Life Sciences, Vol. 31, pp. 2271-2273 Pergamon Press
Printed in the U.S.A.

THE SIGNAL PEPTIDE OF PRO-OPIOMELANCORTIN: VALIDATION OF A SPECIFIC RADIOIMMUNOASSAY

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(Received in final form June 14, 1982)

Summary

The N-terminus portion of the POMC leader sequence (signal peptide) was synthesized, and an antiserum was raised against it. A radioimmunoassay was developed which is effective at a dilution of 1:500,000, and sensitive at less than 1 fmole/tube. Since leader sequences often exhibit structural homologies, and since synthetic peptides are not readily available, we resorted to an unusual procedure to establish specificity. This involved extraction of pituitary RNA, cell-free translation to produce the pre-prohormones, and purification by B-END and signal antibody affinity columns. The eluates were then tested by SDS gel electrophoresis and by multiple immunoprecipitations. All results showed that the signal antibody captured a single molecular species, approximately 30,000 in MW, which was also captured by the B-END column, and was immunoprecipitable by B-END and ACTH antisera. It therefore appears that this antibody selectively measures the POMC leader sequence and should be valuable in measuring the newly synthesized pre-prohormone.

Most measures of B-END in brain and pituitary have been confined to assessing total levels. The other approach, useful in pituitary, is the pulse chase experiment, which is powerful but rather cumbersome. Since the B-END-ACTH precursor, pro-opiomelancortin (POMC) has been shown to have a leader sequence or signal peptide (1), and since this peptide is only thought to be intact while the precursor is on the ribosome (2), we set out to establish an assay for that region. The rationale is that an assay which measures either free POMC signal or the full pre-prohormone (POMC + signal), would measure newly synthesized material. Work in other systems (3) suggests that the half life of the pre-prohormone is very short, on the order of a few minutes. Thus a POMC-signal assay, when coupled with a total POMC and/or B-END assay, can yield an estimate of current synthesis versus total content. As such, it may provide us with

0024-3205/82/202271-03$03.00/0

a dynamic measure of the activity in a POMC system, with the simple device of two radioimmunoassays. It would also allow us to study the kinetics of transformation from POMC pre-prohormone to prohormone in pulse chase experiments.

The main difficulties arising are: 1) the synthesis and handling of the leader sequence, since it contains many leucines and isoleucines, and tends to be highly hydrophobic. 2) the validation of this assay, since many leader sequences posess structural homologies, which render cross reactivity quite likely. Yet, other leader structures are not readily available for such tests. We therefore had to resort to an unusual approach to validate our system and show its usefulness. This report describes this validation process.

Methods

The N-terminus of the POMC leader sequence, and the N-tyrosine version of it (for iodination) were synthesized by solid phase techniques. The peptide was coupled with gluteraldehyde to thyroglobulin, and the antiserum raised in rabbit. The various bleeds ranged in titer from 1:40,000 to 1:500,000 under RIA conditions using the ^{125}I-signal peptide as a tracer, with sensitivity of 0.5 fmoles and IC_{50} of about 10 fmoles/tube. In order to validate the assay, the antibody was affinity purified by passing it through a signal peptide affinity column. Then the purified IGg was itself coupled to sepharose 4B to form the signal antibody column under study. A similar B-END antibody column was also built and had been previously shown (in pulse chase and other experiments) to capture only POMC, B-LPH and B-END.

In order to obtain POMC with the leader sequence attached, we used pituitary cell free translations, since no signal peptidase activity is present in these systems. Total pituitary RNA was extracted with phenol/$CHCl_3$/isoamyl alcohol, using the method of Ross (4). The total RNA was translated using a rabbit reticulocyte lysate system (NEN) in the presence of ^{35}S-methionine. The translation products were then partially purified on Sep Pak to separate protein from free radioactive methione.

At that point we had a mixture of proteins translated from pituitary RNA and presumably containing a number of pre-prohormones with their leader sequences. The translation product was then split in half with one portion applied to the B-END antibody affinity column and the other to the signal antibody affinity column. After extensive washing in NaPB, the material was eluted off both columns with 2N acetic acid. A portion of each eluate (40%) was re-applied to the original column while another portion (40%) was "crossed" i.e., applied to the column it had not been on. Eluates from this second set of runs were tested on disc gel SDS electrophoresis to determine the number of molecular species captured, and their molecular weights.

Finally, a portion of the eluates of the second runs were pooled and used for immunoprecipitation studies. In these, antisera against the signal region, the B-END region and the

$ACTH_{17-24}$ region of POMC were added with or without the appropriate blocking peptide. After double antibody precipitation, the immunoreactive materials were also tested with SDS disc gel electrophoresis.

Results

Gels from the eluates of both the B-END antibody column and the signal antibody showed a single 30,000 MW peak (expected weight of unglycoylated POMC with leader attached). Furthermore, the material captured by the B-END antibody was also captured by the signal antibody and vice versa (recovery in both cases over 95%). This was evidenced by results of the second set of column tests, whereby "crossed" and "uncrossed" materials all showed the same 30,000 MW peak. Finally, the material passed through both columns was immunoprecipitable by B-END leader and ACTH antisera, and these were only blocked by the proper peptide.

These results all show that: 1) The signal antibody column captured a single 30K molecular species out of a mixture of pituitary pre-prohomones, 2) this same molecular species was also captured by the B-END antiserum (and not by a control anti BSA column), and was immunoprecipitable by B-END and ACTH antisera, strongly suggesting that the material is indeed POMC.

Conclusions

The harvesting of an anti-signal peptide antiserum and the use of this approach for validation of assay specificity allows us to measure the signal portion of POMC. Early chromatographic studies using the RIA thus validated shows that the material being assayed is primarily the pre-prohormone (MW:30K) and not the free signal peptide. POMC signal immunoreactivity is differentially distributed in tissue, being highest in pituitary and not detectable in striatum. The levels in general are less than 1% of total POMC levels. This assay should be valuable in studying the dynamics of POMC synthesis.

Acknowledgements

This work was supported by NSF Grant #BNS8004512, NIDA Grant #DA02265.

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