Structure–Function Analysis of the 7B2 CT Peptide

Ekaterina V. Apletalina,* Maria A. Juliano,† Luiz Juliano,† and Iris Lindberg* 1

*Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112; and 1Department of Biophysics, Escola Paulista de Medicina, Rua Tres de Maio 100, 04044-020, Sao Paulo, Brazil

Received December 22, 1999

Prohormone convertases play important roles in the proteolytic conversion of many protein precursors. The neuroendocrine protein 7B2 and its 31-residue carboxy-terminal (CT) peptide potently and specifically inhibit prohormone convertase 2 (PC2). We have analyzed the residues contributing to inhibition using N-terminal truncation and alanine scanning. Removal of more than 3 residues from the amino-terminal end of CT1-18 resulted in a more than 190-fold drop in inhibitory activity, showing that most of the residues between 3 and 18 are required for inhibition. In agreement, an Ala scan indicated that only 4 residues could be replaced with Ala without losing mid-nanomolar inhibitory potency; in particular, Gin7, Gin9, and Asp12 could be Ala-substituted to yield peptides with a similar inhibitory potency to the starting peptide. The all-o-retro-inverso, all-L-inverso, and all-o analogues of CT peptide were completely inactive, indicating that amino acid side chains and the CT peptide main chain interact with PC2. CT peptide inhibition could not be competitively blocked by preincubation with truncated CT peptide forms, supporting an absolute requirement for the Lys-Lys pair in initial binding of the CT peptide to the active site.

The prohormone convertases are eukaryotic subtilisins believed to be involved in a variety of protein maturation processes within the secretory pathway (reviewed in 1). Within this family, the prohormone convertase 2 (PC2) is unique in its requirement for another neuroendocrine protein, 7B2, for maturation to an active enzyme species (reviewed in 2). In addition to its role in facilitating the maturation of proPC2, 7B2 also contains a 31-residue peptide which functions as a potent inhibitor of active PC2 (3–5). This peptide is internally hydrolyzed within cells to generate an 18-residue fragment which is still highly inhibitory; however, removal of the C-terminal Lys–Lys pair results in complete loss of inhibition (6). In the present study, we have undertaken a structure-function analysis of the 18-residue human CT peptide fragment in an effort to understand the inhibitory mechanism of this unusual tight-binding inhibitory peptide.

MATERIALS AND METHODS

Enzyme assay. Recombinant PC2 used in the assay was purified to homogeneity from conditioned medium of CHO cells transfected with expression plasmids encoding cDNAs for rat 21-kDa 7B2 and mouse proPC2 as previously described (7). Duplicate reactions were performed in a polycarbonate microtiter plate in a total volume of 50 μl, containing 100 mM sodium acetate buffer, pH 5.0, 5 mM CaCl2, 0.4% n-octyl glucoside, 22 ng of preactivated mouse PC2, varying amounts of the human CT peptide derivatives, and 0.2 mM fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA. Preactivated mouse PC2 was preincubated with the peptide to be tested for 30 min at room temperature prior to the addition of substrate. The reaction was conducted at 37°C for 30–45 min. Released AMC was measured with a Fluoroscan Ascent fluorometer (Labsystems) using an excitation and an emission wavelength of 355 and 460 nm, respectively. The IC50 values for the peptides were calculated using non-linear regression of the experimental data (fluorescence vs peptide concentration) using the program GraphPad (GraphPad Software, Inc., San Diego, CA).

Peptide synthesis. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM B system from Shimazdu) was used for the solid-phase synthesis of all the peptides by the Fmoc-procedure. The final deprotected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 × 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H2O (1:1000) and (B) TFA/acetonitrile (ACN)/H2O (1:900:100). The column was eluted at a flow rate of 3 ml/min with a 10–80% gradient of B1 over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm. The molecular weight and purity of synthesized peptides were checked by MALDI-TOF–mass spectrometry (ToFSpec-E, Micromass, Manchester, UK) and by peptide sequencing (Sequence PPSQ-23 Shimadzu Tokyo, Japan).

Abbreviations used: CT peptide, 7B2 carboxy-terminal peptide; PC2, prohormone convertase 2; MCA, 4-methyl-coumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; TFA, trifluoroacetic acid; ACN, acetonitrile; Fmoc, 9-fluorenylmethoxycarbonyl.

1 To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, L.S.U. Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112. Fax: (504) 568-6598. E-mail: ililindb@sumc.edu.

The all-$\alpha$-retro-inverso, all-$\alpha$-inverso analogues of CT peptide were synthesized as the other peptides, using Fmoc $\alpha$- or $\alpha$-amino acids, but the sequences in this case are inverted, i.e., the C-terminal amino acid was Ser, followed by all other amino acids of CT peptide till the N-terminal end Lys. Therefore, if these two peptides are aligned with the CT peptide they will have the same sequence, but with inverted peptide bonds. No effort was made to use a malonic acid as well as the CT peptide main chain are further stabilized by the disulfide bridge (IC$_{50}$ = 0.31 $\mu$M for the disulfide-linked peptide compared to 0.10 ± 0.1 $\mu$M for peptide containing Cys[SMe] at the positions 7 and 12).

The all-$\alpha$-retro-inverso, all-$\alpha$-inverso and all-$\alpha$ analogues of CT peptide were completely devoid of inhibitory activity, suggesting that both, the side chain of the amino acids as well as the CT peptide main chain are involved in its interaction with PC2.

In Table II, Alanine Scan (IC$_{50}$ Values for the CT Peptide Derivatives) was presented, showing the results of the alanine scan. The IC$_{50}$ values indicate the potency of the peptides to inhibit the enzyme PC2. The IC$_{50}$ values were determined from two to four independent experiments.
lutely required for binding of the remainder of the peptide to PC2. We speculate that binding of this di-
 basic pair-which clearly occurs at the active site of PC2
since cleavage of the 31-residue peptide occurs at this
position- generates conformational changes within the
binding pocket of PC2 which then permit tight binding
of the remainder of the molecule, in particular, of the
highly conserved amino-terminal heptapeptide.

ACKNOWLEDGMENTS

This work was funded by DA05084 to I.L., who was supported by K02
Award DA00204. L.J. and M.A.J. were supported by Fundação de
Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho
Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We
thank Angus Cameron for critical commentary on the manuscript.

REFERENCES

Chem. 274, 20745–20748.
3. Martens, G. J. M., Braks, J. A. M., Eib, D. W., Zhou, Y., and
5785.
Chem. 270, 14292–14296.
8. Petit, M. C., Benkirane, N., Guichard, G., Du, A. P., Marraud,
Chem. 274, 3686–3692.
Briand, J. P., Van Regenmortel, M. H., Brown, F., and Mascag-
1051–1060.
12. Lindberg, I., Tu, B., Muller, L., and Dickerson, I. (1998) DNA