Characterization of Epitopes Shared by α -Melanocyte-Stimulating Hormone (α -MSH) and the 150-kD Neurofilament Protein (NF150): Relationship to Neurotrophic Sequences

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Immunoblot techniques and immunohistochemistry have been used to investigate epitopes shared by α -melanocyte-stimulating hormone (α -MSH) and the 150-kD neurofilament protein (NF150). Three anti- α -MSH antisera (namely 31H2T, 4394, and M5) from a total of 12 sera were found to react with NF150. The three cross-reacting sera are different in their binding properties to peptide fragments related to α -MSH, suggesting that at least two distinct epitopes are shared by NF150 and α -MSH. The only known sequence common to NF150 and α -MSH, the N-terminal Ac-S-Y residues, appears to be essential for binding of sera 31H2T and 4394. However, the binding of antiserum M5 involves other, currently unknown, similarities between NF150 and α -MSH. This is shown by the binding of M5 to peptides such as ACTH(4-10), which do not contain the N-terminal Ac-S-Y sequence. Binding of M5 to tobacco mosaic virus coat protein (TMV-coat protein), which is homologous with α -MSH and NF150 in its Ac-S-Y residues, was negligible. The peptide structures that are recognized by M5 have previously been shown to exert neurotrophic activity. The data are discussed in the light of the hypothesis that similarity between NF150 and α -MSH, as illustrated by binding to M5, may be significant in the neurotrophic activity of MSH-related peptides.

Abbreviations used: ACTH, adrenocorticotrophic hormone; FITC, fluorescein isothiocyanate; LPH, lipotropic hormone; MSH, melanocyte-stimulating hormone; NF150, intermediate-sized neurofilament protein (MW approximately 150,000 Daltons); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; for amino acids the one-letter code is used as recommended by the IUPAC-IUB Joint Commission of Biochemical Nomenclature (JCBN) [1984].

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INTRODUCTION

A number of immunohistochemical studies have shown that certain antisera to α -melanocyte-stimulating hormone (α -MSH) label nerve processes and cell bodies throughout the central and peripheral nervous system [Swaab and Fisher, 1977; Watson and Akil, 1980; Trojanowski et al, 1985]. This pattern of labeling has been shown to be due to cross-reactivity of the serum with one of the neurofilament triplet proteins, with a molecular weight of approximately 150,000 daltons [Dräger et al, 1983; Shaw et al, 1985; Edwards and Gispen, 1985; Trojanowski et al, 1985]. Binding of the anti- α -MSH antisera to the 150,000-dalton neurofilament protein (NF150) can be removed by preabsorption of the serum by either α -MSH or neurofilament preparations [Dräger et al, 1983; Shaw et al, 1985; Trojanowski et al, 1985]. This indicates that the determinants on α -MSH are also present in NF150.

The ability of α -MSH and related peptides to facilitate nerve repair has been well documented [Bijlsma et al, 1983]. The mechanism by which α -MSH exerts a neurotrophic influence may depend on its similarity to certain regions of NF150 [Edwards et al, 1984]. If this connection is valid, then there should be some correlation between the sequences in α -MSH recognized by the NF150-reacting antibodies and the sequences required for the neurotrophic actions of α -MSH. Structure-activity studies on the ability of α -MSH-related peptides to enhance functional recovery following nerve injury have shown that smaller peptides containing the ACTH(4-10) amino acid sequence also show neurotrophic properties. Previous studies on a single MSH/NF150-reacting serum indicated that the major determinant recognized by these immunoglobulins is in the N-terminal region of NF150, which has been fully sequenced [Shaw et al, 1985]. Comparison of this amino acid sequence with α -MSH indicates homology between NF150 and α -MSH in their N-terminal regions. The similarity in amino acid sequences was lost beyond residue 5 [Shaw et al, 1985].

In order to investigate the similarity between α -MSH and NF150 further, we have examined the NF150 binding of a number of anti- α -MSH antisera. The binding of cross-reacting sera to various peptides related to α -MSH was tested to examine the degree of correlation with sequences known to be active in promoting nerve outgrowth. Binding to tobacco mosaic virus coat protein (TMV-coat protein), which contains only the Ac-S-Y residues common to NF150 and α -MSH, was also investigated.

MATERIALS AND METHODS

Materials

Acrylamide, bisacrylamide, and SDS were obtained from Serva (Heidelberg, Federal Republic of Germany). Nitrocellulose paper (pore size 0.2 μ m) was purchased from Schleicher and Schull (Dassel, Federal Republic of Germany). Peptides were a gift from Dr. H.M. Greven (Organon Int. BV, Oss, Netherlands). Goat anti-rabbit immunoglobulins (IgG's) and rat anti-mouse immunoglobulins conjugated to peroxidase were obtained from Nordic Immunological (Tilburg, Netherlands). Swine anti-rabbit IgG's conjugated to FITC was obtained from Dako (Santa Barbara, CA). Antisera 31H2T and Res 31R2 were purchased from Immuno Nuclear Co. (Stillwater,

OK). The other anti- α -MSH antisera were gifts: 786, 796, and 118 from Dr. A.N. Eberle (Kantonspital, Basel), 4394 (bleeding 23/4) from Dr. D.F. Swaab (Brain Institute, Amsterdam), and M1, M2, M4, M5, M6, and 24 from Dr. A.A. de Rotte (Academic Hospital, Utrecht). The anti-NF150 monoclonal antibody BF10 was a gift of Dr. B.H. Anderson (St. George Hospital, Medical School, London). TMV-coat protein was a gift of Dr. L.C. van Loon (Department of Plant Physiology, Wageningen, Netherlands).

Animals and Surgery

Female rats (120–140 g) of an inbred Wistar strain (TNO, Zeist, Netherlands) were used in all experiments. Crush lesion of the sciatic nerve and marking of the position of the crush was performed as described by Bijlsma et al [1983].

Neurofilament Preparation

Fractions enriched in neurofilaments were prepared from rat spinal cords by a method described by Chiu et al [1980]. About 1.5 g of spinal cord tissue was homogenized by 10 up-and-down strokes of a Potter-type homogenizer (clearance 0.125 mm) in a 50-mM Tris-HCl buffer, pH 6.8, containing 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Triton X-100. The homogenate was centrifuged at 12,000g for 10 min at 4°C. The pellet was homogenized in 30 ml of homogenization buffer containing 30% sucrose and was similarly centrifuged. The pellet, containing cytoskeletal material, was homogenized in 625 μ l double-distilled water and dialyzed overnight against 2 liters of double-distilled water. The dialyzed cytoskeletal proteins were lyophilized and stored at -20°C .

Preparation of Nerve Homogenates and SDS-PAGE

Two sciatic nerves (combined length about 6 cm) were minced in 1 ml ice-cold Tris-HCl buffer (10 mM, pH 7.4). The tissue and buffer were transferred to a Potter-type homogenizer (clearance 0.125 mm) and homogenized by 10 up-and-down strokes. Immediately after homogenization, a sample mix was added to the homogenate to give (final concentrations): Tris-HCl 62.5 mM, pH 6.8; SDS, 1%; glycerol, 10%; bromophenol blue, 0.001%; 2-mercaptoethanol, 5%. Samples of sciatic nerve homogenate or cytoskeletal proteins in sample mix were heated at 60°C prior to electrophoresis in SDS-PAGE slab gels (8% acrylamide; 0.2% bisacrylamide) as described by Zwiers et al [1976]. The final gels were stained with fast green.

Immunoblot Procedures

Transfer of protein from SDS slab gels to nitrocellulose paper was undertaken according to the method of Towbin et al [1979]. Protein transfer was conducted for 2 hr with an average current and voltage of 0.5 mA and 35 V. Nitrocellulose papers with transferred proteins (immunoblots) were rinsed for 30 min in Tris-buffered saline (TBS), pH 7.4. Immunoblots were incubated for 2 hr at 20°C with the anti- α -MSH antisera or monoclonal antibody to the 150-kD neurofilament protein in TBS containing 0.1% Tween-20 and 0.2% gelatin. Subsequently, the nitrocellulose papers were rinsed in TBS containing 0.1% Tween-20 and incubated for another 2 hr with labeled second antibody (peroxidase-labeled goat anti-rabbit or rabbit anti-mouse

IgG). Blots were washed again in TBS 0.1% Tween-20 and the peroxidase was detected by the staining method of Buckel and Zehelein [1981].

Absorption of Antisera

Absorptions of anti- α -MSH antisera were carried out at 4°C overnight. One microliter of antiserum was diluted 1/10 with water and incubated with 1.2 mmol or 6 mmol MSH or an equimolar amount of one of the other peptides.

Immunocytochemical Procedures

The sciatic nerve was carefully dissected, stretched, and tied on a board to prevent shrinkage during fixation. The tied nerve was fixed in periodate lysine paraformaldehyde (2.0% paraformaldehyde, 0.75 M lysine, 0.214% periodate) for 2.5 hr. Subsequently, the nerve was cryoprotected by immersion in graded (7.5–15–25–35%) sucrose in 50 mM phosphate buffer, pH 7.3. Transverse 4- μ m-thick cryostat sections were exposed to anti- α -MSH antiserum for 24 hr at room temperature. Following the incubation with anti- α -MSH antiserum the sections were rinsed with PBS containing 0.2% Triton X-100. The site of antigen-antibody reaction was demonstrated with swine anti-rabbit IgG conjugated to FITC (1:100). The first and second antisera were diluted in PBS containing 0.2% BSA and 0.2% Triton X-100. Sections were mounted in 90% glycerol in veronal-buffered saline, containing 0.1% paraphenylene diamine.

Determination of Protein

Quantitative protein determinations were carried out by the method of Lowry et al [1951] with bovine serum albumin as a standard.

RESULTS

Twelve anti- α -MSH sera from four different laboratories were tested for their ability to recognize NF150 in cryostat sections of rat sciatic nerve and immunoblots of sciatic nerve proteins. In addition to the commercial antiserum 31H2T used in the studies of Dräger et al [1983], Shaw et al [1985] and Trojanowski et al [1985], two other anti- α -MSH antisera, M5 and 4394, stained a protein band with an apparent molecular weight of 150,000 daltons (Fig. 1). None of the other antisera showed binding to a band at the level of 150,000 daltons. The monoclonal antibody BF10, which selectively stains the NF150 protein [Anderton et al, 1982], stained a band at the same height as that stained by 31H2T, M5, and 4394. Staining of this band with BF10, 31H2T, M5, or 4394 could not be detected in blots prepared from degenerating nerves in which the neurofilaments have been degraded (Fig. 1). The anti- α -MSH sera M5 and 4394, which stain the 150,000-dalton protein, also labeled the axoplasm of sciatic nerve fibers (Fig. 2). The serum M2, which did not bind to the protein band, also showed no specific axoplasmic binding.

Preabsorption of antisera M5, 4394, and 31H2T with either α -MSH or a cytoskeletal preparation completely removed the immunolabeling of the axoplasm (shown for M5 in Fig. 2) and from the 150,000-dalton band on immunoblots (Fig. 3, Table I).

Fig. 1. A. Comparison of the ability of anti- α -MSH antisera to stain NF150 on immunoblots of peripheral nerve protein. All sera were used in a dilution of 1/1,000 except 31H2T (1/4,000) and 4394 (1/10,000). Immunostaining of NF150 with BF10, an anti-NF150 monoclonal antibody, is shown on the right-hand track. B. Demonstration of the absence of immunostaining of NF150 in blots of proteins prepared from degenerating nerves 6 days after a crush lesion. The monoclonal antibody, BF10, and the three NF150-binding anti- α -MSH sera were used.

The portion of the α -MSH molecule that is recognized by the NF150 cross-reacting antibodies was examined by studying the staining of NF150 in immunoblots following preabsorption of the antisera with various peptides related to α -MSH. As is shown in Figure 3 and Table I, the antisera clearly differed in their binding properties to the peptides tested. α -MSH, ACTH(1-39), ACTH(1-24), ACTH(1-16) NH_2 , ACTH(1-13) NH_2 and ACTH(1-10) bound to 31H2T and M5 and prevented staining of the 150-kdalton band on immunoblots completely. Peptides structurally unrelated to α -MSH (vasopressin and β -endorphin) or with a marginal C-terminal homology [ACTH(11-24)] were ineffective. None of the other structurally related peptides were able to block immunostaining of NF150 with antiserum 31H2T. In contrast to this, preabsorption of M5 with ACTH(4-10), ACTH(6-10), and ACTH(5-13) NH_2 resulted in diminished staining of NF150 on immunoblots. Absorption of M5 with β -LPH(38-59) blocked immunolabeling of NF150 completely. Samples of ACTH(4-10) and ACTH(6-10) used in these experiments were reanalyzed by HPLC

TABLE I. Summary of Results of the Absorption of Antisera 31H2T and M5 Cross-Reacting With NF150*

Peptide preabsorption	Antiserum ^a	
	31H2T	M5
α -MSH (Ac-ACTH(1-13)-NH ₂)	+	+
ACTH (1-39)	+	+
ACTH (1-24)	+	+
ACTH (1-16)-NH ₂	+	+
ACTH (1-13)-NH ₂	+	+
ACTH (1-10)	+	+
ACTH (5-13)-NH ₂	—	±
ACTH (4-10)	—	±
ACTH (6-10)	—	±
ACTH (1-4)	—	—
ACTH (11-24)	—	—
ACTH (7-16)-NH	—	—
β -LPH (38-59)	—	+
γ^2 -LPH	—	—
β -Endorphin	—	—
Vasopressin	—	—
Cytoskeletal preparation	+	+

*The sera were preabsorbed with α -MSH (1.2 mM) or equimolar amounts of one of the other peptides or with 500 μ g cytoskeletal proteins as described in Experimental Procedures, and were tested for their ability to bind to NF150 on an immunoblot of peripheral nerve homogenate. The dilutions of 31H2T and M5 used during incubation with the blot were 1:4,000 and 1:1,000, respectively.

^aSymbols: +, binds to antibody, no staining of NF150 on immunoblot; ±, binds to antibody, diminished staining of NF150 on immunoblot; —, binds not to antibody, staining of NF150 on immunoblot.

and contained no contamination with α -MSH or its N-terminal fragments (J. Van Nispen, personal communication).

The antisera, 31H2T, M5, and 4394 were compared for their ability to recognize TMV-coat protein (for structure homology between TMV-coat protein, α -MSH, and NF150, see Fig. 5). Antisera 31H2T and 4394 clearly recognized this protein on immunoblots. Furthermore, immunostaining of NF150 could be blocked completely by preabsorption with TMV-coat protein (Fig. 4). Antiserum M5 hardly recognized TMV-coat protein on immunoblots and immunolabeling of NF150 could be diminished but not completely removed when M5 was preabsorbed with TMV-coat protein.

Fig. 2. Transverse cryostat sections of sciatic nerve incubated with antiserum M5, 1:50 (A); antiserum 4394, 1:400 (B); antiserum M2, 1:50 (C); antiserum M5 absorbed with α -MSH, 1.2 mM (D); and antiserum M5 absorbed with cytoskeletal proteins, 500 μ g (E). Note the clear axoplasmic labeling (arrowhead) obtained with M5 and 4394 and the absence of staining with M2 and with M5 preabsorbed with α -MSH or cytoskeletal proteins. Autofluorescence of Schwann cell nuclei (arrow in A) can be seen in all the sections.

Fig. 3. Examples of immunoblots of peripheral nerve proteins incubated with anti- α -MSH antiserum 31H2T (1/4,000, upper panel) and antiserum M5 (1/1000, lower panel) preabsorbed with α -MSH (1.2 mM) and equimolar amounts of several other peptides in accordance with the conditions described in Materials and Methods. Preabsorption of serum 31H2T with ACTH(11-24) is not shown, since this peptide was not tested in this experiment.

Fig. 4. Comparison of the ability of NF150 cross-reacting antisera (31H2T, 4394, M5) to recognize TMV-coat protein. The left panel shows immunoblots of purified TMV-coat protein (2 μ g TMV-coat protein per track). Note the absence of immunolabeling of TMV-coat protein with antiserum M5. The middle panel shows immunolabeling of NF150 on blots of peripheral nerve proteins incubated with the three anti- α -MSH antisera. The effect of preabsorption with TMV-coat protein is shown in the right panel. Note the absence of staining in tracks incubated with 31H2T and 4394, whereas staining with M5 is hardly affected by the preabsorption.

DISCUSSION

A number of investigators have reported that a commercial antiserum to α -MSH (31H2T) recognizes NF150 [Dräger et al, 1983; Edwards and Gispén, 1985; Shaw et al, 1985; Trojanowsky et al, 1985]. In this article we have examined the similarity between α -MSH and NF150 in more detail, using immunochemical and immunocytochemical techniques. The results confirm the cross-reactivity of the commercial α -MSH antiserum 31H2T with a 150,000-dalton protein band [Dräger et al, 1983] and show that only a minority of anti- α -MSH antisera (31H2T, M5, 4394) recognize this band on immunoblots of peripheral nerve homogenates (Fig. 1A). The monoclonal antibody BF10, which selectively stains NF150 [Anderton et al, 1982] labeled a protein band at exactly the same height as the band stained with the three anti- α -MSH antisera. Furthermore, the 150,000-dalton band could not be detected in blots pre-

		1	2	3	4	5	6	7	8	9	10	11	12	13	
α -MSH	Ac	S	Y	S	M	E	H	F	R	W	G	K	P	V	NH ₂
ACTH		S	Y	S	M	E	H	F	R	W	G	K	P	V	...
β -LPH	...	P	Y	R	M	E	H	F	R	W	G	S	P	P	...
γ^2 -LPH			Y	V	M	G	H	F	R	W	D	R	F	G	
TMV	Ac	S	Y	S	I	T	T	P	S	Q	F	V	F	L	...
NF150	Ac	S	Y	T	L	D	S	L	G	N	P	S	S	A	... 436 X X

Fig. 5. Sequence homology between melanocortins, TMV-coat protein, and NF150. X indicates unknown sequences.

pared from homogenates of degenerating nerve with either the anti- α -MSH antisera or BF10 (Fig. 1B). This is further evidence that the 150,000-dalton band labeled with the three anti- α -MSH antisera is NF150, since complete breakdown of the neurofilament triplet proteins following a nerve crush is a well-documented property of these proteins [Schlaepfer et al, 1985]. The observation that many anti- α -MSH antisera do not bind to NF150 suggests that the total MSH amino acid sequence is probably not present in NF150.

Preabsorption of the three cross-reacting antisera with α -MSH or with a cytoskeletal preparation completely blocked immunolabeling of the axoplasm in sciatic nerve sections (shown in Fig. 2 for M5) and of NF150 on immunoblot (Fig. 3), indicating that all three antisera contain antibodies that recognize determinants common to α -MSH and NF150. Absorption by α -MSH was specific, since unrelated peptides were ineffective (Fig. 2, Table I). The immunolabeling of NF150 with 31H2T was not blocked by ACTH(4–10) or by β -LPH(38–59), which is homologous with α -MSH in the (2–10) region, suggesting that the amino acids in the N-terminus of α -MSH are essential for binding to the NF150 cross-reacting antibodies in serum 31H2T. However, ACTH(1–4) (up to a concentration of 6 mM) did not bind to NF150 cross-reacting antibodies, whereas TMV-coat protein, which is homologous in the N-acetylated terminal (1–3) amino acids, and ACTH(1–10) could completely block immunolabeling of NF150. This indicated that both acetylation of the N-terminal serine and C-terminal extension of ACTH(1–4) could enhance the binding of NF150 cross-reacting antibodies to the peptide.

These results are in agreement with those described by Shaw et al [1985]. These authors employed a competitive ELISA with carrier-bound NF150 to test various peptides for antigen competition. α -MSH displayed the best competition, followed by Ac-ACTH(1–4) and ACTH(1–13)NH₂. ACTH(1–4) failed to reach 50% inhibition of binding of NF150 cross-reacting antibodies to NF150 at the highest concentration (100 μ M) tested. Antiserum 4394 possessed characteristics very similar to those of 31H2T, since the 4394/NF150 cross-reacting IgG's did bind to TMV-coat protein (Fig. 4) and did not bind to ACTH(4–10) and β -LPH(38–59) (data not shown).

Antiserum M5, however, differs from 31H2T and 4394 in its binding characteristics, indicating that regions other than the extreme N-terminal of α -MSH and NF150 are involved. First ACTH(4–10), ACTH(6–10), and ACTH(5–13)NH₂ are able to bind NF150 cross-reacting IgG's, since preabsorption with these peptides results in

diminished staining of NF150 (Fig. 3, Table I). Second, binding of M5 to TMV-coat protein is negligible (Fig. 4).

The amino terminal 436 residues of NF150 are known [Geisler et al, 1984]. Studies on the binding of 31H2T to breakdown fragments of NF150 indicated that binding only occurred with N-terminal portions of NF150 [Shaw et al, 1985]. The sequence homology between these fragments and α -MSH is lost after residue 5. However, the extent of the similarity between NF150 and α -MSH is clearly more extensive than suggested by Shaw et al [1985]. The results presented in this article show that the presence of the Ac-S-Y sequence is not a prerequisite for binding of NF150 cross-reacting IgG's present in the anti- α -MSH serum M5. Amino acids in the (4-10) region of MSH also play a key role in the binding potential of these IgG's. The known residues of NF150 do not contain a continuous epitope that is homologous to a sequence on ACTH(4-10). We are currently investigating the binding of M5 to CNBr cleavage fragments of NF150 to determine whether the common determinant is present in unknown sequences in NF150 or whether a discontinuous epitope in the N-terminal region must be considered.

Binding of peptides to IgG's and to protein receptor molecules are analogous processes. Little is known about the characteristics of the putative receptor involved in the neurotrophic actions of MSH apart from what can be deduced from structure-activity studies in whole animals following systemic administration of peptides. The correlation between the structures required for binding to cross-reacting IgG's and neurotrophic activity of α -MSH peptides is quite marked, particularly for the M5 serum. While α -MSH is the most effective peptide in both cases, shorter fragments containing the (4-10) sequences are also active [this article; Bijlsma et al, 1983]. Furthermore, the IgG-binding peptide β -LPH(38-59) is also active in accelerating recovery from peripheral nerve damage (Van der Zee, manuscript in preparation).

The studies described here show that, while NF150 does not contain the whole of the MSH sequence and thus cannot serve as a precursor for the circulating hormone, it clearly does contain regions that bind to IgG's that also recognize MSH-related peptides with neurotrophic activity. If IgG's recognize both α -MSH and NF150, other proteins including the putative MSH receptor may also do so. Thus the data are in support of the hypothesis [Edwards et al, 1984] that the neurotrophic action of injected MSH may be related to its ability to mimic the actions of peptide fragments of NF150.

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