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Studies on co-localization of 7B2 and pancreatic hormones in normal and tumoural islet cells

Cinzia Azzoni[1], Ji-Yao Yu[1], Maria Teresa Baggi[1], Tiziana D'Adda[1], Cathy Timson[2], Julia M. Polak[2], and Cesare Bordi[1]

[1] Institute of Pathological Anatomy, University of Parma, Parma, Italy
[2] Department of Histochemistry, Royal Postgraduate Medical School, London, UK

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Summary. The distribution of protein 7B2, a protein with structural characteristics of GTP-binding proteins, has been studied in normal pancreatic islets and in a series of 70 pancreatic endocrine tumours with emphasis on the co-localization of 7B2 and the different pancreatic hormones. Although all cell types of normal islets were found to store 7B2, variations from intense expression to absence of reaction were seen within each cell type. In particular, B cells showed intense immunostaining for 7B2 in small compact islets and weak or no staining in larger islets with lobular arrangement. Pancreatic polypeptide (PP) cells expressed 7B2 intensely in the PP-rich area of ventral embryological origin, but were mostly non-reactive in the PP-poor area. The A cells, located along intralobular blood vessels, were more frequently immunoreactive for 7B2 than those at the periphery of the islets. Immuno-electron microscopy revealed a preferential localization of 7B2 in secretory granules of islet cells, with more intense localization in the peripheral halo of alpha granules. Benign islet cell tumours more frequently expressed 7B2 than their malignant counterparts. Although often expressed in a lower number of tumour cells than the tumour-specific hormone, 7B2 was usually co-localized with the latter. In contrast, no relationship was found with the localization of proinsulin. It is concluded that 7B2 is a non-permanent component of the cell granule compartment, probably involved in events related to exocytosis and without relationship to intracellular prohormone processing.

Key words: Pancreatic islets – Islet cell hormones – Pancreatic endocrine tumours – 7B2 – GTP-binding proteins

Introduction

In 1982 a protein designated 7B2 was isolated from porcine and human pituitary glands (Hsi et al. 1982; Seidah et al. 1983). Using radioimmunoassay and immunohistochemistry the protein, composed of about 180 amino acids and having an apparent molecular weight of 21000 was subsequently found to be widely distributed in the peripheral diffuse endocrine system (Iguchi et al. 1984; Suzuki et al. 1988). Accordingly, 7B2 was found to be elevated in plasma of patients with various types of endocrine tumours, for which it has been regarded as a potentially useful clinical marker (Suzuki et al. 1986; Iguchi et al. 1989; Ohashi et al. 1990). The protein is found during fetal life and presents high concentrations in plasma and in several tissues – including the pancreas – at term (Suzuki et al. 1985, 1987; Iguchi et al. 1987).

The immunohistochemical distribution of 7B2 in pancreatic islet cells was investigated by Suzuki et al. (1986) and Benjannet et al. (1988). These authors found a co-localization of the protein with insulin (Suzuki et al. 1986; Benjannet et al. 1988), glucagon and pancreatic polypeptide (PP) but not with somatostatin (Benjannet et al. 1988). The production of 7B2 by pancreatic endocrine tumours was well documented in tissue extracts from either a large series of human neoplasms (Suzuki et al. 1986) or experimentally induced tumours of mice (Benjannet et al. 1988), even though pronounced variations were seen not only between different types of tumours (the highest mean concentration of 7B2 being found in insulinomas; Suzuki et al. 1986) but also among individual cases of the same tumour type. The cellular localization of 7B2 in these neoplasms and its relation with intracellular hormone production has not been investigated systematically (Suzuki et al. 1986; Benjannet et al. 1988; Klöppel and In't Veld 1990).

In preliminary staining experiments we have found that the correspondence between immunohistochemical expression of 7B2 and the different types of islet cells was not so clear-cut as previously indicated. Therefore, we have undertaken a systematic comparative analysis on the co-localization of 7B2 and the different types of pancreatic hormones in normal human islets as well as in a series of 70 human pancreatic endocrine tumours.

Correspondence to: C. Bordi, Istituto di Anatomia Patologica, Università di Parma, I-43100 Parma, Italy

Table 1. Antibodies/antisera used in the present study

Antigen	Type and code	Working dilution	Source
7B2	P(r) (lot 29–39)	1:200	Prof. J.M. Polak, London, UK
Insulin	P(gp) A 564	1:1600	Dako, Dakopatts, Copenhagen, Denmark
Proinsulin	M GS-4G9	1:1600	Dr. O.D. Madsen, Gentofte, Denmark
Glucagon	P(r) 05Y	1:800	Dr. R.H. Unger, Dallas, Texas
Somatostatin	P(r) A 566	1:400	Dako
PP	P(r)	1:4000	Dr. R.E. Chance, Indianapolis, Indiana
Gastrin	P(r) 2064	1:1600	Dr. J. Rehfeld, Copenhagen, Denmark
VIP	P(r) 00495	Prediluted kit	Ortho Diagnostic System, Milan, Italy
Chromogranin A	M PHE 5	1:400	Ortho Diagnostic System

PP, Pancreatic polypeptide; VIP, vasoactive intestinal peptide; P, polyclonal (r, raised in rabbit; gp, raised in guinea pig); M, monoclonal

Materials and methods

Grossly and histologically normal specimens of human pancreatic tissue were obtained at surgery in patients operated on for insulinomas or at post-mortem from cadavers, shortly after removal of heart and kidneys for transplantation purposes. Samples of both the pancreatic tail (PP-poor, of dorsal embryological origin) and the PP-rich lobe of the pancreatic head (of ventral embryological origin; Malaisse-Lagae et al. 1979) were included. Tissues were fixed in Bouin's fluid for 18–24 h, dehydrated and embedded in paraffin. Islets of the small, compact type (see Results section) were identified in serial sections to rule out the possibility of a grazing section. For immuno-electron microscopy samples of histologically normal human pancreas removed at surgery were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 or in glutaraldehyde/methanol-free formaldehyde and embedded in Araldite. Thin sections were collected on nickel grids. After permeabilization of the resin with 10% hydrogen peroxide for 10 min the grids were incubated in normal goat serum to reduce non-specific binding. Incubation with the primary antiserum was normally carried out for 24–48 h at 4° C using one of four polyclonal IgG antibodies to 7B2. After rinsing, sections were subsequently incubated for 1 h at room temperature with gold conjugate goat anti-rabbit IgG (gold particle size 20 nm; Bio Clinical Services, Cardiff, UK). They were then counterstained with uranyl acetate and lead citrate and viewed under the electron microscope.

Tissue specimens of 70 endocrine pancreatic tumours obtained at surgery (58 patients) or at autopsy (4 patients) were similarly processed, with the exception of 12 tumours which were fixed in formalin. The tumours were classified on the basis of the associated clinical syndrome and/or the predominant hormonal production as assessed by immunohistochemistry using polyclonal antibodies against insulin, glucagon, somatostatin, PP, gastrin and vasoactive intestinal peptide (VIP) as described previously (Bordi et al. 1979, 1987). All malignant tumours showed lymph node and/or liver metastases except for 2 in which vascular invasion and infiltration of peripancreatic fat was documented. Benign glucagonomas and PP-omas were found in patients with multiple endocrine neoplasia syndrome type I. One glucagonoma was an independent satellite tumour in a patient with a larger insulinoma, and 1 PP-oma was found incidentally at autopsy.

Table 1 lists the antibodies/antisera used in the study and their respective working dilutions. The immunohistochemical procedure was carried out with overnight incubation at 4° C. The immunoreaction was visualized with the avidin-biotin complex (ABC) procedure (Vectastain ABC Kit; Vector Laboratories, Burlinghame, Calif., USA) using diaminobenzidine tetrahydrochloride as a peroxidase substrate. Co-localization of 7B2 and pancreatic hormones was investigated in consecutive 5-μm-thick sections or using the the antibody elution method of Tramu et al. (1978) for successive localization of two antigens on the same section. The elution technique was performed after the first immunocytochemical procedure and photography of recorded areas. Tissue sections were rehydrated in graded ethanols to water. Antibodies were then eluted by immersion with agitation in a potassium permanganate and sulphuric acid solution (1 vol. of 2.5% potassium permanganate and 1 vol. of 5% sulphuric acid in 50 vol. of distilled water). The elution time ranged from 1 to 3 min and the effective disappearance of the reaction was checked every 30 s under the microscope. The sections were then washed in running tap water and reimmersed in buffer before repeating the immunocytochemical technique for the other antigen. Finally, comparative photographs were taken from the areas previously photographed. In post-eluted sections antisera against pancreatic hormones were found to work better than the antiserum against 7B2. For this reason in the elution procedure the latter was always used for the first immunostaining step.

Results

Immunohistochemical staining for 7B2 in pancreatic islets of normal adult subjects showed two basic patterns, which represented the ends of a spectrum of intermediate findings. The first pattern was found in small, compact islets and was represented by a diffuse, homogeneous staining of virtually all islet cells (Fig. 1 A), most of which appeared to be insulin producing, B cells (Fig. 1 B). The second was presented by the larger islets described by Orci (1977) in which entering vascular channels divide the total islet mass into sublobules or trabecular structures. In these islets 7B2 was mostly expressed by cells bordering vascular channels, usually

Table 2. Immunohistochemical expression of 7B2 in 70 pancreatic endocrine tumours

Type of tumours	Number of tumours		Relation 7B2/tumour specific hormone[b]		
	Total	Positive[a]	+	=	−
Benign					
Insulinomas	28	23 (82)	1	16	6
Insulinoma-PPoma	1	1(100)	1		
Insulinoma-somatostatinoma	3	2 (66)			2
Glucagonomas	7	7(100)		7	
PP-omas	5	3 (60)		1	2
Non-functioning[c]	1	1(100)			1
Total	45	37 (82)	2	24	11
Malignant					
Insulinomas	6	5 (83)	1	3	1
Gastrinomas	3	2 (66)		2	
VIP-omas	2	2(100)		2	
Glucagonomas	4	2 (50)	1		1
Somatostatinoma	1	0 −			
PP-oma	1	0 −			
Non-functioning[c]	8	3 (37.5)		1	2
Total	25	14 (56)	2	8	4

[a] In parentheses: percentage of total tumours
[b] Roughly equal proportion of cells immunoreactive for 7B2 and for the specific, main hormone produced by the tumour is indicated by =; + indicates a predominance of 7B2 immunoreactive cells; − indicates a predominance of hormone immunoreactive cells
[c] The proportion of 7B2 immunoreactive cells is evaluated on the number of cells immunoreactive for chromogranin A

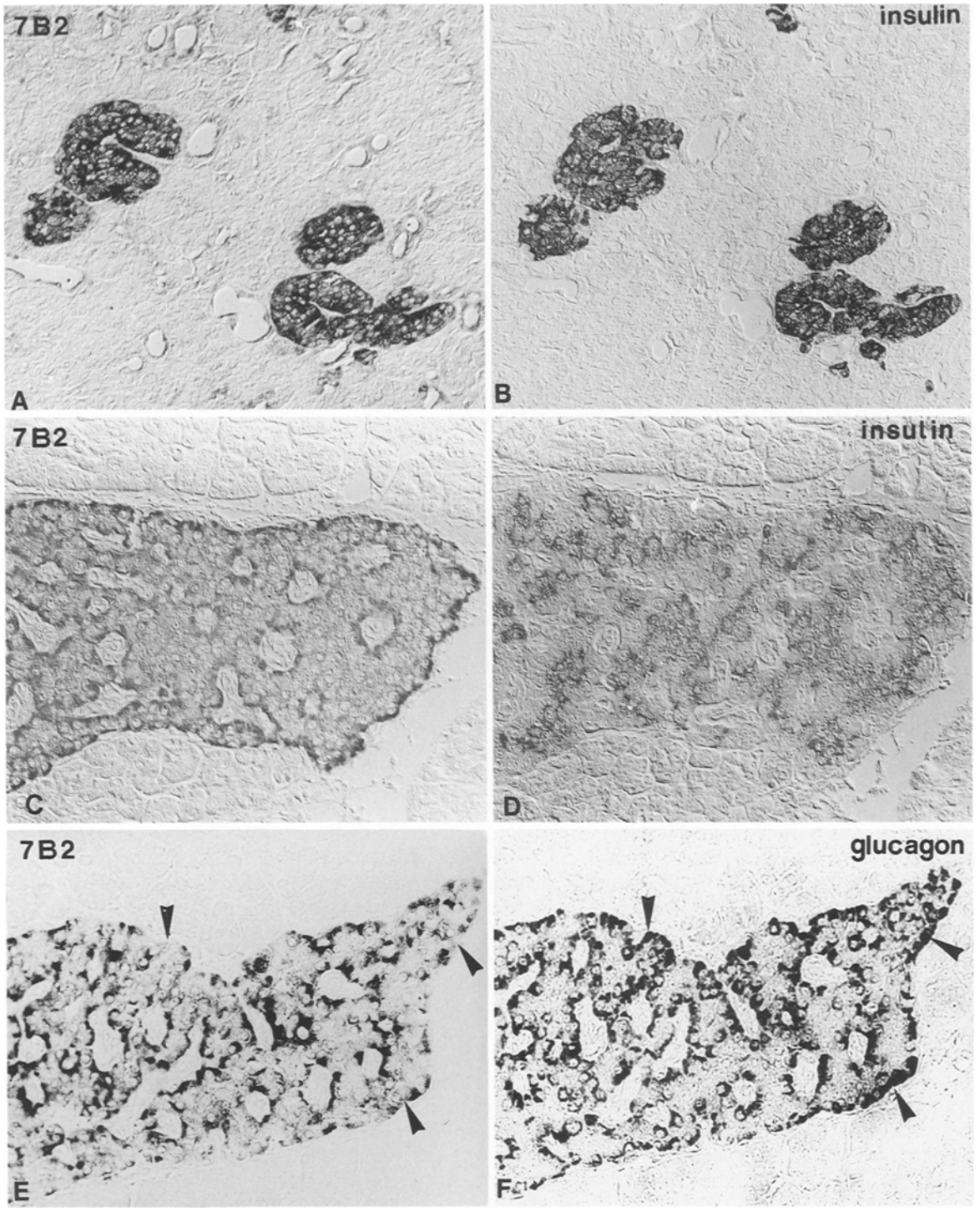
with accumulation in the cell paravascular poles (Fig. 1C). In general, such a distribution did not correspond to that of B cells, which were concentrated in the centre of the sublobules (Fig. 1D) (Orci 1977) and was mostly consistent with that of glucagon-producing A cells (Fig. 1E, F). The latter cells, however, were not always immunoreactive for 7B2, especially when they were located at the periphery of the islets, facing the surrounding acinar tissue (Fig. 1E, F).

Somatostatin-producing D cells were commonly negative for 7B2 and yet a few, scattered cells appeared to be strongly immunoreactive (Fig. 1G, H). Immunostaining of PP cells was largely dependent of the pancreatic region in which they were located. The abundant cells of the PP-rich area were usually immunoreactive (Fig. 1I, J) whereas the scattered cells of the PP-poor region were usually, but again not always, negative (Fig. 1K, L).

Immuno-electron microscopy analysis was focused primarily on the intracellular localization of 7B2 immunoreactive sites and revealed a selective deposition of immunogold particles over cytoplasmic secretory granules. In general A cell granules were more heavily stained than those of B cells with a preferential localization in the less dense peripheral halo of the alpha granule (Fig. 2).

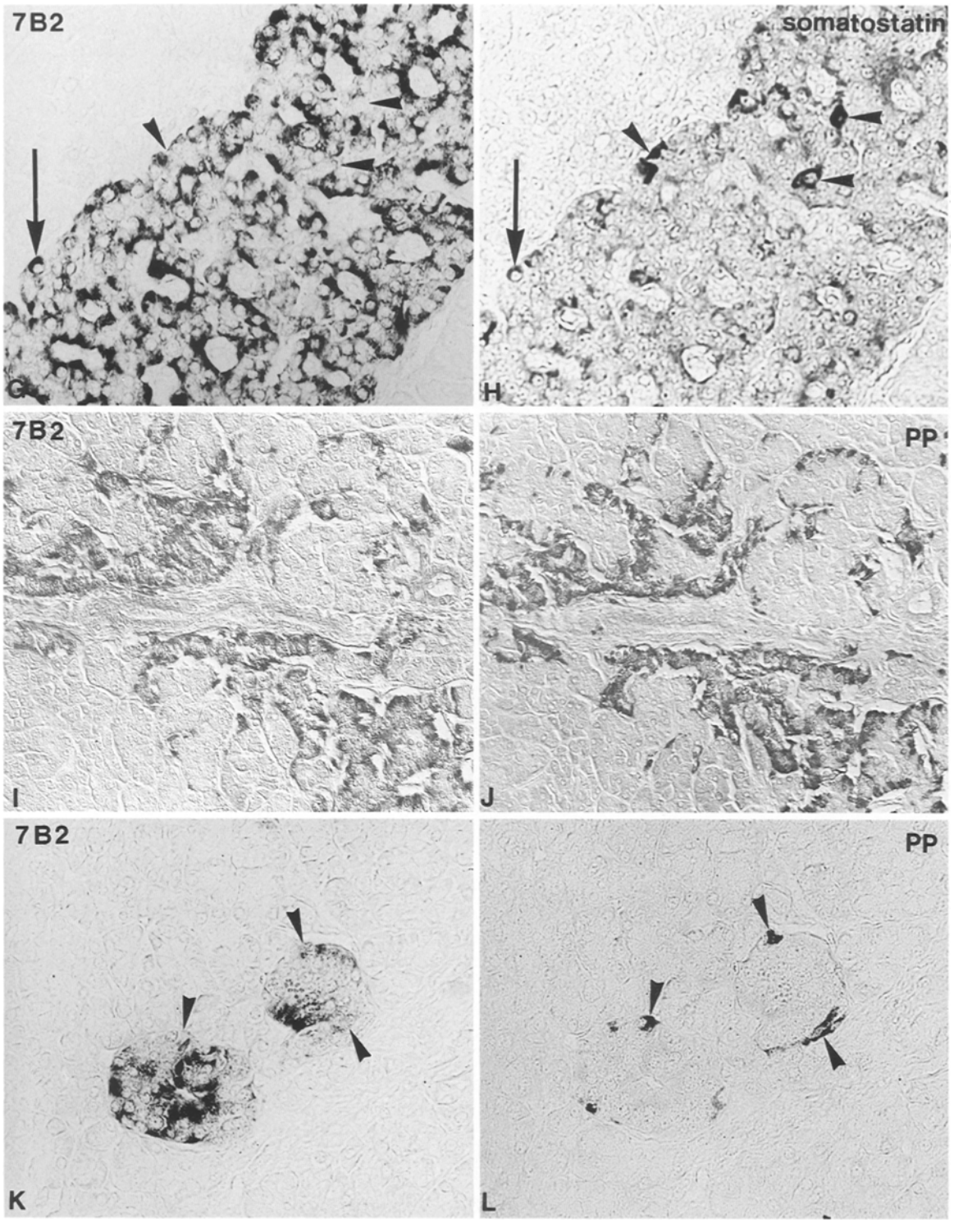
Results of immunostaining for 7B2 in pancreatic endocrine tumours are summarized in Table 2. In general, immunoreactivity for 7B2 proved to be sensitive to the conditions of tissue fixation and preservation, with lower staining in autopsy cases or in formalin-fixed specimens. In contrast, optimal immunostaining of 7B2 was consistently obtained in Bouin-fixed tissues. Cells immunoreactive for 7B2 were found in 82% of benign and 56% of malignant tumours. Immunostaining was usually more intense in insulinomas and gastrinomas even though a diffuse reaction was seen also in PP-omas, glucagonomas and VIP-omas (Figs. 3, 4). Somatostatinomas, either pure or of mixed type, showed the lowest staining intensity. Analysis of consecutive sections revealed that 7B2-immunoreactive cells, closely reproduced the topographic distribution of the latter in the tumour (Figs. 3, 4). although in most cases lower in number than cells immunoreactive for the tumours' specific hormone (Table 2). Within the tumour cells 7B2-immunoreactive material tended to concentrate in the basal pole of the cytoplasm where hormone-storing granules accumulate (Figs. 3A, F; 4A).

Among insulinomas, in particular, cells immunoreactive for 7B2 were found in 23 of 28 benign and in 5 of 6 malignant neoplasms. Their correspondence with tumour cells immunoreacting for insulin, as mentioned before, was seen not only in the diffusely immunoreactive cells of the trabecular, diazoxide-sensitive variant of insulinomas (Fig. 3A, B) but also in the heterogeneous, focally immunoreactive cells of the medullary variant of insulinomas (Fig. 3D, E) known to be diazoxide resistant (Berger et al. 1983). A similar correspondence was also found in metastatic tumour tissue (Fig. 3F, G). In contrast, no correspondence was found



Figs. 1–3. The staining technique is avidin-biotin-complex immunoperoxidase in all figures. Nomarski's interference contrast optics were used in photographs of Figs. 1A, B, I, J, and 2A–I. Elution technique with successive immunostaining of the same section is used for Figs. 1E–H, K and L. In all other figures comparisons are made on the same field of consecutive serial sections

Fig. 1A–L. Immunohistochemical staining for 7B2 (*left*) and pancreatic hormones (*right*) in normal islets. **A, B** Diffuse co-localization of 7B2 and insulin in small, compact islets, ×200. **C, D** Large, lobulated islet with 7B2 mostly concentrated in cells bordering vascular channels and insulin localized to cells in the centre of the sublobules, ×250. **E, F** Extensive co-localization of 7B2 and glucagon in perivascular A cells and frequent absence of 7B2 in peripherally located A cells (*arrowheads*), ×260. **G, H** Several somatostatin-producing D cells are negative (*arrowheads*) and one positive (*arrow*) for 7B2, ×310. **I, J** Diffuse co-localization of 7B2 and pancreatic polypeptide (PP) in islets of the PP-rich region, ×180. **K, L** Absent 7B2 immunoreactivity in the discrete PP cells (*arrowheads*) of the PP-poor region, ×340



with the localization of proinsulin which was mostly concentrated in the Golgi area of tumour cells (Fig. 3C), a finding consistent with the original observation of Roth et al. (1989). In this regard it worth noting that insulinomas characterized by diffuse immunoreactive content of proinsulin but virtually absent insulin accounted for 4 of 5 cases that presented negligible or no 7B2 immunostaining (Fig. 3H, I). Moreover, a very low 7B2 content was found in another tumour with largely predominant proinsulin immunoreactivity.

In non-functioning neoplasms localization of 7B2 was compared with that of cells immunostained for chromogranin A. In all cases but one the latter cells were largely more frequent than those immunoreactive for 7B2. The

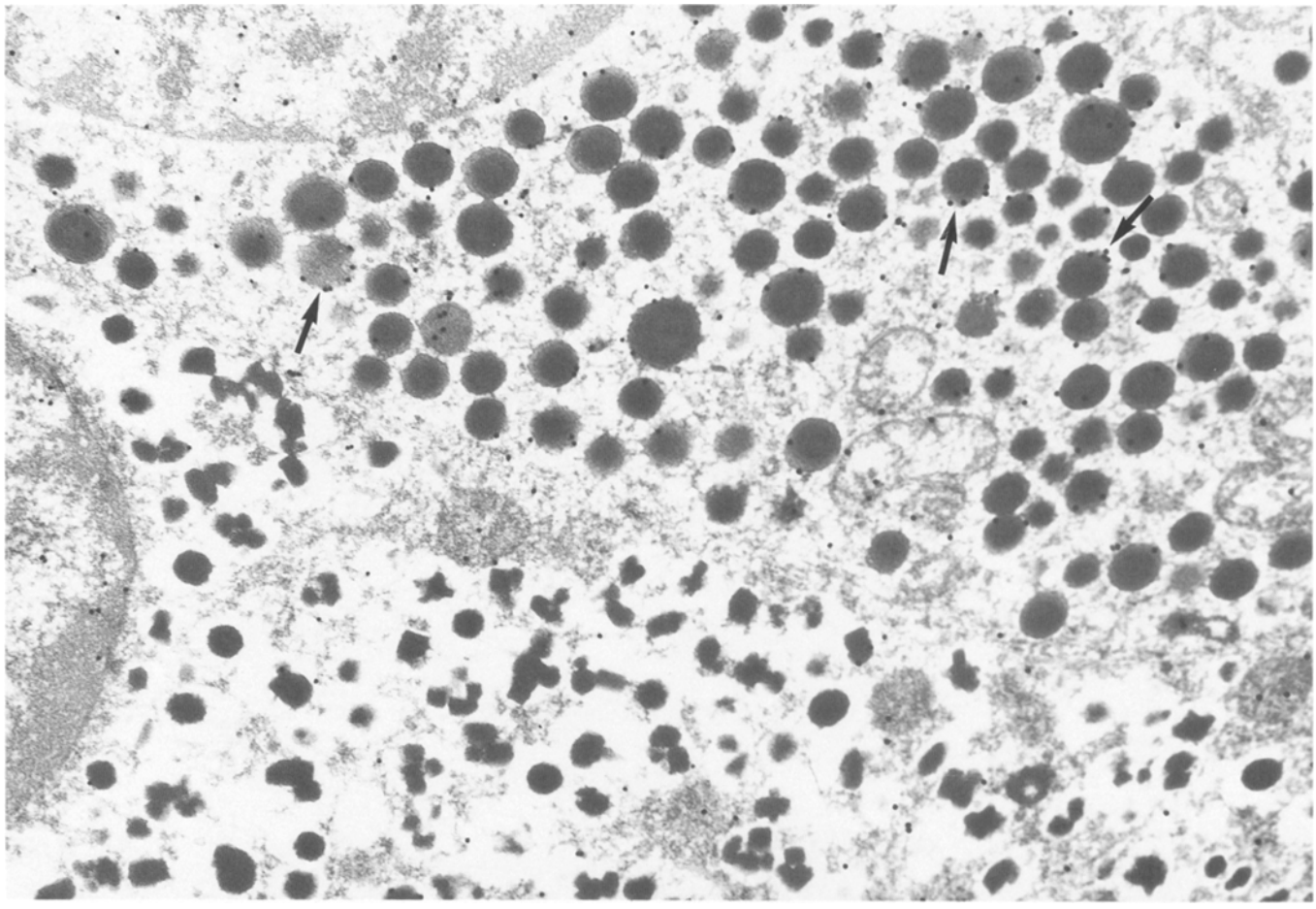


Fig. 2. Immunoultrastructural localization of 7B2 in adjacent A (*above*) and B (*below*) cells of a normal islet. Deposition of gold particles is more abundant on the secretory granules of the A cell, with preferential localization on the granule peripheral halo (*arrows*). Labelling of beta granules does not usually exceed the unspecific background level, ×45000

same result was found in two cases of malignant insulinomas largely composed of non-insulin-containing argyrophil cells.

Discussion

We have found that 7B2 may immunohistochemically be detected in all types of normal islet cells. In this regard our results largely agree with those of Benjannet et al. (1988), the small fraction of somatostatin D cells we have found to be 7B2-immunoreactive explaining the negative result of the previous study.

New information provided by our study is that for every cell type the co-localization of 7B2 and the respective hormone was not complete. The patterns of the observed discrepancies varied from one cell type to another. In particular, insulin-containing B cells were found to express 7B2 intensely in small, compact islets whereas they usually revealed very low or no expression of the protein in large islets with lobular or trabecular structure. In contrast, PP cells were diffusely immunoreactive for 7B2 in the PP-rich pancreatic region but were mostly unreactive in the PP-poor areas. Finally, glucagon-containing A cells were more frequently found to express 7B2 when located along intra-insular blood vessels than when confronting extra-insular acinar tissue at the islet edge.

The reasons for these discordant expressions of 7B2 are probably multiple. PP cells of the PP-rich pancreatic region differ from those of the PP-poor area by their distinct embryological origin (from the ventral or the dorsal pancreatic primordium, respectively; Malaisse-Lagae et al. 1979) and by the ultrastructural appearance of their secretory granules (Fiocca et al. 1983). Whether these variations underlie diverse functional activities of PP cells in different pancreatic regions is not yet clear. However, our results introduce an additional differential characteristic between the two variants of PP cell.

The difference in 7B2 expression of insulin-containing B cells between small, compact and large, lobulated islets suggests some diverging functions of these cells in islets of different size and organization. Our finding of intense 7B2 immunostaining in small islets is in full agreement with the intense immunostaining for 7B2 of pancreatic islets (and related consistently high tissue levels) found in infants with persistent hyperinsulinaemic hypoglycaemia (so-called nesidioblastosis) (Suzuki et al. 1986,

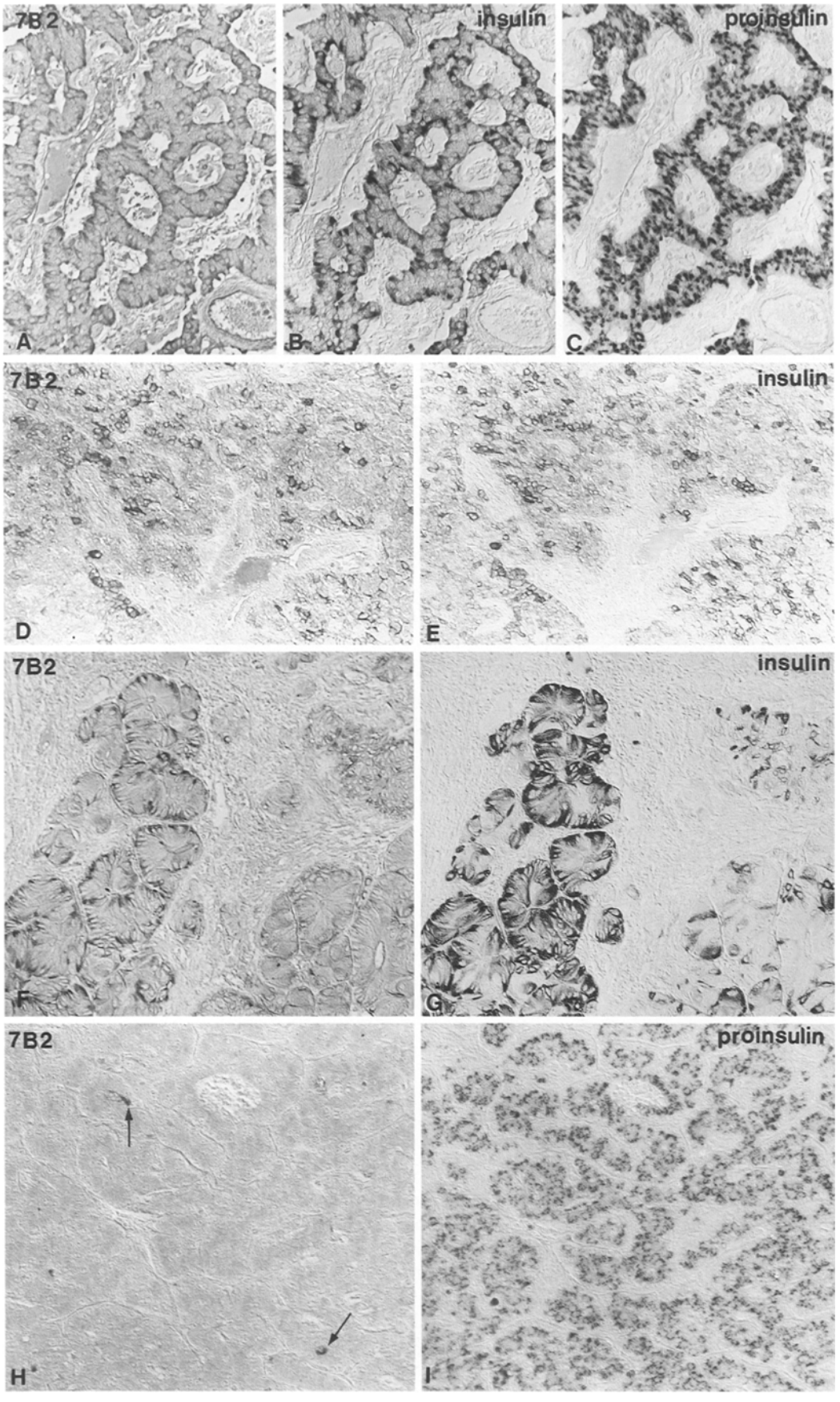


Fig. 3 A–I. Immunohistochemical staining for 7B2 (*left*) in insulinomas. **A–C** Trabecular type tumour with diffuse polarized staining for insulin (**B**) and, to a slightly lesser degree, 7B2 (**A**) whereas proinsulin (**C**) shows dot-like perinuclear staining, ×130. **D, E** Correspondence of 7B2 and insulin heterogeneous immunoreactivity in the medullary variant of insulinoma, ×120. **F, G** Correspondence of 7B2 and insulin localization in a tumour metastatic to the liver. Largely unreactive cells (*right*) are argyrophilic in consecutive sections (not shown), ×150. **H, I** Absent 7B2 immunoreactivity, except for occasional, discrete cells (*arrows in* **H**) in a tumour showing diffuse proinsulin staining (**I**) and virtually no insulin content (not shown), ×250

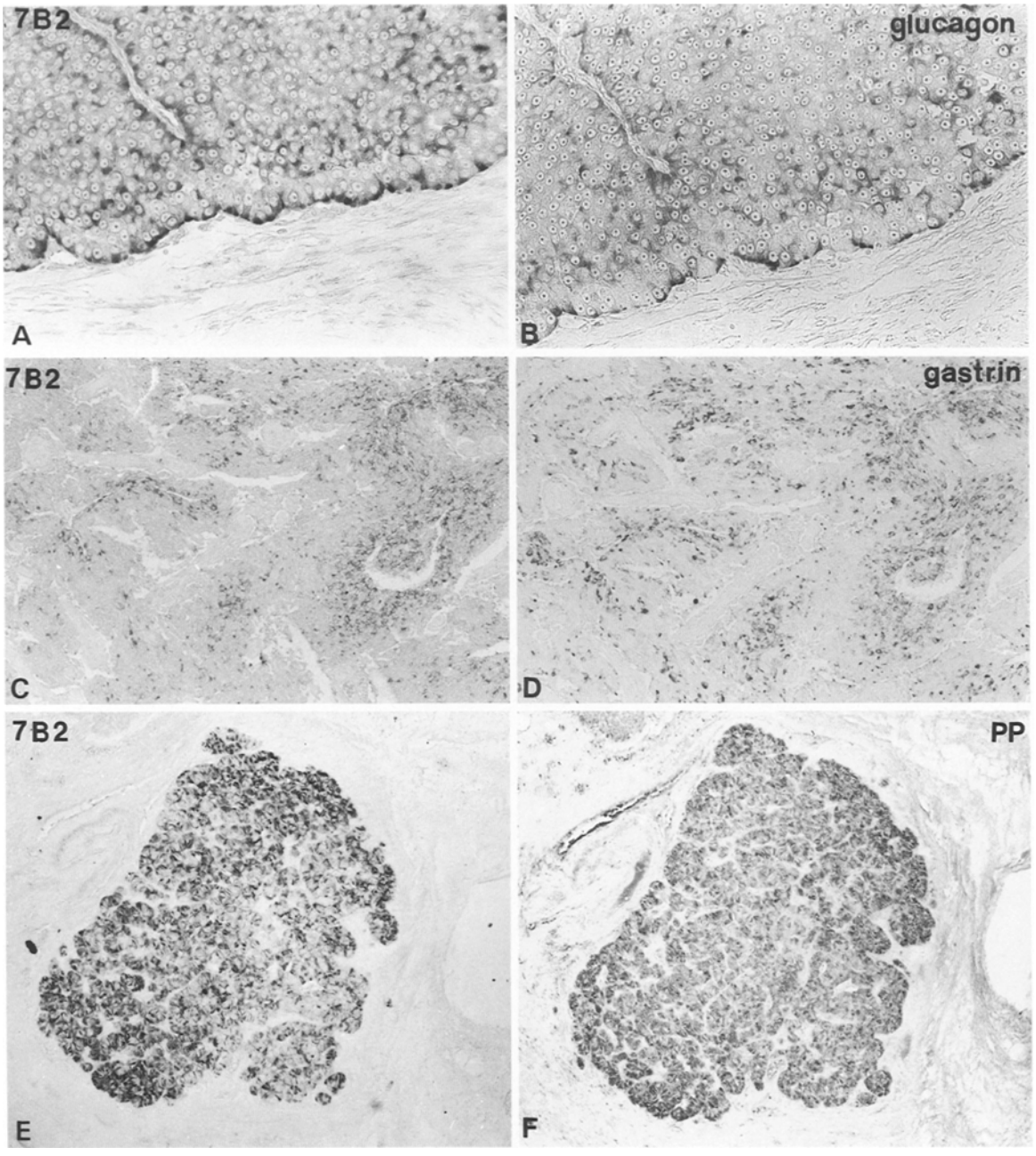


Fig. 4A–F. Immunohistochemical staining for 7B2 (*left*) in non-insulin-producing tumours. **A, B** Co-localization of 7B2, showing a slightly more diffuse distribution, and glucagon in a glucagonoma, ×180. **C, D** Correspondence of 7B2 and gastrin in heterogeneously immunoreacting cells of a gastrinoma, ×55. **E, F** Diffuse 7B2 immunoreactivity of a small PP-oma. ×35

1987). This condition is characterized by an excess of small islet clusters with poorly structured organization (Jaffe et al. 1980). It depends on the persistent excessive formation of new islet tissue due to a development anomaly probably involving defective regulation in the postnatal control of fetal growth (Klöppel and Heitz 1980). This observation, together with the present findings of an intense expression of 7B2 in most insulinomas, suggest a preferential association of this protein with proliferating and/or hypersecreting insulin-producing B cells.

The molecule of 7B2 has been shown to have 30% sequence homologies with duck proinsulin (Hsi et al. 1982), a finding leading to the hypothesis of 7B2 involvement in prohormone transmembranal transport, storage or maturation (Benjannet et al. 1988). The present study in insulinomas, revealing absent 7B2 immunoreactivity in tumours with virtually pure proinsulin content (Fig. 2H, I) and clearly divergent intracellular localization of 7B2 and pro-insulin (Fig. 2A, C), does not indicate a relation between 7B2 and human proinsulin or a role for 7B2 in pro-hormone processing. In contrast, the intracellular co-localization of 7B2 and stored, mature hormones we have seen in normal and in tumour islet cells by light microscopy immunohistochemistry is fully consistent with the results of immuno-electron mi-

croscopy of pancreatic islet cells (Benjannet et al. 1988; present results), pituitary cells (Marcinkiewicz et al. 1987) and thyroid calcitonin cells (Marcinkiewicz et al. 1988) showing that 7B2 is confined in the secretory granule compartment. In particular, 7B2 localization in the peripheral halo of alpha granules differs from that of glucagon, which is mostly confined to the dense central core of the granules (Bussolati et al. 1971), and corresponds to that of glicentin (Ravazzola and Orci 1980).

The human 7B2 structure presents three regions homologous to GTP-binding domains giving 7B2 structural characteristics of a GTP-binding protein (Martens 1988). GTP-binding proteins are presumed to participate in translocation of proteins across the endoplasmic reticulum (Connolly and Gillmore 1986), intracisternal protein transport within the Golgi stack (Melançon et al. 1987) and fusion of secretory granule and plasma membranes during triggered exocytotic secretion (Barrowman et al. 1986). Among these activities the evidence provided by the present study favours a role for 7B2 in exocytotic mechanisms or, as suggested by Benjannet et al. (1988), in post-exocytotic function as a hormone neurotransmitter and/or modulator. Our observation of wide fluctuations in the cell content of this protein even in the presence of an abundant granule content (as revealed by concomitant intense and polarized immunostaining for the specific hormone) suggests a transient role of 7B2 in exocytosis that deserves deeper insight.

In a previous immunohistochemical study of human pancreatic endocrine tumours Suzuki et al. (1986) found intense immunostaining for 7B2 in 5 of 6 insulinomas, less intense immunostaining in 3 of 9 glucagonomas, and no immunoreactivity in 4 VIP-omas. Gastrinomas, somatostatinomas and PP-omas were not investigated. Klöppel and In't Veld (1990) studied 5 benign and 1 malignant insulin-producing tumours. They found a minor population of intensely 7B2 immunoreactive cells in 3 cases and few, weakly positive cells in the remaining 3 neoplasms, including the malignant one. Benjannet et al. (1988) found 7B2 immunoreactive cells in 3 of 7 multihormonal islet cell adenomas induced in rats by streptozotocin-nicotinamide treatment. The protein consistently co-localized with insulin and occasionally with glucagon but not with PP and somatostatin. The present study confirms that 7B2 immunostaining is more intense in insulin-producing tumours and shows strong immunoreactivity in gastrinomas as well. Among other tumour types we have documented 7B2 production in glucagonomas (in agreement with previous data from human and experimental tumours mentioned above) and also in PP-omas and VIP-omas.

As far as the role of 7B2 as a tumour marker is concerned, we are unable to find significant advantages in comparison with immunostaining for specific hormones or for other general neuroendocrine markers such as chromogranin A. Such an assumption, however, has to be regarded as concerning the immunohistochemical domain only. In some tumours, such as glucagonomas and VIP-omas, Suzuki et al. (1986) showed that a low tissue tissue concentration of 7B2 is associated with elevated plasma levels, indicating that the 7B2 is rapidly secreted by tumour cells, with very little storage. Moreover, our observation that 7B2 immunoreactivity is very sparse or absent in B cell tumours containing proinsulin almost exclusively, provides the rationale for the occurrence of negligible amounts of 7B2 in tissue extracts of some insulinomas (Suzuki et al. 1986). A similar condition may explain the low expression of 7B2 in other tumours since prohormonal molecules are predominant over final hormonal forms in some glucagonomas (Bordi et al. 1979) and, probably in other tumour types (Yamaguchi and Abe 1982; Pauwels et al. 1986).

In our study we have found that the 7B2 is more frequently expressed by benign than by malignant neoplasms. Even though possible variations in the rate of secretion versus storage cannot be ruled out, it may be pertinent to note that expression of the gene encoding the 7B2 protein is related to the degree of cell differentiation as shown by analysis of cell lines derived from small cell lung carcinoma (SCLC) (Roebroek et al. 1989): only the classic type of SCLC cell line consistently expressed the gene which, in contrast, was lost in 6 of 7 cells lines of the less differentiated variant type.

In conclusion, the protein 7B2 is found in all cell types of adult human islets although with marked variations within each cell types partly dependent on the type of islet organization or on the regional distribution of individual cell types. The protein appears to be related to the hormone storing granular compartment of the cells but not to be involved in the prohormone processing, a condition regulating its localization in islet cell tumours.

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