The identification of neurotrophic factor as a transferrin

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Received 7 April 1983

Partially purified neurotrophic factor (NTF) from chicken nerves comigrated with transferrin and a component in several preparations known to have neurotrophic effects on cultured skeletal muscle cells. One-dimensional gel electrophoretograms of proteolytic fragments of NTF and fragments obtained from transferrins purified from chicken eggs, serum and embryos were indistinguishable. These purified transferrins, like NTF, all stimulated the incorporation of [^{3}H]thymidine and supported myotube formation to a similar degree as NTF. These studies suggest that NTF is a transferrin-like protein and that both transferrins and NTF act by initially promoting myoblast proliferation and subsequently supporting myogenesis in chick muscle cultures.

Neurotrophic factor *Transferrin* *Myogenesis* *Peptide fragment* *[^{3}H]Thymidine incorporation* *Growth factor*

1. INTRODUCTION

Neurotrophic hormones or factors that promote growth or differentiation of chick muscle cells in vitro have been isolated and characterized in several laboratories [1–9]. These factors were purified by different biochemical methods based on their biological activities. Neurotrophic factor (NTF, [1–3]) was purified from ischiatic-peroneal nerves and assayed by its stimulation of [^{3}H]thymidine incorporation by myoblasts and growth of myogenic colonies at clonal cell densities. Muscle trophic factor (MTF, [7–9]) was isolated from chicken serum, and was found to cause an increase in number of muscle cell nuclei and creatine kinase activity. Another nerve-derived protein, sciatin was purified by following its ability to enhance protein synthesis and morphologic maturation in primary chick muscle cell cultures [4–6].

We have found that NTF has some properties characteristic of transferrins; both NTF and transferrin have reported M_r 78000 ± 2000 [2,3,10]; are salmon colored in concentrated solutions, are relatively heat stable – requiring 80°C for 30 min at pH 8.0 to denature [1,10]; and both precipitate between 50–85% ammonium sulfate and elute from DEAE–Sephadex ion-exchange columns at about 100 mM salt at pH 8.0 [2,10]. In addition as NTF is a requirement for chick muscle cells [1–3], transferrin is a requirement for growth in vitro for a number of mammalian cell lines [11,12] as well as for some primary chick cells [13]. Because of these similarities, we decided to compare NTF and chicken transferrins by limited peptide-fragment analysis in the presence of sodium dodecyl sulfate (SDS), a method which allows us to determine the extent of homology in their primary structures [14]. We have found striking biochemical and biological similarities among three chicken transferrins and NTF, from which we conclude that these are probably the same or very similar proteins, all capable of promoting proliferative activity of chick muscle cells in vitro.

2. MATERIALS AND METHODS

2.1. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was performed as in [15], using the discontinuous buffer system

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00145793/83/$3.00

of Laemmli [16]. Gels contained 7% or 15% total acrylamide. Gels were stained by the simplified silver staining method [17], except that bands to be cut out were stained 1 min in 0.1% Coomassie blue R-250 (Sigma), and then rapidly destained in alcohols.

2.2. *Cell culture and [^{3}H]thymidine incorporation assay*

Dissociated thigh muscle cells (5×10^4 cells/35 mm dish) from 11 day old, specific pathogen free chicken embryos (Larson Labs) were prepared and grown in Ham's F-12, containing bovine serum albumin (10 μg/ml, Sigma), porcine insulin (1 μg/ml, Sigma), and human transferrin (1 μg/ml, Research Plus Labs.) and 20% horse serum (Gibco) as in [3]. After a 6 h attachment period, the medium was changed to 5% horse serum containing [^{3}H]thymidine (0.5 μCi/ml, New England Nuclear) and the purified proteins (20 μg/ml). After incubation for 48 h, radioactivity incorporated into 10% trichloroacetic acid-insoluble macromolecules was determined as in [2].

2.3. *Purification of transferrins*

Transferrins were purified from chicken serum (Gibco), eggs and chicken embryo extract by a modification of methods described for pig [18] and human serum transferrins [19]. Proteins were fractionated with 60–85% ammonium sulfate, 0.3% rivanol (4-amino-dipyridyl-acridine lactate, Winthrop Labs.) and separated on Whatman DE-52, employing a gradient of Tris from 50–200 mM at pH 8.0. All steps were performed in the presence of 1 μM $FeCl_3$ and 1 mM $NaHCO_3$. Pooled column fractions were dialyzed against 10 mM ammonium bicarbonate, 1 nM $FeCl_3$ and concen-

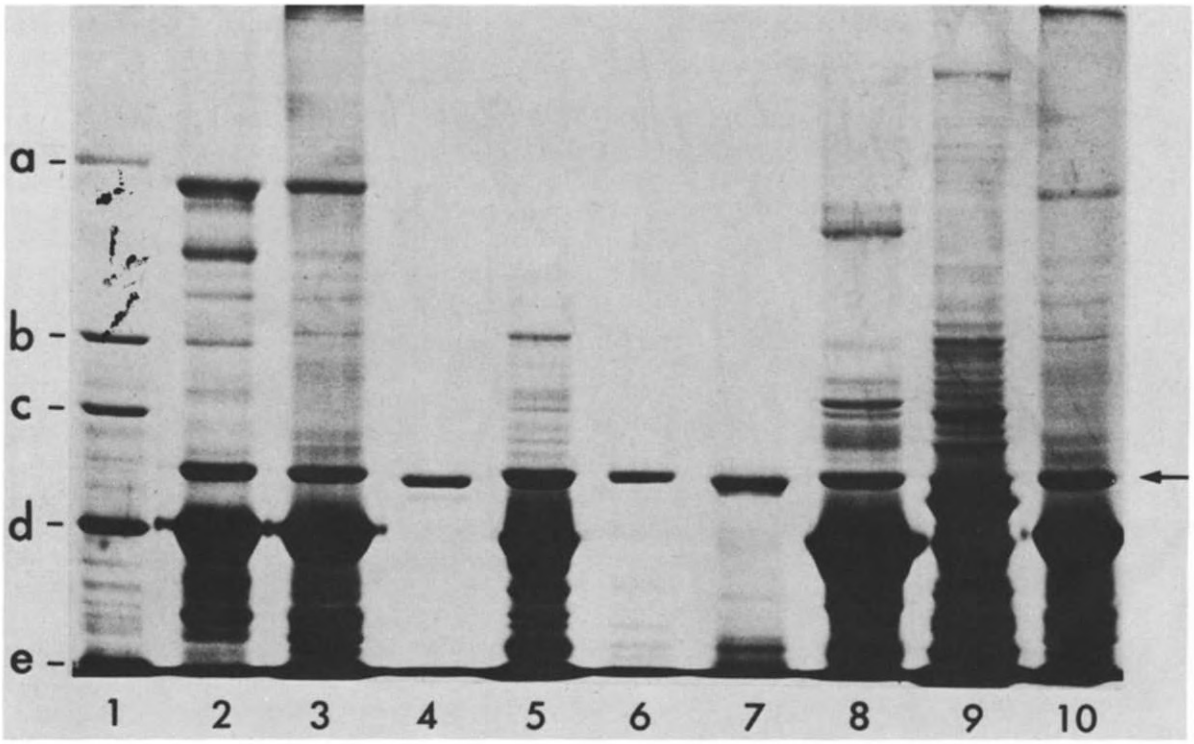

Fig.1. SDS–polyacrylamide gel electrophoresis of fractions which possess neurotrophic activity and purified transferrins. Polyacrylamide gel electrophoresis was performed as in section 2. The gel contained 7% total acrylamide. Lane: (1) M_r markers – (a) myosin, (b) β-galactosidase, (c) phosphorylase B, (d) bovine serum albumin, (e) ovalbumin; (2) rat serum, 40 μg; (3) rat sciatic nerve extract, 42 μg; (4) human transferrin, 1.5 μg; (5) chicken ischiatic-peroneal nerve extract, 37 μg; (6) chicken neurotrophic factor (NTF), 5 μg; (7) chicken ovotransferrin, 9 μg; (8) chicken serum, 75 μg; (9) embryo extract, 60 μg; (10) rat sciatic nerve extract, 42 μg.

trated using an Amicon YM 30 membrane. Neurotrophic factor was purified as in [3].

2.4. *Analysis of peptide fragments*

Peptide fragments obtained after limited digestion of proteins in gel slices in the presence of SDS were analyzed as in [14]. Purified proteins (25 μg) were electrophoresed on 0.75 mm thick, 7% polyacrylamide gels and briefly stained and destained as above. The transferrin bands were cut out, and electrophoresed on 1.0 mm thick 15% gels, in the presence of 1.0 μg *Staphylococcus aureus* V8 protease (Miles) or chymotrypsin (Sigma).

3. RESULTS

3.1. *Electrophoretic comparison of neurotrophic extracts*

We initially compared the electrophoretic mobility of proteins in a number of preparations reported to enhance myogenesis in vitro on SDS–polyacrylamide gels. These preparations all contained a major polypeptide band at M_r 78000 ± 2000 (arrow, fig.1) that closely comigrates with commercially available human transferrin and ovotransferrin (Sigma). Chicken ischiatic-peroneal nerve extract, chicken serum and chicken embryo extract each have a component of the same M_r as transferrin. In the purified neurotrophic factor (NTF), which has 10-times the biological specific activity of crude nerve extract [2,3], this M_r 78000 component becomes the predominant polypeptide. In addition, both rat serum and a trophic extract from rat sciatic nerves [20] also contains a distinct polypeptide of this M_r-value.

3.2. *Comparison of chicken transferrins and NTF*

Since NTF had been purified using a chick muscle culture assay system, we purified transferrins from chicken serum, eggs and embryo extract, as in section 2. All chicken transferrins comigrated with each other and NTF on SDS gels (fig.2A). To compare the structures of these proteins more critically, we subjected the purified proteins to limited proteolytic digestion in the presence of SDS and compared the resulting fragments. Fig.2B shows representative patterns of peptides obtained when NTF, purified from nerves, or transferrins, purified from either chicken serum, embryo extract or eggs, were exposed to two different concentrations of *Staphylococcus aureus* V8 protease.

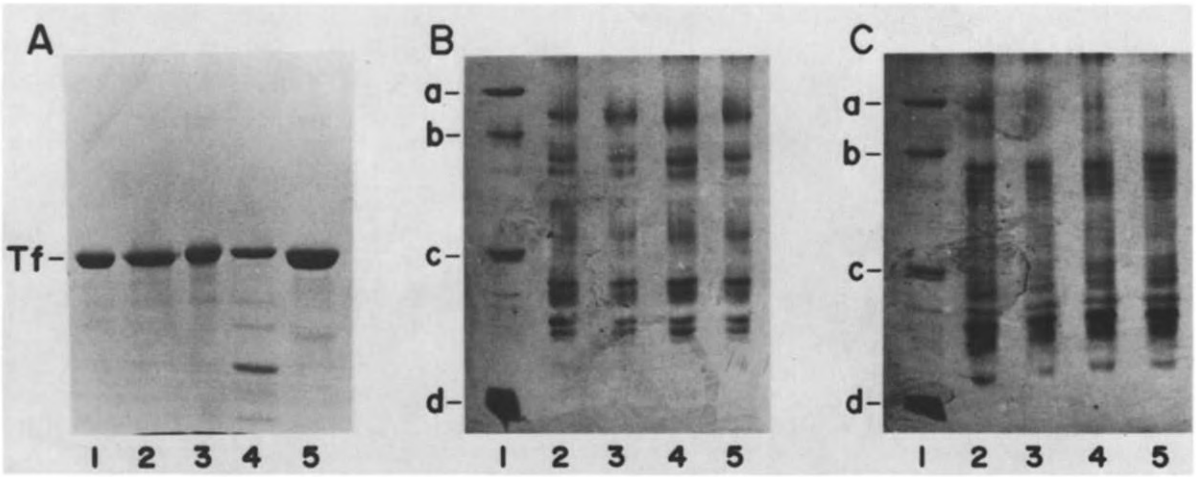

Fig.2. Gel electrophoresis and one-dimensional peptide mapping of purified transferrins and NTF. Transferrins and NTF were purified as in section 2. Analysis of peptide fragments is also described in section 2. (A) SDS–polyacrylamide gels of purified transferrins: (1) human transferrin, 20 μg; (2) embryo extract transferrin, 20 μg; (3) chicken serum transferrin, 20 μg; (4) NTF 10 μg; (5) ovotransferrin, 20 μg. (B) *Staphylococcus aureus* protease digestion fragments: (1) M_r-markers – (a) human transferrin, 76500, (b) ovalbumin, 45000, (c) soy bean trypsin inhibitor, 21500, (d) insulin, 5766; (2) chicken serum transferrin; (3) NTF; (4) ovotransferrin; (5) chicken embryo extract transferrin. (C) Chymotrypsin digestion fragments: lanes as in (B). Total acrylamide is 7% in (A) and 15% in (B) and (C).

Table 1

Biological activity of purified transferrins

Protein added[a]	[^{3}H]Thymidine incorporated[b]	Relative activity[c]	Mature myotubes[d]
NTF	15.7 ± 2.6	1.00	Yes
Ovotransferrin	16.0 ± 2.5	1.02	Yes
Embryo extract transferrin	14.7 ± 2.8	0.94	Yes
Chicken serum transferrin	15.1 ± 5.9	0.97	Yes
None	3.7 ± 0.8	0.23	No

[a] The purified proteins were added at 20 μg/ml
[b] cpm × 10^3
[c] Incorporation relative to NTF
[d] Mature myotubes had multiple nuclei, cross-striations, and were observed to contract

Dissociated muscle cells were grown and assayed for [^{3}H]thymidine incorporation as in section 2

The peptide patterns for the 4 proteins were consistently indistinguishable when examined over a 20-fold range of protease concentration. No fragments unique to any of the proteins were obtained and the relative intensities of the various peptides were similar for all proteins. To further test the homology of primary sequence, we compared the proteolytic fragments produced by digestion of the 4 proteins with chymotrypsin (fig.2C). Again as with V8 protease digestion all fragments were visualized in digests from all four proteins and the relative intensities of the fragments were very similar.

3.3. *Biologic activity of chicken transferrins and NTF*

Once we established a structural homology among NTF, chicken serum transferrin, embryo extract transferrin and ovotransferrin, we next determined if the purified transferrins possessed

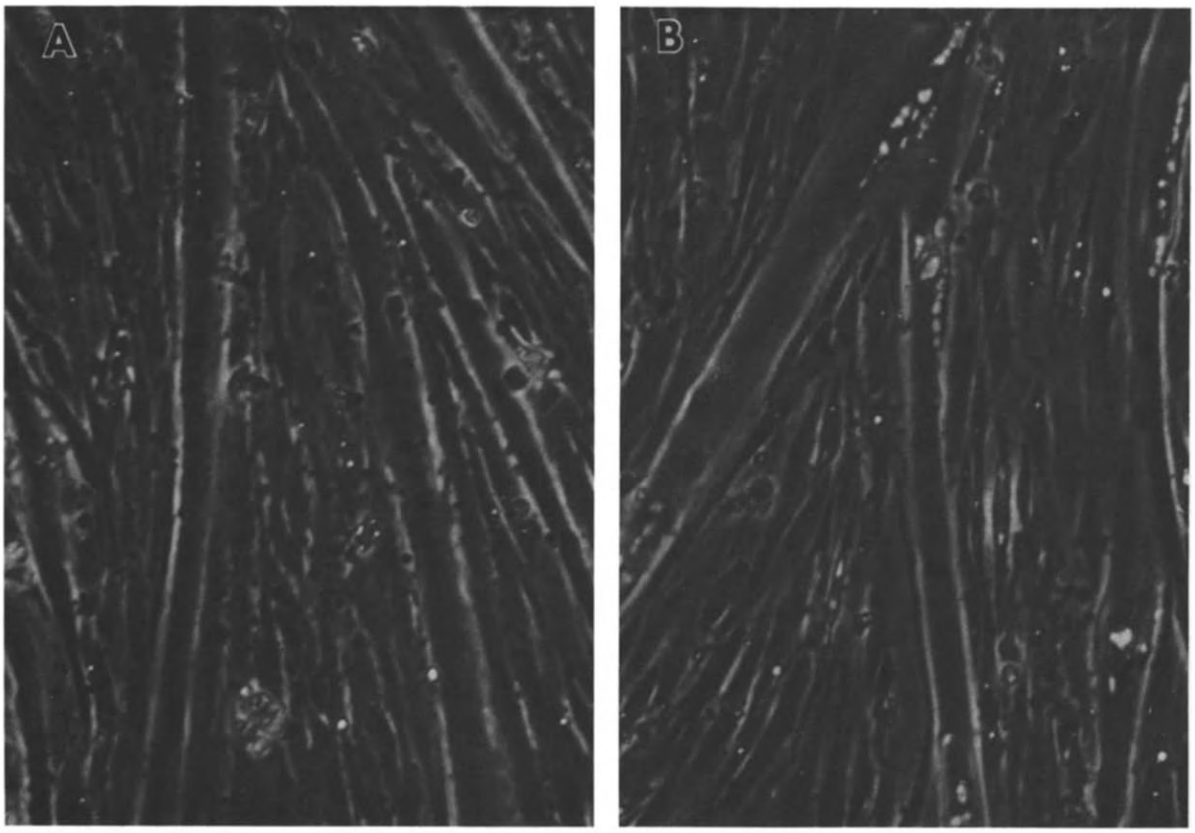

Fig.3. Phase contrast photomicrographs of muscle cell cultures grown 7 DIV in the presence of ovotransferrin (20 μg/ml, A) or NTF (20 μg/ml, B).

similar biological activity to NTF. We compared the purified proteins' abilities to stimulate [^{3}H]thymidine incorporation, the bioassay used to purify NTF [2,3]. At 20 μg/ml, the transferrins and NTF all markedly and equally enhanced [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble macromolecules relative to basal medium (table 1). Maximal levels of incorporation were obtained by all proteins at 16 μg/ml (not shown). The chicken transferrins also supported myogenesis to the same extent as NTF since mature, striated, contractile myotubes developed in cultures to which they were continuously added. Myotubes did not develop in unsupplemented media, although some fibroblasts grew. Representative myotubes from cultures grown 7 days in the presence of ovotransferrin or NTF are shown in fig.3A and 3B, respectively.

4. DISCUSSION

The foregoing data show a high degree of structural similarity between the nerve-derived NTF and transferrins isolated from chicken serum, chicken eggs and chicken embryo extract. In addition, all these preparations possess biological activities associated with NTF: they stimulate [^{3}H]thymidine incorporation by cultured chick myogenic cells and they promote the development of differentiated properties (i.e., myotube formation) by these cells. Furthermore the purified transferrins and NTF have indistinguishable CNBr fragments, have absorption at 465 nm which is dependent on bound iron, and all bind ^{59}Fe (not shown). These facts strongly indicate that NTF is a transferrin, and that it is closely similar or identical to the major form of chicken transferrin found in chicken eggs, serum and embryo extract.

Two other factors which enhance myogenesis in chick muscle cells were also shown to be similar to transferrin. MTF required iron for its activity, and antisera to transferrin cross-reacted with MTF and depleted myotrophic activity from chicken embryo extract and serum [9]. Sciatin has also been demonstrated to closely resemble transferrin by several biochemical criteria, although a monoclonal antibody to chicken transferrin was reported not to react with sciatin [6]. Our data establish the similarity in primary structure between several chicken transferrins and NTF, a mitogenic protein (submitted) which is required for myogenesis [1–3]. The differences in isoelectric point among embryonic MTF and adult serum transferrins [9] probably reflect differences in post translational sialylation [9,21,22]. Further studies with monoclonal antibodies will be required to determine if other subtle differences exist among the transferrin-like proteins. From our current data and these other reports we infer that the 3 factors (MTF, NTF and sciatin) are all transferrins and are very similar if not identical proteins. We suggest that NTF (transferrin) is a mitogen for chick myogenic cells, and that its initial action is a prerequisite for the further development of differentiated muscle cell properties.

Neither human transferrin nor horse transferrin (present in the basal medium) are able to replace chicken NTF. This apparent species or class specificity is in agreement with the requirement for chick, and not human, transferrin reported for chick erythropoietic cells [13]. Although transferrin is present in serum at 2–4 mg/ml [10], one of the transferrin-like proteins, sciatin, is localized in motor neurons and their axons [23]. Neurons with peripheral processes [24] or embryonic neurons [25] contain serum proteins. Nerves may specifically take up proteins like transferrin, and supply them to the muscle target in a selective manner. The role of the neuron could be to chemically modify or to spatially or temporally supply the required proteins in accord with the muscle cell's needs. Whether neurons synthesize, transport and release a NTF-transferrin protein [26], or concentrate it from serum requires further study. Regardless of the origin of this essential protein, the molecular basis of muscle cells' specific requirement for both it and iron [9] are of great interest in relation to neuromuscular diseases and development.

ACKNOWLEDGEMENTS

Support for these studies was provided by NIH (R01NS17197-02), the ALS Society of America, the Medical Research Service of the Veterans Administration, the Charles L. Betzelberger Memorial ALS research fund and the ALS Regional Research Center at the University of Kansas Medical Center. We gratefully thank Mrs Janet Schoeling, Mr Willis Burton and Mrs Carol Flowers for their excellent technical assistance.

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