive pathway could account for poor proinsulin conversion, our data do not support this model. It is therefore probable that lack of CPE activity in granules in some way inhibits activity of the conversion endoproteases per se. Most recently, a study in β-cell lines derived from the Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} mice (NIT-2 and NIT-3 cells) confirmed that this CPE mutation leads to inhibition of proinsulin conversion while suggesting that at least some proinsulin was directed to granules.

The mere presence of proinsulin in a putative storage compartment is not proof in itself of sorting to the regulated pathway. This can only be addressed by following the kinetics of secretion of newly synthesized (labeled) proinsulin/insulin. Very little proinsulin could be detected in the basal medium of either control (Cpe\textsuperscript{fat}+/) or Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets, indicating that constitutive release was minimal in both cases. Essentially no detectable proinsulin was released during the initial 30 min of chase. Thereafter, from 30–90 min of chase under basal conditions, there was 1% proinsulin (of total content) released from the Cpe\textsuperscript{fat}+/ islets and 1.5% from the Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets (Figs. 1A and 2A, Table I). A value of 1% is close to that reported by us previously from rat islets (24) and reflects remarkably efficient proinsulin sorting, notably even in Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} mice.

The hallmark of the regulated pathway is its sensitivity toward secretagogues. Release of proinsulin/insulin was stimulated to a comparable extent from control (Cpe\textsuperscript{fat}+/) and Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets. After 60 min of stimulation, 20% of total (cellular) proinsulin had been released from the islets of both groups (Table I). Furthermore, the HPLC profile for labeled products released following stimulation was essentially identical to that of stored cellular products (Fig. 1, B versus C, and Fig. 2, B versus C). Taken together with the values for constitutive release described above, these data indicate clearly that the overwhelming majority of proinsulin, even in Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} islets, had been correctly sorted to granules.

We conclude that even if proinsulin is not processed rapidly or extensively in the CPE-deficient Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} mice, it is efficiently targeted to the regulated pathway and stored in secretory granules. The lack of CPE does not have any apparent influence on proinsulin routing and therefore it is most unlikely that CPE is the regulated secretory pathway sorting receptor for proinsulin, at least in the Cpe\textsuperscript{fat}/Cpe\textsuperscript{fat} mouse model. In a very recent report, the putative role of CPE as a sorting receptor has been questioned on theoretical grounds (25). It cannot be excluded at this point that different classes of sorting receptors or even different sorting mechanisms can co-exist, responsible for targeting of different subsets of prohormones. Although it remains to be explained why proinsulin is so efficient in displacing POMC from CPE (18), the physiological relevance of this finding must be revisited in the light of the present study, which demonstrates unequivocally that CPE is not indispensable for proinsulin sorting to the regulated pathway.

Acknowledgments—We thank Dr. Edward Leiter (The Jackson Laboratories, Bar Harbor, ME) for sending a copy of the study by Varlamov et al. prior to publication.

REFERENCES

1. Ori, L., Ravazzola, M., Amherdt, M., Madsen, O., Vassalli, J. D., and Perrelet, A. (1985) Cell 22, 671–681
2. Davidson, H. W., Rhodes, C. J., and Hutton, J. C. (1988) Nature 333, 93–96
3. Halban, P. A., and Irminger, J. C. (1994) Biochem. J. 300, 1–18
4. Davidson, H. W., and Hutton, J. C. (1987) Biochem. J. 245, 575–582
5. Fricker, I. D. (1988) Annu. Rev. Physiol. 50, 309–321
6. Guest, P. C., Arden, S. D., Rutherford, N. G., and Petz, L. N. (1993) J. Clin. Invest. 92, 1042–1054
7. Ori, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powell, S. K., Quinn, D. L., and More, H. P. (1987) Cell 51, 1039–1051
8. Sevarino, K. A., Stork, P., Ventimiglia, R., Mandel, G., and Goodman, R. H. (1989) Cell 57, 11–19
9. Stoller, T. J., and Shields, D. (1989) J. Cell Biol. 108, 1647–1655
10. Parmer, R. J., Xi, X. P., Wu, H. J., Helman, L. J., and Petz, L. N. (1993) J. Cell Biol. 120, 294–306
11. Castle, A. M., Stahl, L. E., and Castle, J. D. (1992) J. Biol. Chem. 267, 13093–13100
12. Tam, W. W., Andreasson, K. I., and Loh, Y. P. (1993) Eur. J. Cell Biol. 62, 872–879
13. Cool, D. R., Fenger, M., Snell, C. R., and Loh, Y. P. (1995) J. Biol. Chem. 270, 575–582
14. Roy, P., Chevrier, D., Fournier, H., Chantal, R., Zollinger, M., Crine, P., and Boileau, G. (1991) Mol. Cell Endocrinol. 82, 237–250
15. Creemers, J. W., Usar, E. F., Bright, N. A., Van de Loo, J. W., Jansen, E., Van de Ven, W. J. M., and Huttner, W. B. (1996) J. Biol. Chem. 271, 25284–25291
16. Kizer, J. S., and Tropsha, L. A. (1991) Biochem. Biophys. Res. Commun. 174, 586–592
17. Gep, S. U., and Darling, D. S. (1995) FERS Lett. 25, 5314–5319
18. Davidson, H. W., Normant, É., Chen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1995) Cell 88, 73–83
19. Chen, F. S., and Loh, Y. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5314–5319
20. Nagert, J. R., Fricker, L. D., Varlamov, O., Nishina, P. M., Roushie, Y., Steiner, D. F., Carroll, R. J., Psinger, B. J., and Leiter, E. H. (1995) Nat. Genet. 10, 135–142
21. Fricker, L. D., Berman, Y. L., Leiter, E. H., and Devi, L. A. (1996) J. Biol. Chem. 271, 30619–30624
22. Sisonenok, S. V., and Halban, P. A. (1991) Biochem. J. 278, 621–625
23. Irminger, J. C., Vollenweider, F. M., Neerman-Arbez, M., and Halban, P. A. (1994) J. Biol. Chem. 269, 1756–1762
24. Rhodes, C. J., and Halban, P. A. (1987) J. Cell Biol. 105, 145–153
25. Thiele, C., Gerdes, H. H., and Huttner, W. B. (1997) Curr. Biol. 7, R496–R500

---

2 O. Varlamov, L. D. Fricker, H. Furukawa, D. F. Steiner, S. H. Langley, and E. H. Leiter, submitted for publication.