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Processing of chromogranin A within chromaffin granules starts at C- and N-terminal cleavage sites

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Specific antisera were raised against synthetic peptide fragments of bovine chromogranin A. The soluble proteins of bovine chromaffin granules were subjected to two-dimensional immunoblotting with these antisera. The endogenous breakdown products of chromogranin A gave distinct patterns of immunostaining which enabled us to correlate these peptides with defined regions of the chromogranin A molecule. The results establish that within chromaffin granules degradation of chromogranin A by the endogenous proteases can start either at the C- or the N-terminal site.

Chromogranin A; Chromaffin granule; Proteolytic processing

1. INTRODUCTION

Chromogranin A is a protein originally isolated from chromaffin granules of bovine adrenal medulla [1]. More recently it was discovered that this protein had a widespread distribution in endocrine organs [1] and in brain [2,3]. Chromogranin A is synthesized as a single proprotein [4,5] which is subsequently processed by endogenous proteases within chromaffin granules [6–8]. In mature bovine chromaffin granules about 50% of the proprotein has already been processed [1].

In the present study we used antisera against peptides present in the amino acid sequence [9,10] of chromogranin A in order to characterize the endogenous breakdown products of chromogranin A. This approach was chosen since most of these peptides are so closely related in size and charge that a separation and subsequent sequencing appeared difficult or even impossible. The results indicate that in contrast to proenkephalin, another peptide present in chromaffin granules [11], proteolytic processing of chromogranin A can occur at cleavage points close to either the N- or the C-terminus.

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2. MATERIALS AND METHODS

Soluble proteins from bovine chromaffin granules, isolated as described [12], were separated by two-dimensional non-equilibrium pH gradient electrophoresis [12] followed by immunoblotting with various antisera [12]. Biolytes (pH range 4–6) were from Bio-Rad, Vienna.

Seven peptides present in the amino acid sequence of chromogranin A [9,10] were synthesized by standard solid phase tBOC chemistry and purified by reverse phase HPLC. These peptides were: LE40 (Ch A_{1-40}), HE35 (Ch A_{79-113}), GGa23 (Ch $A_{272-294}$), WE14 (Ch $A_{316-329}$), LL33 (Ch $A_{332-364}$), GE25 (Ch $A_{367-391}$) and PL26 (Ch $A_{403-428}$). The abbreviations indicate the N- and C-terminus by the one-letter code plus the total number of amino acids. WE14 and GGa23 were conjugated to bovine thyroglobulin via carbodiimide (Smith, P. and Eiden, L.E., in preparation). LE40 was conjugated to soybean trypsin inhibitor at sulfhydryl groups [13] in order to leave the N-terminus free. The other peptides were coupled through amino groups using the benzoquinone pro-

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cedure [14]. The conjugates were used to establish peptide antisera in New Zealand white rabbits with a standard immunization protocol. The working dilution for the antisera in immunoblots was 1:200. Experiments with chondroitinase ABC (Sigma, Munich) incubation were performed as described [15].

3. RESULTS

The soluble proteins of bovine chromaffin granules were subjected to two-dimensional electrophoresis followed by immunoblotting. In the

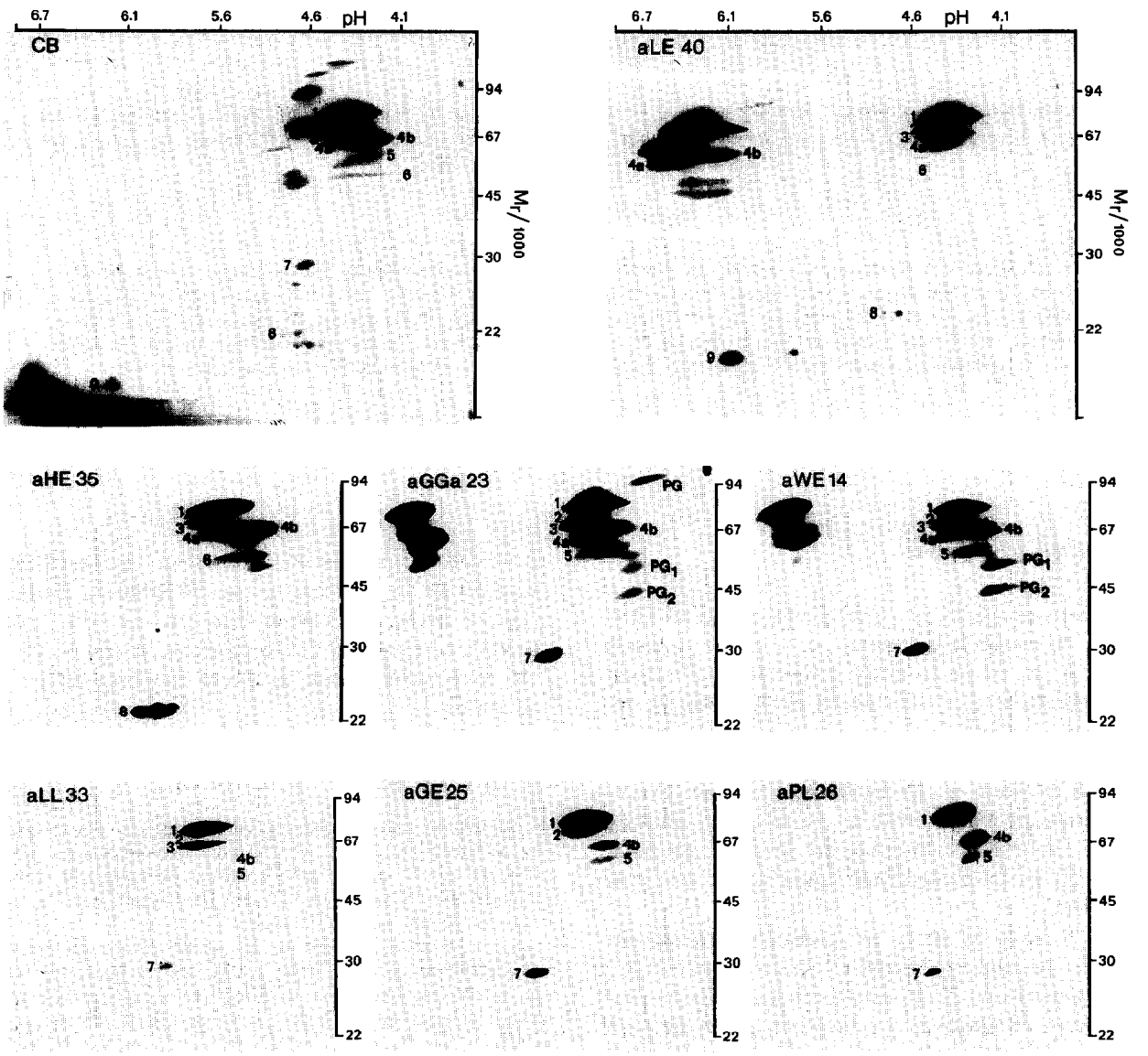

Fig.1. Immunoblots with antisera against peptides present in chromogranin A. Soluble proteins (40 μg) were subjected to two-dimensional electrophoresis followed by Coomassie blue staining and immunoblotting with antisera against several peptides as indicated. Chromogranin A is numbered with 1, the endogenous breakdown products with consecutive numbers. Spot 4 consists of two peptides (a and b) which focus closely together. Antiserum aLE40 reacts only with 4a, but not with 4b. When such an immunoblot is subsequently labeled with aHE35 spot 4b is also stained (see inset in aLE40) demonstrating the distinct properties of 4a and 4b. PG stands for the proteoglycan form of chromogranin A and for other corresponding breakdown products (PG_1, PG_2). When the soluble proteins are treated with chondroitinase ABC before immunoblotting the PG-spots disappear (see insets for aGGa23 and aWE14). In the immunoblot for LL33 spots 2, 4b and 5 were rather weak not allowing photographic reproduction.

Coomassie blue-stained pattern shown in fig.1 the protein spots reacting with antisera against peptides present in chromogranin A [1] are consecutively numbered. In addition to chromogranin A, representing the proprotein [1], several additional spots, i.e. the endogenous breakdown products, are present. The reactivity of these spots with antisera raised against specific peptides of the chromogranin A sequence (fig.2) is shown in fig.1. It can be clearly seen that each antiserum gives a distinct pattern, which was well reproducible. In fig.2 the reactivity of the various protein spots is used to compute the most likely amino acid sequence representing these protein spots. In addition to chromogranin A antisera against this protein stain an additional spot of larger molecular size (PG in fig.1) which has been identified as a proteoglycan of chromogranin A [15–17]. Staining of this PG-chromogranin by antisera is variable (see fig.1). However, two apparent breakdown products of this PG are staining strongly by the antiserum against peptides WE14 and GGa23. Their

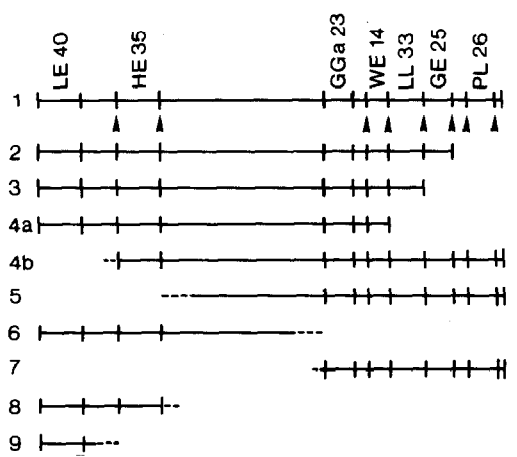

Fig.2. Chromogranin A-immunoreactive proteins in bovine chromaffin granules. The amino acid sequence of chromogranin A is schematically presented together with the various peptides (used for raising antisera) drawn in the proper positions. Possible sites of tryptic cleavage (pairs of basic amino acids) are indicated by arrows. The peptides 2–9 are numbered as indicated in the Coomassie blue stain given in fig.1. Their peptide composition was computed from the immunoblotting results with specific antisera (see fig.1). In some cases where stretches of peptides, for which no antisera were available, are present in an endposition (peptides 5, 6) the length of the peptide could only be deduced from the molecular size of the peptides derived from electrophoresis results. A broken line in the figure indicates that the exact endpoint of the peptide is not known.

proteoglycan nature was identified by chondroitinase ABC digestion which degrades PG and these two additional spots (see insets in fig.1 for aGGa23 and aWE14).

4. DISCUSSION

Proenkephalin is processed in chromaffin granules starting from the C-terminal site [11,18]. Thus all large enkephalin-containing peptides generated in these organelles contain syn-enkephalin, i.e. the peptide on the end of the N-terminal site [18], whereas in brain processing can occur from the N-terminus of the proenkephalin molecule [19]. Therefore, for adrenal chromogranin A, we expected [20] to find processing from the C-terminal site of the molecule and in fact peptides 2, 3 and 4a (in fig.1) are due to processing from this site. However, the results for peptide 4b can only be explained by a processing from the N-terminal site and in fact peptide 9 was found to be the peptide removed by such a cleavage (see fig.2). This peptide was previously not recognized as a product of chromogranin A processing [1,7], which was partly due to its more alkaline p*I*, which is quite different from all the other chromogranins. This p*I* can now be explained rather nicely by the amino acid composition of the sequence of the N-terminus of chromogranin A. In contrast to total chromogranin A the ratio of acidic to basic amino acids of the N-terminus (chromogranin A_{1-76}) is relatively low (1.1 versus 1.6). Thus our results clearly establish that chromogranin A processing can start at two sites. Apparently cleavage at the N-terminal site is a major pathway of processing, since peptide 4b formed by such cleavage represents the major degradation product (see fig.1). This phenomenon is apparently not confined to the adrenal medulla, but also occurs in the pituitary where peptide 9 was found to be present (Fischer-Colbrie, R., unpublished).

The exact cleavage points have not been established in the present study; however, for proenkephalin processing a trypsin-like enzyme is involved and such an enzyme activity has been described in chromaffin granules [21]. Our results are consistent with the action of such an enzyme. Since, however, the exact length of several of these peptides cannot be ascertained by our method, cleavage may also occur at other sites.

We have now established that the processing of chromogranin A can start both at the C- or N-terminal site which is in contrast to adrenal proenkephalin. This may be due to differences in the molecular properties of chromogranin A and proenkephalin. Chromogranin A has a random coil structure [22], a trypsin-like enzyme may therefore find access to several regions of the intact molecule. However, our results may also be explained by different proteases for processing of chromogranin A and proenkephalin in adrenal medulla. Alternatively one might suggest that the co-storage of chromogranin A with neuropeptide hormones dictates the processing of these peptides. This hypothesis is consistent with the data of Seidah et al. [23] that chromogranin A inhibits proenkephalin processing in vitro. It will be interesting to determine whether the presence or absence of chromogranin A in secretory organelles determines C- or N-terminal processing of proenkephalin. These questions, however, can only be answered when the enzymes controlling these processes have been isolated.

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REFERENCES

[1] Winkler, H., Apps, D.K. and Fischer-Colbrie, R. (1986) Neuroscience 18, 261–290.
[2] Somogyi, P., Hodgson, A.J., De Potter, R.W., Fischer-Colbrie, R., Schober, M., Winkler, H. and Chubb, I.W. (1984) Brain Res. Rev. 8, 193–230.
[3] Nolan, J.A., Trojanowski, J.Q. and Hogue-Angeletti, R. (1985) J. Histochem. Cytochem. 33, 791–798.
[4] Kilpatrick, L., Gavine, F., Apps, D. and Phillips, J. (1983) FEBS Lett. 164, 383–388.
[5] Falkensammer, G., Fischer-Colbrie, R., Richter, K. and Winkler, H. (1985) Neuroscience 14, 735–746.
[6] Hörtnagl, H., Lochs, H. and Winkler, H. (1974) J. Neurochem. 22, 197–199.
[7] Fischer-Colbrie, R. and Frischenschlager, I. (1985) J. Neurochem. 44, 1854–1861.
[8] Settleman, J., Fonseca, R., Nolan, J. and Hogue-Angeletti, R. (1985) J. Biol. Chem. 260, 1645–1651.
[9] Iacangelo, A., Affolter, H.U., Eiden, L.E., Herbert, E. and Grimes, M. (1986) Nature 323, 82–86.
[10] Benedum, U.M., Baeuerle, P.A., Konecki, D.S., Frank, R., Powell, J., Mallet, J. and Huttner, W.B. (1986) EMBO J. 5, 1495–1502.
[11] Udenfriend, S. and Kilpatrick, D.L. (1983) Arch. Biochem. Biophys. 221, 309–323.
[12] Fischer-Colbrie, R., Lassmann, H., Hagn, C. and Winkler, H. (1985) Neuroscience 16, 547–555.
[13] Pain, D. and Surolia, A. (1981) J. Immunol. Methods 40, 219–230.
[14] Avrameas, S. and Ternynck, T. (1971) Immunochemistry 8, 1175–1179.
[15] Falkensammer, G., Fischer-Colbrie, R. and Winkler, H. (1985) J. Neurochem. 45, 1475–1480.
[16] Rosa, P., Hille, A., Lee, R.W.H., Zanini, A., De Camilli, P. and Huttner, W.B. (1985) J. Cell Biol. 101, 1999–2011.
[17] Eiden, L.E., Huttner, W.B., Mallet, J., O'Connor, D.T., Winkler, H. and Zanini, A. (1987) Neuroscience 21, 1019–1021.
[18] Patey, G., Liston, D. and Rossier, J. (1984) FEBS Lett. 172, 303–308.
[19] Liston, D., Patey, G., Rossier, J., Verbanck, P. and Vanderhaeghen, J.-J. (1984) Science 225, 734–737.
[20] Fischer-Colbrie, R., Hagn, C. and Schober, M. (1987) Ann. NY Acad. Sci. 493, 120–134.
[21] Lindberg, I.Y.H. (1984) J. Neurochem. 42, 1411–1419.
[22] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 483–492.
[23] Seidah, N.G., Hendy, G.N., Hamelin, J., Paquin, J., Lazure, C., Metters, K.M., Rossier, J. and Chrétien, M. (1987) FEBS Lett. 211, 144–150.