Modulation of Secretory Granule-targeting Efficiency by Cis and Trans Compounding of Sorting Signals*

Received for publication, July 29, 2004, and in revised form, November 23, 2004
Published, JBC Papers in Press, November 29, 2004, DOI 10.1074/jbc.M408658200

Marie-Josée Lacombe‡, Chantal Mercure‡, Jimmy D. Dikeakos, and Timothy L. Reudelhuber§
From the Laboratory of Molecular Biochemistry of Hypertension, Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7, Canada

Several protein domains acting through seemingly different mechanisms have been reported to have the capacity to target proteins to dense core secretory granules. Because proteins enter secretory granules with different efficiencies and because some of these proteins contain more than one granule-targeting motif, we have investigated whether compounding sorting signals could alter the efficiency of protein entry into secretory granules. In the current study we demonstrate that a paired basic cleavage site from human prorenin and an α-helix-containing secretory granule-sorting signal from the prohormone convertase PC1/3 can synergize to increase granule-sorting efficiency not only when located on the same protein, but also when located on distinct proteins that associate in the secretory pathway.

Once proteins in the secretory pathway arrive in the trans-Golgi network (TGN)1 they must be sorted to their appropriate cellular destination. Although certain proteins (e.g. cathepsin B) are sorted to lysosomes, other proteins are recycled into earlier secretory compartments by recognition of a retention signal (e.g. KDEL for the endoplasmic reticulum). Still other proteins are secreted constitutively from the cell at a rate that is only limited by their rate of synthesis. This pathway, called the constitutive secretory pathway, is thought to be the default pathway for proteins that do not contain a specific sorting or retention signal. Finally, certain endocrine and neuroendocrine cells send a specific subset of proteins to electron-dense cytoplasmic granules (dense core secretory granules) where they are stored until the cell receives a signal for their release. This process, called regulated secretion, depends on a highly selective triage of proteins to electron-dense cytoplasmic vesicles, although these vesicles do not contain all of the characteristics of secretory granules (12). Proinsulin, proopiomelanocortin, and engineered proteins containing the C-terminal tail of PC1/3 also form multimers; however, this property alone is insufficient to direct granule sorting (3, 13). A second group of granule-sorting domains may act by anchoring cargo proteins to other granule-resident proteins including carboxypeptidase E (2) and (14). Finally, granule-sorting domains in some granule-resident proteins such as PC1/3 (3, 7), PC2 (6), and carboxypeptidase E (5) bind to membranes raising the possibility that specific lipids in the TGN act to either sequester or retain such proteins in nascent granules. The existence of multiple sorting domains has almost certainly contributed to the historical difficulty in assigning a canonical sorting signal for granule cargo proteins.

The efficiency with which proteins enter secretory granules can also vary significantly. For example, in the juxtaglomerular cells of the kidney it has been estimated that only 25–30% of prorenin is sorted to secretory granules where it is converted to active renin (15). This inefficient sorting is in large part responsible for the fact that circulating levels of prorenin are 5–10 times higher than those of active renin in humans (16). Conversely, proinsulin is targeted almost quantitatively to secretory granules (17) where a high percentage is converted to active insulin. As a result, mature insulin levels are roughly five times higher than those of proinsulin in circulation in normoglycemic humans (18). Because the proteolytic activation of many of these precursors only occurs after their entry in secretory granules (19–21), modulation of the granule-sorting efficiency could represent a key step of evolutionary selection in controlling animal physiology. Although some of the variation in sorting efficiency could be due to differences in the internal machinery of the cell types expressing these proproteins, they could also conceivably be due to the exposure, multiplicity, and the particular combination of sorting signals present on the sorted precursor proteins. In the current study we have tested the latter hypothesis and demonstrate that unique secretory granule-sorting signals can be compounded both in cis and in trans to modulate the efficiency of protein targeting to dense core secretory granules.

* This work was supported by operating Grant MOP-53177 from the Canadian Institutes for Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Clinical Research Inst. of Montreal, 110 Pine Ave. W., Montreal, QC H2W 1R7, Canada. Tel.: 514-987-5716; Fax: 514-987-5717; E-mail: reudel@ircm.qc.ca.

§ The abbreviations used are: TGN, trans-Golgi network; HA, hemagglutinin; TBS, Tris-buffered saline; PC, prohormone convertase.
FIG. 1. A, protein sequence of the secretory granule-sorting domains used in this study. The upper sequence corresponds to the sorting domain at the C terminus of the mouse prohormone convertase 1/3. The overlined sequence is predicted to form an α-helix, and its disruption by mutagenesis (insertion of 2 prolines as depicted below the sequence) abolishes secretory granule sorting (3). The lower sequence depicts the first 16 amino acids of the human prorenin prosegment. Mutation of the two paired basic residues shown in bold disrupts sorting of fusion proteins containing this sequence (14). B, GH4C1 cells were transiently transfected with the constructions shown at the right. The supernatants of the pulse (P), chase (C), and sequential constitutive (Na) and regulated secretion (K) samples were immunoprecipitated, fractionated by SDS-PAGE, and subjected to fluorography. SP, secretory pathway; Proseg, prosegment. C, gels like those shown in B were exposed to storage phosphor screens, and the ratio of fusion protein band intensity in the regulated (K) to constitutive (Na) secretion samples was calculated. The results are shown as the means (±S.E.) of 4–8 independent experiments. *, p < 0.05; **, p < 0.01.
Compounding Secretory Granule-sorting Signals

EXPERIMENTAL PROCEDURES

Recombinant Plasmid Construction—Naturally occurring peptide fragments to be analyzed for secretory granule sorting were derived from the mouse PC1 (GenBank™ accession number NM_013628) and the human prorenin cDNAs (GenBank™ accession number NM_000537). Site-directed mutagenesis used to identify the protein domains used is relative to initiator methionine of both proteins. Protein fragments were tested for their ability to sort heterologous proteins to secretory granules by attachment to a fragment of mouse immunoglobulin IgG2b (referred to as Fc) as described previously (3) and illustrated in Fig. 1. Fusion proteins were constructed by selective amplification of corresponding fragments using polymerase chain reaction. For co-targeting studies (Fig. 3), a peptide encoding the influenza hemagglutinin (HA) epitope (YPYDVPDYA) was inserted between the signal peptide and the 16-amino acid human prorenin prosegment by overlap extension PCR. All of the resulting coding sequences were verified in their entirety by DNA sequencing and were inserted into the pCDNA3 mammalian expression vector.

Cell Culture, Transfection, and Secretion Analysis—Rat somatotroph cell line PC1 (GenBank™ accession number NM_013628) and the human prorenin cDNA (GenBank™ accession number NM_000537) were transfected with the appropriate expression vectors. For confocal microscopy, the cells were fixed with 4% paraformaldehyde in PBS and then washed with TBS and incubated anti-rabbit IgG antibody conjugated to fluorescein (1:200, Roche Applied Science) overnight at 4 °C for 1 h. Slides were incubated with a monoclonal rat anti-HA (Sigma) antibody and the immunoprecipitated proteins were separated by SDS-PAGE. The gels were immunoblotted with protein A-Sepharose (Sigma), and the immunoprecipitated proteins were detected by fluorography as described in the legend to Fig. 1. P, pulse; C, chase; Na, sequential constitutive; K, regulated.

Synergy between Compounded Sorting Domains—Rat somatotroph cell line GH4C1 cells were transfected with a series of plasmid expression vectors in which a fragment of the mouse immunoglobulin heavy chain constant region (Fc) is linked to a signal peptide and combinations of two previously described secretory granule-sorting domains (Fig. 1A and B). These consist of the C-terminal tail of the prohormone convertase PC1/3 (3) and a dibasic protease cleavage site from the human prorenin prosegment (14). As shown in Fig. 1B, all of the fusion proteins can be detected in the supernatant of the transfected cells even during the 2-h pulse labeling period (lane P), confirming that they are efficiently synthesized and secreted by the cells. Likewise, a roughly equivalent amount of the various fusion proteins is secreted from the cells during the first 90-min chase period (lane C). A second chase period in medium supplemented with 50 mM sodium lactate (lane Na) reveals the amount of labeled protein secreted constitutively from the cells during a 20-min time window, whereas a subsequent 20-min incubation in the presence of 50 mM potassium (lane K) will cause the cells to depolarize and release secretory granule contents. Thus, the ratio of the amount of labeled fusion protein secreted in the presence of potassium versus that secreted in the presence of sodium is a reflection of amount of protein stored in secretory granules. Such an analysis (Fig. 1, B and C) reveals that only a very small amount of the Fc fusion protein containing 6 amino acids of the human prorenin prosegment (Pro6:Fc) enters granules, confirming our previous report obtained in AtT-20 cells that this region of the human prorenin prosegment is inactive in secretory granule targeting (14). The sorting efficiency of the prorenin prosegment is increased ~2-fold, however, when it is extended to contain paired basic residues (Pro16:Fc), constituting a weak sorting signal. Addition of the targeting region containing the PC1 C-terminal α-helical region to the C terminus of the Pro6:Fc fusion protein (Pro6:Fc:PC1) leads to a more efficient targeting of the fusion protein to granules than the paired basic residues. Strikingly, combining both the paired basic residues and the PC1 α-helical region in the fusion protein (Pro16:Fc:PC1) leads to an increase in sorting efficiency that appears to exceed the sum of the effects seen with the individual domains (Fig. 1, B and C). Loss of stimulated secretion when the same cells express an analogous protein in which the α-helix in the PC1/3 C-terminal tail is dis-
ruptured by proline substitutions (3) (Pro16:Fc:PC1PP) confirms that this synergy is due to the interaction of the α-helical element and the paired basic cleavage sites. These results suggest that independent sorting domains contained in cis on the same protein can act synergistically to increase secretory granule-sorting efficiency.

**Protein-Protein Association Modulates Sorting Efficiency**—To determine whether protein complex formation could alter the efficiency of either protein entry or retention in secretory granules, various combinations of the fusion proteins described above were co-expressed in GH4 cells. As immunoglobin heavy chains normally form dimers, we expected that the Fc domains of the fusion proteins would promote protein-protein interactions. As shown in Fig. 2, co-expression of the fusion proteins containing either 6 or 16 amino acids of the human prorenin prosegment did not lead to significant targeting of either protein to dense core secretory granules as evidenced by the lack of a detectable band in the potassium-stimulated supernatant (Fig. 2, lane K). Strikingly, when either of these proteins is co-expressed with the fusion protein containing a granule targeting domain from the PC1 C terminus (PC1 C-term), the HA-tagged fusion protein shifts from a predominantly TGN labeling pattern to one that includes both the TGN and the cell periphery where growth hormone-containing dense core secretory granules (G) are stored.

**Compounding Secretory Granule-sorting Signals**

FIG. 3. A, schematic diagram of the fusion proteins used to transfect GH4C1 cells. The HA peptide (YPYDVPDYA) is inserted between the signal peptide and dibasic cleavage site-containing prosegment. B, double labeling of transfected cells with antibody for endogenous growth hormone (contained in dense core granules at the cell periphery) and the HA peptide (detects only HA:Pro:Fc). The merged image shows areas stained by both markers in yellow superimposed on a Nomarski view of the cells. Note that when co-transfected with the fusion protein containing a granule targeting domain from the PC1 C terminus (PC1 C-term), the HA-tagged fusion protein shifts from a predominantly TGN labeling pattern to one that includes both the TGN and the cell periphery where growth hormone-containing dense core secretory granules (G) are stored.

FIG. 4. Heterodimer formation between the Fc fusion proteins. GH4C1 cells transfected with the corresponding expression vectors either alone or in combination were treated with a cell-permeable cross-linker, and the fusion proteins were immunoprecipitated from the lysates. The single asterisks show the migration positions of homodimers, whereas the double asterisks denote the migration of a heterodimer formed between the two fusion proteins. Numbers at the left denote the apparent molecular weights of co-migrating size standards (× 10^3).

plexes that “pull” the prorenin prosegment-containing fusion proteins into secretory granules. Formation of heterodimers between the two fusion proteins is confirmed by intracellular cross-linking experiments (Fig. 4). Although the Pro16:Fc protein shows a slight tendency to form dimers, dimerization is greatly increased when the fusion protein contains the C-terminal tail of PC1/3 (3). When the two proteins are co-expressed, a new dimer species of intermediate molecular weight is formed, confirming the formation of heterodimers in the secretory pathway of the co-transfected cells. In conclusion, trans association of granule targeting signals by protein-protein contact is also capable of increasing protein targeting to dense core secretory granules.
DISCUSSION

The identification of domains involved in targeting secretory proteins to the dense core granules of endocrine and neuroendocrine cells has been the subject of intensive research for many years. This molecular step plays a key role in vertebrate biology because it not only provides for physiological control over protein hormone secretion, but it also provides a measure of control over the efficiency of activation of certain protein precursors (such as proinsulin, proopiomelanocortin, prorenin, and others) that are only proteolytically processed after the precursor is sorted to the nascent granule. Despite the importance of this biological event, it has been difficult to agree on canonical secretory granule-sorting sequences most likely because of three main reasons: First, the protein domains required for secretory granule sorting might be different in different cell types. For example, the C terminus of the prohormone convertase PC2 is capable of directing sorting to secretory granules in Neuro2A cells (6) but is not required for PC2 sorting to granules in corticotrophic ACT-20 cells (22). Likewise, chromogranin A has been reported to require an N-terminal sorting signal for granule entry in PC12 cells, whereas a C-terminal domain is required for granule sorting in GH4C1 cells (23). Second, several distinct secretory granule-sorting signals have been reported suggesting that there is more than one mechanism for sorting proteins to secretory granules. These include paired basic residues that may interact with granule-resident PC enzymes (14, 24–26), N-terminal disulfide-bound loops (27, 28), and other domains that mediate sorting by binding to caboxypeptidase E (2), calcium-binding domains that lead to aggregation (reviewed in Ref. 8), and α-helices that either traverse or interact with membranes (3, 5–7). Finally, proteins may contain more than one single type of sorting domain so that analyses in which any one of these is eradicated will not eliminate sorting in the context of the native protein. Indeed, because insulin has been shown to aggregate in nascent granules (29), form multimers (13), bind CPE (30), and be cleaved by PC enzymes (31), all of which have been shown to direct secretory granule targeting in various systems, it is perhaps not surprising that the eradication of hexameric or the dibasic cleavage sites alone does not prevent its entry into secretory granules (13, 32).

It is puzzling that the mechanisms for sorting proteins to other cellular destinations such as the nucleus (33), the endoplasmic reticulum (34), and endosomes and lysosomes (35) display less heterogeneity than those involved in secretory granule sorting. One possible explanation is that the heterogeneity in granule-sorting signals is the result of evolutionary selection pressure to achieve greater flexibility in modulating granule-targeting efficiencies. In support of this hypothesis proinsulin, which contains multiple sorting motifs (see above), is sorted with far greater efficiency to secretory granules than is, for example, human prorenin the secretory granule sorting of which in model cell lines is dependent on a single pair of basic amino acids (14). The existence of multiple sorting mechanisms might also provide a greater measure of physiological control over sorting efficiency as a means of controlling active hormone production. Indeed, some pathological conditions can lead to increases or decreases in the constitutive release of proinsulin (36) and prorenin (15), suggesting a diversion from the regulated secretory pathway although the exact mechanisms are not yet known.

In addition, our results provide evidence that partnering of proteins in higher order complexes would enhance sorting efficiency because a sorting domain on only one of the unit monomers would be sufficient to direct sorting of associated partners. The aggregation that characterizes many secretory granule cargo proteins (8) could serve a similar function and thereby lead to a dramatic amplification of the primary tethering interactions between granule cargo and sorting acceptor proteins or lipids.

In conclusion, we show that the efficiency of protein sorting to the regulated secretory pathway and dense core secretory granules can be modulated by combining multiple, distinct sorting signals either in cis or in trans. This phenomenon may contribute to the observed differences in sorting efficiencies of proteins destined to the regulated secretary pathway.

Acknowledgments—We thank Drs. Christian Deschepper and Savita Dhavanvari for critical reading of the manuscript.

REFERENCES

1. Chung, K. N., Walter, P., Aponte, G. W., and Moore, H. P. (1989) Science 243, 192–197
2. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Cell 88, 73–83
3. Jutras, I., Seidah, N. G., and Reudelhuber, T. L. (2000) J. Biol. Chem. 275, 40337–40343
4. Blazquez, M., Thiele, C., Huttner, W. B., Docherty, K., and Shennan, K. I. (2000) Biochem. J. 349, 909–913
5. Dhavanvari, S., Arnautova, I., Snell, C. R., Steinbach, P. J., Hammond, K., Caputo, G. A., London, E., and Loh, Y. P. (2002) Biochemistry 41, 52–60
6. Assadi, M., Sharpe, J. C., Snell, C., and Loh, Y. P. (2004) Biochemistry 43, 7789–7807
7. Arnautova, I., Smith, A. M., Coates, L. C., Sharpe, J. C., Dhavanvari, S., Snell, C. R., Birch, N. P., and Loh, Y. P. (2003) Biochemistry 42, 10445–10455
8. Danniels, P. S. (2001) Mol. Cell. Endocrinol. 177, 87–93
9. Jain, R. K., Chang, W. T., Geetha, C., Joyce, P. B., and Gorr, S. U. (2002) Biochem. J. 368, 605–610
10. Canaff, L., Brechler, V., Reudelhuber, T. L., and Thibault, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9435–9437
11. Kim, T., Tso-Cheng, J. H., Eiden, L. E., and Loh, Y. P. (2001) Cell 106, 499–509
12. Beuret, N., Stettler, H., Renold, A., Rutishauser, J., and Spies, M. (2004) J. Biol. Chem. 279, 20242–20249
13. Quinn, D., Ori, L., Ravazolla, M., and Moore, H. P. (1991) J. Cell Biol. 113, 987–996
14. Brechler, V., Chu, W. N., Baxter, J. D., Thibault, G., and Reudelhuber, T. L. (1996) J. Biol. Chem. 271, 20636–20640
15. Pratt, R. E., Carleton, J. E., Richie, J. P., Heusser, C., and Dzau, V. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7837–7840
16. Toffelmire, E. B., Slater, K., Corvol, P., Menard, J., and Schambelan, M. (1989) J. Clin. Invest. 83, 679–687
17. Rhodes, C. J., and Halban, P. A. (1987) J. Cell Biol. 105, 145–153
18. Højlund, K., Wildner-Christensen, M., Ekahg, O., Skjaerbaek, C., Holst, J. L., Koldkaajer, O., Moller, J. D., and Beck-Nielsen, H. (2001) Am. J. Physiol. 280, E50–E58
19. Ori, L., Ravazolla, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J. D., and Anderson, R. G. (1986) J. Cell Biol. 103, 2273–2281
20. Schmidt, W. K., and Moore, H. P. (1995) Mol. Biol. Cell 6, 1271–1285
21. Taugner, R., Kim, S. J., Murakami, K., and Walldorf, R. (1987) Histochemistry 86, 249–255
22. Taylor, N. A., Jan, G., Scougall, K. T., Docherty, K., and Shennan, K. I. (1998) J. Mol. Endocrinol. 21, 209–216
23. Cowley, H. J., Moore, Y. R., Darling, D. S., Joyce, P., and Gorr, S. U. (2000) J. Biol. Chem. 275, 7743–7748
24. Brachk, N., Cohen, P., and Boileau, G. (1994) Biochem. Biophys. Res. Commun. 205, 221–229
25. Pelczarski, S., Kitaghi, P., and Bidard, J. N. (2001) J. Biol. Chem. 276, 6140–6150
26. Bündgaard, J. R., Birkedal, H., and Rehfeld, J. F. (2004) J. Biol. Chem. 279, 5488–5493
27. Thiele, C., and Huttner, W. B. (1998) J. Biol. Chem. 273, 1223–1231
28. Cool, D. R., Fenger, M., Snell, C. R., and Loh, Y. P. (1995) J. Biol. Chem. 270, 8723–8729
29. Ori, L. (1986) Diabetes Metab. Rev. 2, 71–106
30. Cool, D. R., and Loh, Y. P. (1998) Mol. Cell. Endocrinol. 139, 7–13
31. Steiner, D. F., Rouille, Y., Geng, Q., Martin, S., Carroll, R., and Chan, S. J. (1996) Diabetes Metab. 22, 94–104
32. Halban, P. A., and Irnberger, J. C. (2000) Mol. Cell. Biol. 14, 1195–1203
33. Christophe, D., Christophe-Hoberuts, C., and Pichon, B. (2000) Cell. Signal. 12, 337–341
34. Gomord, V., Wee, E., and Fayle, L. (1999) Biochimie (Paris) 81, 607–618
35. Bonifacino, J. S., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447
36. Eyring, D. G. (1999) J. Clin. Investig. 104, 67–72
