Volume 294, number 1,2, 23 26 FEBS 10430 December 1991

Immunological identification and sequence characterization of a peptide derived from the processing of neuroendocrine protein 7B2

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Received 5 August 1991; revised version received 8 October 1991

A newly raised antiserum against the C-terminal region of neuroendocrine protein 7B2 was used to purify a novel peptide from the culture media of the mouse corticotroph cell line AtT-20. Based on partial sequencing, this peptide, which we call Cter-7B2, begins at Ser^{156} and appears to result from the cleavage of pro7B2 after a five-basic-residue sequence. Thus, 7B2 processing may contribute to the diversity of peptides found in neuronal and endocrine cells.

7B2; Precursor protein; Antibody; Proteolytic processing; AtT-20 cell

1. INTRODUCTION

Most regulatory peptides (hormones, growth factors, neuromodulators) derive from specific proteolysis of large precursor proteins, most often at pairs of basic amino acids [1]. 7B2, a highly conserved marker of the secretory granules of neuronal and endocrine cells, carries such potential sites at its carboxyl portion [2–5]. It could thus represent a substrate for proprotein convertases. In this study, we describe an antibody raised against the carboxyl end of 7B2 which has allowed us to purify and characterize a novel peptide derived from 7B2 processing.

2. MATERIALS AND METHODS

2.1. *Production of antisera against the carboxyl terminus of 7B2*

The DNA sequence encoding mouse $7B2_{156-186}$ was amplified by the polymerase chain reaction (PCR) from the cloned cDNA [2]. The 0.1-kb PCR product was digested with *Eco*RI and *Bam*HI and ligated into the plasmid pGEX-2T (Pharmacia) [6] cut with same enzymes, downstream of the gene encoding the *Schistosoma japonicum* glutathione *S*-transferase (GST), in a continuous reading frame with it, under the control of the inducible *Tac* promoter. *Escherichia coli* DH5α were transformed with the recombinant plasmid; plasmid was extracted from several clones and the inserted DNAs were sequenced to select those without PCR-caused missense or nonsense mutations. The production of fusion protein (GST–Cter-7B2) was induced with isopropyl-β-thiogalactopyranoside (IPTG) in a 2-liter culture of one such clone. The protein was purified by affinity chromatography on Glutathione-Sepharose 4B (Pharmacia) [6] and was used to immunize rabbits.

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2.2. *Radioimmunoassay and immunoprecipitation*

Radioimmunoassays (RIA) were performed as previously described for the N-terminal region of 7B2 [7]. For a standard assay, the C7B2 antiserum was diluted 1:600 (final dilution). The tracer, a synthetic peptide $7B2_{156-171}$, was ^{125}I-labelled by the chloramine T method (spec. act. 6×10^5 cpm/ng) and was used at 3×10^4 cpm per tube.

For immunoprecipitation, the samples were diluted 1:1 with a buffer containing 2% Nonidet-P40, 0.2% SDS, 1% sodium deoxycholate, 0.3 M NaCl, 0.1 M Tris-HCl, pH 8.0 (2× IP buffer). The antiserum (20 μl) was added and the mixture was incubated at 4°C for 16 h, after which 50 μl of a 10% suspension of a fixed cell preparation of *Staphylococcus aureus* membranes (IgGSorb, The Enzyme Center) were added. After 30 min at 21°C, the suspended material was sedimented at 6000 × *g* and 4°C, for 10 min, and the pellet was rinsed twice with 1× IP buffer.

2.3. *Purification and sequencing of Cter-7B2 peptide*

As source for the peptide, we used AtT-20 cells infected with the recombinant vaccinia virus VV:m7B2 (to be described elsewhere) and expressing high levels of mouse 7B2. We followed the protocol of Hruby et al. [8] for production of the virus and for its use as expression vector to study prohormone processing in AtT-20 cells. Infected cells (4×10^6–1.5×10^7) were grown in 5–10 ml of Dulbecco Minimal Essential Medium (DMEM) containing 0.5% fetal bovine serum for 16 h. Media were collected and supplemented with phenylmethyl sulfonyl fluoride (PMSF), iodoacetamide (IAA) and EDTA to final concentrations of 1 mM, 1 mM and 2.5 mM, respectively. Cells were harvested in 500 μl of 0.1 M Tris-HCl, pH 7.4, containing PMSF, IAA and EDTA as above, and were disrupted with 2 cycles of freezing and thawing followed by two 30-s sonication bursts on ice. The extracts were centrifuged at 14 000 × *g* for 15 min, and the supernatants collected. Media and cell extracts were acidified with trifluoroacetic acid (TFA) at 0.1%. They were fractionated by high performance liquid chromatography (HPLC) on a Vydac C-18 column (0.78 × 30 cm) using 0.1% TFA for 10 min, then a 0-60% linear gradient of acetonitrile in 0.1% TFA. The fractions were analysed by RIA.

In some experiments, incubation of VV:m7B2-infected cells was done in the presence of [^{35}S]methionine, [^{3}H]phenylalanine, [^{3}H]leucine or [^{3}H]valine. The culture medium was subjected to immunoprecipitation as described above, and the radioactive precipitates were sequenced on a gas-phase sequenator (Applied Biosystem, model 470A [9].

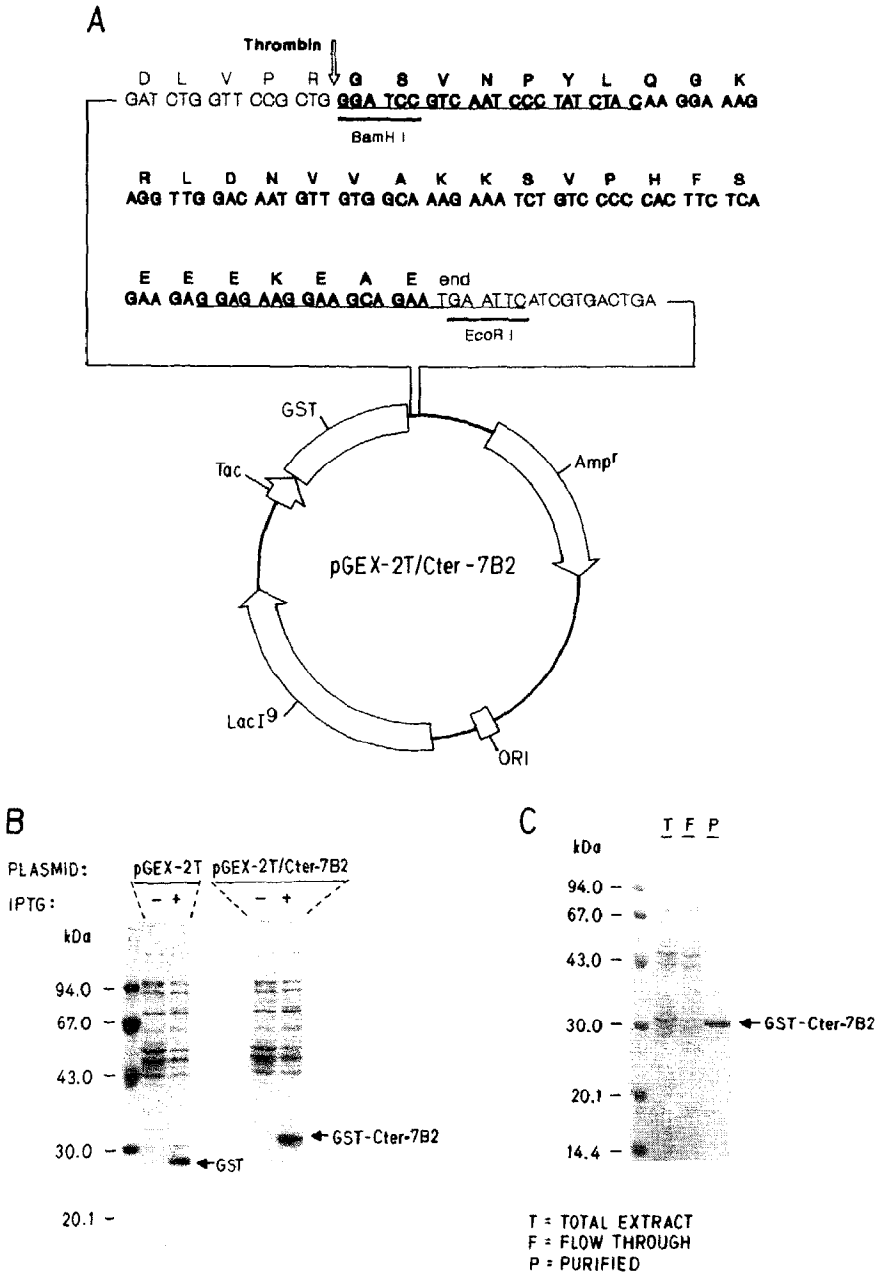

Fig. 1. (A) Plasmid pGEX-2T/Cter-7B2 construct. The inserted DNA sequence and the polypeptide it encodes are printed in bold. The PCR primer segment is underlined. The thrombin cleavage site to release the C-terminal peptide is shown. (B) IPTG induction of GST–Cter-7B2. Proteins extracted from 1 ml bacterial culture grown with or without 0.4 mM IPTG were resolved on a 0.1% SDS–15% PAGE and stained with Coomassie brilliant blue [16]. (C) Purification of GST–Cter-7B2 by chromatography on Glutathione-Sepharose 4B. Aliquots were analysed by SDS-PAGE as in (B). A two-liter culture yielded 16 mg of purified fusion protein.

3. RESULTS AND DISCUSSION

3.1. *Characterization of the C7B2 antiserum*

The plasmid pGEX-2T/Cter-7B2 used to produce GST–Cter-7B2 in *E. coli* is depicted in Fig. 1A. On Coomassie blue-stained SDS-PAGE gels, extracts from pGEX-2T-carrying bacteria incubated with IPTG showed a prominent band of 27 kDa corresponding to GST (Fig. 1B). Extracts from bacteria carrying pGEX-2T/Cter-7B2 contained an IPTG-induced protein of 31 kDa corresponding to GST–Cter-7B2. This fusion protein was purified in a single step by affinity chromatography (Fig. 1C).

The antiserum obtained from rabbits immunized with the fusion protein attached 10 pg (20%) of radioactive tracer under our RIA conditions (Fig. 2A,B). It was specific for the $7B2_{156\ 171}$ peptide and showed no cross-reactivity to other synthetic peptides derived from the C-terminus of 7B2 (Fig. 2B), placing the recognized epitope possibly between residues 156 and 165 of

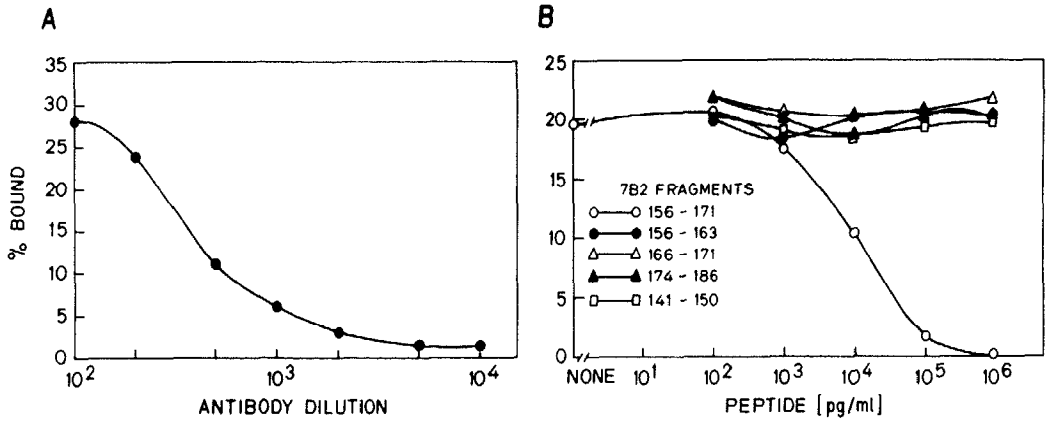

Fig. 2. Characterization of the C7B2 antiserum. (A) Antiserum titration curve (initial dilution). (B) Antiserum binding of the tracer in the presence of increasing amounts of various synthetic peptides.

pro7B2 (see Fig. 4C). ACTH, β-endorphin and the 23-kDa 7B2 purified from porcine pituitary did not cross-react with this antiserum (data not shown).

3.3. *Purification and sequencing of Cter-7B2*

When the antiserum was used for RIA on HPLC fractions of culture media and extracts of VV:m7B2-infected AtT-20 cells, a large immunoreactivity peak was observed at 41 min with both samples (Fig. 3A,B). The Cter-7B2 peptide released by thrombin digestion of GST–Cter-7B2 co-elutes with this peak. A smaller immunoreactivity peak was detected at 57 min with the extracts only (Fig. 3B). Although the 23 kDa pituitary form of 7B2 also elutes at this time [10], it is unlikely to be the immunoreactive material detected here as it is not recognized by the antiserum. We presume this late-eluting peak to be due to pro7B2.

To determine the nature of the immunoreactive protein eluting at 41 min, cells were incubated with [^{35}S]methionine, [^{3}H]phenylalanine, [^{3}H]leucine or [^{3}H]valine and materials recognized by antiserum were immunoprecipitated from the culture medium. Radioactive immunoprecipitates were obtained with [^{3}H]Phe, Val and Leu, but not with [^{35}S]Met. Sequencing

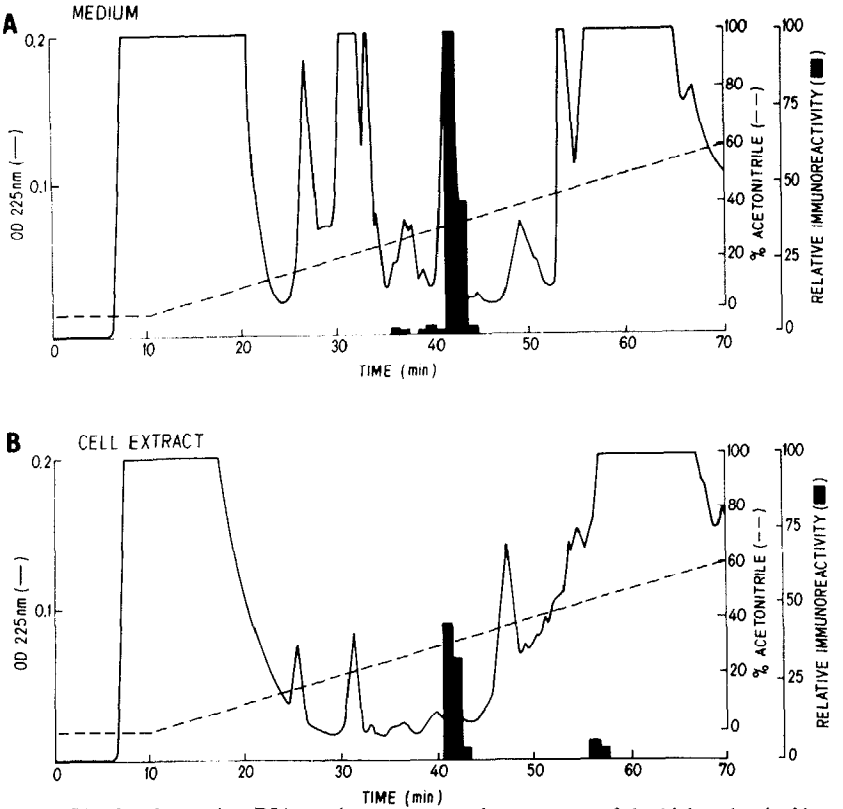

Fig. 3. HPLC fractionation and RIA of 7B2 proteins. RIA results are expressed as percents of the highest level of immunoreactivity detected in the HPLC fractions of the culture medium.

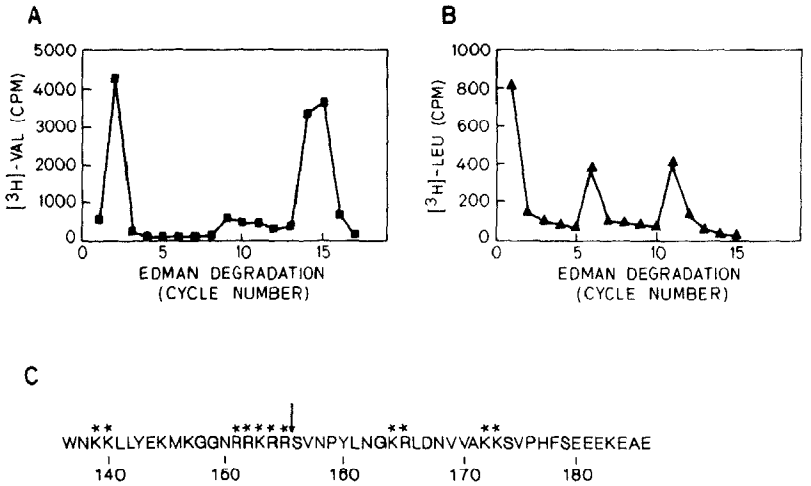

Fig. 4. Sequencing of the C-terminal peptide. Edman degradation cycles of immunoprecipitates labelled with (A) [^{3}H]Val, and (B) with [^{3}H]Leu. (C) Diagram showing potential proteolytic maturation sites (*) in pro7B2. Arrow indicates the deduced processing site.

of the labelled material yielded [^{3}H]Val peaks at cycles 2, 14 and 15 (Fig. 4A), and [^{3}H]Leu peaks on cycles 1, 6 and 11 (Fig. 4B). Except for the [^{3}H]Leu peak on cycle 1, the positions of all the radioactive peaks are predictable for a peptide starting at residue 156 of mouse pro7B2 and generated by its processing after the string of basic residues Arg-Arg-Lys-Arg-Arg$^{151-155}$ (Fig. 4C). The source of [^{3}H]Leu peak on cycle 1 has not been clarified. It may be due to non-specific adsorption of free Leu to the polybrene matrix of the sequenator, to contamination of the Cter-7B2 immunoprecipitates by another protein, or to a Ser-to-Leu mutation during virus propagation. Nonetheless, the absence of any other ambiguities in the deduced sequence indicates that the antiserum recognized the appropriate antigen rather selectively. Because the immunoprecipitate could be [^{3}H]-labelled at the single Phe found in the C-terminal segment and because there is no additional processing site beyond this residue (see Fig. 4C), it is very likely that the peptide extends to the end of the pro7B2 sequence and is 31 residues long.

Taken together, these results confirm the precursor nature of 7B2. In pulse-chase studies with AtT-20 cells, we found that 7B2 was first produced as precursor of 29 kDa which was rapidly converted to a 23 kDa form (Paquet et al., manuscript in preparation). This conversion apparently involves the release of the Cter-7B2 peptide characterized above and could explain the lack of cross-reactivity between this antiserum and tissue-extracted 7B2. In a similar study with explants of *Xenopus* neurointermediate pituitary [11], the maturation site was tentatively placed at the Lys-Lys$^{139-140}$. The variation in processing site may reflect differences among tissues in resident proprotein convertases and in cellular environment [9].

This new antiserum should nicely complement the one against the amino terminal region [7] in studies of 7B2 biosynthesis, maturation and secretion, as well as in the eventual definition of its biological role.

Acknowledgements: This work was supported by grants from the Medical Research Council of Canada and by La Succession J.A. de Sève. L.P. is a Ph.D pre-doctoral fellow and C.L. a scholar of the Fonds de Recherche en santé du Québec.

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