Biochem. J. (1987) 245, 557–566 (Printed in Great Britain)

Production and characterization of monoclonal antibodies to insulin secretory granule membranes

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Monoclonal antibodies to insulin secretory granule membranes were obtained following immunization of mice with granule membranes purified from a rat transplantable insulinoma. The specificities of the antibodies were investigated by using binding assays with different insulinoma subcellular fractions, by indirect immunofluorescence studies with intact and permeabilized cells, and by immunoblotting of granule membrane proteins fractionated by SDS/polyacrylamide-gel electrophoresis. Fifty-six antibodies were characterized initially, and 21 representative cell lines were cloned. The antibodies fell into four categories: (1) binding preferentially to secretory granules, and reacting with a component of approx. 80000 Da on immunoblots (antigen designated SGM 80); (2) binding preferentially to secretory granules, and reacting with components of approx. 110000 and 50000 Da on immunoblots (antigen designated SGM 110); (3) binding preferentially to secretory granules but unreactive on immunoblots; (4) binding to membrane antigen(s) with a widespread intracellular distribution which included granules and plasma membranes. The antigens SGM 80 and SGM 110 were studied in more detail and both were shown to be integral membrane glycoproteins with antigenic determinants located on the internal face of the secretory granule membrane. These antigens were also present in normal rat islets of Langerhans and similar components were detected by immunoblotting in secretory granules from anterior pituitary and adrenal medulla. Proteins which were immunologically related to SGM 80 and SGM 110, but distinct in molecular size, were also identified in liver. It is concluded that secretory granules contain specific components which are restricted in subcellular location but widespread in tissue distribution. The antibodies obtained will be valuable reagents in the further investigation of the biogenesis and turnover of insulin secretory granules.

INTRODUCTION

The secretory granule of the pancreatic β-cell is enveloped in a phospholipid bilayer membrane containing upwards of 100 different components. Clearly many of these proteins are essential to the function of the organelle during processing and storage of the hormone and its secretion, although only a few have been positively identified (Hutton *et al.*, 1982, 1983). These include a Mg^{2+}-ATPase proton pump (Hutton & Peshavaria, 1982), a phosphatidylinositol kinase which is involved in (poly)phosphoinositide metabolism (Tooke *et al.*, 1984) and a peptide of 29000 Da which is phosphorylated by a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) (Brocklehurst & Hutton, 1984). Other activities have been assigned to the granule membrane but with less certainty and based on indirect evidence or results obtained using only partially purified subcellular fractions. These include an adenine nucleotide transporter (Sussman & Leitner, 1977), a Ca^{2+}-stimulated ATPase (Formby *et al.*, 1976), 5′-nucleotidase (Tooke *et al.*, 1984), a NADPH binding site (Watkins & Moore, 1977) and a somatostatin receptor (Mehler *et al.*, 1980).

Given the complexity of granule composition and the small amounts of material available, it is not surprising that the processes involved in granule biogenesis, and the fate of granule membrane constituents after fusion with the plasma membrane during exocytosis, remain poorly understood. In order to study individual proteins which are specific to the granule membrane we have exploited a rat transplantable insulinoma (Chick *et al.*, 1977) as a source of material, and hybridoma technology as a means of generating reagents specific to individual proteins. This paper describes the production and characterization of a range of monoclonal antibodies reacting with insulin secretory granule membrane antigens and the initial investigation of two glycoproteins located in the granule membrane which were identified by this approach. The following paper (Grimaldi *et al.*, 1987) examines the biosynthesis of one of these glycoproteins and its regulation in insulinoma tissue and pancreatic islets.

METHODS

Materials

Laboratory chemicals of analytical grade were obtained either from BDH Chemicals or Sigma Chemical Co. All radioactive isotopes were obtained from Amersham International. Myeloma cell lines P3/NSI/1-Ag4-1 and P3/NSO/1 were a gift from Dr C. Milstein, MRC Laboratory of Molecular Biology, Cambridge. Sheep anti-(mouse IgG) serum was prepared as described by Soos & Siddle (1982).

Abbreviations used: PBS, phosphate-buffered saline; PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin.

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Preparation of secretory granules and membranes

The transplantable insulinoma was propagated by serial subcutaneous implantation in New England Deaconess Hospital (NEDH) strain rats and harvested as previously described (Hutton *et al.*, 1981).

Insulin secretory granules were prepared from homogenates of the tissue by density gradient centrifugation on Percoll gradients according to the method of Hutton *et al.* (1982). Granules (5 mg of protein) were disrupted by sonication in 5 ml of $(NH_4)_2CO_3$ buffer [10 mM-$(NH_4)_2CO_3$/5 mM-EDTA, pH 8] and the membranes collected on a sucrose (60%, w/w) cushion by centrifugation at 4 °C for 1 h at 100000 *g* (33000 rev./min) in a Beckman SW 50.1 rotor (r_{av} 8 cm). The membranes were washed once in $(NH_4)_2CO_3$ buffer, once in 10 mM-Mes/Tris buffer (10 mM-Mes adjusted to pH 6.5 with Tris base), and resuspended in Mes/Tris buffer to a protein concentration of approx. 1 mg/ml.

Chromaffin granules were prepared from NEDH rat adrenal tissue on a continuous sucrose gradient as described by Smith & Winkler (1967). Secretory vesicles were prepared from NEDH rat pituitary on an iso-osmolar continuous sucrose/metrizamide gradient [0.25 M-sucrose/36% (w/v) metrizamide] based on a procedure described by Loh *et al.* (1984). A vesicle preparation enriched in lysosomes was obtained from NEDH rat liver according to the method described by Maguire & Luzio (1985). Membranes were prepared from these fractions in the same way as for insulin secretory granules.

Preparation of insulinoma and islet cell suspensions

Insulinoma tissue was separated from the fibrous capsule of the tumour, chopped into 1–2 mm fragments and rinsed in modified Krebs buffer (120 mM-NaCl, 5 mM-KCl, 24 mM-$NaHCO_3$, 1 mM-$MgCl_2$ and 2.5 mM-$CaCl_2$) containing 1 g of BSA/l (Krebs/BSA buffer). The tissue pieces (1–2 g wet wt) were then forced through a plastic tea strainer (500 μm mesh) with a rubber policeman, and the filtrate resuspended in 40 ml buffer and passed successively through nylon filters of 500 μm and 50 μm mesh (Pharmacia) retained in 2.5 cm Swinney adaptors. The filtrate was layered onto eight discontinuous Percoll gradients each composed of 5 ml of 43% and 1 ml of 60% (v/v) Percoll in Krebs buffer in a 10 ml capacity polystyrene centrifuge tube (Sterilin, Middlesex, U.K.). The tubes were centrifuged at room temperature for 20 min at 1500 *g* in an MSE Minor centrifuge. Large cellular aggregates and dead cells were recovered in the upper portion of the 43% Percoll, while individual and small clusters of intact viable cells collected at the 43–60% interface. Erythrocytes passed through the 60% Percoll to the bottom of the tube. The viable cells were collected from the interface and washed three times by repeated suspension in Krebs/BSA buffer and centrifugation for 5 min at 300 *g*. Samples were tested for viability by Trypan Blue exclusion (Fallon *et al.*, 1962) and typically over 90% of cells excluded the dye.

Rat islets were isolated from the pancreas of 8–10-week-old NEDH rats by the method of Lacy & Kostianovsky (1967). These were then dispersed into individual and small clumps of cells by gently vortex-mixing 100 islets in 100 μl of calcium-free Krebs buffer containing 5 mM-EGTA as described by Lernmark (1974). Cells were washed once and returned to normal Krebs buffer.

Radioiodination

Monoclonal antibodies were iodinated according to the method of Fraker & Speck (1978). Purified immunoglobulin (25 μg) in 50 μl of 100 mM-sodium phosphate buffer, pH 7.5, was incubated with 0.5 mCi of $Na^{125}I$ (IMS 30; Amersham) in a glass tube precoated with 10 μg of iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril; Pierce and Warriner Ltd., Chester, Cheshire, U.K.). The iodinated protein (specific activity 3–6 mCi/mg) was then isolated by chromatography on a column (0.8 cm × 20 cm) of Sephadex G-25 (Pharmacia). Sheep anti-(mouse IgG) antibody was affinity-purified by using a mouse IgG immunoadsorbent, and iodinated (specific activity approx. 1–5 mCi/mg), as described by Hales & Woodhead (1980).

Preparation of monoclonal antibodies

Secretory granule membranes (1 mg of protein/ml) were emulsified in an equal volume of Freund's complete adjuvant (Behringwerke AG, Marburg, Germany) and 50 μg was injected intraperitoneally into Balb/c mice (Bantin and Kingman Ltd., Hull, Yorks, U.K.). At 12 weeks later they were injected intravenously and intraperitoneally with 50 μg of granule membrane protein in phosphate-buffered saline (PBS) comprised of 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, 2.7 mM-KCl and 140 mM-NaCl. At 4 days after the second injection spleen cells prepared from each mouse were fused with P3/NSI/1-Ag4-1 (two mice), or P3/NSO/1 (one mouse) myeloma cells as described by Galfre & Milstein (1981). All three mice possessed good titres of serum antibody, as detected in the standard screening assay (see below), at the time of fusion. Subsequent culture of hybridomas, cloning at limiting dilution and production of ascitic fluid in mice followed standard procedures (Galfre & Milstein, 1981; Goding, 1983). Antibodies were used in experiments either in the form of untreated culture supernatants from cells grown to stationary phase, or after purification from ascites (Bruck *et al.*, 1982).

Immobilization of secretory granule membranes

Routine screening of culture supernatants was performed in flexible poly(vinyl chloride) 96-well microtitre plates (Falcon; Becton Dickinson & Co.) in which secretory granule membranes were immobilized. Each well was precoated for 60 min at 37 °C with 50 μl of poly(L-lysine), 20 mg/l in 10 mM-Mes/Tris buffer, pH 6.5. This was aspirated and 25 μl of granule membrane suspension (50 μg) in the same buffer was added to each well. The 96-well plates were centrifuged for 20 min at 1100 *g* (3200 rev./min) in a Hereus Minifuge GL centrifuge using a Type 3471 microtitre plate rotor. Unbound membrane was aspirated and the wells were washed with veronal buffer (50 mM-sodium barbitone/85 mM-NaCl containing 5 g of BSA and 0.1 g of NaN_3/l, adjusted to pH 8 with HCl) dispensed from a wash bottle. To ensure blockage of remaining binding sites the wells were filled with veronal buffer, incubated for 1 h at 4 °C, then stored at −20 °C.

Screening procedure

Coated plates were thawed, buffer was removed by inversion and 50 μl of undiluted culture supernatant was added to each well and incubated for 1 h at 4 °C. Culture supernatants containing monoclonal antibodies raised

against other irrelevant antigens (human thyrotropin and choriogonadotropin) were used as controls. Wells were washed three times with veronal buffer dispensed from a wash bottle, and then 100 μl of ^{125}I-labelled sheep anti-(mouse IgG) (approx. 30000 d.p.m./well in veronal buffer) was added to each and incubated for 1 h at 4 °C. Wells were washed six times with veronal buffer, cut out from the plate and their radioactivity determined in an NE 1600 γ-counter (counting efficiency 70%).

Specificity of antibody binding

Microtitre plates were also coated with total membrane fraction prepared from NEDH rat liver and with lysed erythrocytes and various insulinoma subcellular fractions designated A_1, A_2, B, C and D, which were prepared by Percoll density gradient centrifugation (Hutton *et al.*, 1982; Docherty & Hutton, 1983). Fraction D (mitochondria) was subjected to further purification by urografin density gradient centrifugation (Tooke *et al.*, 1984). Antibody binding to these membrane fractions was assayed in the same way as for the initial screening of culture supernatants.

Electrophoresis and immunoblotting

Single-dimension SDS/PAGE was performed on 1.5 mm × 150 mm × 150 mm slab gels polymerized from 10% (w/v) acrylamide and 0.26% *NN'*-methylenebisacrylamide using the discontinuous buffer system of Laemmli (1970). Samples (50 μg of protein) were solubilized in 125 mM-Tris/HCl (pH 6.8) containing 0.32 M-sucrose, 2% (w/v) SDS, 65 mM-dithiothreitol and 0.001% Bromophenol Blue and heated at 100 °C for 5 min.

The separated proteins were transferred electrophoretically to nitrocellulose membranes (BA85, 0.45 μm pore size; Schleicher und Schull, Dassel, Germany) for 4 h at 60 V in buffer consisting of 25 mM-Tris base/192 mM-glycine (pH 8.3) containing 20% (v/v) methanol (Towbin *et al.*, 1979). Protein binding sites were blocked in PBS containing 0.05% Tween 20 (Koch Light) for 60 min at room temperature and the membrane was then incubated with shaking for 1 h at room temperature, or overnight at 4 °C in hybridoma culture supernatant diluted 1:10, or ascites diluted 1:1000, in PBS containing 0.05% Tween and 1 mg of BSA/ml. After washing three times in PBS/Tween/BSA the membranes were incubated with shaking for 1–4 h at room temperature with ^{125}I-labelled sheep anti-(mouse IgG) (equivalent to approx. 100000 d.p.m./protein track) in PBS/Tween/BSA. They were then washed six times in PBS/Tween/BSA, air dried and autoradiographed at −70 °C using Cronex-4 X-ray film (Du Pont) and intensifying screens.

Immunofluorescence

Distribution of antibody binding sites was investigated both in intact and permeabilized insulinoma and islet cells. Permeabilized cells were prepared by initial fixation for 3 min in 3% paraformaldehyde in PBS followed by freeze–thawing once in solid CO_2/acetone. Approx. 2×10^5 insulinoma cells or cells from 20 islets, suspended in 100 μl of Krebs buffer, were then incubated with 100 μl of undiluted culture supernatant for 1 h at 4 °C in siliconized glass tubes (0.7 cm × 4 cm). They were washed by resuspension in 1 ml of Krebs/BSA buffer followed by centrifugation for 5 min at 300 *g*. The washing process was repeated twice, then the cells were incubated for 1 h at 4 °C with fluorescein-conjugated rabbit anti-(mouse IgG) serum (Miles Scientific) diluted 1:20 in Krebs/BSA buffer. After washing three times the cells were mounted in 20 μl of 90% (v/v) glycerol in PBS and examined with a Leitz fluorescence microscope.

Epitope analysis

Secretory granule membrane-coated wells in 96-well microtitre plates were incubated for 1 h at 4 °C with 100 μl of monoclonal antibody either in the form of undiluted culture supernatant or ascites diluted 1:1000 with veronal buffer. This was followed by the addition of 50 μl of veronal buffer containing ^{125}I-labelled monoclonal antibody (approx. 50000 d.p.m.) derived from the same or a different hybridoma cell line. Incubation was continued for a further 1 h at 4 °C and the wells were washed six times with veronal buffer, cut out and the radioactivity determined.

Binding to concanavalin A–Sepharose

Granule membranes (1 mg of protein/ml) were solubilized in 1.0 ml of 50 mM-Tris/HCl, pH 7.4, containing 200 mM-NaCl, 1 mM-$MnCl_2$, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 1 mM-phenylmethanesulphonyl fluoride and 1% NP40 for 10 min at 50 °C, then centrifuged for 5 min at 8800 *g* in a microcentrifuge. The supernatant was passed onto a column (0.5 cm × 4 cm) of concanavalin A–Sepharose that had been equilibrated with 20 vol. of the same buffer but with 0.15% NP40 (column buffer) and the flow then interrupted. After 30 min the column was washed with 5 ml of column buffer and eluted with 5 ml of column buffer containing 0.5 M-α-methyl mannoside. The proteins in the unbound and eluted fractions were precipitated overnight at −20 °C with 7 vol. of acetone, then pelleted by centrifugation at 4 °C for 30 min at 1700 *g* before electrophoresis. The separated proteins were transferred onto nitrocellulose and incubated with monoclonal antibody as described above.

Location of epitopes in secretory granules

A suspension of freshly prepared secretory granules (approx. 5 mg of protein/ml) was diluted 10-fold either is iso-osmotic buffer (10 mM-Tris/Mes, pH 6.5, containing 150 mM-KCl and 1 mM-$MgSO_4$) or lytic buffer (10 mM-Tris/Mes containing 1 mM-$MgSO_4$). Granules suspended in lytic buffer were frozen and thawed once to ensure their disruption. Samples (200 μl) were collected by filtration onto a nitrocellulose membrane held in a 96-well microfiltration manifold (BioRad). The nitrocellulose was then incubated for 1 h at 4 °C in iso-osmotic buffer containing 10 g of BSA/l to block remaining protein binding sites and cut into strips corresponding to rows of wells. These were incubated for 1 h at 4 °C in 5 ml of monoclonal antibody solution (ascitic fluid diluted 1:1000 in iso-osmotic buffer containing 10 g of BSA/l), washed three times in the same BSA buffer, and then incubated for 1 h at 4 °C with 5 ml of ^{125}I-labelled sheep anti-(mouse IgG) (equivalent to about 15000 d.p.m./well). Strips were washed six times in buffer, then cut up so that the radioactivity corresponding to each incubation could be determined.

Other assays

Spectrophotometric assays for marker enzymes, and insulin immunoassay, were performed as described by

Table 1. Subcellular localization of antigens

Membranes from the indicated subcellular fractions or cell types (50 μg of protein/well) were immobilized on polylysine-coated microtitre plates as described in the Methods section. Hybridoma culture supernatants (50 μl) were incubated with the immobilized membranes and binding was detected with ^{125}I-sheep anti-(mouse IgG) (100 μl; 20000 d.p.m./well). Results are expressed as d.p.m. bound and each value is the mean of two determinations. The table displays results from the uncloned cell lines; the related cloned lines are shown in parentheses. N.D., not determined.

Cell line	Binding category	Binding (d.p.m.) Insulinoma fraction A (granule/ lysosomal)	Insulinoma fraction B (granule)	Insulinoma fraction D (mitochondrial)	Hepatocyte (unfraction-ated)	Erythrocyte
Non-immune		159	100	127	300	120
4A4 (GM1)	1	811	1152	218	623	82
10A1 (GM2)	2	1061	1227	359	560	109
9A4 (GM3)	2	999	1292	264	412	82
9B5 (GM4)	1	1273	1347	440	826	111
5B1 (GM5)	3	669	1360	305	413	107
4A1 (GM6)	4	977	1591	376	1420	109
4B6 (GM7)	4	1035	1836	372	1340	178
5C6 (GM8)	2	1077	1331	360	655	111
5D4 (GM9)	4	1037	1790	422	1265	212
10A2 (GM10)	2	1132	1314	353	519	112
9cA2 (GM11)	3	665	791	174	462	67
9B4 (GM12)	1	1281	1440	297	953	208
4D3 (GM13)	4	976	1833	436	1377	219
9cC5 (GM14)	3	736	963	226	539	138
9A1 (GM15)	1	567	1047	201	521	112
9D5 (GM16)	1	436	420	185	259	152
9A2 (GM17)	3	681	1061	203	594	178
10A4 (GM18)	2	908	1098	401	502	86
10B2 (GM19)	1	1329	1351	395	638	90
10B4 (GM20)	1	616	793	189	538	119
4B4 (GM21)	2	N.D.	1206	139	332	128

Hutton *et al.* (1982). Protein was determined by the spectrophotometric method of Bradford (1976), except when samples contained Percoll in which case the fluorimetric procedure of Udenfriend *et al.* (1972) was used. Bovine serum albumin or bovine insulin was used as standard.

RESULTS

Preparation of monoclonal antibodies and initial characterization of their specificities

Initial screening of hybridoma culture supernatants revealed that out of 1152 wells from three fusions, 224 contained antibody that bound to insulin secretory granule membrane (binding greater than 400 d.p.m. was considered positive compared with non-specific binding of 53–75 d.p.m. with antibodies of irrelevant specificity). Of these initial positives, 196 were maintained by continuous subculture in 2 ml wells. After 3 months, 56 stable positive cell lines remained. At this stage the culture supernatants were used to determine the specificity of antibodies to different insulinoma subcellular fractions, and their tissue specificity. Also, immunoblotting was used to derive information about the molecular mass of the recognized antigens. Twenty-one cell lines were selected for cloning, representing the range of specificities revealed by the membrane binding and immunoblotting experiments.

Of the antibodies produced by stable cell lines, none bound to erythrocytes but most bound to a variable degree to unfractionated hepatocyte membranes (Table 1). Investigation of their binding to different subcellular fractions from the insulinoma indicated that there was no binding to mitochondria, beyond a very low level which might be accounted for by contamination with other membranes. There was significant binding to the lysosomal fraction (A1) which was either lower than or equivalent to the binding to the pure secretory granule fraction (B). The specific activity of insulin in fraction A1 was 60–80% of that of B, which suggested that the antigens detected in this fraction could be accounted for by its contamination with granules.

Immunoblotting experiments in which culture supernatants from 54 cell lines were tested revealed three patterns of binding (Fig. 1). With 16 supernatants, binding to a polypeptide which ran as a broad band of 80000–90000 Da was seen (category 1). With another 10, binding to two polypeptide antigens, both broad bands, in the ranges 100000–110000 Da and 50000–60000 Da was observed (category 2). With the remaining 28 supernatants no binding was detected (categories 3 and 4). Antibodies displaying each of these immunoblotting specificities were obtained in similar proportions from all three fusions carried out. The polyclonal sera taken from mice at the time of fusion recognized each of these components together with one other component of high

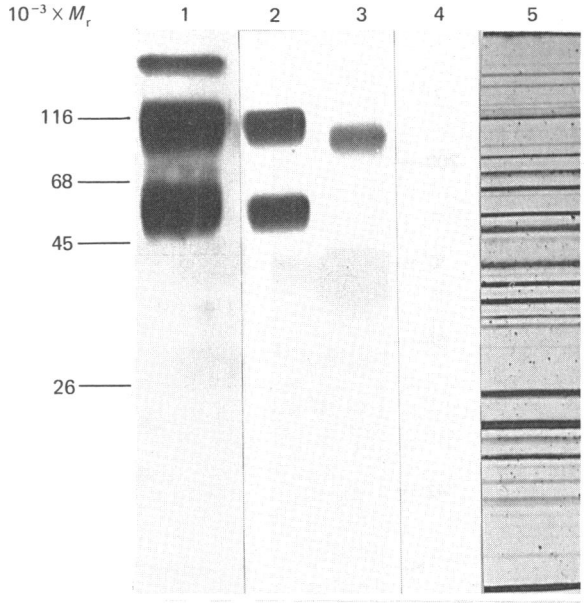


Fig. 1. Binding of antibodies to insulin secretory granule proteins

Secretory granule proteins were separated by SDS/PAGE in 10% acrylamide gels (50 μg of protein/track) and transferred to nitrocellulose membranes. The membranes were incubated with immune mouse serum diluted 1:100 (track 1) or with hybridoma culture supernatants diluted 1:10 which represented category 2 (GM 10) (track 2) or category 1 (GM 15) (track 3) antibodies or antibodies of irrelevant (nonimmune) specificity (track 4). Binding was detected by autoradiography following labelling with ^{125}I-labelled sheep anti-(mouse IgG) (100000 d.p.m./protein track). Track 5 shows an Amido Black-stained replica prepared from secretory granule membrane proteins.

molecular mass which was not recognized by any of the monoclonal antibodies.

Subcellular location of antigens identified by monoclonal antibodies

Subcellular location of antigens was further investigated by immunofluorescence. Antibodies GM 4, 8, 10, 15, 20 and 21 from cloned cell lines showed some cell surface binding to intact insulinoma cells but this was weak and varied from cell to cell. However the same antibodies when incubated with permeabilized cells produced a strong fluorescence which had a punctate distribution within the cell consistent with a granular location of the antigen (Fig. 2). Antibodies GM 9 and 13 by contrast gave a strong surface fluorescence with intact cells, and also a punctate distribution within permeabilized cells consistent with a more generalized distribution of their binding sites (Fig. 2*e*). The latter antibodies were notably those giving a higher binding to non-granule and hepatocyte membranes (Table 1). A similar distribution of antigenic sites was observed with each of these antibodies in permeabilized islet cells, indicating that the antigens are not specifically tumour-related (K. A. Grimaldi, J. C. Hutton & K. Siddle, unpublished work).

During the course of propagation by serial transplantation of the tumour in NEDH rats a cell line developed that grew much more rapidly but contained less than 10% of the insulin of the original insulinoma. The cells of this tumour contained very few granules as judged by electron microscopy and subcellular fractionation, whereas the content of other subcellular organelles as determined by marker enzyme assays was similar to the well-granulated insulinoma lines (K. A. Grimaldi, J. C. Hutton & K. Siddle, unpublished work). The antigens recognized by monoclonal antibodies in the well-granulated insulinoma were barely detectable in the preparation from the insulin-deficient tumour, again consistent with the secretory granule localization of the antigens (Fig. 3).

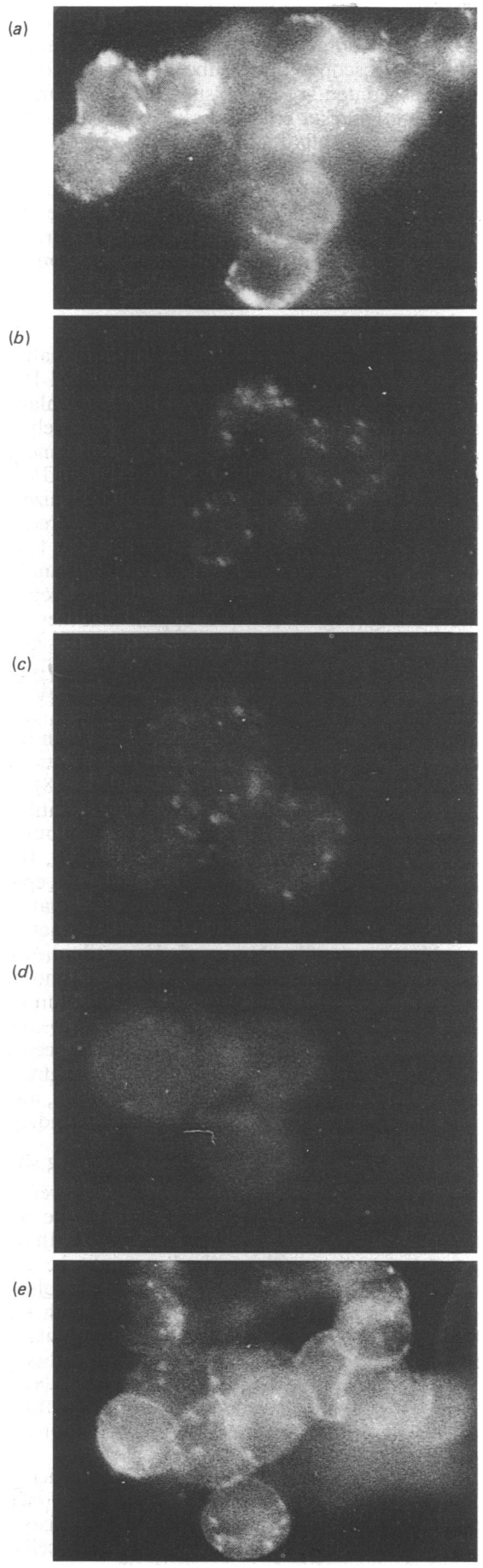
Epitope analysis

Epitope analysis was performed by determination of the inhibition of binding of ^{125}I-labelled GM 15 and GM 10 antibodies to granule membranes by unlabelled monoclonals. These antibodies react respectively with the 80000–90000 Da (SGM 80) antigen and the 100000–110000 Da and 50000–60000 Da (SGM 110) antigen(s) (Fig. 1). The binding site recognized by antibody GM 15 on the SGM 80 antigen appeared to be the same as that seen by GM 20. However GM 1, 4, 12, 16 and 19, which bound similar antigens on immunoblots, produced only a partial, but significant, reduction in GM 15 binding (Fig. 4). It would appear that these latter antibodies recognize the same protein antigen as GM 15 and GM 20 but are binding to different sites, although the possibility cannot be ruled out that the different epitopes are on distinct proteins of very similar electrophoretic mobility. Of the antibodies which bound to the SGM 110 antigen(s) on immunoblots, four inhibited GM 10 binding (GM 2, 3, 8, and 18) while GM 21 did not (Fig. 4). Thus of the six antibodies recognizing the SGM 110 antigen(s), five bound to closely related or identical epitopes (GM 2, 3, 8, 10 and 18) while GM 21 apparently bound to a different epitope on the same protein, it being highly unlikely that there exist two distinct proteins with such a characteristic immunoblotting profile. None of the antibodies that recognized the SGM 80 antigen and none of those for which the antigen could not be defined on immunoblots significantly inhibited GM 10 binding relative to control mouse monoclonal antibodies of irrelevant specificity. Such 'non-immune' antibodies themselves slightly inhibited labelled antibody binding (approx. 20%), indicating a small non-specific component of total binding.

Glycosylation of antigens and topology of binding sites

The polypeptide bands seen on immunoblots were very broad compared to the staining pattern of the major constituent proteins (Fig. 1). This suggested that the components reacting with antibodies might be glycoproteins, the diffuse migration being due to microheterogeneity of the carbohydrate side chains (Leach *et al.*, 1980; Cummings *et al.*, 1983). This possibility was investigated by examining the ability of these antigens to bind to concanavalin A–Sepharose columns. No immunoreactivity was detected in the unbound fraction (Fig. 5) but the antigens were present in the fraction eluted with α-methyl mannoside.

The orientation of the epitopes to which the antibodies bind in the granule membrane was investigated using freshly prepared intact and disrupted granules. These were immobilized onto nitrocellulose which was immersed in iso-osmotic buffer to minimize



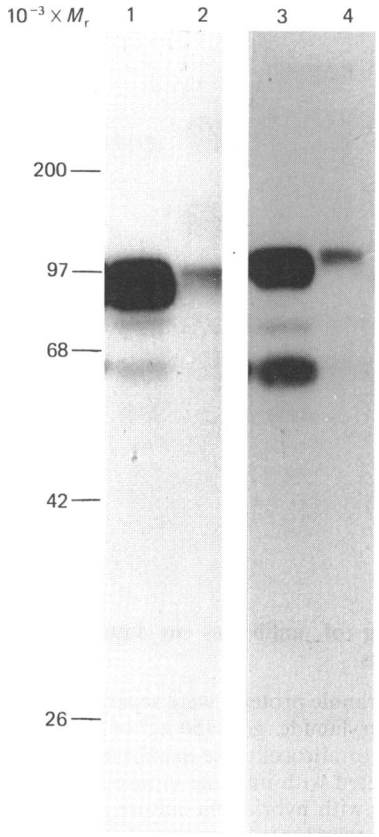


Fig. 3. Antibody binding to granule proteins from well-granulated and insulin-deficient insulinomas

Fractions from a Percoll gradient of densities between 1.10 and 1.13 were prepared from a well-granulated insulinoma (tracks 1 and 3) and an insulinoma of reduced insulin content (tracks 2 and 4), separated by SDS/PAGE (50 μg of protein/track) and transferred to nitrocellulose membranes. The membranes were incubated with monoclonal antibodies GM 4 (tracks 1 and 2) and GM 10 (tracks 3 and 4) and binding was detected by autoradiography following incubation with ^{125}I-labelled sheep anti-(mouse IgG).

lysis of intact granules. It was found that although binding to the intact granule preparation was greater than the control value there was a further significant increase in binding to disrupted granules (Fig. 6). This was also the case for an anti-insulin monoclonal antibody 3B7 (Gray *et al.*, 1987). Since disruption was required for maximum binding it appeared that the epitopes recognized by GM 4, 10, 15 and 21 are exposed on the internal face of the granule membrane. The lesser

Fig. 2. Immunofluorescent localization of monoclonal antibody binding to insulinoma cells

Cells were incubated with undiluted culture supernatant and antibody binding was detected with fluorescein-conjugated rabbit anti-(mouse IgG) antiserum. Binding of monoclonal antibodies GM 9 (*a*), GM 10 (*b*), GM 15 (*c*) and control (*d*) to insulinoma cells fixed in paraformaldehyde (3% in PBS) and permeabilized by freeze–thawing in solid CO_2/acetone is shown together with the binding of monoclonal antibody GM 9 to freshly prepared intact insulinoma cells (*e*). Magnification ×915.

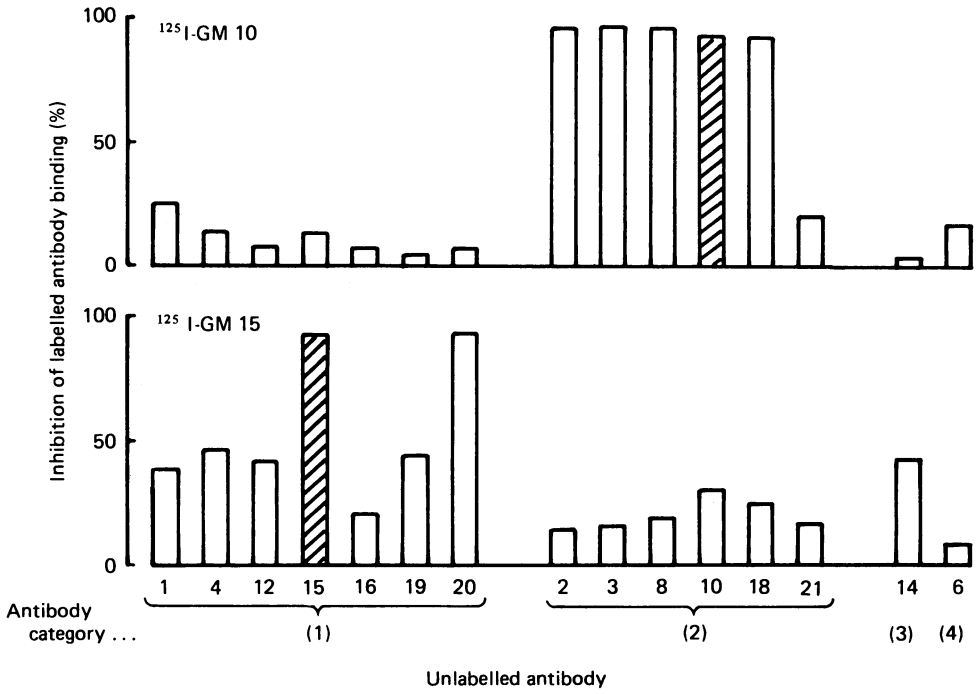


Fig. 4. Epitope analysis of monoclonal antibodies

Wells coated with granule membrane were incubated for 1 h with 100 μl of hybridoma culture supernatant or ascites diluted 1:1000 in veronal buffer, before determining the binding of 50 μl of ^{125}I-labelled monoclonal antibody GM 10 or GM 15 (50000 d.p.m./well). Results were calculated as percentage inhibition of labelled antibody binding by different unlabelled antibodies. Binding in the absence of unlabelled antibody was 3000 d.p.m./well (GM 10) or 1300 d.p.m./well (GM 15). Binding categories for different antibodies, as detailed in the text, were: 1, granule-located 80000 Da antigen; 2, granule-located 110000/50000 Da antigens; 3, granule-located, not detected on immunoblots; 4, widespread distribution, not detected on immunoblots.

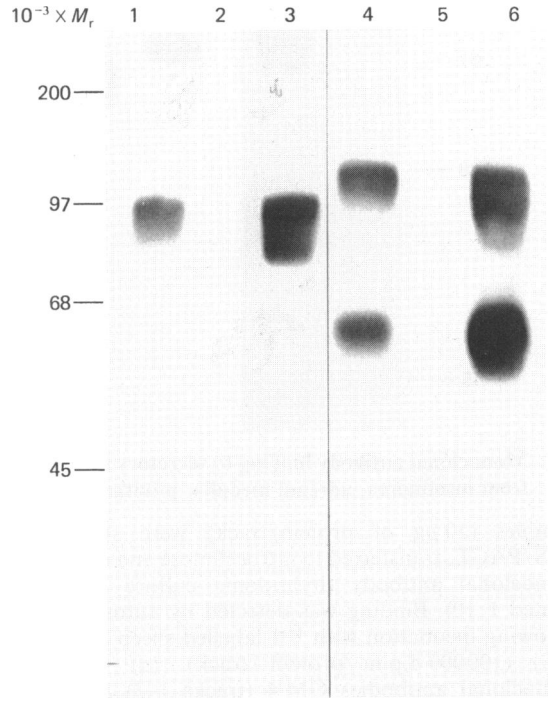


Fig. 5. Binding of insulin secretory granule membrane antigens to concanavalin A

Insulin secretory granule membrane protein (1 mg/ml) was solubilized and passed onto concanavalin A–Sepharose columns. Proteins eluted by α-methyl mannoside (tracks 1 and 4), unbound protein (tracks 2 and 5) and the starting material (tracks 3 and 6) were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with antibodies GM 4 (tracks 1–3) and GM 10 (tracks 4–6). Binding was detected by autoradiography following incubation with ^{125}I-labelled sheep anti-(mouse IgG).

binding to the non-lysed granules is probably accounted for by unavoidable partial disruption occurring during the immobilization procedure.

Using a similar technique, equivalent quantities of granule matrix proteins solubilized by osmotic disruption of the granule and a membrane fraction which had been washed in a solution containing 0.5 M-KCl and 10 mM-Tris/Mes, pH 6.5, were also immobilized on nitrocellulose and probed with the hybridoma culture supernatants. Antibodies recognizing SGM 80 and SGM 110 in every case bound preferentially to the salt-washed membrane fraction, with little or no binding to the matrix protein fraction. This suggested that SGM 80 and SGM 110 were probably integral membrane constituents.

Location of antigens in other tissues

Preliminary immunoblotting experiments revealed that antibodies did not react detectably with whole tissue extracts even from insulinoma. Granule fractions were therefore prepared from various secretory tissues. Antibodies GM 4, 10, 15 and 21 recognized antigens in anterior pituitary that were the same molecular sizes as those seen in insulin secretory granules. All four

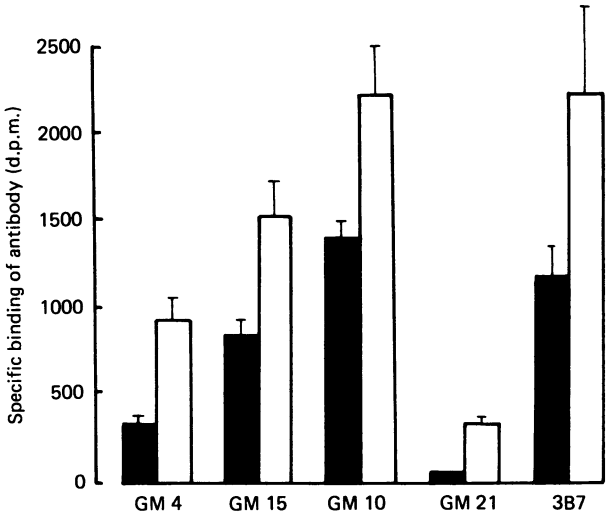


Fig. 6. Binding of monoclonal antibodies to intact and disrupted insulin secretory granules

Monoclonal antibodies as indicated (ascitic fluid diluted 1:1000) were incubated with freshly prepared intact granules (solid bars) or disrupted granules (open bars) which had been immobilized onto nitrocellulose membranes. Binding was detected with ^{125}I-labelled sheep anti-(mouse IgG) and is expressed as d.p.m. bound after subtracting values for non-immune controls (intact, 1422 ± 146 d.p.m.; lysed, 1317 ± 44 d.p.m.). Error bars represent the S.E.M. for four determinations.

antibodies also recognized antigens in chromaffin granules from the adrenal medulla, which migrated on electrophoresis as similar broad bands but which appeared to be of slightly higher molecular mass than those recognized in insulin secretory granules (Fig. 7*a*). With liver vesicles, GM 4 and GM 15 bound strongly to a single band which was much narrower and had a higher molecular mass than the 80000 Da granule antigen (Fig. 7*b*). Antibodies GM 10 and GM 21 recognized a single band in liver that was also narrow and had a slightly higher molecular mass than the 110000 Da component of SGM 110 present in secretory granules. A 50000 Da component was not seen in liver.

DISCUSSION

A library of monoclonal antibodies which reacted with components of the insulin secretory granule membrane has been prepared and two novel granule membrane glycoproteins have been identified. The antibodies were classified into four categories: (1) binding on immunoblots to a polypeptide of approx. 80000 Da (SGM 80) which was preferentially located in granule membranes; (2) binding on immunoblots to two polypeptides of approx. 110000 and 50000 Da (SGM 110) which were also preferentially located in granule membranes; (3) binding to antigens which were preferentially located in granule fractions according to membrane binding assays, which had a punctate, mainly intracellular distribution as determined by immunofluorescence but which were not recognized on immunoblots; (4) binding to antigen(s) which had a widespread intracellular distribution, including expression on the cell surface, but which were not recognized on immunoblots. Although a large

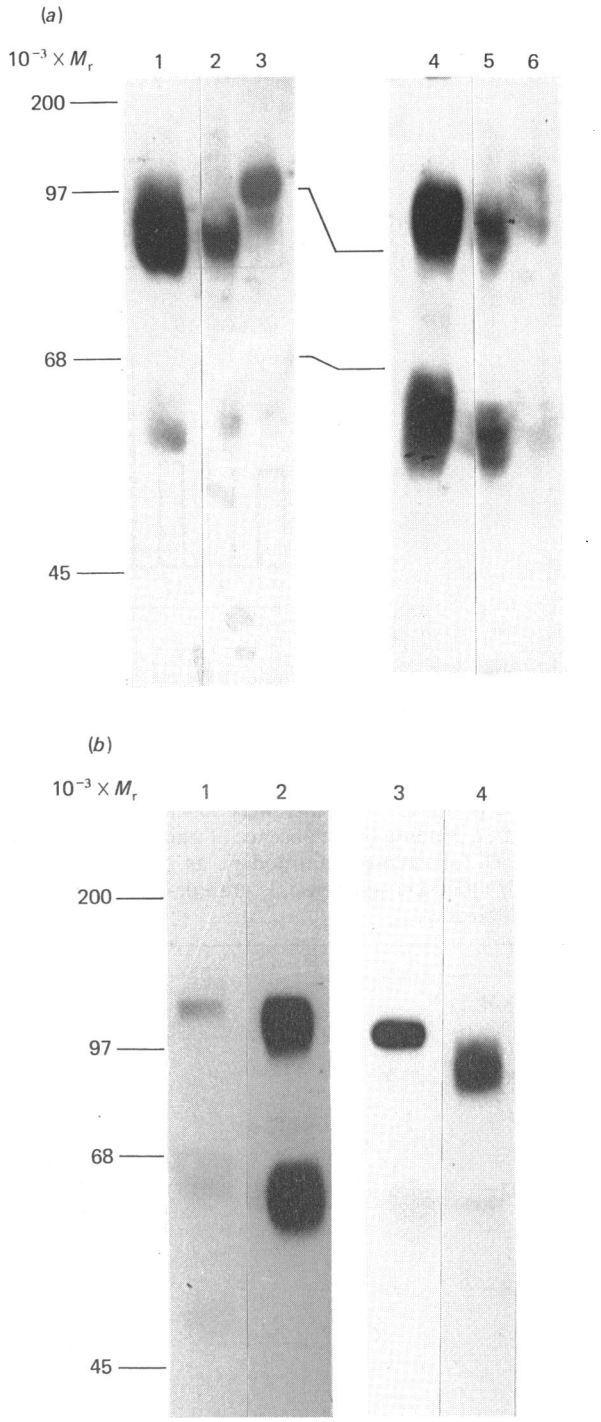


Fig. 7. Monoclonal antibody binding to secretory vesicle proteins from insulinoma, adrenal medulla, pituitary and liver

Samples (50 μg of protein/track) were subjected to SDS/PAGE, transferred to nitrocellulose and reacted with monoclonal antibody (hybridoma culture supernatant diluted 1:10). Binding was detected by autoradiography following incubation with ^{125}I-labelled sheep anti-(mouse IgG) (100000 d.p.m./protein track). (*a*) Binding of monoclonal antibodies GM 4 (tracks 1–3) and GM 10 (tracks 4–6) to insulinoma (tracks 1 and 4), pituitary (tracks 2 and 5) and adrenal medulla (tracks 3 and 6) secretory vesicles is shown. (*b*) Binding of monoclonal antibodies GM 4 (tracks 1 and 2) and GM 10 (tracks 3 and 4) to liver vesicles (tracks 1 and 3) and insulinoma granules (tracks 2 and 4) is shown.

number of antibodies was initially generated in each of three independent fusions, it appeared that only a few dominant antigens were recognized, at least in terms of those which could be characterized by immunoblotting. The monoclonal antibodies obtained reflected the serum antibody response in the mice used for fusions in this respect.

SGM 80 and SGM 110 appear to be relatively minor cellular components because their detection on immunoblots required purification of granules, the antigens not being detected on immunoblots of total cell membrane. Also, no corresponding bands were seen on Coomassie Blue-stained SDS electrophoretograms. The localization of the antigens principally in the secretory granule was supported by the finding that antibody binding correlated with the distribution of insulin among different subcellular fractions. Immunofluorescence microscopy revealed a punctate distribution within permeabilized cells consistent with a granule location. Furthermore, the antigens were barely detectable on immunoblots of a granule fraction prepared from an insulin-depleted insulinoma. Both antigens were glycoproteins, as shown by their binding to concanavalin A and in both cases the antibody binding sites appeared to be exposed to the internal face of the granule membrane.

The possibility cannot be excluded that the antigens were normally present in lysosomes but to a lesser extent than in secretory granules. Lysosomes and secretory granules may share common proteins such as proton pumps which have a similar function in both organelles (Hutton & Peshavaria, 1982; Reeves, 1984; Rudnick, 1985). However there was very little SGM 80 and SGM 110 in the insulin-depleted insulinoma, suggesting that granule membrane proteins might arrive in the lysosome compartment via crinophagy of mature granules or by endocytic retrieval of granule membranes following exocytosis, rather than as normal components of primary lysosomes.

Proteins with a similar electrophoretic mobility to SGM 80 and SGM 110 were detected by antibodies GM 4, 10, 15 and 21 in rat chromaffin granules and anterior pituitary secretory vesicles. The cell types from which these secretory vesicles were isolated share many common features with insulin-secreting cells, in terms of the pathway of granule biogenesis, proteolytic processing of their stored products, and the exocytotic release mechanism (see Poisner & Trifaro, 1982). It is not surprising therefore to find insulin secretory granule membrane proteins in the secretory granules of these other cell types. In addition, related peptides were also detected on immunoblots of liver vesicle membrane proteins but the precise cellular location here was not presently defined.

Previous investigations using gel electrophoresis of the protein composition of secretory granules have not detected proteins common to secretory vesicle membranes from various tissues (e.g. Corcoran *et al.*, 1982) and electrophoretograms of rat insulin secretory granule proteins (Hutton *et al.*, 1982) revealed little similarity to those of bovine chromaffin granule proteins (Abbs & Phillips, 1980). As illustrated here, however, immunological methods of detection may be required for the identification of common granule proteins, particularly minor membrane glycoproteins, whose molecular mass is subject to variation in different cell types. A similar conclusion can be drawn from the studies of Buckley & Kelly (1985), who found that a monoclonal antibody reacting with a cholinergic synaptic vesicle glycoprotein of 100000 Da cross-reacted with various endocrine and neural tissues, but with antigens of variable molecular mass. The relationship of this 100000 Da antigen to SGM 110 is unknown. However, it is of interest that a similar strategy to that presently used has resulted in the generation of monoclonal antibodies to lysosomal membrane glycoproteins in the 100000–120000 Da range by at least three other groups of investigators (D'Souza & August, 1986; Lewis *et al.*, 1985; Lippincott-Schwartz & Fambrough, 1986).

The antibodies obtained in the present study will be valuable reagents in the further investigation of the proteins SGM 80 and SGM 110. Since the epitopes on SGM 80 and SGM 110 are exposed on the luminal surface of the granule membrane and thus on the cell surface during exocytosis, it should be feasible to use antibodies conjugated to suitable markers to investigate the fate of the granule membranes after they have been endocytosed. The antibodies will also be valuable tools for the investigation of granule biogenesis and the regulation of secretory granule protein biosynthesis. They may provide the means to the eventual elucidation of the structures of SGM 80 and SGM 110 which may in turn reveal clues as to the function of these proteins.

We thank Dr. C. Milstein for the gift of myeloma cell lines, Dr. P. Gray for anti-(pro)insulin monoclonal antibody 3B7, and Dr. T. Gard for other antibodies. We are also grateful to the Medical Research Council, the British Diabetic Association and the Wellcome Trust for financial support. K.A.G. was the recipient of an MRC Research Training Scholarship. J.C.H. is a BDA Senior Research Fellow and K.S. is a Wellcome Lecturer.

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Received 15 December 1986/12 March 1987; accepted 9 April 1987