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Application of the Multiple Antigenic Peptides (MAP) Strategy to the Production of Prohormone Convertases Antibodies: Synthesis, Characterization and use of 8-Branched Immunogenic Peptides

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Abstract: Antiserum against an N-terminal sequence of murine prohormone convertase-1 (mPC1) incorporating the sequence immediately following the junction between the putative pro-region and the active enzyme was obtained. This was accomplished using the multiple antigenic peptide (MAP) approach whereupon an 8-branched polylysine core to which are grafted multiple copies of a 16 amino acid peptide representing the N-terminal sequence of mPC1 (positions 84–99) was synthesized by solid-phase Fmoc chemistry. The ensuing peptide was purified and fully characterized by RP-HPLC, ^{1}H-NMR, amino acid composition, peptide sequencing and ion-spray mass spectrometry. The immunological properties of the resulting antibodies in detecting recombinant PC1 in both crude and purified preparations were compared with antibodies raised against a similar N-terminal segment of PC1 but using the conventional method of peptide–carrier protein conjugation and also developed against a C-terminal fusion protein of PC1. Our data indicate that the MAP antibody was as efficient as both the amino and carboxy-terminal antibodies in qualitative as well as quantitative analysis of PC1 encoded protein by radioimmunoassay. Following an identical approach, antibodies against other prohormone convertases like furin, PC5/6 and PACE4 were also developed and subsequently applied to a number of biochemical and immunological studies. In each case, the ease of preparation and high immunogenicity of the MAP approach were confirmed and reside in the simplicity and rapidity with which a potent and useful antiserum is obtained.

Keywords: Prohormone convertase; MAP; polyclonal antibodies; ion-spray mass spectrometry; peptide synthesis

Abbreviations

MAP, multiple antigenic peptide; hPC1, human prohormone convertase 1; mPC1, mouse prohormone convertase 1; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; MCA, 4-methylcoumaryl-7-amide; pGlu, pyroglutamic acid; HOBT, 1-hydroxybenzotriazole; PTH, phenylthiohydantoin; R_t, retention time; OPfp, pentafluorophenyl; ODhbt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine.

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INTRODUCTION

Polypeptide hormones and various biologically active proteins are initially synthesized as larger high molecular weight inactive precursor molecules which must then undergo processing at either single or pairs of basic residues to release their active compo-

nents. The long search for the proteinase(s) responsible for these cleavages culminated in the identification of six different gene products, representing kexin/subtilisin-like serine proteinases (reviewed in [1–4]). These proteinases collectively known as proprotein convertases include furin, PC1/3, PC2, PACE4, PC4 and PC5/6. The involvement of PC1, PC2 and furin in the selective endoproteolysis of a large number of proprotein and prohormone precursor molecules both *in vitro* and *in vivo* has been well documented in the literature. These include prohormone precursor proteins such as proopiomelanocortin and proinsulin, growth factors such as β-nerve growth factor and even viral envelope glycoproteins such as haemagglutinin of influenza virus and gp160 of the human immunodeficiency virus-1 [1–4].

In order to understand better the important role of prohormone convertases in biological processes and their biosyntheses and also to define their structure/function relationships, polyclonal antipeptide antibodies were developed against some regions of these enzymes by conjugating an immunogenic peptide to a carrier protein such as keyhole limpet hemocyanin (KLH) or thyroglobulin, prior to immunization in the animal. This classical strategy proved useful in obtaining antibodies against either the N-terminal or the C-terminal region of prohormone convertases [5, 6]. Another approach consists of the preparation of monoclonal antibodies as done, for example, in the case of furin using a bacterially expressed hybrid protein containing the almost entire furin protein [7]. Finally, polyclonal antibodies against the C-terminal region of PC1 and PC2 were also obtained by using as immunogen a glutathione-S-transferase fusion protein (A. Boudreault *et al.*, submitted); a similar route was used to generate an antibody against the last 100 residues of PC1 [8].

Having experienced the classical approach, we decided to examine other avenues especially in light of the fact that the peptide antigen usually represents only a minor fraction of the total molecular weight of the antigen–carrier conjugate. One alternative is to couple the peptide to itself [9, 10] but this may modify the immunogenic and antigenic epitopes of the peptide since the structure of the resulting peptide after self-conjugation will be changed. In addition, the reagents used for conjugation may also be immunogenic [11]. These limitations and associated problems were circumvented by the group of Tam who introduced the elegant Multiple Antigenic Peptide (MAP) system [12] which yields a large macromolecule exhibiting a high molar ratio of the antigen to core molecule and does not require the use of a carrier protein to elicit an antibody response [13, 14]. A further modification of this approach using half-cystine residues instead of lysine residues as anchoring groups has recently been proposed [15].

In this paper, we describe the synthesis, purification and complete characterization by ^{1}H-NMR, ion-spray mass spectrometry (ISMS), peptide sequencing and amino acid analysis of the mPC1 MAP immunogen. In addition the synthesis of other PC-related MAPs are also described. As a representative model, properties of the MAP anti-PC1 antiserum are illustrated in relation to two other polyclonal antibodies, one raised against an almost identical N-terminal region through conventional peptide–carrier protein conjugation approach and another using a larger C-terminal fusion protein immunogen.

MATERIALS AND METHODS

Materials

All amino acid derivatives (L-form except otherwise mentioned) were purchased from Calbiochem-Novabiochem (San Diego, Ca, USA). The reagents and the solvents for solid-phase peptide synthesis were obtained from Applied Biosystems Inc. (Mississauga, Ont., Canada) whereas other solvents and chemicals were bought from Institut Armand Frappier (Laval, Qué, Canada) and Aldrich Chemical Co. (Milwaukee, Wi, USA). The fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA was obtained from Peptides International (Louisville, Ky, U.S.A.) whereas the substrate acetyl-Arg-Ser-Lys-Arg-MCA was prepared in our laboratory [16].

The recombinant hPC1 enzyme was obtained from the medium of somatomammotroph GH_4C_1 cells following infection with a recombinant vaccinia virus as described [16]. The protease was partially purified through dimethylaminoethyl (DEAE) anion resin chromatography and its enzymatic activity was measured by using pGlu-Arg-Thr-Lys-Arg-MCA and/or acetyl-Arg-Ser-Lys-Arg-MCA as fluorogenic substrates [16].

Reversed-phase High Performance Liquid Chromatography

RP-HPLC was performed with an analytical CSC-Excil A300/ODS column (25 × 0.46 cm, Chromatography Specialty Co., St-Laurent, Qué., Canada). The buffer system consisted of an aqueous 0.1% (v/v)

TFA solution and an organic phase of acetonitrile also containing 0.1% TFA (v/v). The elution was carried out with a linear gradient from 5 to 85% of acetonitrile in 85 min with (gradient A) or without (gradient B) an initial 5 min isocratic step at 5% acetonitrile or a linear gradient of 10–80% CH_3CN in 75 min following a 5 min isocratic at 10% CH_3CN (gradient C), the flow was adjusted to 1.0 ml/min except for preparative runs where a CSC-Excil A300/ODS column (25 × 0.94 cm) was used at a flow rate of 3.0 ml/min. The elution was monitored by measuring UV-absorbency at 225 nm.

^{1}H-NMR Spectrometry and Ion-Spray Mass Spectrometry

The ^{1}H-NMR spectra (in DMSO-d_6 unless otherwise mentioned) and mass spectra for the linear peptide were recorded on a Varian Associate Bruker 400 MHz and a MS-50 HMTCTA (operating in the FAB mode) instruments. Chemical shifts (in p.p.m relative to tetramethyl silane) are reported along with the peak multiplicities: br, broad; m, multiple; t, triplet; d, doublet; s, singlet. For the IS-MS of the MAPs, a triple quadrupole mass spectrometer (API III LC/MS/MS, Sciex, Thornhill, Ont., Canada) was used. The instrument has a mass-to-charge (m/z) range of 2400 and is fitted with a pneumatically assisted electrospray (also referred to as ion spray) interface. The protein sample was dissolved at a 100 μM concentration in 10% (v/v) aqueous acetic acid solution and infused directly into an ion-spray interface at a flow rate of 1 ml/min. Multiply charged protein ions were generated by spraying the sample solution through a stainless steel capillary held at high potential. The voltage on the sprayer was set between +4 to +6 kV for positive ion production.

Amino Acid Analysis

Amino acid analysis were performed following 24 h (for linear peptides) and 24, 48 and 72 h hydrolysis (for the MAPs) in 5.7N HCl at 110 °C *in vacuo*. The hydrolysates were analysed using a modified Beckman 120C autoanalyser equipped with a Varian DS 604 integrator/plotter and using post-column ninhydrin detection. In the case of MAPs, only the results obtained following 72 h hydrolysis are shown.

Amino Acid Sequence Analysis

Aliquots of the monomeric [Tyr_{99}]-mPC1(84–99) peptide (1.37 nmoles, based on weight) and of the [Tyr_{99}]-mPC1(84–99)-MAP (0.25 nmoles, based on weight) were solubilized in 10% aqueous acetic acid immediately prior to loading on a TFA-treated glass fibre filter previously wetted with 30 μl of a Biobrene solution containing 3 mg of polybrene in 0.1 M NaCl (Applied Biosystems). Peptide sequencing was performed using an Applied Biosystems gas-phase sequenator (Model 470A updated to Model 475A specifications) directly coupled to an Applied Biosystems PTH-analyser (Model 120A). Modifications to the normal sequencing procedure include the use of a 100 μl injection loop together with a modified S4 transfer solvent (10% (v/v) acetonitrile : water), TFA (R3) delivery through argon bubbling as well as multiple short S3 deliveries as proposed by Reim and Speicher [17]. Modifications to the PTH-analysis procedure includes the use of PreMix buffer in the aqueous phase as well as the use of 12% (v/v) isopropanol in the organic phase as recommended by the manufacturer (Applied Biosystems). The recovery of the PTH-amino acids for each cycle was normalized according to a PTH-Norleucine standard (R5) added directly prior to separation. The initial and repetitive yields were obtained by linear regression using the yields of selected stable PTH-amino acids.

Preparation of the [Tyr_{99}]-mPC1(84–99)-MAP

The following peptide sequence, Ser-Val-Gln-Lys-Asp-Ser-Ala-Leu-Asp-Leu-Phe-Asn-Asp-Pro-Met-Tyr representing the N-terminal region of mPC1 immediately following the putative junction separating the active form from the prodomain, was selected as the antigenic peptide. In this peptide, Tyr_{99} replaces the normally occurring Trp_{99} residue in the native sequence and was used for radiolabelling purposes. This peptide was synthesized onto a preformed octameric dendritic polylysine MAP core. The synthesis was accomplished by solid-phase Fmoc chemistry on a semi-automatic peptide synthesizer (Pharmacia-LKB Biolynx, Model 4175) with a continuous flow procedure utilizing either OPfp and ODhbt (for Ser and Thr residues only) esters of side chain-protected Fmoc-amino acids. The sidechain protection was afforded by *t*Boc for Lys and *t*But for Tyr, Ser and Asp. Aminotech KA-100 MAP resin (Aminotech KA 100-βAla-Lys_2-Lys_4-$Fmoc_8$, 200 mg containing 0.52 mmol Fmoc group/g resin), was used with 1 mmol of each Fmoc-protected amino acid ester and HOBT. Each amino acid derivative along with HOBT was dissolved in 1.5– 2.0 ml of DMF and loaded on the resin at a flow rate of 2.0 ml/min, whereas for coupling the flow rate was maintained at

3.0 ml/min. The coupling and the deprotection of Fmoc group at each step was monitored by measuring the UV absorbency of the eluent. Following the final coupling, half of the resin was subjected to Fmoc deprotection in order to obtain both the N-terminally protected $Fmoc_8$-MAP and the free MAP. The deprotection and cleavage of the MAPs were accomplished by repeated overnight treatment with reagent K [18], followed by lyophilization and trituration with ether. The crude material was purified by RP-HPLC using gradient A. The purified $Fmoc_8$-MAP and the completely deprotected MAP with $R_t = 60.1$ min (60% CH_3CN) and $R_t = 56.4$ min (56% CH_3CN) respectively were characterized by IS-MS, ^{1}H-NMR and amino acid composition as follows.

(Tyr$_{99}$)-mPC1(84–99)-MAP (Ia). (Ser-Val-Gln-Lys-Asp-Ser-Ala-Leu-Asp-Leu-Phe-Asn-Asp-Pro-Met-Tyr)$_8$-Lys$_4$-Lys$_2$-Lys-βAla, white powder; ^{1}H-NMR: δ 9.8–6.6 (series of m's, ~127H, amide NH), 7.22 (s, 40H, Phe arom.), 6.98 (finely split d, 16H, 8Tyr$_{3,5}$H), 6.62 (finely split d, 16H, 8Tyr$_{2,6}$H), 5.0–3.90 (series of m's, ~135H, 8 × 16 αH + 7 core Lys αH), 3.75–3.50 (m, 32H, 8 × 2Ser βH), 3.05 (br. m, 46H, 15Lys εH + 8Pro δH), 2.8–2.6 (m, 32H, 8Tyr + 8Phe βH), 2.4–2.3 (m, 64H, 8 × 3Asp βH + 8Gln γH), 2.0 (s, 24H, 8Met SCH_3), 2.1–1.1 [m's, ~224H, 8 × (Lys $\beta\gamma\delta$H, Val βH, Ala CH_3, 2Leu $\beta\gamma$H, Pro $\beta\gamma$H, Met $\beta\gamma$H, Gln + Asn βH)], 0.94–0.76 [overlapping s + d's, ~ 144H, 8 × (Val CH_3 + 2Leu $2CH_3$)]; IS-MS: molecular weight 15 577 ± 5.0 (calculated 15 577); amino acid composition: Asx$_{32.4}$(32), Ser$_{13.3}$(16), Glx$_{8.5}$(8), Pro$_{6.4}$(8), Ala$_{9.4}$(8), Val$_{8.3}$(8), Met$_{8.5}$(8), Leu$_{16.5}$(16), Tyr$_{7.4}$(8), Phe$_{8.7}$(8) and Lys$_{14.8}$(15), the β-Ala (1) content was not determined.

Fmoc$_8$-(Tyr$_{99}$)-mPC1(84–99)-MAP (Ib). White powder; ^{1}H-NMR data are similar to that of (**Ia**), except for the additional 88 protons of the 8 Fmoc groups identified at δ 8.95 (d, 16H, 8Fmoc$_{1,8}$ H), 7.4 (d, 16H, 8Fmoc$_{4,5}$ H), 7.32 (dd, 32H, 8Fmoc$_{2,3,6,7}$ H) and 4.32 (s + m, 24H, 8Fmoc$_8$ CH and CH_2); amino acid composition: Asx$_{35.4}$(32), Ser$_{19.6}$(16), Glx$_{7.9}$(8), Pro$_{7.9}$(8), Ala$_{6.9}$(8), Val$_{8.0}$(8), Met$_{6.5}$(8), Leu$_{18.8}$(16), Tyr$_{6.6}$(8), Phe$_{6.3}$(8), Lys$_{13.0}$(15).

Synthesis of the (Tyr$_{99}$)-mPC1(84–99) Peptide (II)

The synthesis of the corresponding monomeric peptide was accomplished by solid-phase Fmoc chemistry using a fully automated peptide synthesizer (ABI model 431A). An unloaded HMP resin (0.25 mmol) and 1 mmol of each side chain-protected Fmoc amino acid derivatives were used. The crude peptide was cleaved from the resin and fully deprotected by two successive overnight treatments at room temperature with reagent K and recovered in 35% overall yield. The peptide was purified by RP-HPLC with gradient B. The major peak eluting at $R_t = 37.4$ min (42% CH_3CN) was characterized; amino acid composition: Asx$_{4.4}$(4), Ser$_{1.9}$(2), Glx$_{1.4}$(1), Pro$_{0.9}$(1), Ala$_{0.8}$(1), Val$_{0.8}$(1), Met$_{0.9}$(1), Leu$_{2.4}$(2), Tyr$_{0.9}$(1), Phe$_{0.8}$(1), Lys$_{1.0}$(1); ^{1}H-NMR, δ 9.2–6.8 (m's, 15H, amide NH), 7.22 (s, 5H, Phe arom.), 6.98 (s, 2H, Tyr$_{3,5}$ H), 6.65 (s, 2H, Tyr$_{2,6}$ H), 5.05–3.95 (m, 16H, αH), 3.55 (m, 4H, 2Ser βH), m's at 3.05 (2H), 2.90 (2H), 2.70, (6H), 2.40 (6H) for δH of Pro and Lys and βH of 3Asp, Tyr and Phe + Gln γH, 1.98 (s, 3H, Met SCH_3), 2.0–1.22 (m's, 21H, Pro $\beta\gamma$H + Lys $\beta\gamma\delta$H + 2Leu $\beta\gamma$H + Val βH + Met $\beta\gamma$H), 1.20 (d, 3H, Ala CH_3), 0.90–0.75 (s + d, 18H, 2Leu CH_3 and Val CH_3); FAB-MS: m/z 1842 $(M+H)^+$.

Radioiodination of (Tyr$_{99}$)-mPC1(84–99) (II)

Radiolabelling of RP-HPLC purified [Tyr$_{99}$]-mPC1(84–99) peptide (**II**) (15 µg, 8.14 nmol) with [I^{125}] was performed with chloramine T following the procedure described earlier [19]. The resulting monoiodinated radiolabelled peptide was purified by RP-HPLC with gradient B and eluted at 38.5 min (44% CH_3CN) as compared with 37.4 min (42% CH_3CN) for the unlabelled peptide under identical elution conditions. There was no significant formation of diiodinated peptide. The purified iodinated peptide had a specific activity of ~7 600 c.p.m/pmol.

Coupling of (Tyr$_{99}$)-mPC1(84–99) (II) to Thyroglobulin via CNBr Activation

Cyanogen bromide (22.8 mg, 0.22 mmol) was dissolved in 0.1N aqueous HCl (2 ml). A mixture containing the unlabelled (3.3 mg, 1.8 µmol) and radiolabelled (20 µl, 6.2×10^6 c.p.m) [Tyr$_{99}$]-mPC1(84–99)peptide (**II**) was added and the resulting mixture was stirred overnight at room temperature. The reaction was followed by injecting a 10 µl aliquot of reaction representing 16 µg and 54 000 c.p.m of radioactivity of the peptide in a RP-HPLC column and measuring the radioactivity and UV absorption of each collected fraction (1 ml). After stirring overnight, the reaction mixture was evaporated completely under vacuum and redissolved in 0.1 N HCl (2.0 ml). A solution of thyroglobulin (10 mg) in 0.1N HCl (1.0 ml) was added and the pH was raised to 9.0 by adding 1N NaOH (300 µl). The reaction was stirred at room temperature for 18 h when it became turbid. The progress of the reaction was monitored by RP-HPLC.

Immunization Protocol

Two New Zealand white female rabbits weighing 2.0–2.5 kg were purchased from a local company. The rabbits were housed in individual stainless steel cages as specified by the Canadian Council on Animal Care (CCAC) guidelines. The room was maintained on a 12 h light and 12 h dark cycle. Following two weeks of quarantine, the rabbits were injected with 200–500 μg of [Tyr_{99}]-mPC1(84–99)-MAP in complete Freund adjuvant. Booster injections were repeated each time with 200 μg of the above-mentioned MAP. Quadriceps muscles were used for intramuscular injection, and intrascapular regions were used for subcutaneous injection. A small blood sample was obtained from each rabbit 10 days post-immunization to test antibody titre. After the 7th injection, the animals were killed and antisera were collected. Similar procedure was followed for other MAPs.

Synthesis of Other Convertase-related MAPs

Apart from [Tyr_{99}]-mPC1(84–99)-MAP, other MAPs were prepared for furin, PC5 and PACE4 using the recently introduced FastFmoc solid-phase chemistry in a fully automated peptide synthesizer (ABI, model 431A) using 0.1 mmol MAP Wang resin (0.4–0.6 mmol Fmoc/g) and 1 mmol of each Fmoc-protected amino acid. Some physico-chemical data on these peptides are presented below.

h or mfurin(108–120)-MAP. The sequence of the 13 amino acids linear peptide corresponds to Asp-Val-Tyr-Gln-Glu-Pro-Thr-Asp-Pro-Lys-Phe-Pro-Gln; RP-HPLC (gradient B): broad peak at $R_t = 33.7$ min (39% CH_3CN); the computed molecular weight is 13 352; amino acid composition: $Asx_{16.3}(16)$, $Thr_{7.7}(8)$, $Glx_{24.7}(24)$, $Pro_{24.5}(24)$, $Val_{6.7}(8)$, $Tyr_{6.4}(8)$, $Phe_{8.5}(8)$ and $Lys_{16.4}(15)$.

rPC5(83–98)-MAP. The sequence of the 16 amino acids linear peptide is Asp-Tyr-Asp-Leu-Ser-Arg-Ala-Gln-Ser-Thr-Tyr-Phe-Asn-Asp-Pro-Lys; RP-HPLC (gradient B): broad peak at $R_t = 36.7$ min (42% CH_3CN); the computed molecular weight is 16 203; amino acid composition: $Asx_{33.7}(32)$, $Thr_{8.4}(8)$, $Ser_{13.8}(16)$, $Glx_{9.7}(8)$, $Pro_{7.4}(8)$, $Ala_{8.9}(8)$, $Leu_{7.8}(8)$, $Tyr_{11.1}(16)$, $Phe_{9.5}(8)$, $Lys_{14.8}(15)$ and $Arg_{9.7}(8)$.

(Tyr$_{555}$)-hPACE4(536–555)-MAP. The sequence of the 20 amino acids peptide is Ala-Ala-Ser-Asp-Lys-Arg-Pro-Arg-Ser-Ile-Pro-Leu-Val-Gln-Val-Leu-Arg-Thr-Thr-Tyr; the computed molecular weight is 19 016; amino acid composition: $Asx_{8.3}(8)$, $Thr_{17.5}(16)$, $Ser_{12.3}(16)$, $Glx_{9.8}(8)$, $Pro_{17.4}(16)$, $Ala_{13.0}(16)$, $Val_{18.1}(16)$, $Ile_{8.4}(8)$, $Leu_{19.9}(16)$, $Tyr_{8.3}(8)$, $Lys_{19.2}(15)$ and $Arg_{28.2}(24)$.

Enzymatic Activity and Radioimmunoassays for Prohormone Convertase

The enzymatic activities of PC1 and furin were measured using pGlu-Arg-Thr-Lys-Arg-MCA and/or acetyl-Arg-Ser-Lys-Arg-MCA as fluorogenic substrates [16]. The released AMC was detected using a Perkin-Elmer MPF3L spectrofluorimeter. The amount of immunoreactive prohormone convertases in various samples were determined employing different antibodies by a radioimmunoassay procedure (RIA) using the corresponding iodinated linear peptides as tracer molecules.

Gel Electrophoresis and Western Blotting

All gel electrophoreses were made on 1.5 mm sodium dodecyl sulphate containing polyacrylamide gels in a vertical gel apparatus (Hoeffer Scientific Instruments Inc.). For Western blotting, gels were immersed for 30 min in a transfer buffer composed of 25 mM tris–HCl pH 8.3 containing 192 mM glycine and 20% (v/v) methanol. The proteins were then electrotransferred at 33 volts for 15 h at 4 °C onto an Immobilon-P membrane (Millipore). At the end, the residual sites on the membrane were blocked for 1 h at room temperature in 5% (w/v) non-fat skim milk in phosphate-buffered saline prior to incubating the membrane for 1 h at room temperature with the N-terminal MAP-derived mPC1 antiserum at a final dilution of 1/500 in milk solution. Following repeated washings (5 × 5 min) with 0.1% (w/v) tween-20 in phosphate-buffered saline, the immune complex was detected using the streptavidin-biotin horseradish peroxidase detection system (Amersham). Staining was done using nickel chloride and DAB (3,3′-diaminobenzidine) as described by the manufacturer but with 0.5 mg/ml of substrate.

RESULTS

Characterisation of [Tyr_{99}]-mPC1(84–99) Peptide

This peptide was recovered following cleavage and complete deprotection by two successive treatments with TFA in the presence of 8% (v/v) anisole. The RP-HPLC purified material exhibited the correct molecular mass 1842 $(M)^+$ as determined using various

ionization modes such as FAB, CI and IS. In FAB/CI modes, its mass spectrum also displayed peaks at *m/z* 1865 ($M^+ + Na$), 1828 ($M + H^+ - CH_3$), 1796 ($M^+ - SCH_3$), 1781 ($M^+ - CH_3 - S - CH_3$) and 1756 ($M + H^+$—Ser—C=O), all in full agreement with its structure. A single treatment of the peptide bound-resin with either TFA/anisole or reagent K, yielded, after RP-HPLC purification, materials with molecular masses of 1955 and 1899 corresponding to the correct peptide (*m/z* 1842) but with the addition of two and one *t*But groups respectively. Complete elimination of these groups thus required more drastic conditions and multiple treatments with the deprotection reagent.

Chemical Characterization of [Tyr_{99}]-mPC1(84–99)-MAP (Ia)

The average molecular mass of **(Ia)** is calculated to be 15 577 based upon its amino acid sequence and is well beyond the resolution of normal mass spectrometry techniques as described above. However, ion spray mass spectrometry, by allowing detection of proteins with masses up to 100 kDa [20], permits the characterization of the above MAP. Thus, [Tyr_{99}]-mPC1(84–99)-MAP **(Ia)** exhibited several multiply charged peaks, as shown in Figure 1A, all indicating an *m/z* value of 15 577 ± 5, fully consistent with the molecular formula. The unusual broadening of the observed peaks, normally not observed with usual peptides or proteins, could possibly be due to the complex geometrical and highly branched nature of the MAP, its association to metal ions and/or to non-homogeneity of the desired product, in which case it may be contaminated with small amounts of less branched MAPs not separated during the RP-HPLC step. Despite this difficulty, ES-MS combined with amino acid analysis is very useful for the characterization of MAPs as it allowed detection of the presence of, for example, remaining protecting groups. Indeed, as mentioned above, we observed that following a single 12 h treatment of [Tyr_{99}]-mPC1(84–99)-MAP

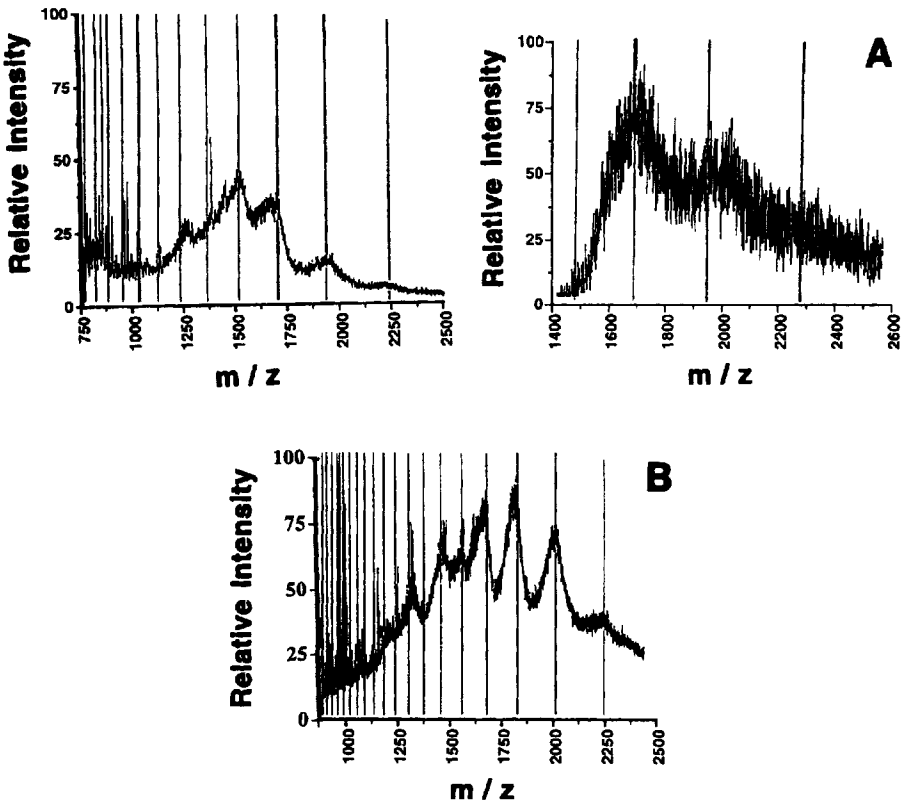

Figure 1 Electron-spray ionization mass spectral analysis of [Tyr_{99}]-mPC1(84–99)-MAP. **(A)** Mass spectrum of the RP-HPLC purified MAP following two consecutive treatment with the deprotection reagent. The multiply charged ions peaks are shown in the *m/z* region from 750 to 2250. **(B)** Mass spectrum of the RP-HPLC purified MAP following a single deprotection step yielding a deconvoluted mass of 15 686 m.u. indicative of the presence of remaining protecting groups.

bound-resin yielded upon RP-HPLC, a material with a molecular mass of 15 686 ± 5 as revealed by ES-MS (Figure 1B). This estimate is 109 m.u. more than the expected mass and could possibly be accounted for by the presence of two *t*But groups remaining on the peptide. This excess mass disappeared following another 12 h treatment of this material. This suggests that, in certain cases, some protecting groups may be located in a sterically hindered and less accessible environment. This unusually long period of time needed to deprotect this peptide fully may also be related to the exceptionally large number of protecting groups (64 in this case).

While purifying the crude [Tyr_{99}]-mPC1(84–99)-MAP, we also isolated a compound which can easily be mistaken for a peptide in view of its spectral and chromatographic properties. This compound yielded a nicely resolved peak eluting with a R_t of 39.9 min (45% CH_3CN) (gradient B) and, as evidenced by amino acid analysis was of non-peptidic nature. Upon analysis by FAB-MS, this compound was found to be of low molecular mass (m/z 439) and upon further characterization by ^{1}H-NMR as a phenolic derivative exhibiting resonance peaks at δ 9.75 (s, 2H), 7.38 (d, J=9.8 Hz, 2H), 6.80 (d, J=9.8 Hz, 2H), 3.30 (s, 2H) and generated by self-condensation of the scavengers used in the deprotection procedure. Interestingly, a closely related material with a reported molecular mass of 455 (16 m.u. more, likely to correspond to an oxidized form) was described in a correction article [21] as it had originally been wrongly characterized and identified as synthetic chromostatin, a 20 amino acid peptide derived from chromogranin A.

Amino Acid and Sequence Analysis of MAPs

During this study it rapidly became evident that, for optimum amino acid analysis of MAPs, one should resort to the longer hydrolysis condition such as 72 h hydrolysis and even then, in certain cases, the molar ratio thus computed may not be ideal. This long period of time needed to ensure as near as possible complete hydrolysis of the peptide bonds does complicate analysis of these compounds but, on the other hand, yields useful information concerning the integrity of the peptide. However, considering that amino acid analysis cannot provide information on the presence of remaining protecting groups and that computation of the proper molar ratios can be troublesome, this type of analysis for MAPs should whenever feasible be complemented by mass spectral analysis and/or sequence analysis.

In spite of the fact that both the monomeric peptide and the MAP are built upon, in the case of PC1, a small 16-residue long sequence, both were sequenced up to the COOH-terminus. However, inclusion of this sequence onto a polylysine core greatly facilitated sequence analysis as it leads to improvements in both the initial yield estimated as 26.4% for the MAP compared with 15.9% for the monomeric peptide, even though five times less peptide on a molar basis was used and the repetitive yield obtained through linear regression computed as 83.7% (correlation coefficient of 0.991) and 76.9% (correlation coefficient of 0.970) respectively. Furthermore, it was possible to observe the presence of an $N-1$ sequence representing, in both cases, 20% of the estimated initial yield as well as the presence of the sequence Pro-Met-Tyr in the first three cycles. The latter tripeptide sequence represented 21% of the estimated initial yield in the case of the monomeric peptide but only 1.3% in the case of the MAP; this sequence more than likely arises from the known susceptibility of the internal Asp-Pro bond to be cleaved in acidic conditions. Finally, it should be noted that peptide sequencing of the MAP continued right into the polylysine core as evidenced by the presence, albeit in low yields, of the three successive Lys residues. It can thus be proposed that, in addition to derivatized membranes, such a polylysine core could be used to attach peptides following activation of the carboxyl moiety prior to sequencing. Thus, as illustrated here with a PC1-MAP, peptide sequencing in conjunction with amino acid analysis and mass spectrometry represents a very useful tool in order to characterize and assess the chemical integrity of MAPs.

Production of Polyclonal Antibodies to MAPs

A minimum of two separate rabbits was used for raising antisera against the various MAPs. In the case of anti-PC1 antibodies, the progress of the immune response was carefully monitored using the corresponding [I^{125}]-labelled monomeric [Tyr_{99}]-mPC1(84–99) peptide as the tracer, as illustrated in Figure 2. Thus, it can be seen that after only two or three injections, a relatively potent antiserum from either of the two rabbits used was obtained. The immune response did not increase significantly upon further injections, even when the immunization was continued up to seven injections in both the rabbits. The first rabbit (Figure 2, right) responded rapidly since the antibody titre, following the second injection, raised sharply to a maximum and then

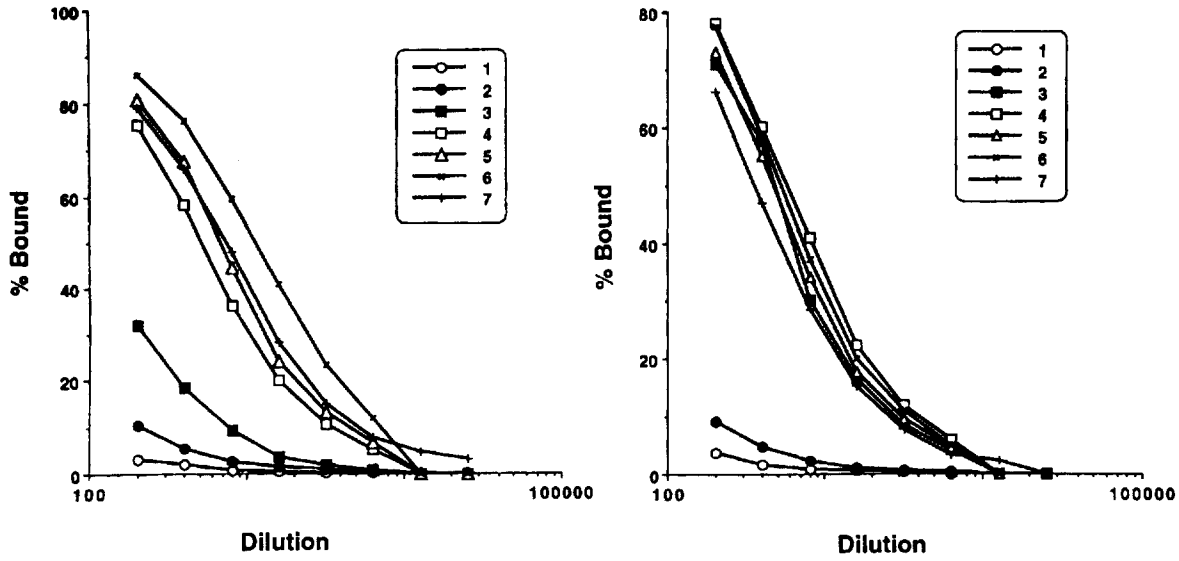

Figure 2 Representative displacement curves of the antibody response of two individual rabbits immunized with the [Tyr_{99}]-mPC1(84–99)-MAP as described in Materials and methods. Sera from various bleeds numbered 01 to 07 were serially diluted and the ability to bind mPC1-related antigen determined using a radioimmunoassay procedure with the [^{125}I]-labelled monomeric [Tyr^{99}]]-mPC1(84–99) peptide as tracer.

remained almost stationary until the seventh injection. For the second rabbit (Figure 2, left), the antibody titre after each immunization increased steadily for first three injections before reaching an almost steady level. Thus, we found in the case of the [Tyr_{99}]-mPC1(84–99)-MAP, a very fast immune response occurring within two or three injections; similar results were also obtained with the other MAPs that we have prepared and reported in this article (data not shown). In contrast, injection of the conjugate prepared by reacting the monomeric peptide with thyroglobulin using CNBr failed to generate any immune response against the peptide. It is possible that such a result could be due to the low content of immunogenic peptide (less than 10%) with respect to the carrier protein despite the fact that 40–50% of the peptide, based upon radioactivity measurement, was coupled to the carrier (data not shown).

Comparative Abilities of N-terminal MAP- and Carbodiimide-generated Antibodies in Detecting Immunoreactive PC1 Protein

Two N-terminally-directed PC1 antisera were obtained: (i) a MAP-generated antiserum, henceforth referred to as MAP-antiserum and (ii) an antiserum, henceforth referred to as N_{term}-antiserum, obtained by immunizing a rabbit with mPC1(84–100) coupled to KLH as previously described [6]. Both antisera displayed almost identical displacement curves for the same synthetic [Tyr_{99}]-mPC1(84–99) peptide but at two different dilution levels (Figure 3). The N_{term}-antiserum was used at a 1 : 50 000 dilution whereas the MAP-antiserum was used at a 1 : 10 000 dilution. The ED_{50} values (concentration of the antigen peptide necessary to displace 50% of the bound immunoreactive PC1 protein to the antibodies) were calculated based on the two curves and were found to be 2.7 ng/ml for the N_{term}-antiserum and 2.3 ng/ml for the MAP-antiserum respectively. Both antisera were used successfully to detect and measure PC1 immunoreactive protein in either crude or partially purified samples. Thus, using the MAP- and N_{term}-antisera and assuming that the molecular weight of the major form of mPC1 is 80–85 kDa, it can be estimated that there is 45 ± 1.5 and 29 ± 2.6 μg of immunoreactive PC1 protein respectively per 200 ml of recombinant vv:hPC1 infected GH_4C_1 cell medium. Using a C_{term}-PC1 antiserum, developed against a mPC1(626–729) carboxy terminal fusion protein, the same 200 ml medium yielded 30 ± 4.3 μg of immunoreactive PC1 protein. Thus, it appears that the N_{term}-, MAP- and C_{term} antisera provide almost identical estimates of the level of recombinant PC1. The above estimates were computed by averaging data obtained with three or more independent enzyme preparations and also compared with the control wild type medium which exhibited undetectable levels of immunoreactive PC1 protein.

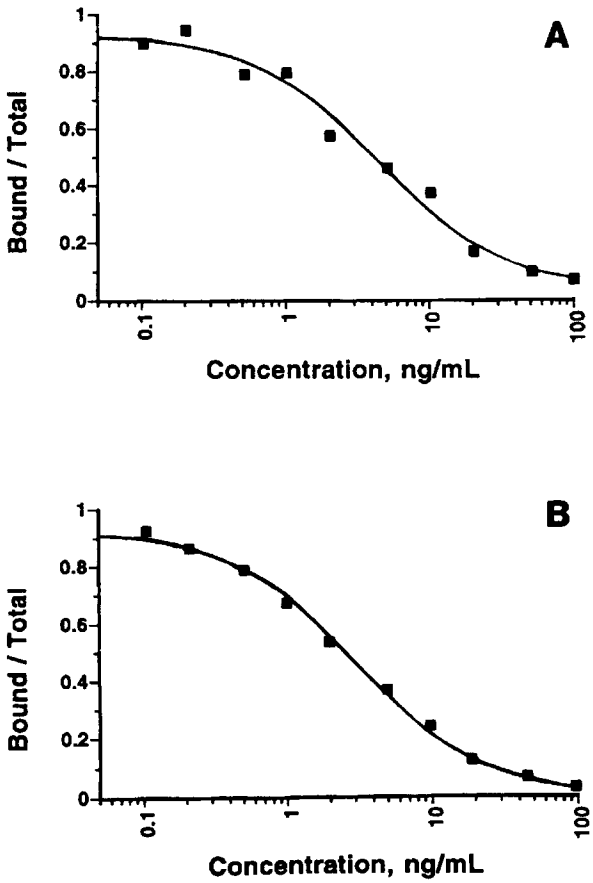

Figure 3 Representative displacement curves using labelled and unlabelled [Tyr_{99}]-mPC1(84–99) peptide as determined using **(A)** the N_{term}-antiserum and **(B)** the MAP-antiserum.

The purification of recombinant hPC1 upon chromatography through DEAE anion exchange resin [16] was monitored by radioimmunoassay using the three antisera described above. Such monitoring, as summarized in Table I, allows the determination of the immunoreactive PC1 protein content in each chromatographic fraction (1 ml size) and can thus be coupled with determination of the total protein content and enzymatic activity. Similar information can also be obtained by screening PC1 containing fractions using Western blotting, as shown in Figure 4. Indeed, using total cell extracts or total medium from insect cells infected with recombinant mPC1 baculoviruses, one can readily detect expression of mPC1 as either the proenzyme form in cell extracts or as two molecular forms of 80–85 and 66 kDa in the cell culture medium. In each case, the use of the MAP-antiserum corroborated the results obtained with antisera raised through classical procedures as well as with antisera obtained through the GST–fusion protein protocol.

Antisera Against Furin, PC5 and PACE4

Besides PC1, other antipeptide antibodies were developed against furin, PC5 and PACE4 by preparing MAPs; the various monomer peptide sequences were in the range of 13–20 amino acids. In each case, the number of injections required prior to reaching an optimum immune response was found to be from one to three even though inoculations were continued right to the end of the injection protocol (seven injections). The antibodies thus generated were able to detect the corresponding macromolecules in 1 to 10 000 or 20 000 dilutions and also in many cases proved useful for radioimmunoassay, immunoprecipitation and/or immunocytochemical studies; studies conducted using these antibodies are currently in progress.

DISCUSSION

By eliminating the necessity of conjugating the antigen peptide to a carrier protein, a step which often results in complex mixture of products contain-

Table I. mPC1 Content of Cell Culture Medium as Determined by Immunological Procedures

Sample	Enzymatic activity (nmol/h)	Protein content (μg)	Immunoreactivity N_{term}-antibody (μg)	Immunoreactivity MAP-antibody (μg)	Immunoreactivity C-term antibody (μg)
Conc. medium	25.3 ± 2.7	3250 ± 250	29 ± 2.6	45 ± 1.5	30 ± 4.3
DEAE-pool A (unretained pool)	5.6 ± 0.3	2085 ± 225	4.9 ± 1.9	8.5 ± 1.3	18.4 ± 2.8
DEAE-pool B (retained fraction)	2.4 ± 0.2	527 ± 62	11.3 ± 2.6	11.2 ± 1.1	8.8 ± 1.0
% Recovery (pool B)	9.5	16.2	39.0	25.0	29.3

Note: the crude medium from vv:mPC1 infected GH_4C_1 cells (200 ml) was first concentrated then chromatographed on a DEAE-Biogel-A column. Fractions were analysed for enzymatic activity using acetyl-Arg-Ser-Lys-Arg-MCA (100 μM) at pH 5.5 in the presence of 5.0 mM calcium, for protein content using the Bradford assay and for immunoreactive content using mPC1 N-terminal and C-terminal-directed antibodies.

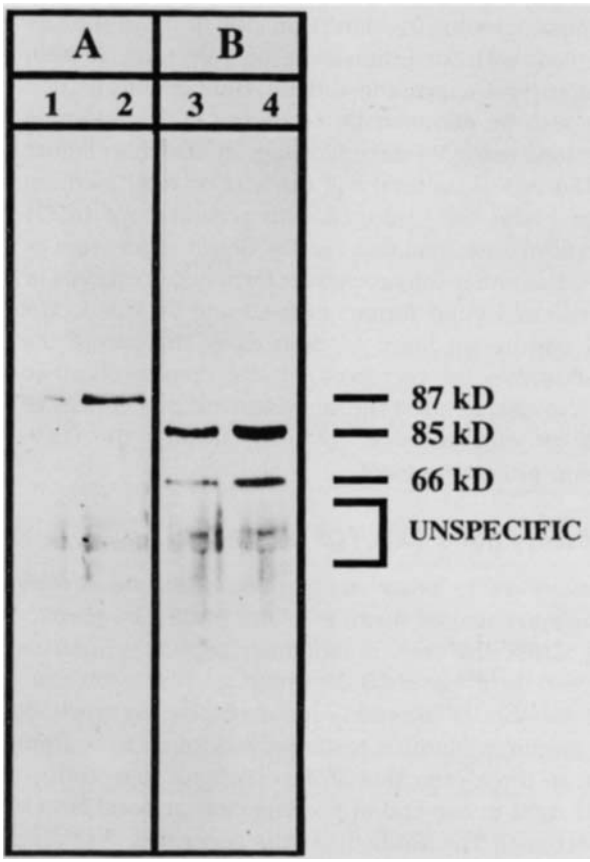

Figure 4 Western blot analysis of the different molecular forms of the mPC1 produced in Sf9 insect cells infected with the recombinant mPC1 baculovirus. Following 43 h post-infection, aliquots of total cell extracts (part A) corresponding to 450 cells (A1) and 900 cells (A2) and of the medium (part B) corresponding to 25 µl (B3) and 50 µl (B4) were separated by electrophoresis on a 7% SDS-PAGE and the immunoreactive proteins were detected as described in Materials and methods using the [Tyr_{99}]-mPC1(84–99)-MAP antiserum. The position of molecular weight standard proteins (Bio-Rad) is indicated on the left side of the gel.

ing little immunogenic material, we have successfully employed the MAP strategy to obtain polyclonal antibodies against various prohormone convertases. We hereby demonstrate that numerous techniques of protein chemistry including mass spectral analysis, peptide sequencing and amino acid analysis can be used effectively to characterize the ensuing immunogens. In addition, we show that an immunogen containing 93.7% immunogenic peptide representing an N-terminal PC1 segment was able to elicit a rapid antigenic response. In fact, no more than two or three injections at the concentration levels reported and at three-week intervals, are sufficient to generate a potent antiserum. By comparison, the usual peptide–protein conjugate method can at times be quite labour- and time-intensive while requiring more elaborate immunization protocol. Our data also indicate that the antiserum thus obtained possess properties and characteristics quite similar to those antisera raised against identical monomeric N-terminal fragment or against C-terminal located epitopes. Indeed, they can be used to detect and quantify the immunoreactive PC1 protein efficiently in either crude or purified samples while being complementary in terms of antigenic recognition.

This study also demonstrated that synthesis of MAPs can be accomplished with solid-phase Fmoc chemistry following the OPfp/ODhbt esters of Fmoc-protected amino acids and commercially available MAP-resin using a semi-automated continuous flow peptide synthesizer or more efficiently by FastMoc chemistry using the Fmoc-protected amino acids and HBTU as the coupling agent in a fully automated peptide synthesizer. Some of these MAPs, incorporating a linear chain containing 16–20 amino acids, may appear as very broad peaks in RP-HPLC chromatograms and can often be difficult to purify. The use of the various analytical techniques, as described herein, permits their characterization at both their crude and purified levels. The crude material was often found pure enough so as to be used directly as immunogen. The presence of any remaining protecting groups which could obscure some epitopes can be observed by ion-spray mass spectrometry and even ^{1}H-NMR whereas problems arising from the synthesis can be investigated through peptide sequencing. It should be noted that some MAPs, especially those incorporating a significant number of hydrophobic residues, can lead to solubility problems which can seriously jeopardize their analysis by some of the techniques described above.

In conclusion, it may be stressed that the MAP strategy is an efficient alternative method of developing antibodies against any segment of bioactive macromolecules. The synthesis of MAPs can be achieved through the use of an automated solid-phase peptide synthesizer. The deprotection and cleavage of the MAPs from the bound resin may require multiple treatment with deprotection reagents and should be monitored carefully. The resulting MAP can be purified by RP-HPLC and characterized by various techniques. Finally, the use of MAPs to generate antiserum against different prohormone convertases has proved advantageous both in terms of synthesis and of abilities to generate an immune response.

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