

Nonconverted, Amino Acid Analog-modified Proinsulin Stays in a Golgi-derived Clathrin-coated Membrane Compartment

LELIO ORCI, PHILIPPE HALBAN,* MYLENE AMHERDT, MARIELLA RAVAZZOLA, JEAN-DOMINIQUE VASSALLI, and ALAIN PERRELET

*Institute of Histology and Embryology and *Institute of Clinical Biochemistry, University of Geneva Medical School, Geneva, Switzerland*

ABSTRACT The secretion of insulin by the pancreatic B-cell involves a passage of the newly synthesized (pro)insulin polypeptides across the Golgi apparatus, at the *trans* pole of which secretory proteins are released as a population of secretory granules characterized by a clathrinlike coat on segments of their limiting membrane. When the conversion of radiolabeled proinsulin to insulin was inhibited by replacing arginine and lysine with the amino acid analogs, canavanine and thialysine, the nonconverted radioactive material remained associated with Golgi-derived, coated secretory granules. The coat was characterized as clathrin-containing by immunocytochemistry. Under analog treatment, the noncoated, storage secretory granules did not become markedly labeled during the pulse-chase experiment. These data are compatible with the hypothesis that in normal conditions, the maturation of the coated compartment into noncoated granules is linked to the effective conversion of the prohormone.

Insulin formation involves the synthesis of pre-proinsulin (1) by ribosomes on the rough endoplasmic reticulum, the cotranslational removal of the "pre" or signal sequence and the posttranslational conversion of proinsulin to insulin. The conversion process has been suggested to begin in the Golgi complex (2, 3), and the comparison of the ratio of radiolabeled cellular proinsulin/insulin with the time course of the intracellular migration of radiolabeled protein, as detected by autoradiography, has confirmed that proinsulin to insulin conversion is initiated when the labeled polypeptides reach the Golgi area (4–6). The Golgi complex releases at its *trans* pole (for review, see reference 7) a population of newly formed secretory granules that are identified morphologically by coated membrane segments. The coated membrane, as the dense secretory granule core, originates from the pinching off of the extremities of dilated coated Golgi cisternae (6). The coated secretory granules are the first granule population to become autoradiographically labeled during a pulse-chase experiment (6); in order to further probe the relationship between proinsulin to insulin conversion and the transit of the hormone through the Golgi complex and into coated secretory granules, we have used an experimental procedure designed to interfere with the normal processing of proinsulin

polypeptides. This consisted in rendering the proinsulin molecule resistant to cleavage by the converting enzyme(s) upon replacing the amino acids arginine and lysine with their respective analogs, canavanine and thialysine (8, 9). Such analog-modified proinsulin was shown not to be converted by lysed islet cell secretory granules (8). Gel chromatography and radioimmunoassay were used to measure the extent of conversion of biosynthetically labeled proinsulin into insulin and the release of these peptides from the B-cell; high resolution autoradiography allowed the visualization of the labeled polypeptides in their intracellular location. The analog-modified proinsulin, which was secreted at a rate comparable to that of insulin (9), was found to remain associated with coated Golgi cisternae and coated granules derived therefrom. Coated granules did not mature into noncoated granules as when proinsulin was normally converted to insulin.

MATERIALS AND METHODS

Isolated islets of Langerhans from adult rats were obtained by the collagenase method (10). Batches of 200 isolated islets were incubated for 1 h at 37°C in Dulbecco's modified Eagle's medium, (Gibco Biocult, Paisley, Scotland) containing 5 mg/ml BSA, deprived of arginine and lysine but supplemented with 1 mM *S*-2-aminoethyl cysteine (4-thialysine) and 1 mM canavanine (Sigma

Chemical Co., St. Louis, MO); control islets were incubated in Dulbecco's modified Eagle's medium containing 1 mM arginine and 1 mM lysine. After incubation, analog-treated and control islets were labeled for 5 min with L-[4,5-³H]leucine (1 mCi/ml, 62 Ci/mmol, Amersham Corp., Amersham, England) in 0.5 ml Krebs' Ringer bicarbonate buffer (KRB) containing 10 mM HEPES

buffer, pH 7.4, 5 mg/ml BSA in the continued presence of analogs or native amino acids, respectively. Glucose concentration was 8.3 mM throughout the preincubation and labeling periods.

At the end of the labeling period, the tubes were placed on ice and the islets washed three times with ice-cold KRB-HEPES buffer containing 8.3 mM

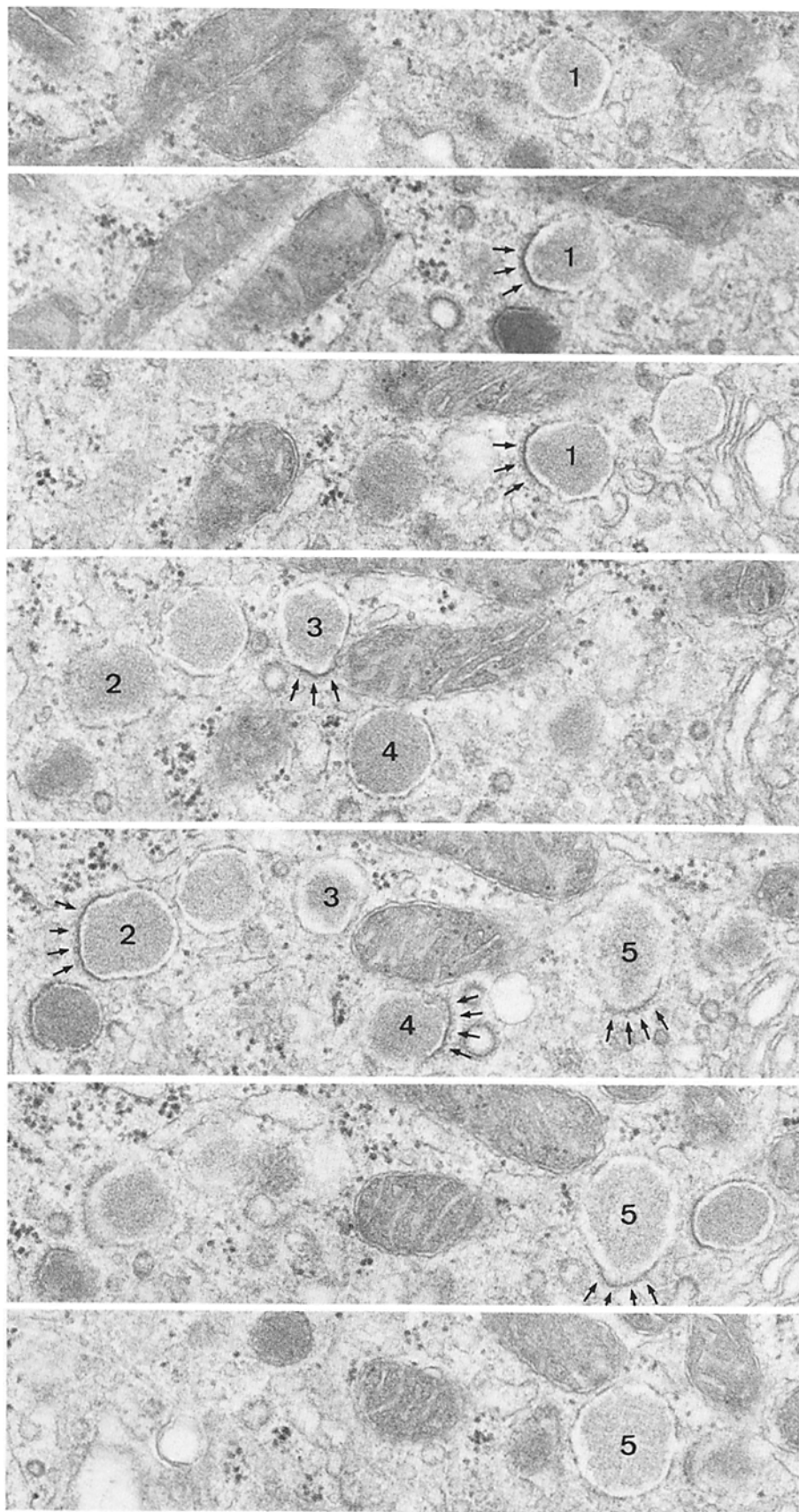


FIGURE 1 Series of seven successive sections of the Golgi region of a control B-cell. Serial sectioning shows several secretory granules (the same granule is identified by the same number) on two or three successive sections: a coat (indicated by arrows) will almost invariably be present on segments of each granule limiting membrane. This demonstrates that virtually all granules in the Golgi area are coated. Besides the coat, coated granules are characterized by a relatively pale content and a narrow electron lucent halo. $\times 42,000$.

glucose. After being washed, batches of islets were taken for biochemical and morphological analysis (see below), while the remaining islets were replaced in complete Dulbecco's medium (10% newborn calf serum) containing 8.3 mM glucose but without analogs for a chase period of 85 min at 37°C. At the end of the chase period, islets were washed and processed for biochemical and morphological analysis. The culture medium was also analyzed biochemically following centrifugation. Approximately 40 islets were taken for the biochemical analysis and 20 for autoradiographic evaluation.

Biochemistry: To determine the proinsulin/insulin content, aliquots of islets were suspended in 1 ml 0.2 M glycine containing 2.5 mg/ml human serum albumin, pH 8.8, and sonicated (11). Both sonicated islets and chase culture media were centrifuged to remove cell debris. The respective amounts of labeled proinsulin and insulin were determined by quantitative immunoprecipitation as detailed previously (11). To separate proinsulin from insulin, immunoprecipitated products were displaced from the immune complex with 1 M acetic acid containing 2.5 mg/ml BSA, 0.5% (vol/vol) Nonidet P-40

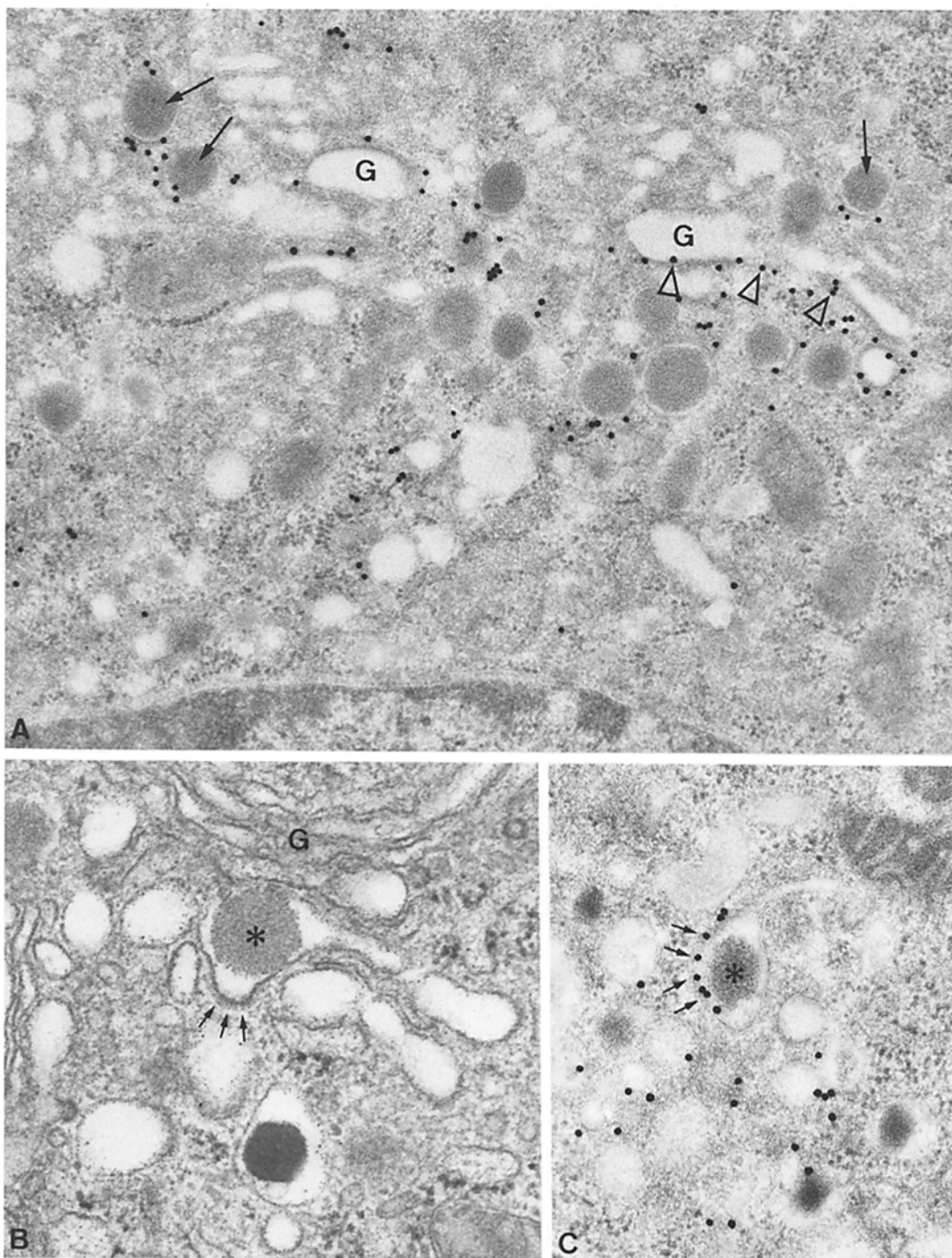


FIGURE 2 (A and C) Immunocytochemical demonstration of clathrin in the Golgi area of analog-treated B-cells. In A, gold particles revealing the clathrin immunoreactive sites decorate the periphery of several secretory granules (indicated by arrows) in the vicinity of Golgi cisternae (G). Arrowheads indicate the membrane of a *trans* Golgi cisterna labeled by gold particles. B is a section without immunostaining showing a Golgi complex with secretory granule corelike material (arrows) in an expanded cisterna characterized by a coat (asterisk) on part of its limiting membrane. The pinching off of the expanded region of the cisterna is assumed to give rise to a coated secretory granule. C is a region comparable to that shown in B in a thin section treated with an anticlathrin antiserum revealed by the protein A-gold. The membrane limiting the expanded Golgi cisterna that contain secretory granule corelike material (asterisk) is distinctly labeled by gold particles (arrows). The other gold particles in the field label coated vesicles. (A) $\times 33,000$. (B) $\times 50,000$. (C) $\times 41,000$. Size of gold particles $\cong 15$ nm.

(Fluka, Buchs, Switzerland) and chromatographed on Sephadex G-50 Fine (Pharmacia Fine Chemicals, Piscataway, NJ) (9).

Autoradiography: For autoradiography, aliquots of control and analog-treated islets were pelleted, fixed in 2.5% cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon. For each time point, three or four isolated islets were thin-sectioned. Thin sections were prepared and autoradiographed using Ilford L₄ emulsion (12). Exposure time was 4 wk. Following development of the emulsion with Microdol X (Eastman Kodak), 12 pictures of B-cells were photographed randomly in each islet at $\times 21,600$ to record autoradiographic grains (the magnification was calibrated with a reference test grid, 2160 lines/mm, Fullam Inc., Schenectady, NY). The distribution and frequency of the grains over the various intracellular compartments were evaluated quantitatively by the probability circle method (13) coupled to morphometry of the cell compartments (14). The radioactivity was expressed as specific radiation label density (R_{vi}^*), which represents the autoradiographic grain count per unit volume of a given compartment: $R_{vi}^* = G_i/P_i(5 \cdot d^2 \cdot T)$, where G_i is the number of points of the 95% probability circle falling on compartment i , P_i the number of points of the morphometric test lattice falling on the compartment i , d is the point spacing of the morphometric lattice, and T the section thickness. The data were based on the evaluation of a total of $\sim 8,000$ silver grains. Background labeling was two to five grains per $1,000 \mu\text{m}^2$.

Immunocytochemistry: The immunocytochemical localization of clathrin (15) was carried out as follows: control and analog-treated islets fixed with 1% cacodylate-buffered glutaraldehyde were processed for Lowicryl K4M

low temperature embedding (16). Thin sections picked on nickel grids were floated overnight at 4°C in a moist chamber on drops of anticlathrin antiserum (17) diluted 1:50. The antiserum (polyclonal) is directed against pig brain coated vesicles (17). After washing, sections were incubated with the protein A-gold solution (18) for 1 h at room temperature, washed again, and stained with uranyl acetate and lead citrate.

RESULTS

Previous morphologic and autoradiographic studies of B-cells in a pulse-chase experiment after [^3H]leucine labeling (6) have shown that the transit of labeled (pro)insulin polypeptides from the compartment of synthesis (the rough endoplasmic reticulum) to the compartment of hormone storage (the secretory granules) involves a passage through the Golgi apparatus and that the *trans* cisternae of this complex give rise to a specific population of secretory granules characterized by the presence of a clathrinlike coat (see below). These coated granules are maximally labeled 30 min after a 5-min pulse, and they subsequently shed their coat to mature into non-coated storage granules who show a maximal labeling at 90 min postpulse (6). The time points evaluated in the present paper (i.e., 5 and 90 min after the beginning of the pulse)

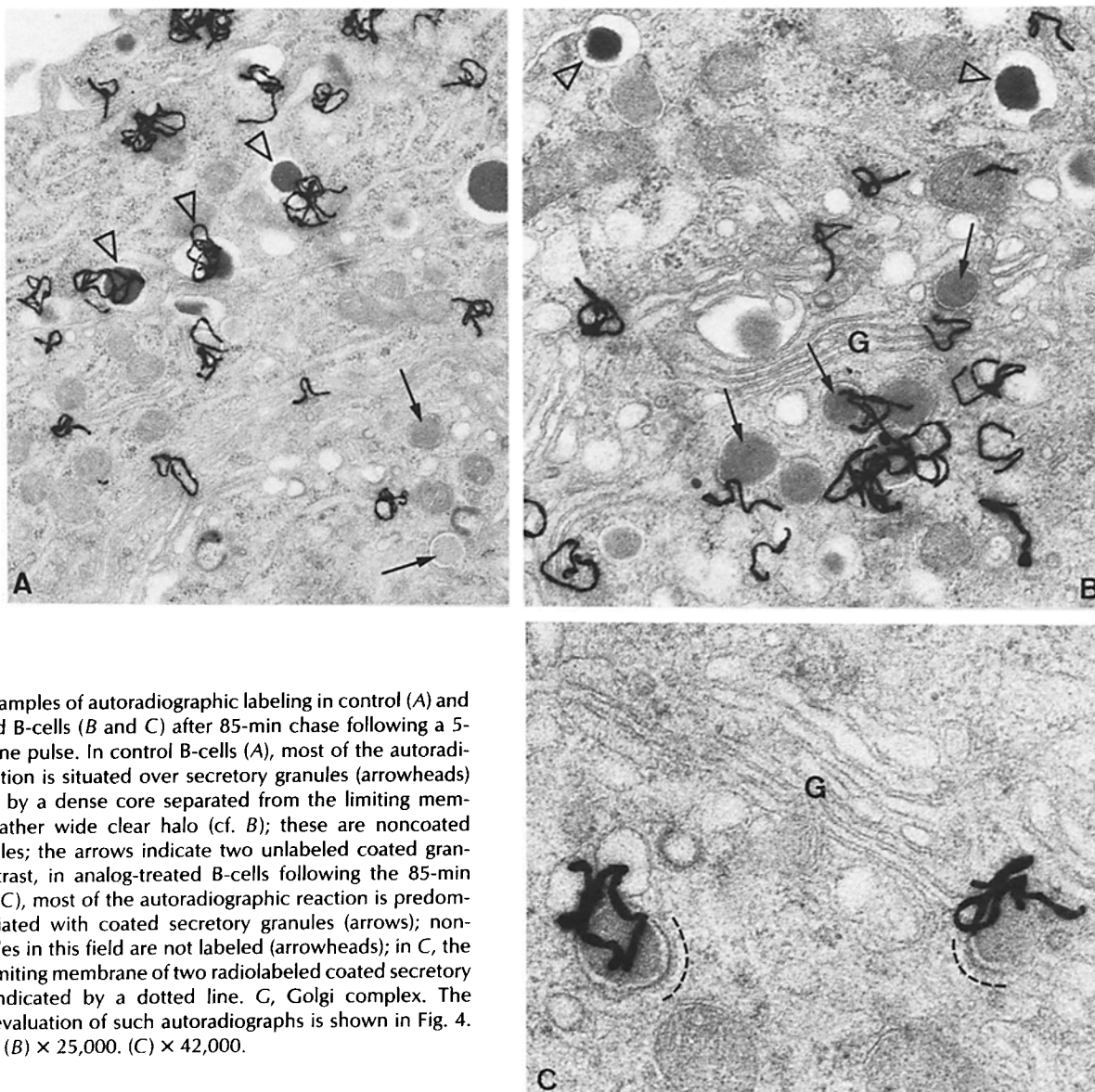


FIGURE 3 Examples of autoradiographic labeling in control (A) and analog-treated B-cells (B and C) after 85-min chase following a 5-min [^3H]leucine pulse. In control B-cells (A), most of the autoradiographic reaction is situated over secretory granules (arrowheads) characterized by a dense core separated from the limiting membrane by a rather wide clear halo (cf. B); these are noncoated storage granules; the arrows indicate two unlabeled coated granules. By contrast, in analog-treated B-cells following the 85-min chase (B and C), most of the autoradiographic reaction is predominantly associated with coated secretory granules (arrows); non-coated granules in this field are not labeled (arrowheads); in C, the coat on the limiting membrane of two radiolabeled coated secretory granules is indicated by a dotted line. C, Golgi complex. The quantitative evaluation of such autoradiographs is shown in Fig. 4. (A) $\times 17,000$. (B) $\times 25,000$. (C) $\times 42,000$.

correspond to conditions at which proinsulin to insulin conversion is virtually nil and maximal, respectively.

Biochemistry

Analog treatment induced a marked block of the conversion of proinsulin to insulin, in confirmation of previous experiments with a 30-min pulse and a 3-h chase (9). While in controls 78% of the immunoprecipitable material in the islets was in the form of native insulin, this percentage decreased to 11% in analog-treated islets. Also in agreement with previous experiments (9), the analog treatment did not affect the release of either total (labeled and unlabeled) immunoreactive insulin, or of labeled proinsulin/insulin products in the medium (as compared to controls).

Autoradiography and Immunocytochemistry

Analog-treated B-cells showed no detectable ultrastructural changes of the compartments involved in insulin processing (i.e., RER, Golgi, coated granules, and noncoated granules) as compared to controls; this was confirmed by morphometry yielding in both conditions similar values of volume density that were used to calculate the specific radiation label density of these different compartments. The coated granules in control B-cells, as determined on the basis of the morphological identification of the coat, represented ~10% of the total (coated plus noncoated) secretory granule population (the size of the sample evaluated was about 9,000 secretory granules in 100 different cells). Serial sectioning of the Golgi area of control and analog-treated B-cells showed that virtually all granules present in this area were of the coated type (Fig. 1). The immunocytochemical demonstration, with an anticlathrin antiserum (17) revealed by the protein A-gold method (18), of the clathrinlike nature of the coat on coated Golgi cisternae and coated secretory granules is illustrated in Fig. 2. The pattern of radioactive labeling of the various intracellular compartments evaluated, except at the rough endoplasmic reticulum level, was altered in analog-treated B-cells (Fig. 3). The quantitative evaluation of the radioactivity revealed an accumulation of radioactivity in the Golgi apparatus at the end of the 85-min chase, instead of its normal emptying observed in controls (Fig. 4). The coated granule compartment of analog-treated B-cells showed likewise a much more intense radioactive labeling than that of controls at the end of the 85-min chase. The noncoated granules, by contrast, presented only a slight increase in labeling at the end of the chase period, while at that time, in control B-cells, noncoated granules represented the most intensely labeled intracellular compartment (Fig. 4).

DISCUSSION

Following a pulse-chase experiment in presence of amino acid analogs that become incorporated in the newly synthesized, ^3H -labeled proinsulin molecule and render it resistant to proteolytic conversion (8, 9), high-resolution autoradiography revealed that a clathrin-coated compartment of the B-cell, comprising both coated Golgi cisternae with condensing granule core material and coated secretory granules, remains significantly labeled for up to 85 min postpulse. At this time, in controls, most of the radioactivity is detected over the noncoated, storage secretory granules; these granules show only a low level of radioactive labeling during analog treatment. This suggests that when the conversion of proinsulin

SPECIFIC RADIATION LABEL DENSITY

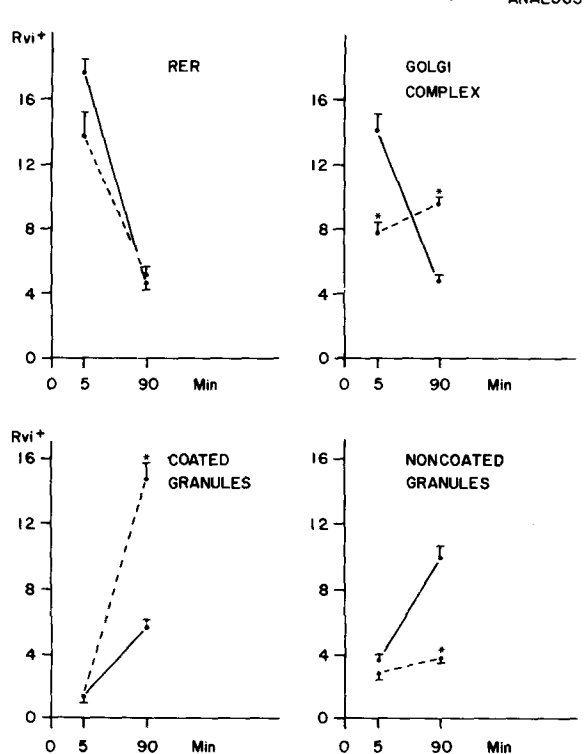


FIGURE 4 Quantitative evaluation of radioactive labeling of intracellular compartments involved in the processing of (pro)insulin in control and analog-treated B-cells. The compartment identified as Golgi complex comprises all cisternal/vesicular elements of the Golgi apparatus. The coated granules were selected on the basis of the morphological identification of a clathrinlike coat. Radioactivity in each compartment was expressed as specific radiation label density (R_{vi}^+) that represents the radioactivity per unit volume of the compartment considered (14). The asterisks on a given time point of analog experiments indicate a value significantly different ($P < 0.001$) from that of controls. In R_{vi}^+ plots of controls, all 5-min values are significantly different from those of 90 min. In analog experiments, the difference of the noncoated granule labeling between 5 and 90 min was at the limit of significance ($P < 0.05$). Data are means \pm SEM, and the differences between groups were assessed with the Student's t test for unpaired groups. The evaluation of the radiation label density (R_{vi}^+) measuring the radioactivity in each compartment irrespective of its contribution to the cell volume (14) gave similar results.

to insulin is prevented, the maturation of coated granules into noncoated granules (upon shedding of the coat) is inhibited. The finding that shedding of the coat from the coated granule population failed to occur during analog treatment is compatible with the hypothesis that in normal condition, the maturation of a coated membrane compartment into noncoated secretory granules is linked to the conversion of proinsulin. The inhibition of conversion by the monovalent ionophore monensin that blocks intracellular migration of proteins also reveals the association of nonconverted proinsulin with a clathrin-coated compartment similar to that described in the present study (19). Biochemical data have previously suggested that conversion proceeds at the secretory granule stage (for review, see reference 20), and it is possible that the signal for coat shedding is the attainment of a specific ratio between precursor (proinsulin) and product (insulin, C-peptide) when conversion normally takes place. This ratio would not be reached with the protease-resistant, analog-modified

proinsulin. The ultimate proof that the coated compartment is the converting compartment will depend upon its isolation and chemical characterization. The fact that the prospective converting compartment appears marked by a clathrin coat should render this feasible, as recently shown for a population of coated vesicles isolated from pig brain by affinity separation using anticlathrin-coated *Staphylococcus aureus* as a solid immunoadsorbant (21). The present data also suggests that conversion is not needed for the sorting out and release of proinsulin polypeptides from the *trans* pole of the Golgi apparatus into the coated secretory granules; furthermore, the observation that analog-treated B-cells are able to release nonconverted proinsulin at the same rate as untreated B-cells secrete native proinsulin and insulin implies that the coated granules can be involved in exocytosis.

We thank Danielle Boghikian, Martine Lavanchy, Anne-Marie Lucini, and Gorana Perrelet, for technical assistance, Isabelle Bernard for typing the manuscript, and P.-A. Rüttimann for photographic work. The anticlathrin antiserum was a gift of D. Louvard, Pasteur Institute, Paris.

This work was supported by grants 3.246.82 (P. Halban) and 3.460.83 (L. Orci) from the Swiss National Science Foundation.

Received for publication 15 April 1984, and in revised form 3 July 1984.

Note Added in Proof: In K_4M -embedded human pancreas we have been able to localize, by immunocytochemistry with the protein A-gold method, a human proinsulin-specific antiserum (Madsen O. D., et al., *Endocrinology*, 1983, 113:2135-2144), which does not cross-react with insulin. With this proinsulin-specific antiserum, an intense immunolabeling was observed on clathrin-coated secretory granules (326 ± 10 gold particles/ μm^2 ; three islets, 59 pictures evaluated) as well as on the condensing secretory material in clathrin-coated, expanded *trans* Golgi cisternae. By contrast, the labeling of noncoated storage secretory granules (including pale granules) was very low (27 ± 6 particles/ μm^2 ; three islets, 67 pictures evaluated). This data represents a direct evidence that the proinsulin is a major constituent of the coated granules and that the clathrin-coated compartment is a critical site of proinsulin conversion. A similar pattern of immunolabeling was observed with this antiserum on rat tissue

(Orci L., M. Ravazzola, and O. D. Madsen, unpublished results). The proinsulin-specific antiserum should represent a useful marker for the identification of the coated compartment to be isolated by immunoadsorption on anticlathrin-coated *S. aureus*.

REFERENCES

1. Patzelt, C., A. D. Labrecque, J. R. Duguid, R. J. Carroll, P. S. Keim, R. L. Heinrikson, and D. F. Steiner. 1978. Detection and kinetic behavior of preproinsulin in pancreatic islets. *Proc. Natl. Acad. Sci. USA*. 75:1260-1264.
2. Steiner, D. F., W. Kemmler, H. S. Tager, and J. D. Peterson. 1974. Proteolytic processing in the biosynthesis of insulin and other proteins. *Fed. Proc.* 33:2105-2115.
3. Docherty, K., and D. F. Steiner. 1982. Post-translational proteolysis in polypeptide hormone biosynthesis. *Annu. Rev. Physiol.* 44:625-638.
4. Orci, L., A. A. Like, M. Amherdt, B. Blondel, Y. Kanazawa, E. B. Marliss, A. E. Lambert, C. B. Wollheim, and A. E. Renold. 1973. Monolayer cell culture of neonatal rat pancreas: an ultrastructural and biochemical study of functioning endocrine cells. *J. Ultrastruct. Res.* 43:270-297.
5. Orci, L. 1974. A portrait of the pancreatic B-cell. *Diabetologia*. 10:163-187.
6. Orci, L. 1982. Macro- and micro-domains in the endocrine pancreas. *Diabetes*. 31:538-565.
7. Farquhar, M. G., and G. E. Palade. 1981. The Golgi apparatus (complex)—(1954-1981)—from artifact to center stage. *J. Cell Biol.* 91(3, Pt. 2):775-1035.
8. Noe, B. D. 1981. Inhibition of islet prohormone to hormone conversion by incorporation of arginine and lysine analogs. *J. Biol. Chem.* 256:4940-4946.
9. Halban, P. A. 1982. Inhibition of proinsulin to insulin conversion in rat islets using arginine and lysine analogs. Lack of effect on rate of release of modified products. *J. Biol. Chem.* 257:13177-13180.
10. Lacy, P. E., and M. Kostianovsky. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes*. 16:35-39.
11. Halban, P. A., C. B. Wollheim, B. Blondel, and A. E. Renold. 1980. Long-term exposure of isolated pancreatic islets to mannoheptulose: evidence for insulin degradation in the β -cell. *Biochem. Pharmacol.* 29:2625-2633.
12. Caro, L. G., and R. P. van Tubergen. 1962. High resolution autoradiography. I. Methods. *J. Cell Biol.* 15:173-199.
13. Whur, P., A. Herscovics, and C. P. Leblond. 1969. Radioautographic visualization of the incorporation of galactose- 3H and mannose- 3H by rat thyroids in vitro in relation to the stages of thyroglobulin synthesis. *J. Cell Biol.* 43:289-311.
14. Stäubli, W., W. Schweizer, J. Suter, and E. R. Weibel. 1977. The proliferative response of hepatic peroxisomes of neonatal rats to treatment with SU-13437 (nafepopin). *J. Cell Biol.* 74:665-689.
15. Pearce, B. M. F. 1976. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc. Natl. Acad. Sci. USA*. 73:1255-1259.
16. Armbruster, B. L., E. Carlemalm, R. Chiovetti, R. M. Garavito, J. A. Hobot, E. Kellenberger, and W. Villiger. 1982. Specimen preparation for electron microscopy using low temperature embedding resins. *J. Microsc. (Oxf.)*. 126:77-85.
17. Louvard, D., C. Morris, G. Warren, K. Stanley, F. Winkler, and H. Reggio. 1983. A monoclonal antibody to the heavy chain of clathrin. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1655-1664.
18. Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074-1081.
19. Orci, L., P. Halban, M. Amherdt, M. Ravazzola, J.-D. Vassalli, and A. Perrelet. 1984. A clathrin-coated, Golgi-related compartment of the insulin secreting cell accumulates proinsulin in the presence of monensin. *Cell*. 39:39-47.
20. Steiner, D. F., K. Docherty, and R. Carroll. 1984. Golgi/granule processing of peptide hormones and neuropeptide precursors: a minireview. *J. Cell. Biochem.* 24:121-130.
21. Merisko, E. M., M. G. Farquhar, and G. E. Palade. 1982. Coated vesicle isolation by immunoadsorption on *Staphylococcus* cells. *J. Cell Biol.* 92:846-857.