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Functioning human insulinomas

An immunohistochemical analysis of intracellular insulin processing

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Abstract Sixty-seven insulinomas were investigated by immunohistochemistry using site-directed antibodies against insulin, proinsulin, chromogranin A, HISL-19, and four proteins directly or indirectly involved in the proteolytic processing of proinsulin: the prohormone convertases PC2 and PC3, carboxypeptidase H (CPH) and 7B2. Results were expressed in a six-grade score according to the frequency of immunoreactive tumour cells. Insulin was expressed by all tumours, appearing in either a diffuse or a polarized pattern and being detected in more than 30% of tumour cells in all cases but three. Proinsulin was also expressed in all tumours, with more than 50% of tumour cells immunoreactive in all cases but 5. It was consistently localized in the Golgi apparatus. In about half the cases, moreover, it also showed diffuse cytoplasmic staining, usually with a very sparse distribution. Trabecular and solid insulinomas did not present specific, homogeneous patterns of insulin immunostaining. However, insulin immunoreactivity was much more abundant in trabecular than in solid neoplasms, being present in virtually all tumour cells (score 6) in 50% and 8% of cases, respectively. Virtually all insulinomas expressed PC2, PC3, CPH and 7B2, usually in 30–100% of tumour cells, with a frequency significantly related to that of insulin. However, detection of PC2 and 7B2 was slightly less frequent than that of PC3 and CPH. In consecutive sections these proteins were found to be mostly co-localized with insulin and chromogranin A but not with proinsulin. They were heavily expressed in all 10 tumours with more than 10% of cells showing cytoplasmic proinsulin immunoreactivity, indicating that the leakage of proinsulin from the Golgi compartment is not associated with faulty expression of converting enzymes and possibly reflects a saturated processing capacity. HISL-19 immunoreactivity was found in both Golgi apparatus and insulin stores, indicating that the relevant antigen is different from all other proteins investigated. These results do not support a defect in expression or localization of proinsulin-processing enzymes in most insulinomas.

Key words Insulinomas · Prohormone convertases · Carboxypeptidase H · Insulin · Proinsulin

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Introduction

Insulinomas are functionally characterized by inappropriate release of insulin and/or precursor molecules, usually resulting in the hypoglycaemic syndrome. The classic view supports that the inability of insulin-producing tumour cells to control insulin release in spite of the concomitant severe hypoglycaemia is the main functional defect responsible for the clinical syndrome [7]. However, a potential role for defective mechanism(s) in the intracellular insulin processing has also been considered [9, 18, 19].

In recent years, our knowledge of intracellular mechanism(s) governing the proteolytic conversion from proinsulin to insulin has increased significantly, and the role of the specific endo- and exoproteases involved has been clarified (for a review see [24]). However, immunohistochemical studies of insulinomas with respect to the proteins directly or indirectly involved in this process have

Table 1 Primary antibodies used in the present study (*M* monoclonal, *P* polyclonal)

Antigen	Type	Code	Dilution	Source
Insulin (INS)	M	Mab INSUL #5	1:8000	Authors' own [6]
Proinsulin (P-INS)	M	GS4-G9	1:1600	Authors' own [14]
PC2	P	PC2-P4	1:1000	Dr. D.F. Steiner, Chicago, Ill. [20]
PC3	P	RS20	1:1000	Dr. D.F. Steiner, Chicago, Ill. [22]
CPH	P	CPH-2-2-7	1:1000	Authors' own[a] [5]
7B2	P	7B2-2-3-7	1:1000	Authors' own[a] [5]
Chromogranin A (CgA)	M	LK2H10	1:250	Biogenex Laboratories, San Ramon, Calif.
HISL-19	M		1:800	Dr. G. Eisenbarth, Boston, Mass. [23]

[a] These antibodies were raised and characterized by one of us (L.S.) in 1991, while he was a Senior Researcher at the Division of Pathology, the National Tumour Institute, Milan, Italy

Table 2 Distribution of immunoreactivity scores for different antigens in 67 human insulinomas (*INS* insulin, *P-INS* proinsulin, *PC2* prohormone convertase 2, *PC3* prohormone convertase 3; *CPH* carboxypeptidase H, *CgA* chromogranin A)

Score[a]	INS (*n*=67)	P-INS* (*n*=67)	PC2 (*n*=67)	PC3 (*n*=67)	CPH (*n*=65)	7B2 (*n*=65)	CgA (*n*=67)	HISL-19[b] (*n*=67)
6	18	60	18	20	21	18	18	48
5	36	2	16	30	30	24	29	6
4	10	2	16	13	12	9	10	6
3	1	1	7	2	3	8	4	5
2	1	1	5	3	1	4	2	2
1	1	1	4	1	0	3	2	0
0	0	1	1	0	0	0	0	0
P vs INS			0.0001	0.0001	0.0016	0.0001	0.0001	

[a] 6, >90%; 5, 60–90%; 4, 30–60%; 3, 10–30%; 2, 1–10%; 1, <1%
[b] Includes both perinuclear and diffuse staining

been few. Indeed, these studies are restricted to the analysis of prohormone convertases PC2 and PC1/PC3 (hereinafter referred to as PC3) in a cumulative analysis of neuroendocrine tumors of various organs [21] or in a small series of three insulinomas [9] and of 7B2 in cumulative studies on neuroendocrine tumours of various organs [25] or of the pancreas [1].

We have performed a systematic, immunohistochemical analysis on the distribution of four substances related to the mechanisms of proinsulin processing, i.e. PC2, PC3, carboxypeptidase H (CPH) and 7B2, in a large series of insulinomas. We have compared their expression with that of proinsulin and insulin, as shown by site-directed monoclonal antibodies, and with that of other antigens previously found in insulinomas, such as chromogranin A [4, 13], and HISL-19 [4].

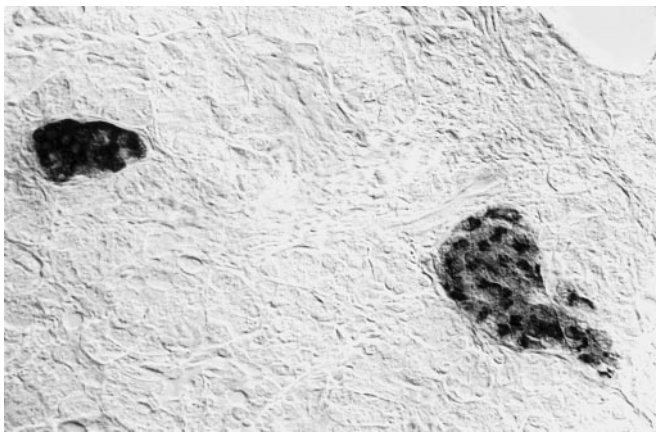

Fig. 1 Discordant patterns of proinsulin immunostaining in contiguous islets of a pancreatic lobule surrounding an insulinoma. The islet on the *right* shows the typical dot-like staining corresponding to the Golgi apparatus of B cells, whereas that on the *left* shows abnormal, diffuse cytoplasmic staining. ×210

Materials and methods

Specimens of tumour were obtained at surgery from 67 patients (23 male, 44 femals; mean age 44.8 years; range 15–80 years) affected by the hypoglycaemic syndrome. Peritumour pancreatic tissue was available in 54 cases. In addition, specimens of pancreatic tissue from 10 patients not affected by insulinoma were also available. All tissues were fixed overnight in Bouin's fluid and routinely processed for paraffin embedding. Consecutive 5-µm-thick sections were mounted on silanized slides and were stained with haematoxylin and eosin for identification of the histological features of the tumours and with immunohistochemistry using a panel of primary antibodies listed in Table 1, with or without pre-exposure (three times, 5 min each) in a microwave oven in citrate buffer (pH 6.0). Biotinylated goat anti-rabbit (code BA-1000; working dilution, 1:200) or horse anti-mouse (code BA-2000; working dilution, 1:100) immunoglobulins (both from Vector Laboratories, Burlinghame, Calif.) were then used as a secondary antibody, followed by the avidin–biotin complex peroxidase technique (Vectastain ABC Kit, Vector Laboratories) with diaminobenzidine tetrahydrochloride as a chromogen substrate. Sections of normal pancreatic tissue were used for positive controls, and preadsorption of primary polyclonal antibodies with the synthetic peptides used for their generation or substitution of the primary monoclonal antibodies

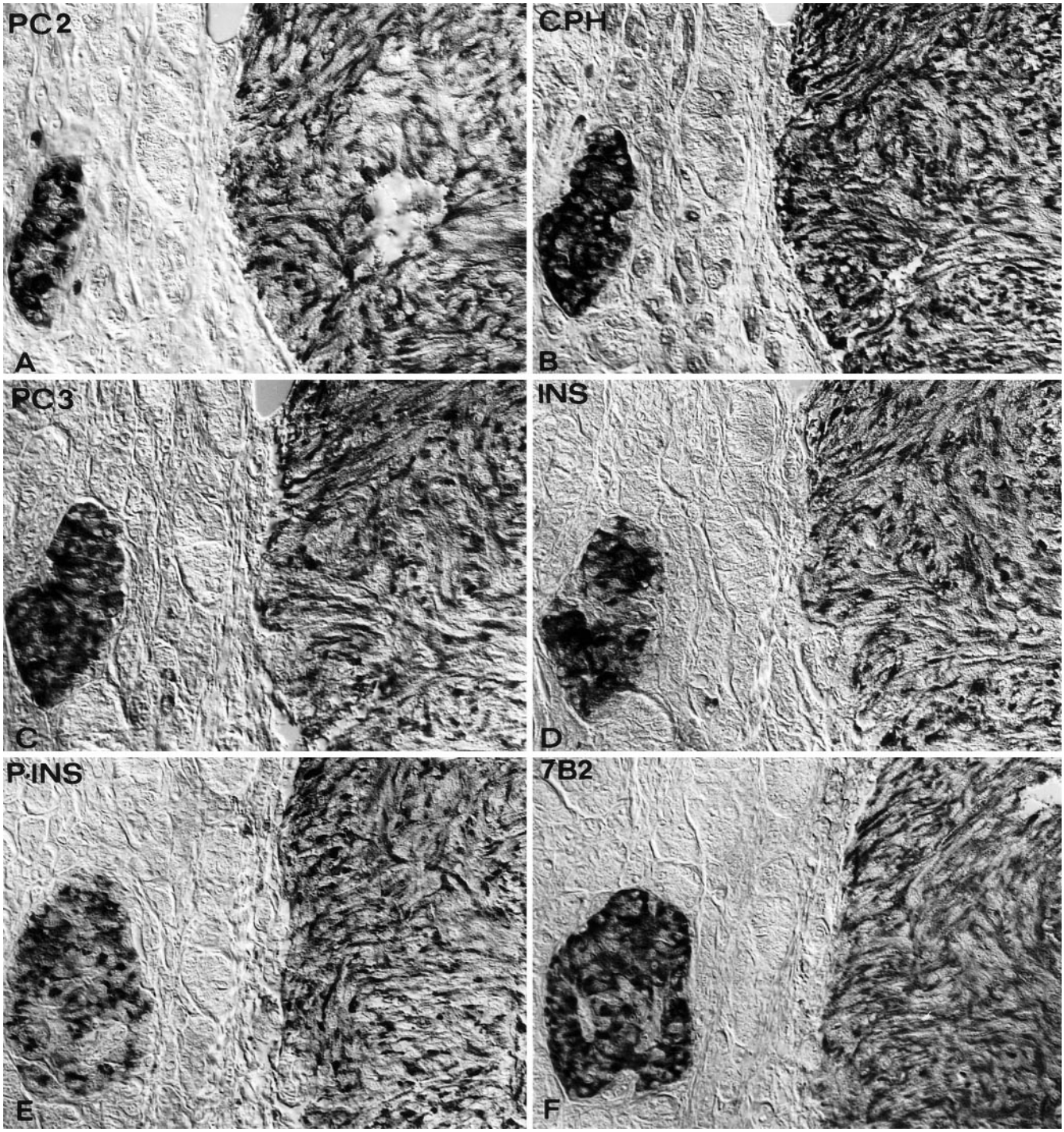

Fig. 2A–F Immunolocalization of proinsulin (*P-INS*), insulin (*INS*), *PC2* and *PC3* convertases, carboxypeptidase H (*CPH*) and 7B2 in a solid insulinoma with spindle-shaped cells and in a peritumour islet. In the latter the mixed pattern of proinsulin immunostaining (E) and the preferential location of PC2 in peripheral cells, mostly corresponding to A cells, are apparent. Consecutive sections presented in order (**A–F**) of cutting. ×210

with immunoglobulins of the same class and concentration were used as negative, specificity controls.

Classification of the tumours according to histological structure (trabecular, solid, glandular and mixed) [18] was achieved in haematoxylin-eosin-stained sections. However, some tumours that had a solid appearance in these sections actually disclosed tight apposition of trabeculae when immunostained for insulin. They were classified as trabecular.

The immunohistochemical results were evaluated according to the six-point score used in this laboratory and are based on a semiquantitative evaluation of the percentage of immunoreactive tumour cells [1]: 1, <1%; 2, 1–10%; 3, 10–30%; 4, 30–60%; 5, 60–90%; 6, >90%. Scores 1 and 6 were separated (out) to account for findings likely to be of no biological significance (score 1) or of potential specific biological significance (score 6, virtually all cells immunoreactive, see later, in the "Discussion").

The monoclonal antibody against human insulin, described in detail elsewhere [6], showed no interference of proinsulin up to a concentration of about 1 pmol proinsulin/ml. The monoclonal an-

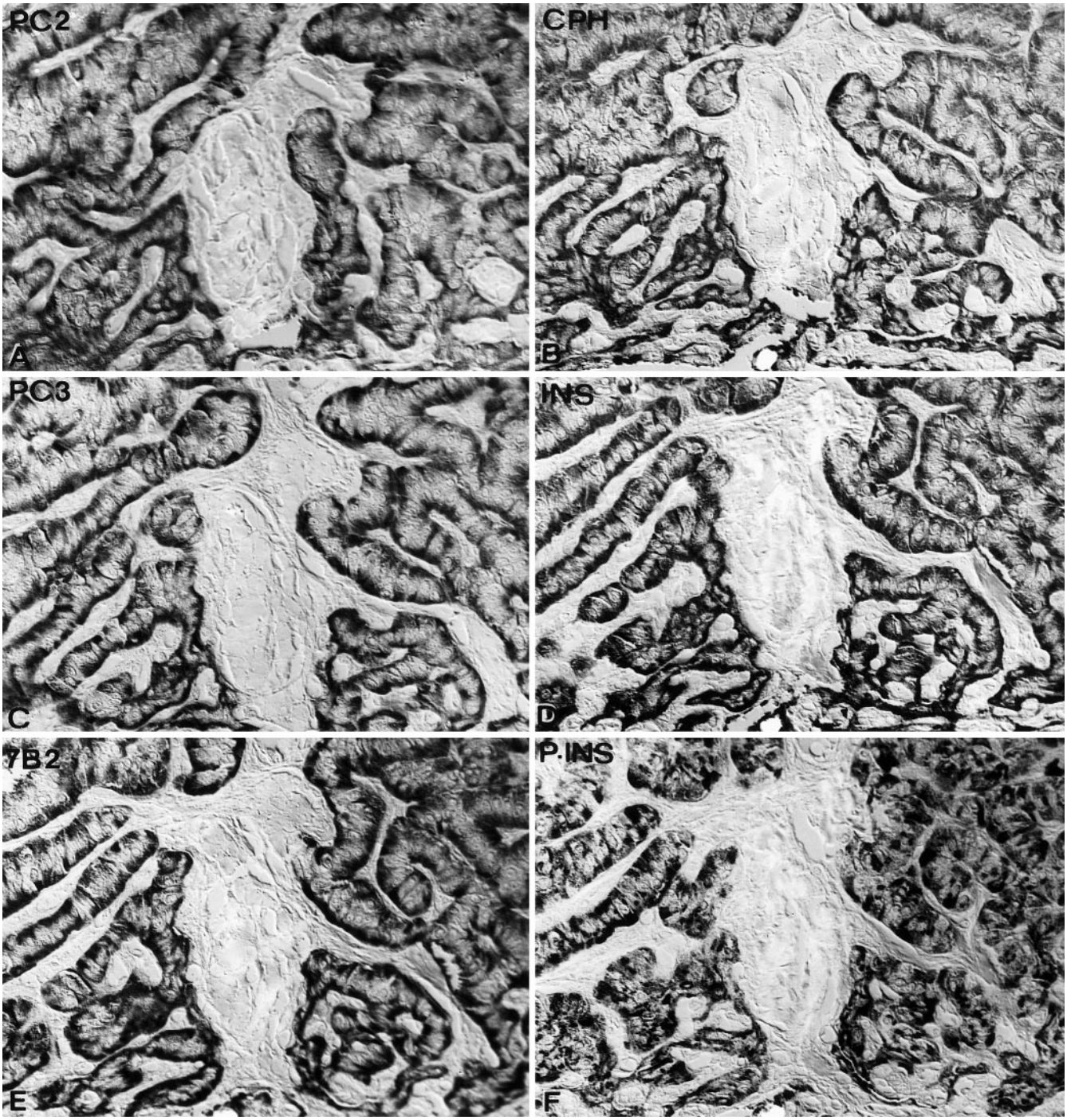

Fig. 3A–F Representative patterns of immunostaining in a trabecular insulinoma. As is characteristic for this type of tumour, cytoplasmic immunoreactivity for P-INS in several tumour cells coexists with the polarized, mutually overlapping immunostaining for the other substances involving virtually all tumour cells. Consecutive sections in order (**A–F**) of cutting. ×210

tibody GS-4G9 against proinsulin recognizes an epitope encompassing the dibasic processing site Arg_{31}–Arg_{32} [14]. The polyclonal antibodies directed against short synthetic segments of the molecule of PC2 (prepro$PC2_{611–630}$; code: P4) [20], PC3 (prepro$PC3_{Cys95–108}$+prepro$PC3_{110–122}$; code: RS20) [22], CPH (human $CPH_{440–453Cys}$; code: CPH-2-2-7), and 7B2 (human $7B2_{1–14Cys}$; code: 7B2-2-3-7) [5] have previously been characterized in detail. The monoclonal antibody HISL-19 was produced after immunization of BALB/c mice with human islet cells isolated from cadaveric pancreatic specimens [23]. It reacts with many neuroendocrine cells and neurons of human, bovine and porcine origin but not with those of rodents or of the angler fish [1, 2]. Its antigenic epitope is present in four islet cell proteins with molecular weights in the region of 120, 69, 67, and 56 kDa [10, 23] likely representing related products resulting from sequential posttranslational cleavage and processing of a common precursor protein [10, 23]. Although the 67-kDa (35/32 dimer) protein shares many biochemical and molecular features with the chromogranin proteins it differs from the latter in tissue distribution, molecular weight and immunological cross-reactivity [11].

Data were analysed using the nonparametric correlation of Spearman. *P*-values less than 0.05 were considered significant.

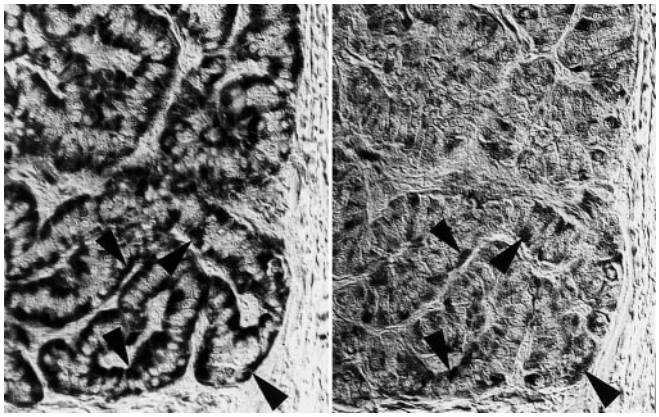

Fig. 4 Comparison of consecutive sections of a trabecular insulinoma showing colocalization of abnormal cytoplasmic proinsulin (*right panel: arrowheads*) with insulin (*left panel: arrowheads*). ×230

Results

The normal islets in peritumour pancreas showed diffuse cytoplasmic immunostaining for insulin and dot-like or crescent-shaped perinuclear spots, corresponding to the Golgi complex [16], that were immunoreactive for proinsulin. In addition, some degree of diffuse cytoplasmic immunostaining for proinsulin was seen in 8 cases (Figs. 1, 2), a finding absent from the pancreatic islets of patients without insulinoma. Diffuse cytoplasmic immunostaining for PC2, PC3, CPH and 7B2 was consistently found in non-tumour islets even though 7B2 and, in particular, PC2 yielded strong staining of peripheral glucagon A cells and weak staining of central insulin B cells (Fig. 2).

The immunohistochemical results obtained in our series of insulinomas are summarized in Table 2. Insulin was expressed in all tumours, being found in more than 30% of tumour cells in all cases except 3. The immunostaining showed either uniform distribution in virtually all tumour cells (score 6; Figs. 2–4) or heterogeneous distribution in discrete or clustered cells variously intermingled with nonreactive cells (Fig. 5). When the tumours were subdivided according to the histological structure (Table 3), the trabecular tumours consistently showed the greatest abundance of immunoreactive cells (Figs. 3, 4) which in no case accounted to less than 60% of the tumour cells. However, tumors with immunostaining in virtually all cells (score 6) were also found among solid, mixed solid-trabecular or glandular histological variants, including 2 cases of insulinoma composed of spindle cells (Fig. 2). Two intracellular patterns of insulin immunostaining were presented by tumour cells in either pure or combined forms: (a) a polarized pattern reflecting insulin accumulation at the basal cell pole facing blood capillaries (Figs. 3, 4); (b) a diffuse pattern involving the whole cytoplasm (Fig. 5). The former was more commonly found in trabecular tumours and the latter was the usual finding in solid tumours with a heterogeneous content of immunoreactive cells. Proinsulin was also expressed by all tumours, usually in the form of paranuclear, Golgi-related aggregates (Fig. 5). In 34 tumours (50.7%) diffuse cytoplasmic proinsulin immunoreactivity was also found (Table 4, Figs. 3, 4). However, in 19 of these cases the finding was rare (<1% of tumour cells), and only in 10 cases did it occur in more than 10% of tumour cells.

Immunostaining of PC2, PC3, CPH and 7B2 was observed in virtually all insulinomas and, as detailed in Table 2, was mostly found in a range of 30–100% of tumour cells. Semiquantitative scoring of immunoreactive cells in individual cases showed a significant relation with that of insulin for all proteins (Table 2). Detection of PC2 and 7B2 was slightly less frequent than that of PC3 and CPH. Moreover, the immunostaining of PC3, CPH and 7B2 was of an intensity comparable to that of insulin, whereas that of PC2 was weak (Fig. 5), except in cases with immunoreactivity of virtually all cells (score 6), in which the staining was intense (Fig. 3).

When these proteins were compared with each other and with insulin for immunostaining in consecutive sections a remarkable overlapping in terms of both intratumour topographic distribution and cytoplasmic pattern (polarized vs diffuse) was observed (Figs. 2, 3, 5). Only occasionally did single or clustered cells show immunoreactivity for convertases, CPH or 7B2, in the absence of insulin or vice versa. In contrast, no immunostaining of the Golgi area of tumour cells for PC2, PC3, CPH, and 7B2 was seen, a major difference with respect to the intracellular localization of proinsulin. It is worth noting that all 10 cases showing diffuse cytoplasmic immunoreactivity for proinsulin in at least 10% of tumour cells (score 3–6) showed the maximal expression (score 6) both of all proteins related to proinsulin conversion (including PC2) and of insulin (Figs. 3, 4).

Table 3 Distribution of cells showing diffuse cytoplasmic staining for proinsulin and HISL-19 in 67 human insulinomas

Score	P-INS	HISL-19
6	1	4
5	1	3
4	2	6
3	6	11
2	5	18
1	19	16
0	33	9

Table 4 Distribution of immunoreactivity scores for insulin in 66 human insulinomas subdivided according to the histological structure*

Score	Trabecular (*n*=26)	Solid (*n*=25)	Glandular (*n*=4)	Mixed (*n*=11)
6	13	2	1	2
5	13	14	1	7
4	–	8	1	1
3	–	1	–	–
2	–	–	–	1
1	–	–	1	–
0	–	–	–	–

* not evaluable in 1 case

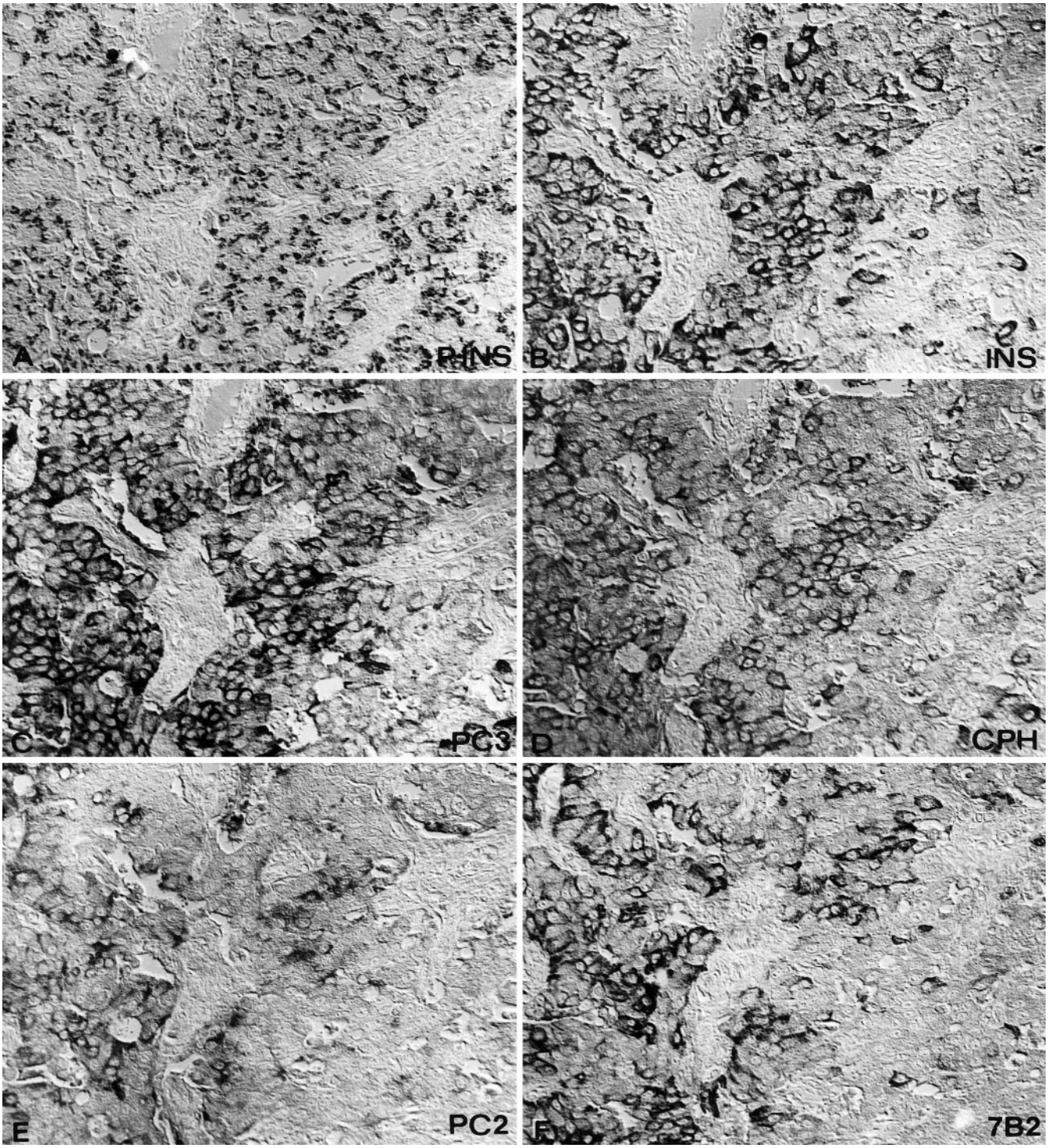

Fig. 5A–F Representative patterns of immunostaining in an insulinoma with solid histological pattern. P-INS is overall found in tumour cells, showing dot-like staining corresponding to the Golgi areas. In contrast, INS, PC3, CPH, and 7B2 obviously reveal cytoplasmic localization and are heterogeneously distributed in contiguous areas with topographic overlapping. PC2 is less expressed. Consecutive sections in order (**A–F**) of cutting. ×210

Immunostaining for the monoclonal antibody HISL-19 was found in all cases, usually involving more than 60% of tumour cells (Table 1). As previously reported [4], the most common pattern was represented by focal localization in the Golgi areas (not shown). Diffuse or polarized cytoplasmic was also present in all tumours except 9, but usually found in minor cell populations (Table 4). The extent of cytoplasmic staining was significantly related to that of proinsulin ($P<0.0001$). Immunoreactivity for chromogranin A showed both diffuse and

polarized cytoplasmic patterns and was topographically consistent with that of insulin.

Discussion

When 67 functioning insulin-producing tumours were examined, insulin and proinsulin were found in all of them. In particular, insulin was expressed in more than 30% of tumour cells in all cases but 3, and proinsulin in more than 50% of tumour cells in all but 5 cases. In the only previous similar study comparing insulin and proinsulin in a large series of 76 insulinomas [18], 8 and 15 tumours unreactive for insulin or proinsulin, respectively, were found. We suggest that the lack of nonreactive cases in our series may depend on the exclusive use of Bouin fixation, which is known to allow better preservation of intracellular hormonal peptides [12].

Each of the two peptides presented two characteristic intracellular patterns of immunostaining in insulinomas. Immunoreactive proinsulin showed either focal, perinuclear staining, occurring in virtually all cases, or diffuse cytoplasmic staining, which occurred in about half the cases, usually with a very sparse distribution. Immunoreactive insulin, in contrast, showed either diffuse or polarized cytoplasmic localization. Roth et al. [18] pointed out that the perinuclear staining of proinsulin, corresponding to its localization in the Golgi compartment, and the diffuse cytoplasmic staining of insulin in insulinomas reflect the usual distribution of these peptides in normal human islets, as originally described by Orci et al. [16, 17]. In contrast, diffuse cytoplasmic staining of proinsulin and polarized staining of insulin are patterns peculiar to insulinoma cells, a finding substantially confirmed by our results. Based on these observations, Roth et al. [18] subdivided four groups of insulinomas according the different combinations of proinsulin and insulin immunostaining: (a) normal (found in 3.9% of cases); (b) near-normal, characterized by polarized immunostaining for insulin and by absence of diffuse immunostaining for proinsulin (32.9%); (c) intermediate, characterized by diffuse immunostaining for proinsulin (39.5%); and (d) abnormal, with heterogeneous irregular patterns (23.7%). These different patterns of immunohistochemical expression were found to be related to neither the histological structure nor the clinical characteristics of the tumours. In our current experience diffuse and polarized immunostaining of insulin represented the ends of a spectrum and it was not rare for the two to be associated within the same cells, making their semiquantitative assessment very difficult. For this reason a simplified semiquantitative evaluation of insulin immunostaining based on the frequency of immunoreactive cells irrespective of the intracellular localization of the hormone was adopted in the present study.

A relation between the histological structure of insulinomas and their pattern of insulin immunostaining was originally proposed by Woodtli and Hedinger [26] using polyclonal antiserum raised in the guinea pig. These authors subdivided two groups of neoplasms, one with trabecular architecture showing equal, polarized insulin immunofluorescence in all tumour cells and the other with a solid or medullary structure with unevenly distributed immunofluorescent cells scattered among unreactive cells. In a subsequent ultrastructural morphometric study of 10 insulinomas, Berger et al. [2] found that tumours with a high frequency of cells containing typical beta granules (group A) clinically responded to insulin inhibitors such as diazoxide or somatostatin, whereas tumours with a low number of the same cells (group B) were nonresponsive to both agents and associated with elevated circulating levels of proinsulin. Immunofluorescent analysis of three cases in group A consistently revealed a trabecular pattern according to Woodtli and Hedinger [26], whereas the four cases in group B showed a purely or predominantly solid pattern. On the basis of these observations it was proposed that trabecular and solid insulinomas may represent two distinct types of tumour having distinctive histological and functional characteristics [2, 3]. After the introduction of site-directed monoclonal antibodies, however, Roth et al. [18] were unable to find a definite relation between tumour histological structure and type of immunohistochemical insulin expression, and found heterogeneous patterns of immunoreactivity in all types of human insulinomas. The present study, also using a site-directed monoclonal antibody in a large series of cases, confirms that trabecular and solid insulinomas do not represent homogeneous groups with respect to the pattern of insulin immunostaining. However, some differences can still be appreciated among the two groups. Trabecular tumours showed insulin immunoreactivity in virtually all tumour cells (score 6) in 50% of cases, and no cases showed less than 60% of immunoreactive cells (score <5). In contrast, only 8% of solid tumours were scored as 6, whereas 36% of them had scores <5. As previously noted [18], the difference could be even more clearly appreciated in insulinomas showing a mixture of trabecular and solid structures, the former pattern presenting consistent, polarized immunostaining of all cells and the latter pattern mostly showing haphazard mixtures of insulin-nonreactive and -reactive (mostly nonpolarized) cells. The issue is made even more complicated by the observation that several tumours presenting a solid appearance in conventionally stained sections appear to be actually composed of tightly apposed trabeculae after insulin immunostaining. In sum, it appears that the histological structure is not a reliable marker of insulinomas with different biological properties. Ultrastructural assessment of tumour cells containing typical beta granules remains the most representative morphological counterpart of tumour cell responsiveness to inhibitors of insulin secretion in vivo [2]. Studies are now in progress to evaluate whether, irrespective of the histological structure, tumours with virtually universal cell expression of insulin behave biologically in a different manner from those with marked heterogeneity of insulin immunoreactivity even in contiguous cells. It is noteworthy that the former tumours were found to include 2 in-

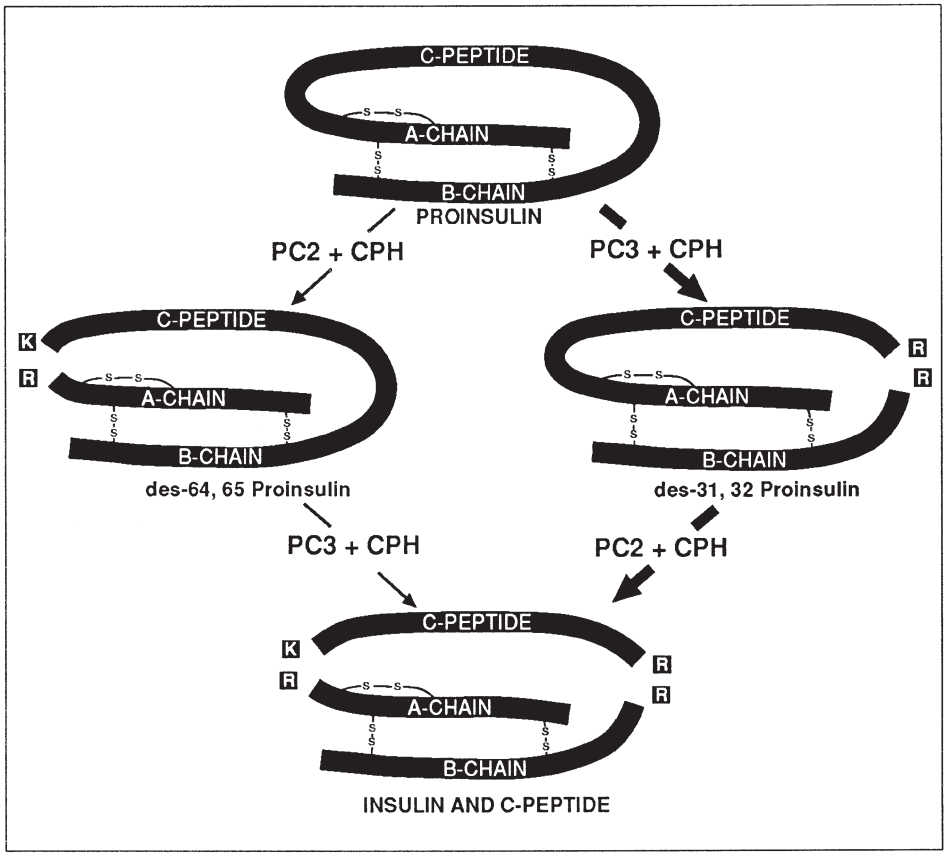

Fig. 6 Scheme showing the role of PC2, PC3 and CPH in the two possible pathways of proinsulin processing in pancreatic B cells. The route on the *right* is probably more dominant (*thicker arrows*). CPH removes the basic residues after endoprotelytic cleavage of the proinsulin molecule by PCs. (Modified from [24])

sulinomas composed of spindle cells, which are traditionally reputed to reflect a lesser degree of differentiation.

Our study demonstrated that PC2, PC3, CPH and 7B2 are well expressed in insulinomas, being found in virtually all tumours, usually in a range of 30–100% of all tumour cells. In consecutive sections the pattern of immunostaining of all these substances was found to overlap remarkably in terms of both intratumour topographic distribution and intracellular pattern of immunostaining. Such co-localization is explained by their close functional relationship in the proteolytic processing of proinsulin leading to its conversion to insulin (Fig. 6). PC2, in fact, is the endoproteolytic enzyme selectively cleaving proinsulin at the C-peptide/A chain junction (Arg_{31}–Arg_{32}), whereas PC3 has a similar selective activity at the C peptide/B chain junction (Lys_{64}–Arg_{65}) [24]. CPH removes the C-terminal basic residues left after endoproteolytic cleavage by the PCs. It is reputed that PC2 and PC3 act in sequence, the pathway involving PC3 action first probably being the dominant one in normal conditions (Fig. 6) [24]. However, recent experiments indicate that at high concentrations both PC2 and PC3 are able to split proinsulin at both junctions in vivo [8]. Finally, the complex interrelationships between PC2 and 7B2 have been elucidated at least in part. The amino terminus of the 7B2 molecule acts as a chaperone in maturation of the PC2, while its carboxyl terminal portion inhibits PC2 [27]. On the other side, PC2 cleaves, and hence inactivates, the 7B2 carboxyl terminal peptide [28].

In our series of insulinomas PC2 and 7B2 were found to be less frequently expressed than PC3 and CPH. Moreover, the immunohistochemical signal of PC2 often was less intense than that of other antigens, including its chaperone 7B2, even in the presence of strong immunoreactivity of extratumour normal islets. In this latter location, however, PC2 was strongly expressed by peripheral glucagon-containing A cells. Functionally, PC2 is the most important convertase in these cells [24], whereas the immunostaining of insulin B cells was consistently weak, a result already reported by others [9]. It may, therefore, be assumed that the weak immunostaining signal of PC2 is a general feature of B cells and does not imply a specific defect of insulinoma cells, as was recently suggested in a study of three insulinomas, two of which lacked PC2 immunreactivity [9], possibly because of formalin fixation. A potential explanation for the weak immunocytochemical signal of PC2 in this study may be inherent in a reduced affinity of the antiserum used for its specific antigen. However, the strong signal observed in extratumour A cells and in most trabecular insulinomas is not in keeping with this hypothesis and may reflect an actual defect in the expression of PC2 in some, particularly solid, insulinomas, an interpretation supported by the concomitant weak expression of the PC2 chaperone protein 7B2.

Analysis of serial sections immunostained for insulin or proinsulin revealed that the topographic intratumoral

distribution and the intracellular pattern of PC3, CPH, PC2 and 7B2 closely corresponded to that of the mature hormone, without aspects of focal perinuclear immunoreactivity similar to that of proinsulin. Such co-localization of convertases and related molecules with insulin but not with proinsulin is not specific to insulinoma cells. Indeed, immunoelectron microscopic investigations in normal endocrine cells have demonstrated the both PC2 and PC3 are localized within mature secretory granules but not in the Golgi stacks [9, 21]. These findings are consistent with the fact that proteolytic processing of proinsulin in a post-Golgi event occurring in the clathrin-coated maturing secretory granules after they have been formed in the *trans*-Golgi compartment [17].

The association of the diffuse cytoplasmic immunoreactivity for proinsulin, indicating post-Golgi proinsulin leakage, with intense expression of all proteins of the proinsulin processing found in our study is worth noting. Roth et al. regard such a peculiar pattern of proinsulin immunostaining as a pathologic finding specific to insulin-producing tumour cells, reflecting a disorder in the intracellular conversion mechanism from proinsulin to insulin [18]. In a further elegant study using immunoelectron microscopy with site-directed antibodies, Roth's group provided additional evidence for a derangement of the conversion pathway in insulin-producing tumour cells by showing the occurrence of immunoreactive insulin as early as in the Golgi stacks, and of proinsulin in the late-occurring mature secretory granules [19]. Our study did not yield any information on the subcellular localization of cytoplasmic proinsulin, but confirmed that diffuse proinsulin immunostaining is a common finding in insulinomas, although there were only 10 of them in which it was expressed by more than 10% of tumour cells. However, these 10 cases invariably showed maximal expression of insulin and of all conversion-related molecules, including PC2. These results indicate that the significant leakage of proinsulin from its regular localization in the *trans*-Golgi compartment found in a subset of insulinomas is associated with an otherwise apparently efficient processing pathway, and may rather reflect a saturated processing capacity. The potential responsible mechanisms include suboptimal pH and Ca^{2+}, which may hamper the processing rate or shorten the average life time of secretory granules (increased average excretion rate) leaving shorter time available for the processing to be complete.

In contrast with previous results [18], we also found that cytoplasmic proinsulin immunostaining occurred in at least a few islets of normal appearance located in the peritumour acinar tissue of 8 of 54 patients, but not in those of patients without insulinoma. Whether such an abnormal proinsulin expression of peritumour islets depends on intracellular derangements secondary to tumour influences or, rather, has histogenetic implications for tumour development is uncertain.

HISL-19 is a monoclonal antibody generated using an extract of human islets as immunogen [23]. In accordance with previous results [4], insulinoma cells presented two patterns of intracellular HISL-19 immunostaining: one, almost universally expressed, topographically corresponding to the Golgi apparatus and showing co-localization with proinsulin in serial sections; the other, less frequently expressed (only 13 cases with more than 30% immunoreactive cells in our study), corresponding to the secretory granules and showing co-localization with insulin and related convertases. The former pattern indicates that the cytologically interesting but functionally still elusive HISL-19 antigen does not correspond to any known protein involved in the proinsulin to insulin conversion. However, its expression by normal or tumour islet cells producing other hormones, such as glucagon, PP, gastrin and VIP [4], precludes its identification with proinsulin.

We have shown that faulty expression of the proteins involved in the proteolytic conversion from proinsulin to insulin does not occur in most insulinomas, particularly in those with extensive immunohistochemical content of insulin and with intracellular leakage of proinsulin beyond the Golgi compartment. In contrast, prohormone convertases and related molecules are not expressed in those insulinoma cells that do not contain immunodetectable insulin. Indeed, these cells have very recently been found by in situ hybridization to contain abundant insulin mRNA [15]. An investigation is now in progress to evaluate whether tumours largely composed of such non-immunoreactive cells behave in a different way, functionally, from those showing full expression of insulin and of peptides involved in the intracellular processing of proinsulin.

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