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Immunohistochemical evidence for the derivation of a peptide ligand from the amyloid β-protein precursor of Alzheimer disease*

(monoclonal antibody/synthetic peptide/binding sites/pancreas/adrenal gland)

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ABSTRACT **A monoclonal antibody to a synthetic peptide consisting of residues 8–17 of the amyloid β protein of Alzheimer disease was used in immunohistochemical studies to reveal binding sites for this peptide in vesicular elements in the islets of Langerhans of the pancreas and the zona reticularis of the adrenal gland. These binding sites may represent a specific membrane receptor. These results, together with similarities in structural features between the precursors for epidermal growth factor and β protein, suggest that the β-protein precursor may be processed to release an active peptide ligand rather than acting as a membrane receptor. In Alzheimer disease, abnormal processing of this active peptide precursor may result in the deposition of β-protein amyloid fibrils in the brain.**

Alzheimer disease is the commonest cause of dementia in old people and presently afflicts an estimated two million such individuals in the United States (1). This number is likely to rise alarmingly over the next several decades as the proportion of the elderly in the population increases (2). The obvious impact of this fact on health care has stimulated interest in research aimed at understanding the etiology and pathogenesis of this disease in detail. At present, the initial cause of Alzheimer disease is unknown, and the processes leading to the formation of the hallmark brain lesions (neuritic or "senile" plaques and neurofibrillary tangles) are poorly understood. Substantial research progress has been achieved over the last few years (reviewed in refs. 3–6). In 1984, Glenner and Wong (7) isolated a 4200-dalton peptide (which they termed "β protein") from cerebrovascular amyloid. Amino acid sequence data (8) subsequently indicated that β protein is also a component of plaque amyloid fibrils, and this has been substantiated by immunohistochemical studies with monoclonal (9) and polyclonal (10) antibodies raised against synthetic peptides corresponding to various segments of β protein. An anti-β-protein monoclonal antibody (1G10/2/3) has recently been shown to label both cerebrovascular and plaque amyloid fibrils at the ultrastructural level (11), leaving little doubt that this protein is an intrinsic fibril component. Whether or not the paired helical filaments that form the neurofibrillary tangles are also composed of β protein is still a matter of considerable dispute (5). The availability of amino acid sequence data has led to the isolation and sequencing of a full-length cDNA clone that encodes a putative 695 amino acid β-protein precursor (12). The posttranslational cleavage mechanisms that generate the amyloid β protein are unknown. It has been deduced from the nucleotide sequence of the cDNA clone that this precursor is probably a membrane receptor glycoprotein (12). Here we present immunohistochemical evidence suggesting that the β-protein precursor is instead processed to release a peptide ligand. These results, together with the observation that the precursors for β protein and epidermal growth factor (EGF) share common structural features, lead to the proposal that β-protein amyloid filbrils in Alzheimer disease may be derived from an aberrant or abnormally processed active peptide precursor.

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MATERIALS AND METHODS

Tissues. Samples of adrenal gland and pancreas were removed at autopsy from four patients with histologically verified Alzheimer disease and four adult patients with Down syndrome. The tissues were fixed for 3–5 days in buffered 10% formalin, dehydrated through graded ethanol solutions into toluene, and embedded in paraffin. Sections (6 μm) were cut and mounted on glass slides with Hitostik (Accurate Chemicals, Westbury, NY).

Synthetic Peptides. These were synthesized by the solid-phase method of Marglin and Merrifield (13) with a Beckman system 990C synthesizer. The peptides employed for this study were OP1 (residues 1–10 of β protein: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr) (10), AL1 (residues 8–17 of β protein: Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu) (9), OP3 (residues 21–28 of β protein: Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys), OP7 (residues 587–596 of the β-protein precursor: Lys-Tyr-Glu-Glu-Ile-Ser-Glu-Val-Lys-Met) (12), and OP8 (residues 314–322 of the β-protein precursor: Ala-His-Phe-Gln-Lys-Ala-Lys-Glu-Arg) (12). A cysteine residue (for coupling purposes) was added to the carboxyl terminus of each of these peptides except for OP3, which was synthesized instead with a cysteine residue at the amino terminus.

Antibodies. The production of monoclonal antibody 1G10/2/3 (to AL1) has been described (9). Monoclonal antibody 2F5, raised against the A chain of ricin (14), was used as a control. Both 1G10/2/3 and 2F5 are IgG type 1 antibodies made from parent P3 NS1-Ag4 myelomas.

Immunohistochemical Staining. Sections were dewaxed in xylene, immersed for 30 min in 3.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase, and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS: 0.14 M NaCl/0.01 M phosphate, pH 7.4). Nonspecific protein binding sites were blocked by a 30-min incubation with 10% (wt/vol) ovalbumin in PBS. Conventional immunostaining was carried out by the peroxidase-antiperoxidase (PAP) method. The tissues were incubated

Abbreviations: EGF, epidermal growth factor; LH-RH, luteinizing hormone-releasing hormone; PAP, peroxidase-antiperoxidase.

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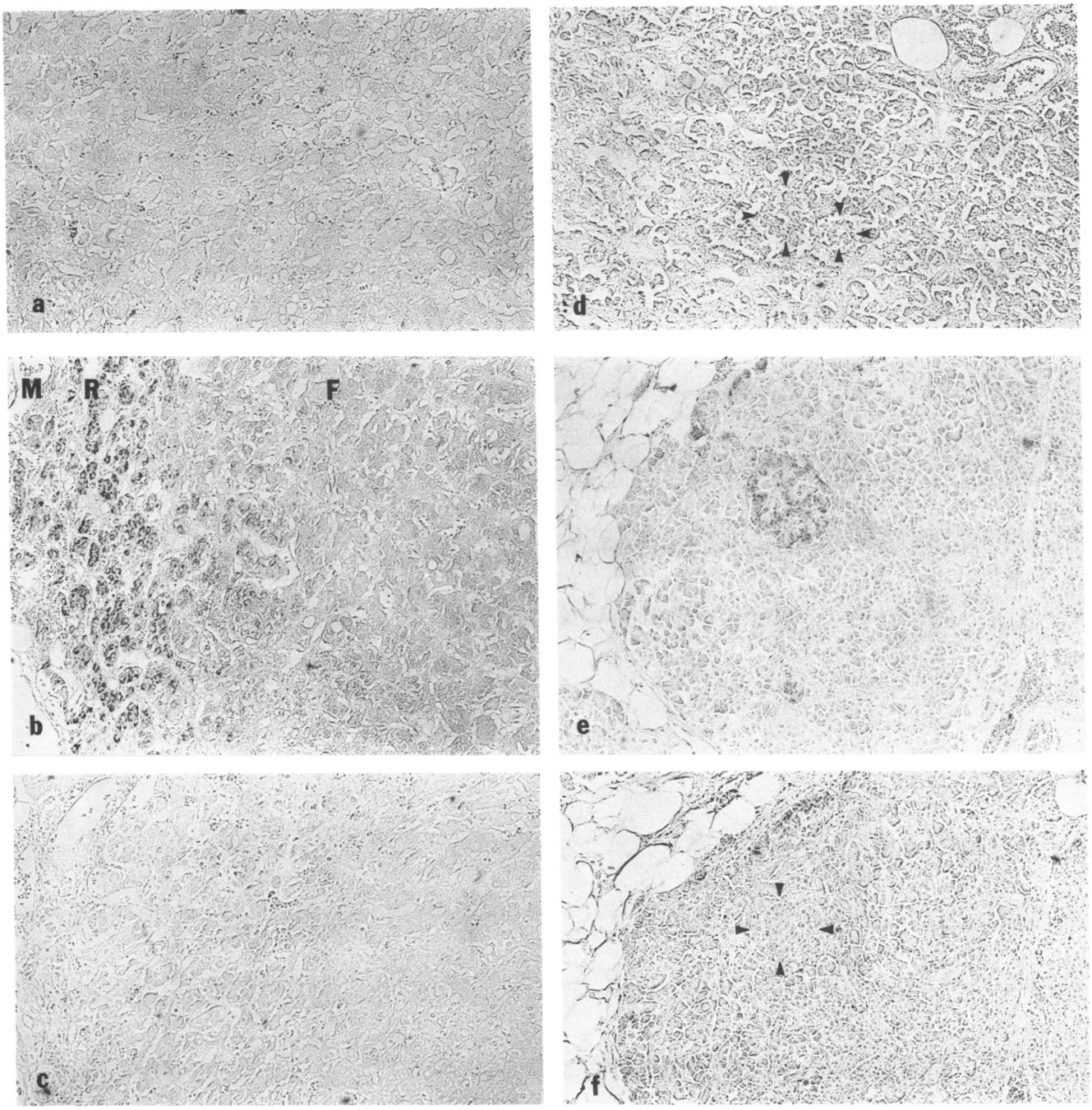

FIG. 1. Binding of AL1 peptide to sections of adrenal gland (*a–c*) and pancreas (*d–f*) demonstrated by immunostaining with 1G10/2/3. After pretreatment of tissue sections with no peptide or with peptide OP1, OP3 (*a* and *d*), OP7, or OP8 (10 μg/ml), there was no detectable stain. However, if the sections were pretreated with AL1 (5 μm/ml) either on its own or in the presence (0.5 mg/ml) of OP1, OP3 (*b*), OP7 (*e*), or OP8, positive staining was observed. This staining was blocked by preincubation of 1G10/2/3 with 1 mM AL1 (*c* and *f*) and was not seen with control 2F5 antibody. All sections were immunostained with 1G10/2/3 at 1:10,000 dilution. Sections *a–c* and *d–f* are from similar areas of the same tissue blocks. M, R, and F refer respectively to adrenal medulla, zona reticularis, and zona fasciculata. Arrowheads demark unstained pancreatic islets. (×90.)

overnight at 5°C with primary antibody and treated sequentially for 30–60 min at room temperature with each of the following (purchased from Dako, Santa Barbara, CA): (*i*) 1:1000 rabbit anti-mouse IgG, (*ii*) 1:100 swine anti-rabbit IgG, and (*iii*) 1:100 rabbit PAP. All immunoreagents were diluted in PBS containing 1% ovalbumin (dilution buffer). Positive immunoreaction was detected with 0.06% 3,3′-diaminobenzidine (Sigma) and 0.03% hydrogen peroxide in 10 mM Tris·HCl (pH 7.5).

For the peptide-pretreatment experiments, the sections were exposed overnight at room temperature to the synthetic peptide (or mixture of two peptides) in dilution buffer and then washed in three changes of PBS (5 min per wash with stirring) prior to immunostaining by the above PAP procedure. In the experiments designed to block PAP staining, the primary antibodies were preincubated with the synthetic peptide in dilution buffer for 3 hr at room temperature.

RESULTS

The 1G10/2/3 antibody was tested on pancreas and adrenal tissue sections by a conventional PAP immunostaining technique using ascites fluid diluted in the range 1:1000–1:10,000.

This failed to produce any convincing staining in either tissue. At low dilution (less than 1:4000) some nonspecific staining of pancreatic acinar cells was observed, and so all subsequent experiments described below were carried out with 1G10/2/3 ascites fluid diluted 1:10,000.

When these tissues were pretreated with peptide AL1 at 0.5–10 μg/ml (0.38–7.7 μM) prior to PAP staining with 1G10/2/3, clear positive staining was observed in zona reticularis cells of the adrenal cortex (Fig. 1*b*) and in endocrine cells in the islets of Langerhans of the pancreas (Fig. 1*e*). In the latter case, the pattern of staining suggested labeling of the islet beta cells, although this was not demonstrated rigorously. These results suggested that AL1 might be binding with high affinity to sites in these tissue regions (material binding with low affinity would presumably be lost during the extensive washing and staining procedures). When the concentration of AL1 was varied from 0.5 to 10 μg/ml the intensity of this staining increased accordingly, although the higher levels of peptide also produced a significant increase in the amount of background staining. No further increase in the staining intensity of the pancreatic islet and adrenal zona reticularis cells was observed at concentrations greater than 10 μg/ml. When AL1 was replaced by peptide OP1, OP3, OP7, or OP8, no staining was observed (Fig. 1 *a* and *d*); this is as expected, since none of these peptides showed any significant reaction with 1G10/2/3 in ELISA tests. The above staining was immunospecific, since it was not observed in AL1-treated sections tested with either antibody 2F5 (1:1000 ascites fluid) or 1G10/2/3 blocked by preincubation with 1 mM AL1 (Fig. 1 *c* and *f*).

To test the specificity of these tissue binding sites for the AL1 peptide, immunostaining experiments were carried out with 1G10/2/3 on tissue sections pretreated with a saturating amount (5 or 10 μg/ml) of AL1 peptide in the presence of a 100-fold molar excess of a second peptide (OP1, OP3, OP7, or OP8). If both peptides could bind to the same tissue sites, the second peptide would competitively displace AL1, and the staining pattern reported above would be considerably reduced. Such a reduction was not observed; in fact, the presence of a second peptide resulted in very little or no background on the sections, and so the AL1-dependent staining was more easily demonstrated. This was presumably due to occupancy of nonspecific peptide binding sites with the competing peptide. The immunospecificity of the staining produced under these conditions was verified by control experiments similar to those described above (Fig. 1).

Examination of the adrenal tissue under a Nikon Microphot-FX microscope with Nomarski differential interference contrast optics revealed that the AL1-dependent staining was deposited in granules, suggesting labeling of cytoplasmic vesicles (Fig. 2*a*). In the pancreatic tissue, the stain seemed to be located on the membranes of larger vesicular structures in the cytoplasm (Fig. 2*b*). There was no evidence of significant labeling of the plasma membrane, or the nucleus, in any positively stained cell. Determination of the precise sites of AL1 peptide binding must await further immunohistochemical staining results at the ultrastructural level.

DISCUSSION

In this study we used monoclonal antibody 1G10/2/3 to demonstrate the presence of synthetic peptide AL1 binding sites in the islets of Langerhans of the pancreas and the zona reticularis of the adrenal gland. This is based on the finding that at low concentrations of antibody, there was no detectable staining of these tissues unless they were first exposed to a small amount of AL1. Treatment with other peptides

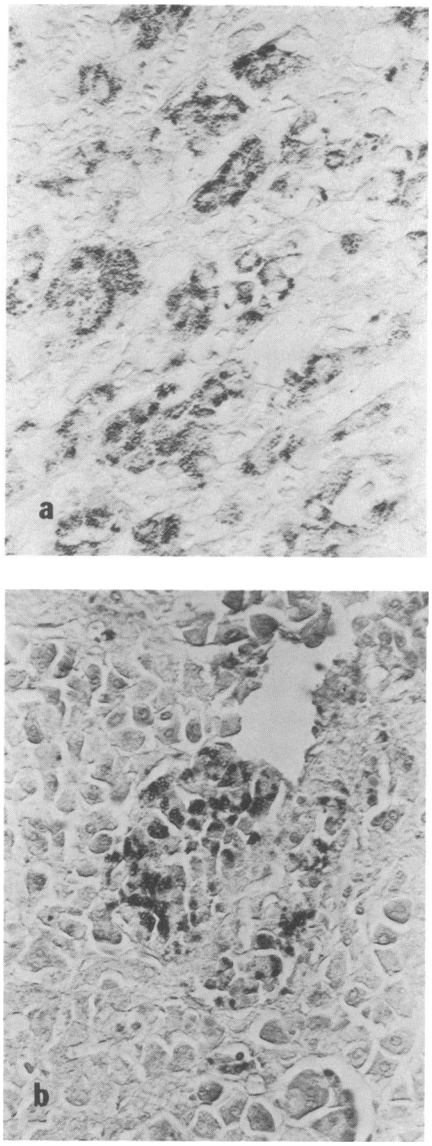

FIG. 2. AL1 peptide-dependent staining (obtained as in Fig. 1 *b* and *e*) at higher magnification. Note granular staining in the cytoplasm of the adrenal zona reticularis cells (*a*) and labeling of membranes of cytoplasmic vesicles in the pancreatic islets (*b*). (× 267.)

failed to reproduce this phenomenon. The immunospecificity of this peptide-dependent staining was confirmed by the fact that it was not observed when 1G10/2/3 was replaced by either 2F5 or 1G10/2/3 antibody preincubated with AL1. Furthermore, peptide binding to these tissue sites showed specificity for AL1, since bound AL1 was not competitively displaced by a large excess of a second peptide.

We propose from these results that the β-protein precursor is processed to release a peptide ligand, such as a neuroendocrine hormone or a growth factor. The peptide-dependent staining can then be explained by the binding of AL1 (which we presume to contain at least part of the receptor-binding region of this putative peptide) to a specific membrane receptor in the tissues. To explain the results, the AL1 peptide must be able to bind to this receptor without sterically hindering the subsequent immunochemical reaction with 1G10/2/3. Also, the receptor involved must not lose its binding capacity after formaldehyde fixation and paraffin embedding. This situation would be analogous to that described in detail by Sternberger (15) for a number of hormone/receptor systems. For example, antisera to luteinizing hormone-releasing hormone (LH-RH; a 10-residue

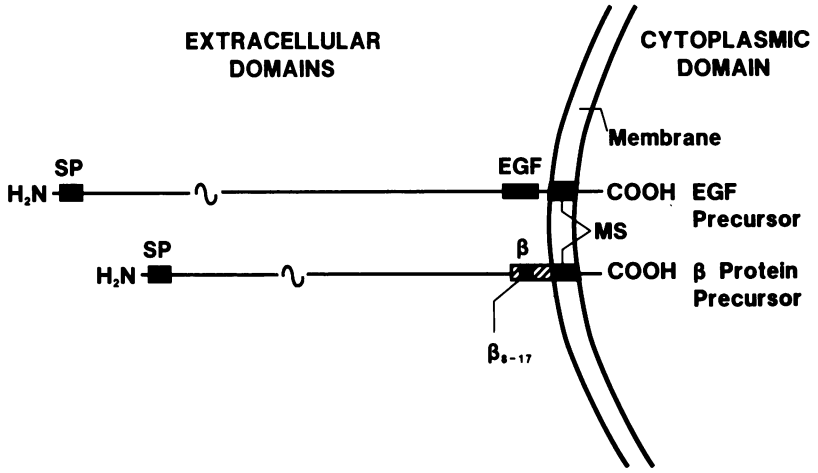

FIG. 3. Schematic illustration of EGF and β-protein precursors, stressing similarities in structural features. The EGF precursor (1168 residues) has an amino-terminal signal-peptide sequence (SP), with EGF itself at residues 977–1029 and a predicted membrane-spanning region (MS) at residues 1039–1059 (25). In the case of the β-protein precursor (695 residues), the sequence for β protein (β) commences at residue 597, and the potential transmembrane sequence occurs at residues 625–648 (12). $β_{8-17}$ marks the position of the synthetic peptide (AL1) that binds to pancreatic and adrenal tissue.

peptide) have been used to detect LH-RH receptors in picric acid/paraformaldehyde-fixed sections of rat pituitary (16, 17). Staining with antibody alone was minimal, reflecting a low occupancy of the LH-RH receptor with endogenous hormone. When LH-RH and anti-LH-RH were added in sequence, this staining was considerably amplified due to the binding of exogenous hormone to its receptor. Whether or not the AL1 binding sites demonstrated here represent a physiologically relevant, pharmacologically acceptable receptor remains to be established.

The observation that the AL1-dependent staining was confined to cytoplasmic vesicles is also consistent with the idea that the β-protein precursor is processed to release a peptide ligand. Although early studies of membrane-bound receptors stressed their location on the cell surface, it has recently become apparent that many receptors are in fact found in a higher concentration in vesicular elements ("receptosomes" or "endosomes"), particularly in the Golgi complex (15, 18–20). Consequently, a number of membrane receptors have been localized by immunocytochemistry to this type of structure (15–22). Sussman *et al.* (23) demonstrated the presence of somatostatin receptors on isolated pancreatic islet secretory granules by ligand-binding techniques. Sternberger (15) employed immunohistochemical methods to locate intracellular LH-RH receptors in the zona fasciculata and reticularis of the adrenal gland. The absence of plasma-membrane staining has been noted in the immunocytochemistry of membrane receptors and could be due to the prolonged fixation of the tissues in formalin (15). Thus both of the AL1 tissue binding sites reported here are potential target areas for a putative hormone or growth factor. Preliminary observations (D.A. and G.G.G., unpublished) have indicated that AL1 peptide binding sites may also be present in collecting tubules in the kidney and in certain neuronal cell bodies in the cerebral cortex and the hippocampus.

Recently, Kang *et al.* (12) isolated and sequenced a cDNA clone that codes for a possible 695 amino acid precursor to the amyloid β protein. From the deduced amino acid sequence, they proposed that this precursor is a membrane-spanning glycoprotein receptor. Our results are more consistent with the idea that a fragment of this precursor functions as a ligand. This idea is further supported by the fact that the overall structural features of the β-protein precursor show some parallels to those of the precursor for EGF (24, 25) (Fig. 3). Both of these precursors are predicted to contain a single membrane-spanning region, with a short carboxyl-terminal domain that projects into the cytoplasm and a long extracellular amino-terminal segment. The EGF sequence is found close to the probable membrane-spanning segment on the amino-terminal side, and consequently it has been proposed that the EGF precursor is anchored to the outer membrane of its cells of origin, with limited extracellular proteolysis resulting in the release of active EGF (25). In the case of the β-protein precursor, the sequence for β protein itself lies in an equivalent position to EGF, suggesting that posttranslational processing may release an active peptide from the β-protein region.

We conclude that β-protein amyloid fibrils in Alzheimer disease may be derived from an abnormal or abnormally processed active peptide precursor. It is possible that a segment of this precursor circulates in the bloodstream, in accord with previous proposals of a serum amyloid precursor (26, 27). If abnormal processing of the β-protein precursor occurs in Alzheimer disease, producing a nonfunctional ligand ("hormone" or "regulator"), then it is possible that introduction of the normal ligand might be an approach to the therapy of this disease. This may also be true if its pathogenesis is due to depletion of the ligand by metabolic divergence to amyloid fibril formation.

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