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Chromogranin A and B and secretogranin II in bronchial and intestinal carcinoids

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Summary. Carcinoid tumours (bronchial and intestinal) were analyzed by immunoblotting for the presence of chromogranin A, B and secretogranin II. In all tumours an antigen corresponding in electrophoretic behaviour to adrenal chromogranin A was present. Lung carcinoids (3 out of 5) contained a relatively high concentration of a proteoglycan form of this antigen in addition. Chromogranin B was found in all tumours. In one and two dimensional immunoblotting it appeared identical to the corresponding adrenal antigen. Secretogranin II was also present, however concentrations (especially in intestinal carcinoids) were low and variable. Furthermore, in intestinal tumours it differed from the adrenal antigen by having a slightly higher molecular size and a more alkaline pI. Immunohistochemistry revealed that the tumour tissues stained positively for all three antigens. For secretogranin II the staining in intestinal tumours was relatively weak and quite variable. These results should provide a defined basis for immunohistochemical screening of carcinoids for the chromogranin/secretogranin antigens.

Key words: Carcinoids – Bronchial carcinoids – Intestinal carcinoids – Chromogranin A – Chromogranin B – Secretogranin

Introduction

We have recently shown (Hagn et al. 1986) that human chromaffin granules contain three families of acidic proteins, the chromogranins A and B and secretogranin II (or chromogranin C). We use these terms according to a recent nomenclature proposal (Eiden et al. 1987). Chromogranin A was originally isolated from bovine chromaffin granules (Helle 1966; Smith and Winkler 1967; Smith and Kirshner 1967), later found to be similar to secretory protein I (SP I) present in parathyroid gland (Cohn et al. 1982) and finally established to have a widespread occurrence in endocrine tissues (O'Connor et al. 1983; Wilson and Lloyd 1984; Cohn et al. 1984; Facer et al. 1985; Varndell et al. 1985; Lassmann et al. 1986; Rindi et al. 1986), in brain (Somogyi et al. 1984; Nolan et al. 1985), in tissues of the immune system (Hogue-Angeletti and Hickey 1985) and in tumours of endocrine origin (Lloyd and Wilson 1983; Wilson and Lloyd 1984; O'Connor et al. 1983; Said et al. 1985; Walts et al. 1985; Bussolati et al. 1985). Chromogranin B and secretogranin II also have a widespread distribution in endocrine organs of ox and rat (Fischer-Colbrie et al. 1985; Lassmann et al. 1986; Rosa et al. 1985; Rindi et al. 1986; Rundle et al. 1986) and we have already established their presence in human pituitary and endocrine pancreas (Hagn et al. 1986), phaeochromocytomas (Schober et al. 1987) and in medullary carcinomas of the thyroid (Schmid et al. 1987).

For carcinoid tumours immunohistochemical data for chromogranin A only have been published. Positive staining was obtained in carcinoid tumours of the lung, thymus and intestine (Walts et al. 1985; Nash and Said 1986; Wilson and Lloyd 1984). In the present study we have investigated carcinoids from the lung and the small intestine by immunoblotting. Here we establish that these tumours contain chromogranin A which behaves in a manner identical to the adrenal protein but can have a relatively high proportion of chromogranin A in the proteoglycan form (PG-chromogranin A: see Eiden et al. 1987). Chromogranin

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B and secretogranin II were also present in these carcinoids. These results define the chromogranin/secretogranin antigens present in these tumours. In agreement with these findings positive immunohistochemical staining was obtained for all three antigens in carcinoid tumours.

Materials and methods

Antisera against bovine chromogranins A, B and secretogranin II were obtained as already described in detail (Fischer-Colbrie and Frischenschlager 1985; Fischer-Colbrie et al. 1986). The specificity of the antisera and the cross-reaction with human tissue was established by two-dimensional immunoblotting (Hagn et al. 1986). In addition a rabbit antiserum was also raised against human chromogranin A. The antigen was isolated from human phaeochromocytoma by high performance liquid chromatography as described by Fischer-Colbrie and Schober (1987) for bovine and rat chromogranin but with some modification necessary for the human material (Schober et al. 1987). Both antisera (against human and bovine chromogranin A) gave identical results in immunoblotting.

Lung carcinoids were trabecular tumours with uniform cell component and roundish, slightly hyperchromatic nuclei (3 cases). Mitoses were numerous in two additional cases which also showed aggressive behaviour with mediastinal lymph node metastases and, in one case, blood vessel invasion. These latter tumours were diagnosed as atypical carcinoids (according to Arrigoni et al. 1972) or well differentiated neuro-endocrine carcinomas, as suggested by Gould et al. (1983). All cases gave positive reaction when tested for argyrophila with the Grimelius' silver impregnation.

Tissue from small intestinal tumours (2 primary tumours, 1 lymph node and 1 liver metastasis) showed the classical light microscopical picture of a mid-gut carcinoid, with an insular growth pattern or anastomosing cords (Williams and Sandler 1963). The peripheral tumour cells often showed a pallisade arrangement against the surrounding stroma. There was a slight nuclear polymorphism with a few mitoses. Almost all cells displayed an argyrophil reaction and the majority of cells also showed an argentaffin reaction.

In addition to the tumours described above, further cases of carcinoids were retrieved from paraffin embedded material and used for immunohistochemistry.

For immunoblotting experiments a heat-stable protein fraction was prepared from the tumour tissue in order to enrich the chromogranins (Rosa et al. 1985). Tumours were immediately frozen in liquid N_2, lyophilized, sliced with a razor into small pieces and homogenized with distilled water in a Potter-Elvejhem homogenizer. After immediate boiling for five minutes insoluble material was sedimented for 45 min at 120000 *g* (see Schober et al. 1987). The supernatant was lyophilized, resuspended in a small volume of distilled water, aliquots were subjected to one-dimensional sodium dodecylsulfate electrophoresis (Laemmli 1970) with an acrylamide gradient ranging from 10 to 17% or to two-dimensional electrophoresis (O'Farrel 1975). Immunoblots were obtained following the protocol of Burnette (1981) with some minor modifications (Fischer-Colbrie et al. 1985). Protein was measured after precipitation of proteins with perchloric acid (3% v/v) using bovine serum albumin as a standard (Lowry et al. 1951).

For immunohistochemistry the tumours were fixed in buffered paraformaldehyde (4%, 24 h) and embedded in paraffin. 4 μ thick sections were mounted on poly-L-lysine coated slides, deparaffinized after placement in the oven (56° C, 50 min) in xylene and rehydrated. After blocking endogenous peroxidase activity with 1% H_2O_2 in methanol (30 min) chromogranin A, B and secretogranin II were detected by an indirect immunoperoxidase method including normal swine serum incubation (10%; 10 min) and Tween 20 (0.5%) in rinsing buffers for reducing background staining. Incubation with primary antisera diluted 1:250 in TRIS-HCL (0.05 M; pH 7.2) for 60 min at room temperature was followed by incubation with a peroxidase-labelled swine-antirabbit antibody (1:40; 45 min; DAKO, Copenhagen, Denmark) and development by diaminobenzidine (DAB; Sigma Ltd., Munich, FRG). The sections were counterstained with haemalaum. Negative controls – replacement of the primary antibodies by normal rabbit serum – were included. Additionally, sections of normal human pituitary gland served as a positive control.

Results

Figure 1 demonstrates the results obtained with one dimensional immunoblotting. In adrenal medulla the antisera against chromogranin A, B and secretogranin II react with three distinct antigens differing in molecular weight (compare Hagn et al. 1986). In addition to the respective largest component additional bands representing endogenous breakdown products can be seen (see Fig. 1). In carcinoid extracts the antiserum against chromogranin A stains a component corresponding in molecular weight to the adrenal chromogranin A. In addition (see Fig. 1 b) a slower moving band stains strongly in some bronchial carcinoids (3 out of 5). It has previously been shown that a proteoglycan form of chromogranin A (PG-chromogranin A) migrates to this position (Rosa et al. 1985; Falkensammer et al. 1985). This PG-chromogranin A can be identified by two-dimensional electrophoresis by its more acidic pI (see Falkensammer et al. 1985). In extracts of adrenal medulla this component can only be immunostained, when much higher concentrations of proteins than those used for Fig. 1 are subjected to electrophoresis. As shown in Fig. 2 tumour extracts can contain significant amounts of this PG-chromogranin. Analogous results were obtained for two other bronchial carcinoids. In the intestinal carcinoids (see Fig. 1 c) the chromogranin A staining was similar to the adrenal medulla in four cases, in one case some staining for PG-chromogranin A was present.

The antiserum against chromogranin B (see Fig. 1) stained in the carcinoid extracts a band corresponding to the slowest moving antigen in adrenal medulla (in six cases). Furthermore in two-dimensional immunoblotting (see insert a in Fig. 2) chromogranin B could be identified by its typical position relative to chromogranin A (compare Schober et al. 1987; Hagn et al. 1986). In three of the nine tumours investigated only faster moving bands were immunostained for chromogranin

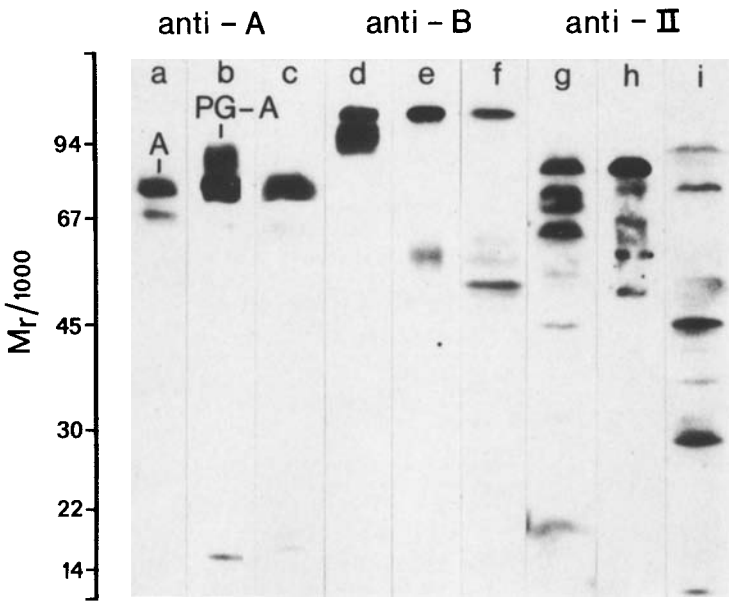

Fig. 1. Characterization of chromogranins/secretogranins by one-dimensional immunoblots. Boiled extracts from human adrenal medulla (a, d, g: 15–100 μg protein), from a bronchial carcinoid (b, e, h: 100–200 μg protein) and from an intestinal carcinoid (c, f, i: 200–420 μg protein) were subjected to one-dimensional electrophoresis followed by immunoblotting with antisera against chromogranin A (a–c), chromogranin B (d–f) and secretogranin II (g–i).
The apparent M_r is indicated on the left

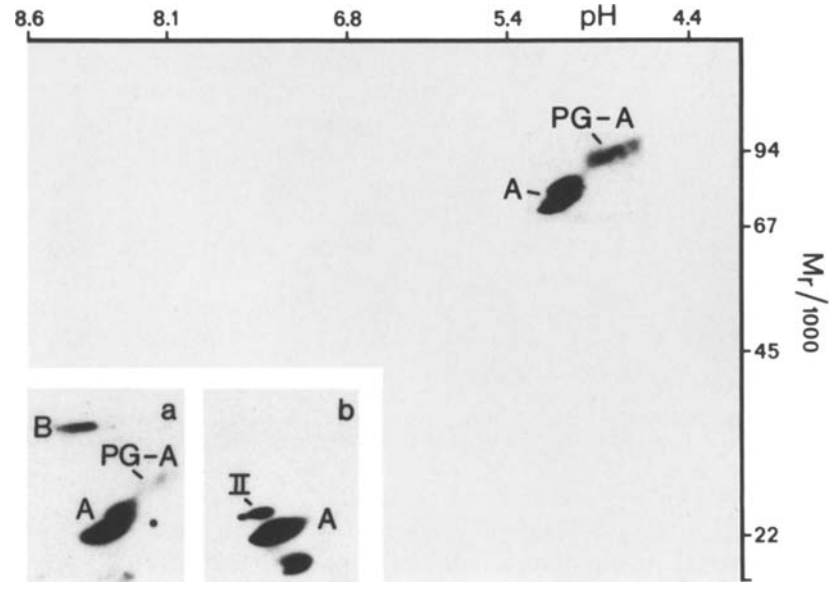

Fig. 2. Two-dimensional immunoblotting of proteins from bronchial carcinoids. A boiled extract from bronchial carcinoid (300 μg protein) was subjected to two-dimensional electrophoresis followed by immunoblotting with an antiserum against chromogranin A. The pI and apparent M_r are indicated. The inserts (a, b) demonstrate the relevant regions of the immunoblots of two additional experiments. For a the immunoblot shown in the main figure was subsequently immunostained with the antiserum against chromogranin B, therefore both A and B components are stained. For b an immunoblot (anti-chromogranin A) of another bronchial carcinoid was subsequently immunostained with an antiserum against secretogranin II. In this case PG-A was not prominent, in addition to chromogranin A a breakdown product of this protein is also found (see fastest moving spot). *A*: chromogranin A; *PG-A*: PG-chromogranin A (see Eiden et al. 1987); *B*: chromogranin B; *II*: secretogranin II

B (results not shown). This most probably represents an artifact due to proteolysis after removal of the tumour.

The antiserum against secretogranin II stained several bands both in adrenal medulla and in carcinoids (see Fig. 1 g–i). However, the staining intensity for this antigen in the tumours was quite variable and only in three of the five lung carcinoids a reliable reaction was observed, whereas in the two atypical cases this was not the case. The slowest moving band in bronchial carcinoid was found in the same position as the corresponding adrenal

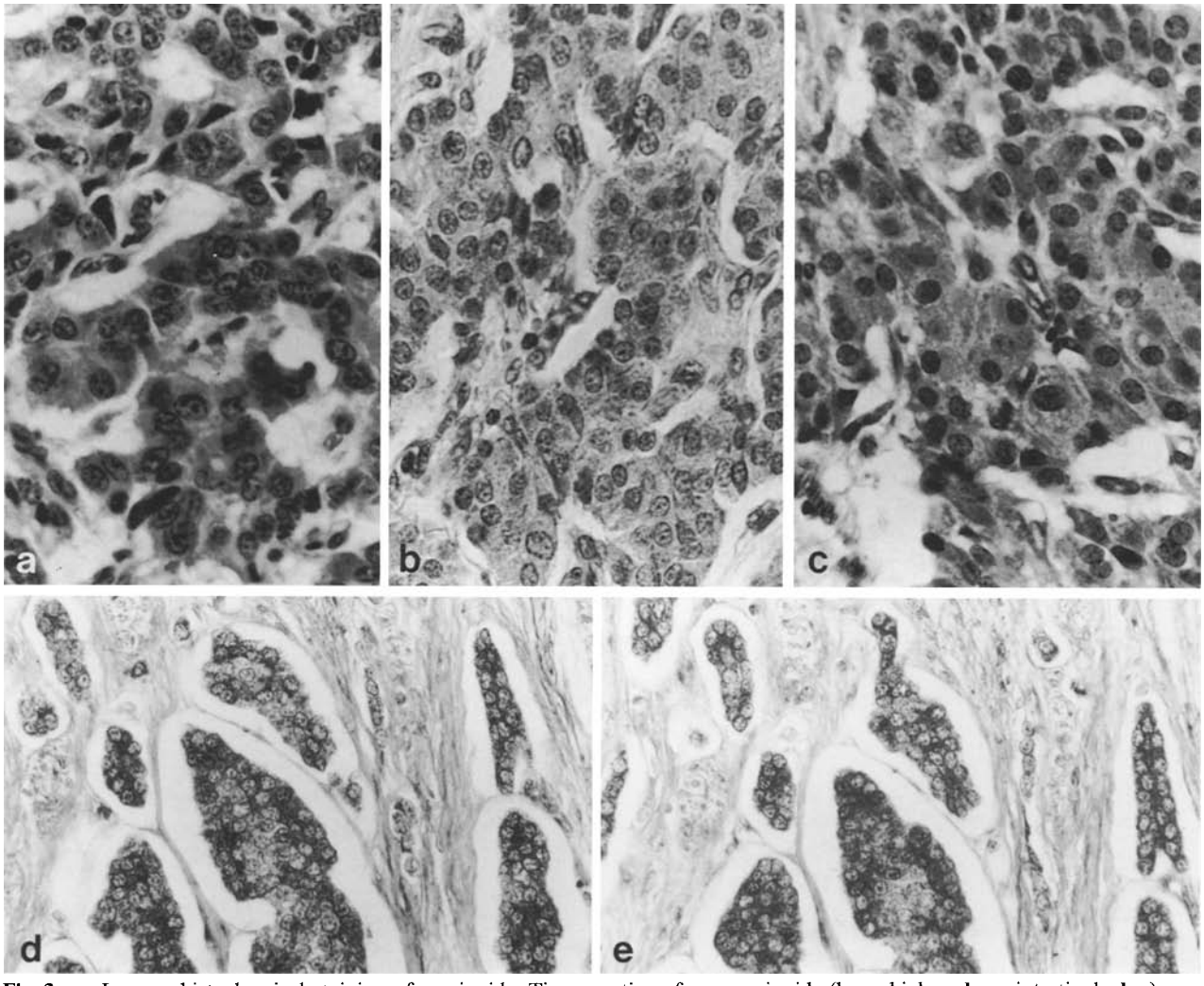

Fig. 3a–e. Immunohistochemical staining of carcinoids. Tissue sections from carcinoids (bronchial: **a**, **b**, **c**; intestinal: **d**, **e**) were immunostained with antisera against chromogranin A (**a**), B (**b**, **d**) and secretogranin II (**c**, **e**) and finally counterstained with Haemalaum. Magnification: ×630 (**a**, **b**, **c**) ×400 (**d**, **e**)

antigen. The faster moving bands representing at least in adrenal medulla endogenous breakdown products (see Winkler et al. 1986) were apparently different (compare g with h in Fig. 1). In two dimensional immunoblots the slowest migrating secretogranin II spot could be identified in its typical position just above chromogranin A (see Fig. 2 insert b).

In intestinal carcinoids staining in immunoblots for secretogranin II was weak and it was difficult to obtain reliable results. A positive staining was obtained in two of the four cases. The slowest moving immunostained band migrates slower than the corresponding band in adrenal (compare i with g in Fig. 1). This was also observed (results not shown) in two-dimensional blots where in addition the secretogranin II spot appeared at a slightly more alcaline pI than the adrenal antigen.

Figure 3 gives representative examples of the immunostaining of carcinoid tissue for chromogranins A, B and secretogranin II. Positive staining occurred throughout the tumour tissue. The results of the immunohistochemical studies (bronchial and intestinal carcinoids) are compiled in Table 1. Nearly all tumours were positive for chromogranins A, B and secretogranin II. However for the latter antigen the staining especially in the intestinal carcinoids was variable, and often only a few scattered cells were stained.

Discussion

Previous immunohistochemical studies have shown that carcinoid tumours stain positively for chromogranin A (see Introduction). However, immunohistochemistry does not define the molecular

Table 1. Presence of chromogranins A, B and secretogranin II in bronchial and intestinal carcinoids

	immunoblot		immunohistochemistry	
	bronchial	intestinal	bronchial	intestinal
Chromogranin A	5/5	4/4	14/14	12/13
Chromogranin B	5/5	4/4	14/14	12/13
Secretogranin II	3/5	2/4	14/14	8/13

Immunoblotting for secretogranin II was variable (see text). A strong staining was observed only for some of the tumours, in the other ones some reaction was seen but it varied too much to be considered reliable.
In immunohistochemistry the strongest staining was observed for chromogranin A and to a slightly lesser degree for B. Staining for secretogranin II was less intense and more variable, especially in some cases of intestinal carcinoids only a few scattered cells were stained

properties of the antigen. In adrenal chromaffin granules, the richest source for this secretory protein (see Winkler 1976), chromogranin A is present as a major component together with several smaller proteins, produced within the secretory granules by endogenous proteases (see Winkler et al. 1986). Furthermore, in addition a small amount of chromogranin A is present in a highly sulfated form representing a proteoglycan (Falkensammer et al. 1985; Rosa et al. 1985) which has been called PG-chromogranin A (Eiden et al. 1987). Immunoblotting is a suitable method to define such complex antigens and we have therefore analyzed carcinoid tumours by this approach.

In all carcinoids (bronchial and intestinal) investigated chromogranin A was demonstrated by immunoblotting. The electrophoretic behaviour of the major band was identical to that of adrenal medulla. In some lung carcinoids (3 out of 5) and in one intestinal carcinoid a relatively high concentration of a slower moving band was present which we identified as the proteoglycan form of chromogranin A (PG-chromogranin A) by two-dimensional immunoblotting. Thus immunoblotting has revealed that the properties of the chromogranin A antigen can differ significantly between normal and some tumour tissues. Further tumours from different origin should be analyzed for the presence of PG-chromogranin A. In medullary carcinomas of the thyroid PG-chromogranin A was not prominent (Schmid et al. 1987). For chromogranin B no previous data on carcinoids were available. We have now shown that this antigen is present in carcinoids (bronchial and intestinal). In six cases the major component of the tumour antigen moved like the largest band in adrenal medulla, apparently endogenous proteolytic processing in the tumour was limited. In three tumours only faster moving B-antigens were found. It seems likely that this is a proteolytic artifact occurring in the interval between the removal of the tumour and freezing. We have recently shown (for phaeochromocytomas: Schober et al. 1987) that great care has to be taken to prevent proteolytic artifacts especially for chromogranin B and secretogranin II. In fact this can be best avoided if tumours are immediately frozen, freeze-dried, cut into pieces and homogenized in distilled water followed by boiling in order to prevent proteolysis and to enrich the heat stable chromogranins. This method was employed in the present study and apparently prevented artifacts in most cases. However this method does not prevent proteolytic artifacts in the interval between removal of the tumour and freezing. In any case we can conclude that carcinoids contain both chromogranin A and B.

Immunohistochemical data established that the antigens are present in the tumour cells and give a positive staining throughout the tumour tissue. Thus the expression of these two antigens in tumour tissue corresponds with their presence in normal enterochromaffin cells of several species (O'Connor et al. 1983, Rindi et al. 1986; Lassmann et al. 1986; Rosa et al. 1985; Fischer-Colbrie et al. 1985; Facer et al. 1985).

For secretogranin II it has been reported that intestinal enterochromaffin cells do not stain for this antigen (Rindi et al. 1986; Lassmann et al. 1986; Rosa et al. 1985). However we have now shown that carcinoid tumours both of lung and intestine contain secretogranin II. The apparent lack of secretogranin II in normal enterochromaffin cells may be caused by the fact that due to the capricious behaviour of this protein during fixation (Rindi et al. 1986) small amounts of secretogranin II present in these cells may have escaped detection. Due to the scarcity of these cells immunoblotting of normal intestinal tissue is inadequate

for demonstrating this antigen. However, in homogenous tumour tissue derived from these cells the antigen could be demonstrated by this method but its quantity was apparently low and variable. In lung carcinoids secretogranin II appeared identical to that of the adrenals, whereas in intestinal tumours secretogranin II appears slightly larger and more alkaline than the adrenal antigen. Further studies are required to define the reasons for the different behaviour of this antigen in intestinal tumours. At present an altered processing of the *m*-RNA or changes in the glycosylation pattern may be offered as an explanation. Such differences are apparently rare since in all normal tissues so far investigated the proforms of the antigens appeared to be identical (Fischer-Colbrie et al. 1985; Somogyi et al. 1984; Rundle et al. 1986; Hagn et al. 1986). However for tumour tissues it has already been observed that chromogranin B in medullary carcinomas of the thyroid appeared smaller than the adrenal antigen (Schmid et al. 1987). These results emphasize that immunoblotting experiments are essential to define the basis for immunohistochemical studies.

We can conclude that for carcinoids in general secretogranin II appears a less reliable marker than chromogranin A or B. One has to consider the possibility that this antigen may only be present in tumours derived from certain enterochromaffin cells containing specific peptides. In endocrine pancreas secretogranin II is apparently concentrated in PP-cells (Yoshie et al. 1987). Further studies on the possible co-localisation of secretogranin II with certain peptides in carcinoids may therefore be of interest.

What can we at present conclude about the co-storage of the chromogranins/secretogranins? Some tumours (medullary carcinoma of the thyroid: Schmid et al. 1987; phaeochromcytomas: Schober et al. 1987) and carcinoids as shown in this study contain all three antigens. Unpublished experiments (R. Weiler and H. Winkler) indicate that parathyroid adenomas contain only chromogranin A. Thus the occurrence of these secretory proteins exhibits a distinct pattern. This has also become clear from studies on normal tissue. In many cases all three antigens are stored together. However, parathyroid gland contains only chromogranin A (Lassmann et al. 1986; Hagn et al. 1986), posterior lobe of the pituitary gland contains only secretogranin II (Rundle et al. 1986) and in bovine endocrine pancreas secretogranin II is mainly concentrated in PP-cells (Yoshie et al. 1987). When we finally discover the definite function of these peptides (see Winkler et al. 1986; Eiden et al. 1987) we may be able to interprete the distinct localizations in normal and tumour tissues in functional terms.

The present study has established that lung and intestinal carcinoids contain chromogranin A, B and secretogranin II and has defined the molecular properties of these antigens by one- and two-dimensional immunoblotting. These results should provide a defined basis for future studies which should further elucidate the role of these antigens as distinctive markers of neuro-endocrine tumours.

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