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ARTICLES

Sharing of Antigenic Epitopes Between Synaptophysin and Granulophysin

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Abstract The immunological crossreactivity between the two granule-specific membrane glycoproteins, synaptophysin and granulophysin, was studied using a series of site-specific monoclonal and polyclonal antibodies. The epitope relatedness of six monoclonal antibodies against granulophysin was examined by competitive ELISA. The antibodies are shown to recognize distinct, but overlapping epitopes within a compact region that is constructed by the three-dimensional configuration of the molecule. All these antibody clones also recognize rat neuronal synaptophysin. Two monoclonal antibodies against synaptophysin, of which one is the well-characterized SY38 antibody, directed against the carboxy terminal of the molecule, are also shown to react with granulophysin. Characterized polyclonal antibodies against different peptide antigens of synaptophysin failed to recognize granulophysin. Synaptophysin and granulophysin are distinctly recognized in brain cell (white matter) and the pituitary both qualitatively and quantitatively. Based on these and other observations, it is suggested that the repeat motif in the cytoplasmic tail of synaptophysin represents an immunodominant construct that is the target for the observed crossreactive antibodies and that a similar tertiary construct has been preserved in granulophysin and in other transmembrane proteins.

Key words: granules, epitope mapping, platelets, dense granules, synaptic vesicles

Synaptophysin, a multimeric glycoprotein of 38 KDa and 307 amino acid residues, is a major component of mammalian neuronal synaptic vesicles. The molecule has been characterized in terms of its DNA sequence, structure, membrane topology and cellular distribution [1–8]. The cellular and tissue distribution of synaptophysin has previously been studied by immunocytochemistry or immunohistochemistry using the SY38 monoclonal antibody and polyclonal antibodies [3,6]. The presence of the molecule has been demonstrated in a variety of cells of neuroendocrine and tumor origin as well as in chromaffin granules [3,11–13]. The target sequence for the SY38 antibody has been mapped recently to amino acids 269–289 in the cytoplasmic tail of the molecule, and it was shown that four other monoclonal clones were directed to the same region [16]. Besides synaptic vesicles and neuroendocrine cells, the presence of synaptophysin was also reported in the pancreas (A and B cells), thyroid and skin (Merkel cells) [reviewed in 3]. These claims have, however, not been substantiated at the molecular level. Tissue samples from kidney, liver, small intestine, muscle, thymus, testis, heart, and ovary were found to be negative by either immunohistology or RNA blots [3,6–8]. It was postulated *that* synaptophysin is a voltage dependent channel within the vesicle membrane which resembles a gap junction protein [9], and that the native molecule could participate in the formation of a fusion pore during exocytosis [reviewed in 14].

Recently, a novel platelet dense granule (DG) membrane glycoprotein of 40–45 KDa has been described [17]. This protein, granulophysin, has been shown to be weakly crossreactive with synaptophysin [17]. In the present study we characterize the antigenic relatedness of a series of monoclonal antibodies against granulophysin and their crossreactivity to synaptophysin.

MATERIALS AND METHODS

Antibodies and Conjugates

The production of a series of monoclonal antibodies specific for human platelet dense gran-

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ules has been previously reported [17]. Seven of these antibodies (denoted by the prefix D) were shown to recognize a major antigen of approximately 40 KDa in the membrane of the DG. Monoclonal antibodies against synaptophysin were purchased from Boehringer Mannheim (SY38) and Bio-Makor, Israel (SVP-38). Antibodies to granulophysin were purified by ammonium sulphate precipitation, Sephadex G-200 column, and protein-A column. Several clones were conjugated with peroxidase by the periodate method essentially as described by Tijssen *and Kurstak* [10]. These conjugates have been calibrated in terms of activity and IgG content and stored frozen until use. The rabbit antisera against defined peptides of synaptophysin were a gift of Dr. T.C. Südhoff, Howard Hughes Medical Institute, University of Texas, Dallas.

Granules and Tissues Preparation

DGs from human platelets were prepared as previously described [17]. Crude synaptic vesicles (CSV) from rat brain were prepared as described elsewhere [6]. All granules and vesicles were stored at −70°C for long term storage. Human tissues were obtained from an autopsy of a normal male within hours of death. The tissues were collected into tubes on ice, washed extensively to remove blood remains, and frozen immediately. These tissues were processed by cutting them into fine pieces on ice and homogenizing in 0.1% SDS for 1 min at high speed. The solution was then clarified by centrifugation at 2,000*g* (10 min at 4°C), followed by a second centrifugation at 10,000*g* for 15 min. Finally, the solution was collected and cleared through a 45μ sterile disposable filter and stored frozen until use. Protein calibration for all samples was done by the Bradford assay (Bio-Rad), using BSA as standard.

ELISA Techniques

A sandwich-type antigen-capture ELISA assay was developed with one monoclonal antibody as a capture antibody and a second one, labeled with peroxidase, used for the detection of the bound molecules. The full details of the assay, including statistics of inter- and intra-assay variability, are presented elsewhere [18].

Polystyrene flat-bottom plates (Falcon 3912) were coated overnight with 150 μl of 20 μg/ml of capture antibody in carbonate/bicarbonate buffer. Following one wash with wash buffer (phosphate buffered saline, PBS-Tween-20 0.05%), the tested samples and standards (purified and solubilized DG) were diluted to appropriate concentrations in PBS containing 1% BSA and added to the wells (100 μl/well). The samples were then left for 75 min and the plate was washed twice with wash buffer. The conjugate, diluted in PBS-Tween containing 1% BSA (normally at 1:400 dilution), was then added to all wells and left for 1 h at room temperature. The plate was then washed three times in wash buffer and once in water and the substrate (OPD, Sigma) was added. The reaction was stopped after 5–10 min by adding 25 μl/well of 3 M sulfuric acid.

The reaction time with the substrate was prolonged up to 1 h in the case of crossreaction studies. The results were read either at 450 nm (reactions exceeding 2.5 OD at 490 nm) or at 490 nm with a reference reading at 600 nm. The left row of the plate was routinely left as blank/control and contained all reagents except the conjugate. This reading was automatically subtracted from the relevant reactions.

The competitive ELISA inhibition assay was essentially the same with a serial dilution (1:2 factor) in PBS-Tween-BSA (1%) of the blocking antibody made in the wells (50 μl/well), before adding the conjugate (50 μl/well at double concentration).

Western Blots

Samples were run in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using the procedure of Laemmli [19]. Amounts of loaded proteins are indicated in the specific figures. Nitrocellulose blots were prepared by transferring under constant current of 450–500 mA for 60 min. The paper was blocked with either 10% skim milk or 5% BSA (blocking solution). Incubations with the primary antibody (at 10 μg/ml concentration of purified IgG) was done in PBS + 1% BSA + 0.05% Tween-20 for 2 h at room temperature or overnight at 4°C. Following extensive washing in PBS-Tween 0.05%, the blots were incubated with the secondary antibody (anti-mouse IgG-peroxidase) at the recommended dilution for 1 or 2 h. Prestained standards were from either Bio-rad or Sigma. The blots were developed with either DAB (Sigma) on nitrocellulose or on film (enhanced luminescence, ECL system, Amersham). For higher sensitivity we also used biotinylated goat anti-mouse IgG (Jackson, 1:3000), and following washing, reincubated with streptavidin conju-

gated peroxidase (Jackson 1:2000). The detection was performed as described above. Controls in which the primary antibody was omitted were included routinely on each set of samples. Samples were treated with sample buffer with (reduced) or without (non-reduced) 10% β-mercaptoethanol and heated (95°C) for 5 min immediately before loading. Affinity purified and serum absorbed peroxidase conjugated $F(ab')_2$ fragment of sheep-anti-mouse IgG (Sigma) was used as the secondary reagent unless indicated otherwise.

RESULTS

Epitope Mapping by Monoclonal Antibodies Against Granulophysin

Six monoclonal antibodies against granulophysin were examined, in a checker-board type table, for their potency to compete with four different peroxidase-conjugated monoclonals (D545, D519, D503, D531) against granulophysin. In an earlier study it was found that the nature of capture antibody had very little or no effect on the final outcome. Therefore, this variable was largely ignored, although as a rule the capture antibody differed from the conjugate. Two non-relevant monoclonal antibodies against the platelet alpha-granule protein GMP-140, were also used as controls. These gave no significant inhibition values at concentrations up to 200 μg/ml (not shown). A representative inhibition curve of the D545 conjugate by all six different non-labeled anti-granulophysin antibodies is shown in Figure 1. The various antibodies display large variations in their inhibition capacities in all cases. The pooled results of 50% inhibition and maximum inhibition (at 50 μg/ml of inhibiting antibody) values from the curves is presented in Figure 2 as a measure of similarity. Each bar represents the relative similarity of the competing antibody clone to the homologous (same as conjugate, 100% similarity) antibody. For instance, if 4 times as much of the antibody was required to obtain the same 50% inhibition value, we refer to it as a relative similarity of 25%. The ratio of the inhibiting antibody to the conjugated antibody was 7:1 at 50 μg/ml of the inhibitor. As shown, no two of the antibodies were found to be identical by these criteria, although in some instances, the final inhibition value may have reached close to 100% (Fig. 2B). While the D545 antibody was strongly inhibitory to D519 and D503 (similarity index of 62.5 and 55, respectively), the reverse combinations

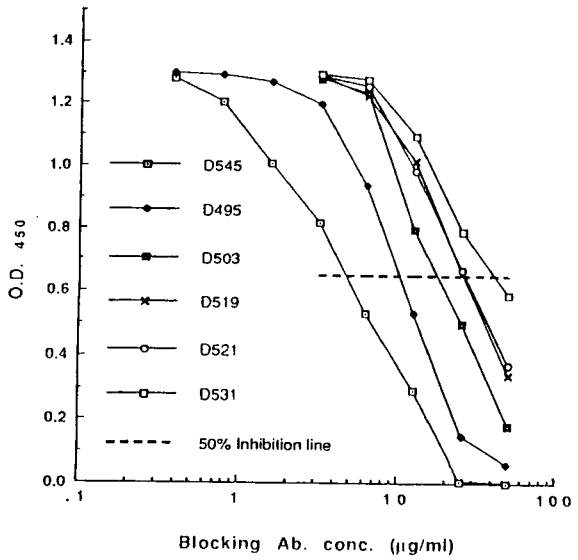

Fig. 1. Inhibition curves of the D545 anti-granulophysin peroxidase conjugate by different anti-granulophysin unlabeled antibodies. The abscissa is a logarithmic scale. The assay was performed as described in Materials and Methods. The 50% inhibition line is marked.

were much less inhibitory (similarity index of 17 and 28.5, respectively) (Fig. 2A). The D495 antibody was strongly inhibitory to all four conjugates, suggesting that its epitope is central and overlapping with all the other epitopes. The most remote epitope was that recognized by D531, as indicated by its low values for similarity (for D521 and D545, 3.2 and 1.1, Fig. 2A). Based on these data, a Venn diagram of the epitopes inter-relationships has been drawn (Fig. 3). Although the two dimensional presentation of the recognized epitopes cannot truly represent the full three-dimensional spheric interactions, relative affinities, or the minor interactions between the antibodies (such as between D531 and D521). The Venn diagram clearly points out that the recognized epitopes are colocalized within a very compact and immunodominant antigenic region on the granulophysin molecule.

Crossreactivity Between Synaptophysin and Granulophysin

The reactivity of six anti-granulophysin antibody clones with rat synaptic vesicles was compared with the monoclonal anti-synaptophysin antibody SVP-38 in both ELISA assay (Fig. 4) and in Western blots (Fig. 5). All anti-granulophysin antibodies recognized the typical synaptophysin molecule in the rat synaptic vesicle

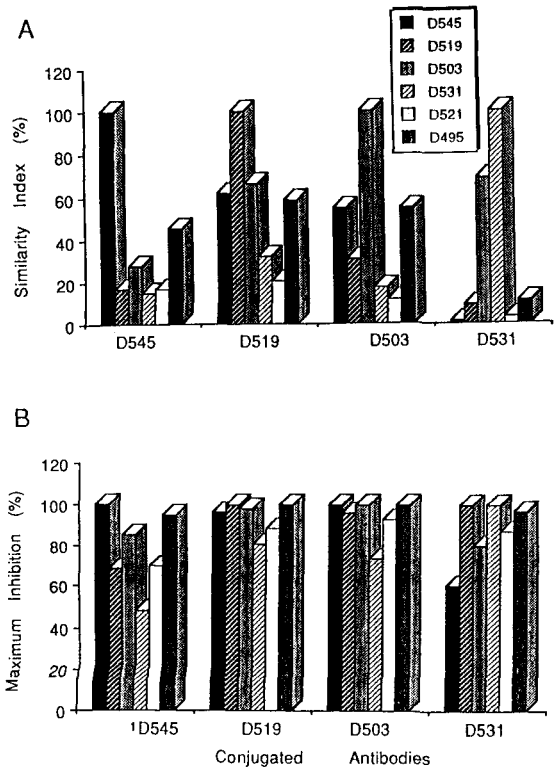

Fig. 2. Relative inhibitory capacity of the different monoclonal antibodies against granulophysin tested against 4 conjugated antibodies. **A:** The 50% inhibition point is represented by a bar. The similarity index represents (in percentage) the inverse value of the amount of antibodies added to meet the 50% inhibition of the conjugated antibody tested. **B:** The maximum inhibition capacity at 50 μg/ml antibody. The data were extracted from graphs such as the one presented in Figure 1.

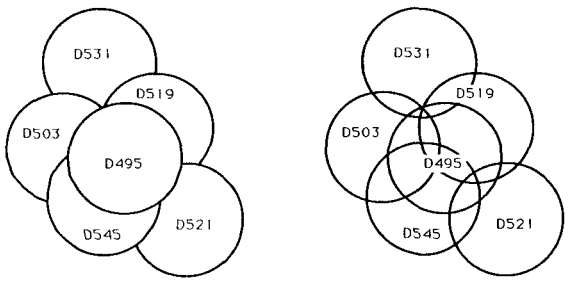

Fig. 3. A Venn diagram of the epitopes recognized by six monoclonal antibodies against granulophysin with (right) and without (left) the overlapping regions: This diagram is based on the similarity indices of Figure 2.

preparation. In the ELISA assay, higher reactivity with synaptic vesicles, and lower reactivity with DGs was observed when anti-synaptophysin was used as a capture antibody, and vice versa when anti-granulophysin was used (Fig. 4). It is important to note that a ten times higher concentration of the rat synaptic vesicle preparation compared to DG, was required to

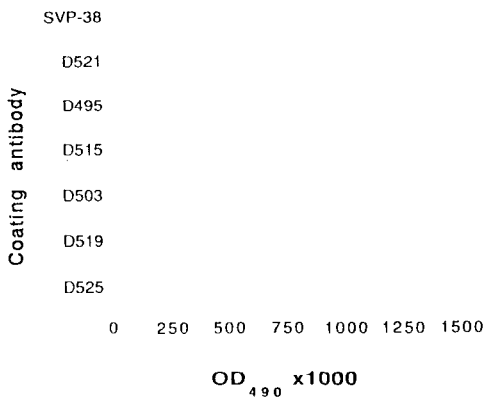

Fig. 4. Crossreactivity between synaptophysin and granulophysin demonstrated by ELISA. The assay was performed as described in Materials and Methods; 10 μg/ml of dense granule material or 100 μg/ml of rat synaptic vesicles were used in the antigen capture assay. The capture antibody is indicated in each case and the recognizing conjugate was D545-peroxidase in all cases. CSV = crude synaptic vesicles. DG = platelet dense granules.

obtain readings within the same range. The D525 antibody (not included in the epitope mapping analysis), also reacted like the other antibodies. The crossreactivity of D545 with synaptophysin has been previously reported [17].

The reactivity of two anti-synaptophysin antibodies (SY38 and SVP-38) with DG was also examined by Western blotting. Both anti-synaptophysin monoclonals recognized a typical granulophysin molecule in DG, although at a considerably lower intensity than the anti-granulophysin monoclonals (Fig. 5B). These differences are in accord with the ELISA results (Fig. 4). The reactivity of four polyclonal antibodies against defined peptides of the rat synaptophysin molecule [6] was examined under high sensitivity conditions in order to test the possibility that these molecules are homologous in regions other than the presumed immunodominant epitopes. These antibodies are directed against peptides that do not overlap with the SY38 epitope [6]. None of these antibodies recognized the DG granulophysin, whereas the SY38 monoclonal antibody did (Fig. 6). Two earlier attempts to react an antiserum against the N-terminal peptide with DG in an amplified avidin-biotin system gave negative results as well (not shown).

Selective Recognition of Synaptophysin and Granulophysin

The recognition of a granulophysin-like molecule in brain cells (white matter) and in the

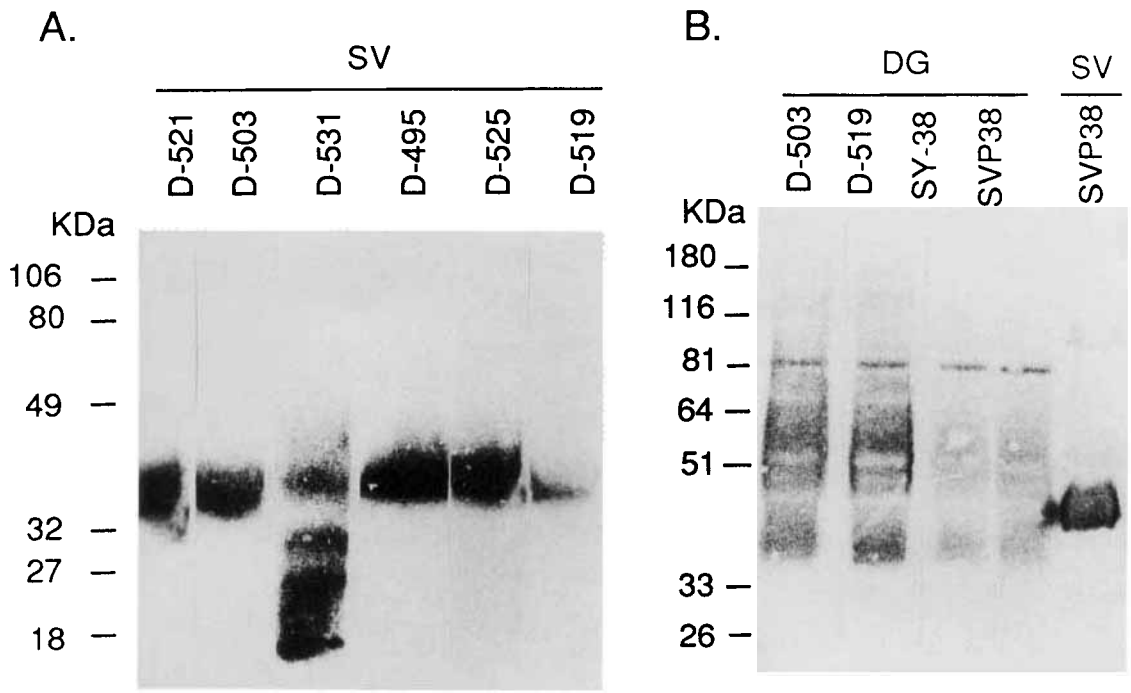

Fig. 5. **A:** Reactivity in Western blots of various anti-granulophysin antibodies with rat synaptic vesicles. Primary antibodies were incubated for 2 h and secondary conjugate was incubated for 1 h. Twenty micrograms of protein were loaded per lane. The blot was developed using the ECL system (Amersham). **B:** Crossreactivity between synaptophysin and granulophysin in Western blots. Purified 10 μg of DG were loaded on each gel. Each slot was incubated with the antibody marked on top and further processed with secondary biotinylated goat anti-mouse IgG (1:3000 dilution, Sigma) and then with streptavidin conjugated peroxidase (1:2000, Jackson). The blot was developed with diaminobenzidine (DAB, Sigma). SV represent 20 μg of rat synaptic vesicles. The sharp band at about 65 KDa also appeared in controls developed without the primary antibody as a non-specific marker.

pituitary is presented in Figure 7. Typical monomers and dimers of synaptophysin were revealed by the SY38 anti-synaptophysin antibody in the brain, whereas the D545 anti-granulophysin antibody recognized a somewhat diffused and higher MW band. A typical strong granulophysin reaction was observed in the pituitary extract. In contrast SY38 was weakly reactive to the pituitary extract suggesting that synaptophysin is in minute quantities in the pituitary compared to granulophysin. These experiments were carried out concomitantly with equivalent amounts of proteins and reacting antibodies.

DISCUSSION

Granulophysin is a recently described molecule which has not been characterized in terms of its sequence or structure. In the present study, we show that a conformationally dependent immunodominant region on this molecule is responsible for the elicitation of at least six independent antibody clones that recognize closely overlapping epitopes. It is striking that none of the seven monoclonal antibodies which recognize this molecule identified the fully reduced form of the molecule (using different reducing agents). Further, it was observed in ELISA that the loss of reactivity was proportional to the extent of denaturation of DG, and in Western blots some reactivity could be maintained with mild or partial reduction treatments (unpublished data). We therefore conclude that the immunodominant region of granulophysin is created by the secondary and/or tertiary folding of the molecule. Synaptophysin, which can be recognized by all the anti-granulophysin antibodies, has also been reported to posses an immunodominant region, which is included within amino acids 269–289 of rat synaptophysin (the SY38 target) [16]. In contrast to the granulophysin super-epitope, however, the SY38 antibody also recognizes the primary sequence (denatured and reduced synaptic vesicles). While we cannot ascertain with confidence that these immunodominant regions in the two molecules are in fact responsible for the observed crossreactivity, this notion is supported by several lines of evidence. First, two monoclonal anti-synaptophysin antibodies (SVP-38 and SY38) recognize the granulophysin molecule but not any other polyclonal

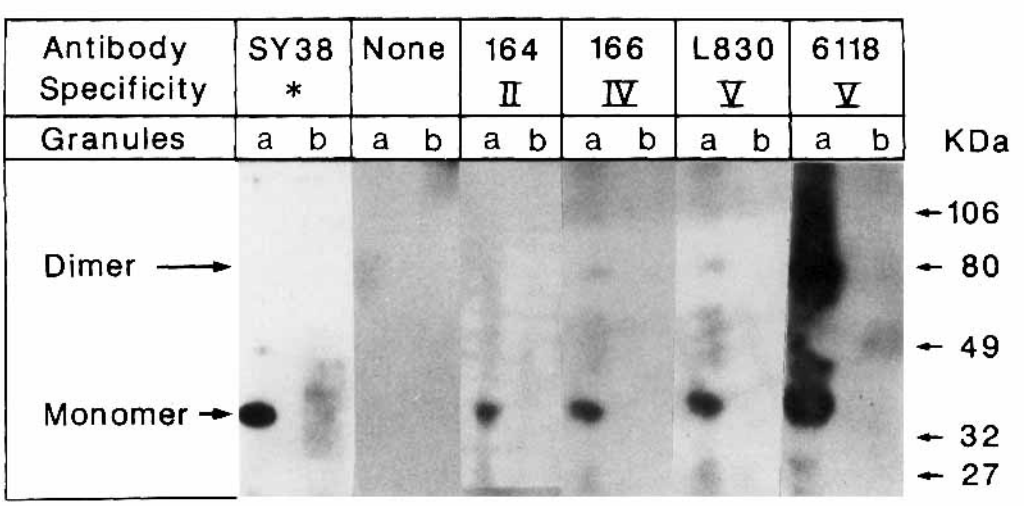

Fig. 6. Reactivity of rabbit polyclonal antibodies against defined peptides of synaptophysin with synaptophysin (**A**) and granulophysin (**B**). Ten micrograms of CSV and DG were loaded on each lane and blotted as described. The antisera and the monoclonal anti-synaptophysin antibody SY38 were incubated for 2 h with the blot at dilutions of 1:200 or 10 μg/ml respectively. Protein A–peroxidase (1:5000) or peroxidase conjugated $F(ab')_2$ fraction of sheep anti-mouse IgG (1:3000) were used as secondary antibodies. The negative control was incubated with protein A–peroxidase alone. The blot was developed in the ECL system. The indicated regions of specificity have previously been published [6]. Epitope II corresponds to the first intraluminal loop of synaptophysin. Epitope IV is part of the second intraluminal loop and epitope V is part of the C-terminus which is different from that of the SY38 epitope. *The epitope of SY38 is confined to amino acids 269–289.

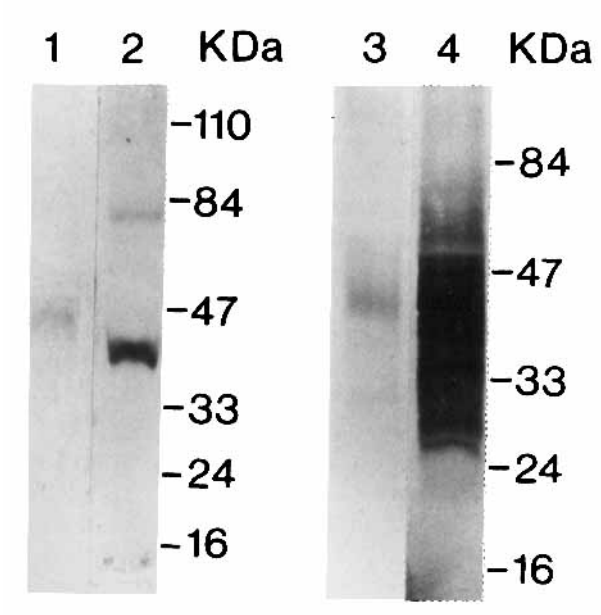

Fig. 7. Selective recognition of synaptophysin and granulophysin in brain white matter (lanes 1, 2) and pituitary (lanes 3, 4) extracts. Twenty micrograms of each tissue extract was run on 10% SDS-PAGE. Antibody concentrations were the same for all treatments (10 g/ml primary antibody and 1:3000 secondary antibody). Lanes 1 and 4 were reacted with anti-granulophysin antibody D545 and lanes 2 and 3 with anti-synaptophysin antibody SY38. Note the presence of the dimer in brain extract recognized by anti-synaptophysin and the difference in MW of the molecule recognized by anti-granulophysin.

antibodies directed against peptides at different sites of the molecule ([6] and Fig. 7). Second, the SY38 epitope is part of the imperfect repetitive pentapeptide YGP(Q)QG which recurs ten times in the cytoplasmic tail of synaptophysin [16]. Since remote molecules such as the Octopus rhodopsin (8 repeats) and a membrane associated 24 KDa molecule of Dictyostelium (5 repeats) share the same sequence, it is probable that the sequence is evolutionally conserved and of importance to a special granule-related function. Finally, recent data further supports the conservation of this region in relation to the observed crossreactivity. Thus, in addition to the leukophysin [20] that is also recognized by SY38, a gene which shares considerable homology with the repeat sequence of synaptophysin at the C-terminal region has been cloned from a lymphoid line, using anti-granulophysin antibodies as a recognition probe (M. Abdelhaleem et al., unpublished data).

While our results point to the conservation of crossreactive epitopes in the two molecules, they are distinct from each other in terms of pattern and tissue distribution. First, the pattern and molecular weight of granulophysin in Western blots is different from that of synaptophysin. Synaptophysin is identified as a sharp band of 38 KDa (neurons) or multiples of 38 KDa. Granulophysin invariably displays a broad band that

can range with major intensity at 40–45 KDa. This is also true for a variety of other tissues besides DG of platelets and is not influenced by the presence of a variety of protease inhibitors or deglycosylation protocols [17 and unpublished data]. Second, granulophysin has a wider and different tissue distribution and has been observed in neutrophils, plasma cells, testis, kidney, heart, muscle, and exocrine tissues, which apparently lack synaptophysin [17,20]. In this study we demonstrated the preferential recognition of these two molecules in brain cells and the pituitary. Although both are present, synaptophysin is dominant in the brain and minor in the pituitary; the reverse is true for granulophysin. Our results also suggest that the observed crossreactions may be significant when performing immunohistological or other studies involving antibodies without direct observation of the molecular mass. It is likely that in some previous studies crossreactive molecules, and not synaptophysin, were detected. For instance, it was reported that the pituitary contains large amounts of synaptophysin [see 3], a finding which we did not substantiate. Rather, the earlier results can be interpreted as a crossreactive reaction to granulophysin (or indeed to other putative membrane associated molecules which share the same repetitive sequence). The same could also be true for the detection of synaptophysin in thyroid and pancreatic cells.

It has recently been reported that serotonin organelles (= dense granules of platelets) in the rabbit contain synaptophysin [21]. The authors of the report could not detect similar molecules in platelets of guinea pig, rat, or human. Likewise, we have not been able to detect a synaptophysin-like molecule in the platelets of human, cow, or sheep, but these species do contain the granulophysin homolog. It would be of interest to examine other species in this respect and the possible functional relationship between these two molecules.

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