Proc. Natl. Acad. Sci. USA
Vol. 78, No. 9, pp. 5661–5664, September 1981
Cell Biology

Insulin immunoreactive sites demonstrated in the Golgi apparatus of pancreatic B cells

(insulin/Golgi apparatus/immunocytochemistry)

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Communicated by Donald F. Steiner, May 22, 1981

ABSTRACT **Insulin immunoreactive sites were localized in the Golgi apparatus of pancreatic B cells by light and electron microscopy. Identification of the Golgi apparatus by immunofluorescence required the prior degranulation of B cells with glibenclamide to reduce the insulin immunostaining due to secretory granules. In such cells, insulin immunofluorescence revealed brightly stained, crescent-shaped strands with form and location super-imposable on that of Golgi complexes seen in thin sections of the same cells. With the electron microscope, the insulin immunoreactive sites revealed by the protein A/gold technique were localized in the cisternae and vesicles of the Golgi apparatus of glibenclamide-treated and control B cells and over maturing and mature secretory granules. The quantitative evaluation of the intensity of the insulin immunoreactive sites in the Golgi apparatus revealed a density of sites 4 times more than cellular background values. The demonstration of insulin immunoreactivity in the Golgi apparatus provides direct evidence for the involvement of this compartment in the transport and maturation of proinsulin into insulin.**

As in many other polypeptide-secreting cells (reviewed in ref. 1), three distinct membrane compartments are sequentially involved in the synthesis, intracellular transport, and release of insulin by the pancreatic B cell. These are the rough endoplasmic reticulum (RER), the Golgi apparatus, and the secretory granules; the implication of these compartments in the insulin biosynthetic pathway derives from the correlation between gel filtration of labeled secretory polypeptides in pulse–chase experiments and their intracellular localization by high-resolution radioautography (reviewed in ref. 2). This approach showed that the appearance of immunoreactive insulin in the chromatographic pattern occurred at a time during the pulse–chase experiment at which radioautographic labeling was predominantly over the Golgi area (cisternae, vesicles, and maturing secretory granules). On the basis of these data and of biochemical experiments in which the conversion of proinsulin into insulin was inhibited by blocking (with antimycin A) the transport of labeled proinsulin distal to the RER, it was inferred that the Golgi area was the site of the initiation of conversion of proinsulin into insulin and of insulin packaging into secretory granules (3). This paper presents direct evidence for this sequence of events by showing, by means of both light- and electron-microscope immunocytochemistry, the presence of insulin immunoreactive sites in the cisternae of the Golgi apparatus.

MATERIALS AND METHODS

Tissue Preparation. Adult female rats were used. The degranulation of B cells [which is accompanied by a marked development of the RER and Golgi apparatus (4)] was induced by 5 or 6 intraperitoneal injections of 0.5 mg of glibenclamide (J. Scholtholz, Hoechst AG, Frankfurt, Federal Republic of Germany) at 12-hr intervals, the last dose being administered 1 hr before sacrifice. Pieces of control and treated pancreas were fixed with 2–4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, dehydrated in ethanol, and embedded in Epon 812 without postfixation in osmium tetroxide.

Immunocytochemistry. *Light microscopy.* Semithin sections (1 μm) containing islets of Langerhans were treated to remove the embedding medium (5) and then processed for immunostaining with the indirect immunofluorescence method (6). Sections were exposed for 2 hr at room temperature in a moist chamber to guinea pig anti-insulin antiserum (P. H. Wright, Indianapolis, IN) at 1:200 dilution, washed several times in phosphate-buffered saline, and then incubated for 1 hr with fluorescein isothiocyanate-labeled rabbit anti-guinea pig IgG antiserum (Pasteur Institute, Paris, France). After washing, sections were counterstained with 0.01% Evans blue and observed in a Leitz Ortholux fluorescence microscope equipped with a Pleomopak L.2 illuminator. The specificity of the immunostaining was controlled by incubating sections with the anti-insulin antiserum preabsorbed with an excess (4 units of undiluted antiserum per ml) of purified insulin (L. Heding, Novo Research Institute, Bagsvaerd, Denmark).

Electron microscopy. Ultrastructural immunostaining was performed according to the protein A/gold technique (7). For this purpose, thin (600–1000 Å) sections of glutaraldehyde-fixed, Epon-embedded material were collected on nickel grids, incubated for 24–48 hr at 4°C in a humid atmosphere by flotation on a drop of anti-insulin antiserum (1:400 dilution), carefully washed with phosphate-buffered saline, and further incubated for 1 hr at room temperature with the protein A/gold complex (1:10 dilution). After rinsing with phosphate-buffered saline and distilled water, sections were contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope. In addition, thin sections of the same islets as examined by immunofluorescence were cut, stained with uranyl acetate and lead citrate, and observed in the electron microscope.

Quantitative evaluation. The density of the protein A/gold labeling following incubation of the thin sections with anti-insulin antiserum over the different compartments of the B cells was evaluated in three pancreatic islets from untreated rats fixed by vascular perfusion. The labeling was measured over the nucleus, the mitochondria, the RER, the Golgi complex, the maturing secretory granules, and the mature secretory granules as follows: 54 micrographs (18 for each islet, 3 for each cell) were taken on 70-mm film at the primary magnification of 14,000,

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Abbreviation: RER, rough endoplasmic reticulum.

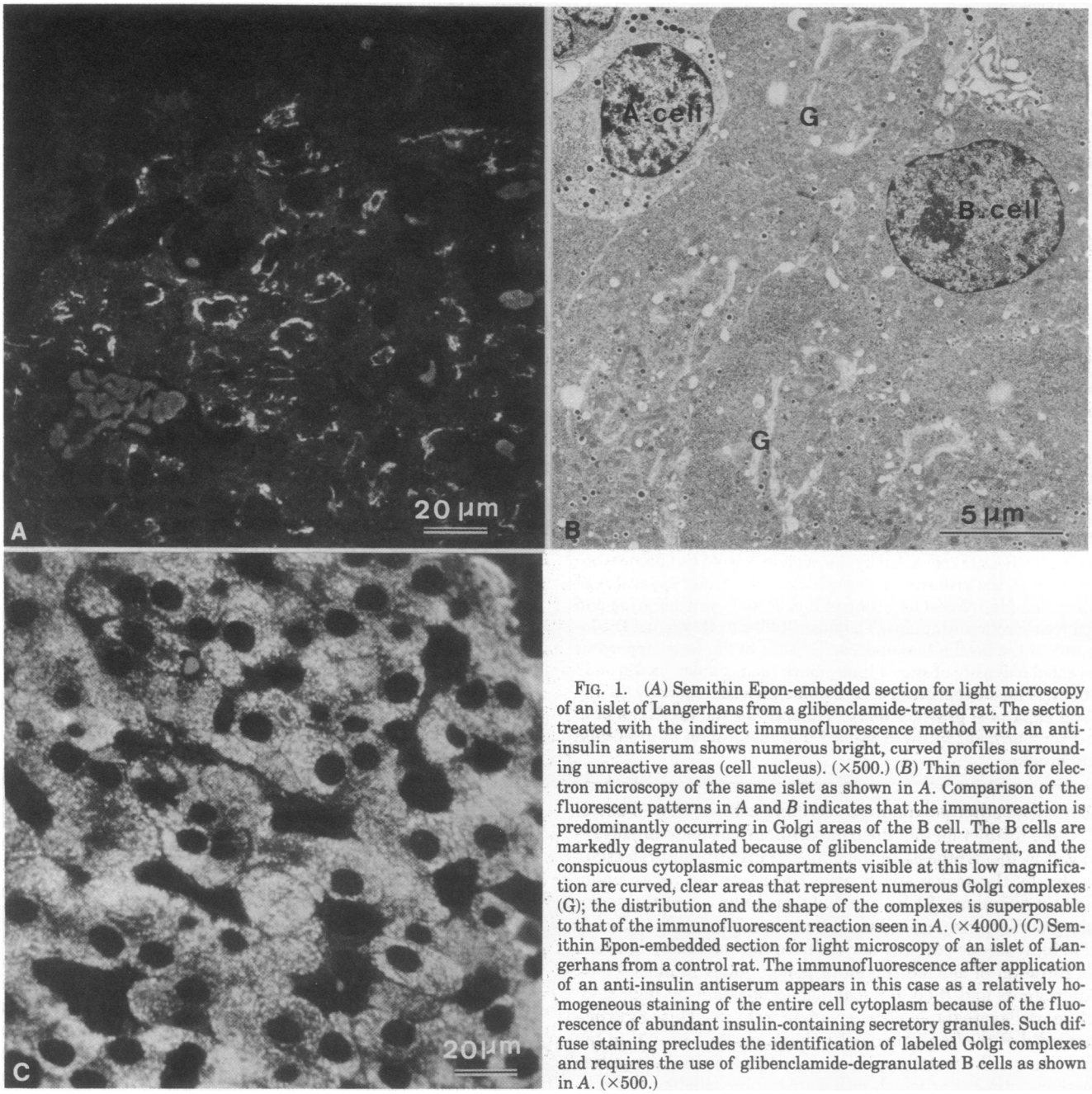

FIG. 1. (*A*) Semithin Epon-embedded section for light microscopy of an islet of Langerhans from a glibenclamide-treated rat. The section treated with the indirect immunofluorescence method with an anti-insulin antiserum shows numerous bright, curved profiles surrounding unreactive areas (cell nucleus). (×500.) (*B*) Thin section for electron microscopy of the same islet as shown in *A*. Comparison of the fluorescent patterns in *A* and *B* indicates that the immunoreaction is predominantly occurring in Golgi areas of the B cell. The B cells are markedly degranulated because of glibenclamide treatment, and the conspicuous cytoplasmic compartments visible at this low magnification are curved, clear areas that represent numerous Golgi complexes (G); the distribution and the shape of the complexes is superposable to that of the immunofluorescent reaction seen in *A*. (×4000.) (*C*) Semithin Epon-embedded section for light microscopy of an islet of Langerhans from a control rat. The immunofluorescence after application of an anti-insulin antiserum appears in this case as a relatively homogeneous staining of the entire cell cytoplasm because of the fluorescence of abundant insulin-containing secretory granules. Such diffuse staining precludes the identification of labeled Golgi complexes and requires the use of glibenclamide-degranulated B cells as shown in *A*. (×500.)

calibrated with a carbon-grating replica containing 2160 lines per mm. Three micrographs (1 for each islet) of the embedding medium alone were also evaluated to assess the nontissular background. The surface of each selected compartment and the number of gold particles present in this compartment were recorded on a graphic tablet (Tektronix, type 4973) connected to a microprocessor system (IMSAI, type 8080) programmed to calculate the number of particles per unit area (μm^2) of the compartment (D. Bertrand and M. Amherdt, personal communication). Statistical comparison of the values was done with the Student *t*-test.

RESULTS

Fig. 1 *A* and *C* show the immunofluorescence pattern of semithin sections stained with anti-insulin antiserum in islets from glibenclamide-treated and control rats. In B cells of control rats (Fig. 1*C*), the bulk of immunofluorescence is due to secretory granules, and the specific morphological appearance of the Golgi complex is usually not recognizable. This led us to study glibenclamide-treated B cells depleted from their secretory granule stores. In such cells (Fig. 1*A*), insulin immunostaining occurred predominantly in the form of bright fluorescent strands surrounding the nucleus. The comparison of such immunofluorescent patterns with thin sections of the same islets (Fig. 1*B*) showed that it corresponded to numerous, crescent-shaped Golgi complexes. The immunostaining was considered specific because it could be completely abolished by the use of the same anti-insulin antiserum previously absorbed with purified insulin.

At the electron microscopic level, the specific appearance of the Golgi apparatus and the good resolution of the protein A/gold technique allowed the localization of insulin immunoreactive sites in control B cells, although the pattern of labeling in

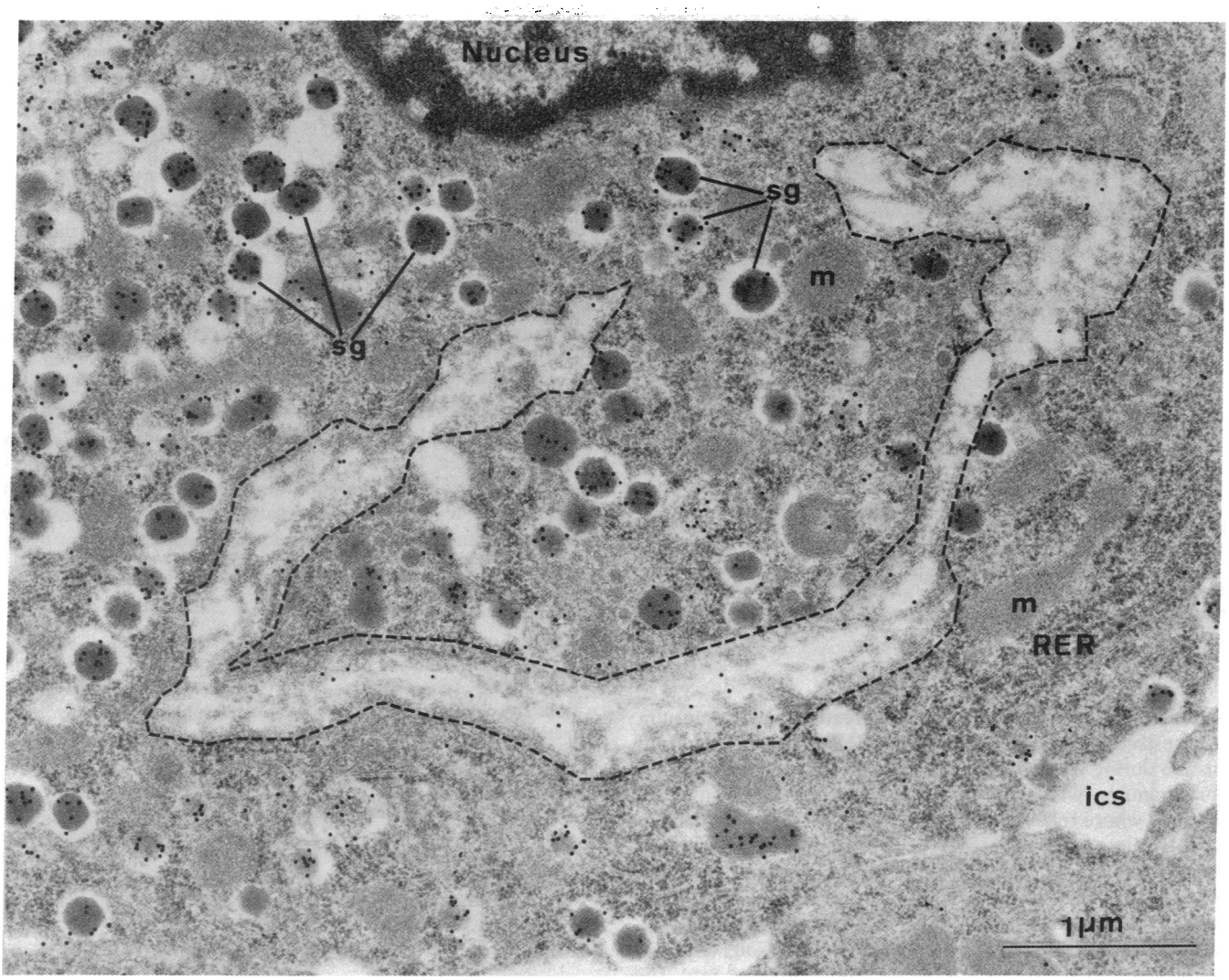

FIG. 2. Thin section of part of a B cell treated by the protein A/gold technique to reveal insulin immunoreactive sites. The area of the cytoplasm illustrated comprises a part of the nucleus, numerous secretory β-granules (sg) and a large, curved electron-lucent region (delineated by the dotted line). This region corresponds to the Golgi area, whose general morphological characteristics are well preserved, although the membrane boundary of the cisternae are not demonstrated with the fixation protocol used for immunocytochemistry. The individual black dots represent gold particles deposited over insulin immunoreactive sites, and these appear particularly numerous over the cores of the secretory granules. Gold particles are scattered over the Golgi region, and their number in the area (comprised within the dotted line) was recorded for the quantitative evaluation shown in Table 1. ics, Intercellular space. A few gold particles appear over the remaining components of the cytoplasm, including RER and mitochondria (m). (×18,000.)

glibenclamide-treated material was entirely superimposable. In control B cells (Fig. 2) gold particles revealing insulin immunoreactivity were evident over the entire extent of the Golgi apparatus. Particles were detectable either over the cisternal space, or close to the boundary of the cisternae, which, owing to the lack of osmium postfixation, did not show a clear-cut membranous structure. As might be expected, gold labeling was particularly abundant over the cores of the mature secretory granules.

On the other hand, as shown by quantitative evaluation of the labeling intensity in control B cells (Table 1), the degree of labeling present over the RER was not above cellular background as represented by mitochondrial or nuclear, non-specific immunostaining. Maturing secretory granules still associated with the Golgi complex (Fig. 3) showed a degree of labeling intermediate between Golgi cisternae and mature secretory granules (Table 1). This result suggested a progressive concentration of the insulin immunoreactive sites from the Golgi apparatus to the mature secretory granules.

Table 1. Density of insulin immunolabeling over various cellular compartments of the B cell in control rats

B-cell compartments	Immunoreactive sites, gold particles per μm^2 ± SEM
Mature secretory granules	116.26 ± 5.32 (n, 46)
	$P < 0.001$
Maturing secretory granules*	88.42 ± 4.58 (n, 36)
	$P < 0.001$
Golgi complex	19.43 ± 1.21 (n, 36)
	$P < 0.001$
RER	5.13 ± 0.45 (n, 26)
	NS
Mitochondria	7.14 ± 0.79 (n, 52)
	NS
Nucleus	4.09 ± 0.43 (n, 25)

Gold labeling over mitochondria and nucleus are considered as cellular background. Noncellular background (i.e., gold labeling over embedding medium is approximately two particles). n, Number of experiments; NS, not significant.

* Associated with Golgi elements.

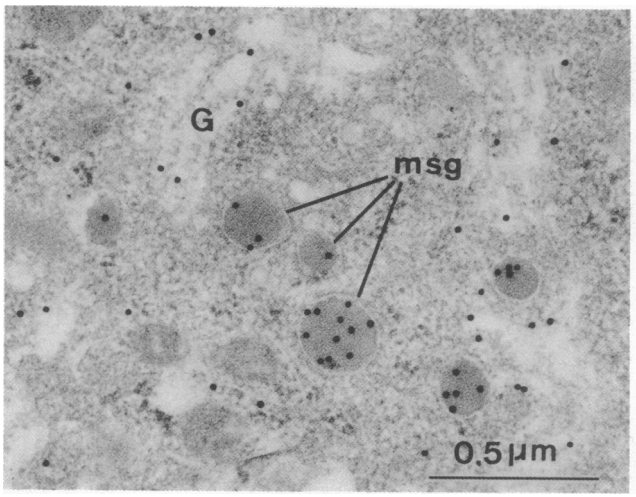

FIG. 3. Field of a B cell cytoplasm incubated with anti-insulin antiserum followed by the protein A/gold technique. This picture shows maturing secretory granule (msg) in the Golgi area (G). Maturing secretory granules have a higher intensity of gold labeling than does the Golgi area (G), suggesting a concentration of the immunoreactive sites between the two compartments. (× 46,000.)

DISCUSSION

In the secretory sequence of the pancreatic B cell, proinsulin synthesized by the ribosomes of the RER is transformed into insulin during its transport from the RER to the mature secretory granules, which are known to contain mainly insulin (8). The site where transformation is initiated has been assumed to be the Golgi area, but direct evidence for the presence of proinsulin or insulin in this region of the cell has not been available. The use of an anti-insulin antiserum for immunostaining in light and electron microscopy now allows the demonstration of a significant level of insulin immunoreactivity in the Golgi cisternae of B cells in both control and glibenclamide-treated rats. The requirement for glibenclamide pretreatment of the B cells in order to reduce the blurring effect of secretory granule immunofluorescence on the faint Golgi labeling does not appear to influence *per se* the level of insulin immunoreactivity: in thin sections of control and glibenclamide-treated material, the degree of labeling with the same antiserum as revealed by the protein A/gold technique appears to be essentially the same. The single glutaraldehyde fixation needed for thin-section immunocytochemistry tends to induce some swelling of the Golgi cisternae and prevents the clear visualization of the membranous structure limiting the Golgi cisternae and vesicles. This difficulty did not seriously hamper the quantitative evaluation of the immunostaining in this region. We could demonstrate specific labeling in the Golgi cisternae that was four-fold above background staining. However, the same technique does not reveal any labeling above background at the RER level. This finding suggests either that the concentration of proinsulin in the RER may be still lower than in the Golgi region or that our antiserum reacts sufficiently less well (9) with proinsulin, as opposed to insulin, so as to prevent its detection in the cisternae of the RER. If the latter is correct, then we may infer that insulin is limited to the Golgi apparatus and secretory granules. The very high number of insulin antigenic sites observed over the latter as compared to the Golgi cisternae is suggestive of a concentration process occurring between these two compartments.

In summary, the detection of insulin immunoreactive sites over the Golgi cisternae represents direct evidence that the formation of granules and the initiation of conversion of proinsulin into insulin occurs in this compartment. The mechanism by which insulin-related polypeptides are sorted out from other RER-derived products and delivered to the B cell secretory granules remains to be established.

We gratefully acknowledge the technical assistance of M. Sidler, G. Perrelet, A.-M. Lucini, and I. Bernard. This work is supported by the Swiss National Science Foundation Grant 3.668.80, by a grant-in-aid from Hoechst Pharmaceuticals (Frankfurt, Federal Republic of Germany), and by the Sandoz Foundation, Basel, Switzerland.

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