α-MSH and neurofilament M-protein share a continuous epitope but not extended sequences

An explanation for neurofibrillary staining with α-MSH antibodies

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The long recognized neurofibrillary immunoreactivity of the nervous system with α-MSH antibodies arises from an epitope on neurofilament M-protein which we have now characterized. It is situated in the amino terminal residues where M-protein and α-MSH exhibit similar but not identical sequences. Their divergence past residue 5 precludes a physiological significance of the crossreactivity which seems to have arisen fortuitously. Our results question previous speculations as to the existence of extrapituitary α-MSH-like hormones.

α-MSH *Neuropeptide* *Neurofilament* *Neuron* *Epitope* *Crossreactivity*

1. INTRODUCTION

The main source of α-melanocyte-stimulating hormone (α-MSH), an acetyl-tridecapeptide amide, is the pituitary gland where it is processed from a large polyprotein precursor [1]. However, many studies dating back at least to 1977 documented strong neurofibrillary α-MSH immunoreactivity in most parts of the nervous system (see, e.g., [2–6]). Radioimmunoassays were used to quantitate this material and various speculations as to extrapituitary α-MSH-related hormones have been advanced. The neurofibrillary specificity of the reaction was partially explained when Dräger et al. [2] found that α-MSH antibodies stain neurofilaments, the neuronal form of intermediate filaments. Immunoblotting revealed reactivity only for the middle-sized M-protein, one of the three filament components: L (68 kDa), M (160 kDa), and H (200 kDa) [7]. This and previous observations [2–6] raised the possibility that neurofilaments may contain peptide hormone-like sequences, and we note that intermediate filament proteins have been postulated to be related to steroid hormone receptors [8]. With the recent availability of partial neurofilament protein sequences [9,10], we felt in a good position to follow up these earlier findings.

2. MATERIALS AND METHODS

Chicken desmin, porcine neurofilament M-protein, and fragments of M covering residues 1–102 (headpiece) and the carboxy-terminal tailpiece extension were isolated [9,10]. Rabbit antiserum to α-MSH was obtained from Immunonuclear Corp. (Stillwater, MN, USA). Initial studies used lot no.31261 as in [2]. As this serum was later not available we made use of lot no. 8410018. Competitive ELISA between immobilized M-protein, α-MSH antibody and various peptides was performed as described for another antigen-antibody system [11]. Western blotting was performed as in [12]. Peroxidase-conjugated second antibodies were used. α-MSH, desacetyl α-MSH, ACTH, $ACTH_{1-10}$, and the tetrapeptide S-Y-S-M were from Bachem (Bubendorf, Switzerland). Serine-tyrosine was obtained from

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Serva. Acetylation was performed with acetic anhydride using standard techniques. Acetylated peptides were purified by HPLC and characterized by amino acid composition and electrophoresis. Tobacco mosaic virus (TMV) coat protein and a rabbit antiserum known to react with the amino-terminal 10 residues of this protein [13] were kindly donated by Dr Van Regenmortel, CNRS, Strasbourg, France. Sequences are given using the one-letter system. Ac refers to the *N*-acetyl group.

3. RESULTS AND DISCUSSION

Fig.1 confirms the specific recognition of porcine neurofilament M-protein by anti-α-MSH serum reported by Dräger et al. [2]. Current sequence data on porcine M-protein do not cover the large carboxy-terminal CNBr fragment (tailpiece) although the amino-terminal 436 residues are known [9]. Western blots of cyanogen bromide-cleaved M-protein showed no reactivity of the large carboxy-terminal fragment or the fragment corresponding to residues 68–285 (fig.1). Strong decoration of an 8-kDa fragment indicated the amino-terminal fragment (residues 1–67) which was corroborated using a pure preparation of a proteolytic fragment covering residues 1–102 (headpiece). Having localized the α-MSH-like antigen to residues 1–67, a sequence comparison with α-MSH indicated only one candidate for a continuous epitope, i.e., the amino-terminal residues. These are Ac-S-Y-T-L-D- and Ac-S-Y-S-M-E- in M-protein and α-MSH, respectively (see table 1).

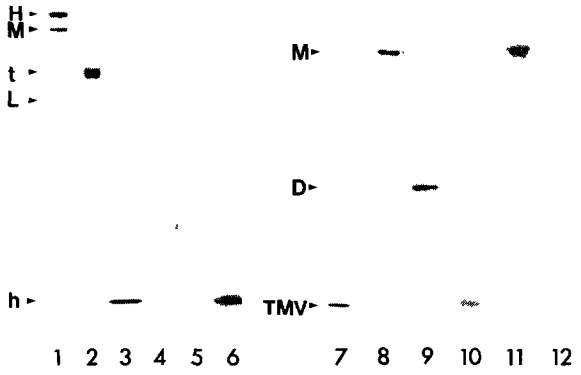

Fig.1. Lanes 1–6, 12.5% polyacrylamide gel (1–3) and corresponding Western blot (4–6) of purified neurofilament proteins (H, M and L), M-protein tail piece (t) and M-protein head piece (h) with α-MSH antibody. Both the intact protein (lanes 1 and 4) and the small amino-terminal moiety (lanes 3 and 6) are strongly stained. Lanes 7–12, 10% polyacrylamide gel (7–9) and corresponding Western blot (10–12) of TMV coat protein (TMV), neurofilament M-protein (M), and chicken desmin (D). Blot was stained with α-MSH antibody. α-MSH antibody recognizes M and TMV, but not chicken desmin.

A modified ELISA with carrier-bound porcine M-protein was used to test various peptides for antigen competition. α-MSH consists of the first 13 residues of ACTH but is acetylated at the amino terminus and amidated at the carboxyl end. The much lower reactivity of ACTH and desacetylated α-MSH in comparison to α-MSH implicated the acetylated end. The amino-terminal tetrapeptide S-Y-S-M displayed very poor competition under our assay conditions, whereas its *N*-acetyl derivative showed significant reaction (table 1). The *N*-acetylated dipeptide S-Y exhibited slight inhibition at very high concentrations, though S-Y alone showed no inhibition. Acetylated $ACTH_{1-10}$ inhibited about as strongly as α-MSH, whereas the non-acetylated form inhibited much more weakly. Both lots of α-MSH antibody we examined behaved in an almost identical manner in these experiments. A previous study made use of four different α-MSH antisera, all of which exhibited staining of non-pituitary structures [6], suggesting that many α-MSH antibodies may be able to recognize the M-protein.

Purified chicken desmin (fig.1, table 1) is not recognized, indicating a requirement for tyrosine at position 2. The combined results suggest that strong antibody binding requires acetyl-serine-tyrosine, followed by at least two and preferably more amino acids the identity of which can, at least within limits, be somewhat variable. The identification of the antigenic site on the M-protein explains the neuronal specificity of α-MSH staining seen in histological samples, since various intermediate filament [9] and other cytoskeletal proteins (see below) differ from M in their amino-terminal sequence [9].

We searched the National Biomedical Research Foundation protein sequence data bank for other proteins exhibiting an acetylated amino-terminal serine followed by a tyrosine and a serine or threonine. This requirement was fulfilled by TMV

Table 1

Summary of results from competitive ELISA and Western blotting experiments

Peptide used in competitive ELISA	Concentration required for 50% inhibition
S-Y	>333 μM[a]
Ac-S-Y	300 μM
S-Y-S-M ($ACTH_{1-4}$)	>100 μM[a]
Ac-S-Y-S-M (acetylated $ACTH_{1-4}$)	235 nM
S-Y-S-M-E-H-F-R-W-G-K-P-V-amide (desacetyl α-MSH)	60 μM
Ac-S-Y-S-M-E-H-F-R-W-G-K-P-V-amide (α-MSH)	380 pM

N-terminus of protein used in Western blotting experiments	Result of Western blotting experiment
Ac-S-Y-T-L-D-S-L-G-N-P-S-S-A... (M protein)	+ + +
Ac-S-Y-S-I-T-T-P-S-Q-F-V-F-L... (TMV coat protein)	+ + +
Ac-S-Q-S-Y-S-S-S-Q-R-V-S-S-Y... (chicken desmin)	–

[a] The peptide failed to reach 50% inhibition at the highest concentration employed

coat protein. The sequence Ac-S-Y-S-I-T- [14,15] shows good homology to α-MSH and M-protein for the first four residues. Western blotting (fig.1) revealed that TMV coat protein was also reactive with α-MSH antibodies. In addition, a rabbit antiserum known to recognize the amino-terminal 10 residues of coat protein [13] recognizes M-protein on Western blots, although very weakly (not shown).

The amino-terminal region of TMV coat protein belongs to the increasing list of linear or continuous epitopes thought to be prime antigenic targets because of their segmental mobility as revealed by X-ray crystallography [13,16]. Such sites are very often situated in loops and can also occur at the termini of different proteins [11,13,16]. Current concepts of intermediate filament structure based on biochemical and sequence considerations already suggest the presence of loops in the amino-terminal region of M-protein as in other intermediate filament proteins [9,10]. The crossreactivity of α-MSH antibodies documented here confirms the general principle of a linear epitope situated in a flexible loop at the amino-terminal end. Tolerable sequence variations in this region account for the crossreactivity of the α-MSH antibodies with proteins as divergent as α-MSH, neurofilament M-protein, and TMV coat protein. As the sequence relationship between α-MSH and neurofilament M-protein is lost past residue 5 (see table 1) we conclude that neurofilament proteins cannot serve as a precursor or storage form of α-MSH-like hormones. This should lay to rest previous speculations on extrapituitary α-MSH-like hormones in the nervous system [3–6] based solely on immunological crossreactivity.

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