

# Chick embryo spinal cord neurons synthesize a transferrin-like myotrophic protein

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Highly enriched cultures of chick embryo spinal cord neurons synthesize and secrete a protein which is immunoprecipitable by anti-ovotransferrin. Ovotransferrin, an iron-binding glycoprotein of  $M_r$  80000, is also shown to stimulate in vitro myogenesis of cultured chick embryo myotubes as measured by saturable dose-dependent increase in acetylcholine receptors. This effect is probably dependent on ovotransferrin's ability to donate iron to the cells. In many respects ovotransferrin is similar to 'sciatin', a myotrophic protein isolated from chicken sciatic nerves [Proc. Natl. Acad. Sci. USA (1980) 77, 6922–6925].

*Muscle development*

*Acetylcholine receptor*

*Cell culture*

*Sciatin*

*Transferrin*

## 1. INTRODUCTION

A glycoprotein from adult chicken sciatic nerves which is necessary for the development and maintenance of myotubes cultured in the absence of chicken embryo extract has been purified to homogeneity from chicken sciatic nerves [1,2]. This glycoprotein ( $M_r$  80000) has been given the name 'sciatin'. We have purified sciatin according to [2]. In characterizing it we have demonstrated that it behaves very similarly, quite possibly identically, to the well-characterized iron-binding protein of egg white, ovotransferrin. We describe results which indicate that a transferrin-like protein is synthesized and secreted by cell cultures highly enriched in embryonic spinal cord neurons.

## 2. MATERIALS AND METHODS

Iron-free and iron-saturated ovotransferrin were purchased from Sigma. Rabbit mono-specific anti-ovotransferrin serum was the generous gift of Dr Richard Palmiter (University of Washington).

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Muscle cell cultures were prepared from pectoralis muscles of 10–12-day-old embryos as in [3]. Spinal cord nerve cell cultures were prepared from 6- or 7-day-old embryos as in [4]. SDS gel electrophoresis was performed as in [5] on 10% SDS–acrylamide slab gels. The gels were then stained with Coomassie blue, destained, treated with Enhance (New England Nuclear) and dried. Fluorography at  $-70^\circ\text{C}$  was performed.

## 3. RESULTS

To demonstrate the neuronal synthesis and secretion of the transferrin-like protein we grew cultures highly enriched in spinal cord neurons. The cultures were totally devoid of flattened cells, although small numbers of small birefringent cells, probably Schwann cells, were attached to neuronal processes.

The media and cell layers from cultures labelled for either 2 h or 48 h with [ $^{35}\text{S}$ ]methionine were separately collected. Fig. 1A shows an autoradiogram of a SDS gel containing the medium and cell material from 48 h labelled cells. A polypeptide having the mobility of transferrin

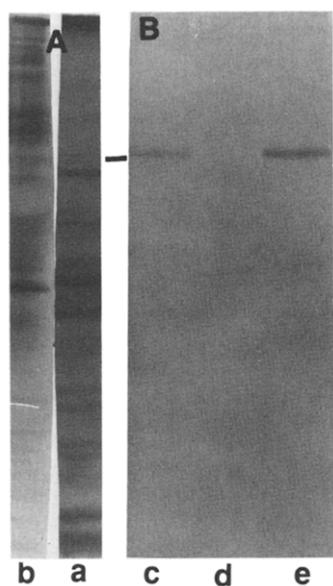


Fig.1. Sciatin is synthesized and secreted by cultured embryonic spinal cord neurons. (A) Fluorograph of SDS gel of the medium and cell layer from spinal cord neuronal cells labelled for 48 h with [ $^{35}$ S]methionine. Cell layer and medium were prepared from 9-day-old cultures. Samples were treated with 10% trichloroacetic acid and the precipitated proteins were collected by centrifugation and redissolved in SDS sample buffer. Of each sample 100000 cpm were applied to the gel: (a) cell homogenate; (b) medium. Fluorograph was for 24 h. (B) Fluorographs of SDS gels containing immune and non-immune precipitates of [ $^{35}$ S]methionine-labelled cell extracts and medium. Immune and non-immune precipitates were obtained by two-step immune precipitation [12] from cell extracts (250000 cpm of trichloroacetic acid precipitate) and medium (100000 cpm of trichloroacetic acid precipitate) obtained from [ $^{35}$ S]methionine-labelled spinal cord neuronal cultures. The precipitates were dissolved in SDS sample buffer and subjected to SDS gel electrophoresis and fluorography: (c) immune precipitate of the cell extract; (d) non-immune precipitate of the cell extract; (e) the immune precipitate of the medium. Fluorography was for 3 days. The bar indicates the mobility of sciatin.

was found in both cells and media. To more definitively identify the protein as transferrin-like we subjected aliquots of [ $^{35}$ S]methionine labelled neurons and medium to two-stage immune precipitation with anti-ovotransferrin. A radioactive polypeptide with the mobility of ovotransfer-

rin was specifically precipitated by anti-ovotransferrin from both cells and medium from cultures which had been pulsed for 48 h (fig.1B).

We have quantitated the amount and percentage of transferrin-like protein synthesized by neuronal cultures labelled for either 2 or 48 h. We found an appreciable amount of this protein in cells pulsed for 2 h while none was found in the medium (table 1). By 48 h a similar amount is found in the cells while 3 times as much is now found in the medium. This result together with the gel patterns in fig.1A, which indicate very different distributions of newly synthesized polypeptides in cells and medium from 48 h labelled cultures, strongly suggest that transferrin-like protein is synthesized in the neuronal cells and is secreted into the medium.

We grew cell cultures depleted in neurons. These flattened cells which appeared fibroblastic produced no detectable transferrin-like protein (table 1).

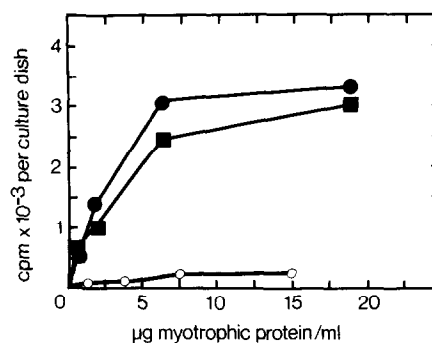


Fig.2. The effects of ovotransferrin and sciatin on acetylcholine receptor number. Myogenic cells were grown in 35 mm tissue culture dishes for one day in minimal Eagle's medium (MEM) containing 10% horse serum (HS), 2.5% chick embryo extract and then maintained in MEM containing 10% HS, or in this medium supplemented with various amounts of either iron-saturated ovotransferrin or sciatin or iron-free ovotransferrin or sciatin. Six-day-old muscle cell cultures were assayed for acetylcholine receptors [8], using 0.005  $\mu$ g/ml  $^{125}$ I-labelled  $\alpha$ -bungarotoxin ( $\alpha$ -BGT) (New England Nuclear; 17 Ci/mmol). Data expressed as cpm/culture dish represent the averages of triplicate determinations of  $^{125}$ I-labelled  $\alpha$ -BGT binding activity. Non-specific binding (12%) was determined by preincubating some cultures with 200  $\mu$ M tubocurarine chloride. Specific binding (cpm/culture dish) is the difference between total binding and non-specific binding: (●) ovotransferrin +  $\text{Fe}^{3+}$ ; (■) sciatin +  $\text{Fe}^{3+}$ ; (○) ovotransferrin or sciatin, iron free.

Table 1

The amount of transferrin-like protein synthesized by cultures of spinal cord neurons or non-neuronal cells (fibroblasts)

Cell type	Labelling time (h)	Trichloroacetic acid -precipitated cpm ( $\times 10^{-3}$ )	Transferrin-like protein (cpm)	% Total cpm in transferrin-like protein
Neuronal cell	2	1 536 000	3225	0.21
Neuronal medium	2 <sup>a</sup>	128 000	0	0
Neuronal cell	48	6 257 000	10 112	0.16
Neuronal medium	48	2 760 000	33 120	1.2
Fibroblast cell	48	7 632 000	0	0
Fibroblast medium	48	3 465 000	0	0

<sup>a</sup> The total medium (5 ml) was lyophilized and then redissolved in 0.5 ml IP buffer

Aliquots of [<sup>35</sup>S]methionine labelled cell extracts and media containing 200 000 cpm trichloroacetic acid-precipitable material were immune precipitated with anti-ovotransferrin serum as in [12]. The resulting precipitates were dissolved in SDS sample buffer. 3  $\mu$ g ovotransferrin was added to each sample and SDS gel electrophoresis performed. After Coomassie blue staining and destaining, the ovotransferrin bands, as well as bands in the two adjacent portions of each gel lane to serve as background controls, were removed from the gel, dissolved in 0.25 ml 30% H<sub>2</sub>O<sub>2</sub>. After air drying at 60°C the samples were dissolved in ACS (Amersham) and counted by scintillation counting. The cpm in adjacent bands were averaged and subtracted from the counts in the ovotransferrin band. The results shown here are the averages of 3 independent determinations. The SEM in each case was < 10%

We compared sciatin and ovotransferrin and determined that the two proteins have identical electrophoretic mobility in SDS gels and that both proteins migrate more rapidly on SDS gels when run under non-reducing conditions, presumably due to the large number of intramolecular disulfide bridges.

A band of identity is obtained when sciatin and ovotransferrin are tested against anti-ovotransferrin in an Ouchterlony immunodiffusion assay (unpublished).

Since the demonstrated function of ovotransferrin is to bind and transport iron, we compared the iron-binding capacity of both proteins as in [6]. Both proteins bound similar amounts of iron:  $E_{1\text{ cm}}^{1\%}$  ovotransferrin = 5.78;  $E_{1\text{ cm}}^{1\%}$  sciatin = 6.00 at 465 nm.

A profound effect of sciatin was reported on chick myotube development in culture [7]. Therefore, we utilized a sensitive quantitative bioassay for the number of acetylcholine receptors (AChR) [8], to compare the effects of increasing amounts of iron-saturated ovotransferrin and sciatin on myotube development. Fig.2 shows the results of this assay. Both proteins gave a very similar concentration-dependent increase in

AChR. This figure also shows that iron-free ovotransferrin or sciatin was much less effective. We also monitored the cultures by phase microscopy. Cultures maintained with either iron-saturated protein contained similar numbers of striated, actively contracting myotubes (unpublished).

#### 4. DISCUSSION

We have demonstrated that transferrin-like protein is synthesized and secreted by spinal cord neurons and also serves as a myotrophic factor for in vitro myogenesis. Our results also provide evidence that sciatin, the myotrophic protein originally isolated and characterized in [1,2] is structurally and functionally very similar, if not identical, to ovotransferrin. In [9] structural similarity was reported between the two proteins. A myotrophic factor in chick serum has also been identified as transferrin [10,11]. It is probable that the observed ability of these proteins to stimulate myotube differentiation in vitro is a result of their iron-binding and iron-transporting activity. Since myotubes contain a large amount of myoglobin it is not surprising they have a special need for iron.

Thus, a transferrin-like protein may play an important role in skeletal muscle maintenance in vivo as well.

Investigators working with cultured myotubes no longer need to supplement their medium with either the totally undefined embryo extract or with sciatin. Ovotransferrin is quite inexpensive, can be purchased in a relatively pure form and appears to be just as effective as sciatin in inducing myotube differentiation.

#### ACKNOWLEDGEMENT

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