METABOLISM OF THYROTROPIN RELEASING FACTOR IN TWO CLONAL CELL LINES OF NERVOUS SYSTEM ORIGIN

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Summary. Immunoreactive thyrotropin releasing factor (TRF) was detected in homogenates of two clonal cell lines, BN1010-1 and BN1010-3, derived from a rat central nervous system tumor. TRF was present in logarithmically-growing cells; daily medium changes with slightly acid culture medium (pH 6.8) greatly increased the TRF content of these cells. In contrast, TRF could not be detected in stationary phase cells. TRF peptidases were <1% as active in homogenates of BN1010 cells as those in homogenates of guinea pig brain or hypothalamus. It is expected that these cells will provide an excellent model system for the study of various aspects of TRF metabolism.

INTRODUCTION

The hypothalamic tripeptide thyrotropin releasing factor (TRF) is widely distributed among vertebrate as well as some invertebrate species (1,2); its localization within the central nervous system is not confined to the hypothalamus (3,4). The wide distribution of this peptide, together with its reported effect on mammalian behavior (5,6), indicate that TRF may be a regulator of neuronal function in addition to its role in the control of the adenohypophysis.

Studies on the mechanism of biosynthesis of TRF have been plagued by the presence of an extremely active TRF degrading principle which is ubiquitously present in the central nervous system (McKelvy, unpublished, 7,8,9). Thus, studies on the mechanism of biosynthesis of TRF would be greatly facilitated if a model system were available which produced and stored a large amount of TRF, but which were devoid of the TRF degrading principle. Here we describe the presence of immunoreactive TRF in two clonal cell lines derived from the rat central nervous system. These lines contain a minimal amount of TRF peptidases, and are capable of incorporating labeled proline into TRF.

MATERIALS AND METHODS

Reagents: pGlu-His-[^{3}H]ProNH$_2$(40 Ci/mmol), L-[2,3-^{3}H]proline (20 Ci/mmol), and ^{125}I were obtained from the New England Nuclear Corporation, Boston, Mass. Synthetic TRF was obtained from Peninsula Laboratories, San Carlos, Ca., Sephadex LH-20 from the Pharmacia Fine Chemicals Inc., Piscataway, N.J., egg albumin (grade III) from Sigma, St. Louis, Mo, and acid alumina (Ag4, 100-200 mesh) from Biorad Laboratories, Richmond, Ca. The horse and fetal calf sera were purchased from Grand Island Biological Co. and Reheis Chemical Co., respectively.

BN1010-1 and BN1010-3 clones: Lines BN1010-1 and BN1010-3 were derived from an ethylnitrosourea-induced central nervous system tumor in a CDF rat (10). In addition to the synthesis of TRF reported in this paper, these cell lines respond to isoproterenol by elevating their intracellular cAMP, and exhibit sodium flux responses to certain neurotoxins in a manner characteristic of action potential sodium channels.

Culture conditions: Cells were plated in DME medium (GIBCO, powered) supplemented with horse serum (5% v/v) and fetal calf serum (5% v/v). In experiments in which the pH of the medium was lowered, the bicarbonate containing medium was titrated with HCl prior to incubation in a CO_2 incubator. Details of the culture conditions have been described previously (11,12).

Preparation of the cells for TRF determination: Cells were grown in 150 mm plastic tissue culture dishes (Falcon). At the time of harvest, the culture medium was removed from the dish and replaced with a HEPES-buffered wash medium of the following composition: NaCl: 135 mM, KCl: 5mM, $CaCl_2$: 5mM, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid): 28 mM, titrated to pH 7.2 with NaOH. This wash medium was removed and replaced by a small volume of the same medium. The cells were freed from the culture dish with a rubber policeman and transferred to a 50 ml conical tube. The cells were centrifuged at 800 rpm for 5 min, resuspended in 1.0 ml distilled water, and homogenized in a Potter Elvejhem type tissue grinder with a teflon pestle. After the addition of 9.0 ml of absolute methanol (redistilled), the methanol-soluble material was extracted by centrifugation at 1000 x g for 20 min. The methanol phase was dried under nitrogen. For direct radioimmunoassay of the methanolic extract, the dry residue was suspended in an appropriate amount of radioimmunoassay buffer (see below).

LH-20 column chromatography: The dried methanolic extracts were suspended in 1.0 ml of 90% methanol and applied to a Sephadex LH-20 column (100 x 0.7 cm) and eluted with 90% methanol. The elution volume of TRF was 0.63 and the recovery of standard TRF 92-95%.

Carboxymethylcellulose paper chromatography: The combined peak fractions corresponding to the elution volume of TRF from the LH-20 column were dried under nitrogen and suspended in a small volume of absolute methanol and applied to a 20x20 cm sheet of carboxymethylcellulose paper (CM-82). The chromatogram was developed in 5 mM NaCl in water. The R_F of standard synthetic TRF under these conditions was 0.25. The section of the chromatogram corresponding to the R_F value of standard TRF was eluted with 1.0 ml of radioimmunoassay buffer according to the method of Eshdat and Mirelman (13). The efficiency of extraction of TRF from the paper under these conditions was 95%.

Radioimmunoassay of TRF: Antibody to TRF was obtained after coupling TRF to bovine serum albumin by the diazotized benzidine method (14) and immunization with this antigen in complete Freund's adjuvant and was used at a final titer of 1:15,000. Synthetic TRF was labeled with I^{125} using the method of Hunter and Greenwood (15) and was purified on Sephadex G-10. The assay was carried out as follows: All dilutions were made in 0.01 M sodium phosphate buffer containing 2% NaCl and 0.25% egg albumin (pH 7.4). 100 µl of the sample of standard TRF were added first, then 100 µl of antibody in the above buffer and 100 µl of [^{125}I]TRF (5000 cpm) also in the above buffer

Table I. SPECIFICITY OF TRF ANTIBODY

COMPOUND	% CROSSREACTIVITY
PCA-HIS-PRO-NH_2	100.0000
PCA-HIS-PRO	0.0100
GLU-HIS-PRO-NH_2	0.0100
GLU-HIS-PRO	0.0025
HIS-PRO-NH_2	<0.0010
GLY-HIS	<0.0010
GLY-HIS-GLY	<0.0010
HIS-ALA	<0.0010
GABA-HIS	<0.0010
LRF	<0.0010
CARNOSINE	<0.0010

were added. The mixture was allowed to reach equilibrium for 48 hr at 4°C. The bound and free hormone were separated by the addition of 2.0 ml of ice-cold absolute ethanol followed by centrifugation 1000 x g for 30 min at 4°C. All TRF standards were assayed in triplicate, and the samples in duplicate at minimum of 3 dilutions. Only samples in which the displacement of [^{125}I]TRF from the antibody diluted out parallel to the dilution of standard synthetic TRF were considered to contain immunoreactive TRF. The coefficient of variation of the assay was 2.4%. The specificity of the antibody is shown in Table I.

Assay for enzymatic degradation of TRF. Aliquots of 10,000 g-min supernatants of cell homogonate and media were incubated in 50mM Tris-Cl, pH 7.4, 1 mM dithioerythrol, 50μM [^{3}H]TRF (273 pCi/pmole) at 37° for varying periods of time and the decrease in TRF radioactivity determined by cellulose acetate thin layer electrophoresis at pH 4.2, as previously described (16). All homogenates were assayed at several concentrations to obtain an estimate of enzyme activity in the linear range of the enzyme concentration curve.

Determination of TRF binding: The ability of the cells to bind TRF was tested by the method of Grant et al (17). The [^{3}H]TRF used in the binding studies was purified prior to the assay by the method of McKelvy (18).

TRF biosynthesis: One 15 cm plate of mid-log phase cells (10^{8} cells) was incubated with 11 ml of incubation medium at pH 6.8 containing 33 μCi/ml of [^{3}H]proline: for 16 hr. The cells and the medium were then scraped and transferred to a plastic conical centrifuge tube and centrifuged at 800 rpm for 5 min at 4°C. The medium was decanted and the cells suspended in 1.0 ml of distilled water. Next 500 μg of synthetic TRF were added and the cells homogenized. The homogenate was extracted with 9.0 ml of absolute methanol and centrifuged at 12,000 x g for 20 min. The supernatant was concentrated to 500 μl and subjected to sequential purification during which the mobility of added carrier TRF (visualized by the Pauli reagent (22)) and standard [^{3}H]TRF in each separation system was correlated with radioactivity (Table II).

RESULTS

When methanolic extracts from several cell lines of central nervous system origin were subjected to radioimmunoassay, it was found that immunoreactive material was present in a number of clones of both glial and

Table II. PURIFICATION SCHEME USED TO ISOLATE [^{3}H]Pro-LABELED TRF

Methanol Extract of Tissue

↓

Acid Alumina Column Chromatography
(0.7 x 2 cm in 90% MeOH)
Pool effluent. Concentrate under N_2

↓

Sephadex LH-20 Column Chromatography
(0.8 x 105 cm in 90% MeOH)
Pool Ve_{TRF}. Concentrate under N_2

↓

Preparative CM-cellulose paper Chromatography, H_2O
Pool TRF region. Concentrate

↓

Preparative Cellulose Acetate Electrophoresis, pH 4.2
Pool TRF region. Concentrate

↓

Preparative High Voltage Paper Electrophoresis, pH 1.9

neuronal origin. The highest amount of immunoreactive material in this initial screening test was found in two recently developed clones (10) named BN1010-1 and BN1010-3. Because many buffer ions, in addition to TRF, are capable of dissociating the antibody-antigen complex under radioimmunoassay conditions (Y. Grimm-Jørgensen, unpublished observation), the identity of the immunoreactive material was further investigated. After LH-20 chromatoraphy of a methanolic cell extract the majority of the immunoreactive material was found in the pooled fractions corresponding to the elution volume of [^{3}H]TRF. A significant amount of apparent immunoreactive material also eluted after [^{3}H]TRF, corresponding to the elution volume of several buffer salts. The identity of the material present in the elution volume of standard TRF was further characterized by chromatography on carboxymethylcellulose paper. The TRF content of an extract from BN1010-3 cells was 21.7 pg/mg alkali soluble proteins after LH-20 column chromatography. After LH-20 chromatography and carboxymethylcellulose chromatography, the content was 20.3 pg/mg alkali soluble protein. A region of the chromatogram adjacent to the

Table III. IMMUNOREACTIVE TRF IN BN1010 CELLS AS A FUNCTION OF GROWTH AND CULTURE CONDITIONS

Cell Line	Growth Condition	TRF, pg/mg alkali soluble protein
BN1010-1	Stationary Phase*	0
	Logarithmic Phase*	35.8
	Solid Tumor	18.3
BN1010-3	Stationary Phase*	0
	Logarithmic Phase*	21.7
	Logarithmic Phase†	123.2
	Logarithmic Phase, pH 6.8†	208.6
	Solid Tumor	116

* Medium changed every 2 days, † Medium changed every day.

TRF area, similarly eluted and assayed, contained no immunoreactive material. All subsequent determinations were made after LH-20 column chromatography of the samples.

The TRF content of BN1010-1 and BN1010-3 cells in the logarithmic and stationary growth phases was examined as a function of culture conditions. In both clones, detectable amounts of TRF were found only during log phase (Table III). Changing the medium daily instead of every other day increased celluar TRF content 5 - 6 fold, from 21.7 pg/mg to 123.2 pg/mg alkali soluble protein. A further 2-fold increase to 208.6 pg/mg was observed when the medium was rendered slightly acid (pH 6.8). Since non-specific assay interferences of buffer ions make it impossible to quantify the TRF content of the incubation medium at the present time, it is not possible to determine whether the altered TRF contents during the growth cycle of the cells, and in response to altered culture conditions, reflect changes in the rates of biosynthesis, degradation, or release of TRF. When the cells were grown as solid clonal tumors in CDF rats, TRF contents of 18.3 and 116 pg/mg alkali soluble material were found to BN1010-1 and BN1010-3, respectively (Table III).

Table IV. DEGRADATION OF TRF BY BN1010 CELL AND GUINEA PIG BRAIN HOMOGENATES

Cell Line	Material	TRF Degraded (pmoles/min/mg alkali soluble protein)
	Stationary Phase Cells	0.10
BN1010-1	Logarithmic Phase Cells	0.11
	Medium	0
BN1010-3	Stationary Phase Cells	0.07
	Logarithmic Phase Cells	0.07
	Medium	0
Guinea Pig	Whole Brain	250
	Hypothalamus	98

When BN1010-3 cells were incubated for 16 hr in the presence of 33 μCi/ml L-[^{3}H]proline and the methanolic extract from these cells purified as outlined in Table II, a peak of Pauli-positive material whose migration matched that of synthetic [^{3}H]TRF was obtained in yields of from 280 - 1200 cpm (not corrected for a 10 - 40% recovery) per 10^8 cells.

The activity of TRF peptidases is low in both clones when compared to the activity present in whole guinea pig brain or guinea pig hypothalamus (Table IV). No TRF peptidase activity was detected in conditioned culture medium from either clone.

Neither BN1010-1 nor BN1010-3 cells bound [^{3}H]TRF specifically in either logarithmic or stationary growth phase, suggesting that these cells do not possess a TRF receptor as has been postulated for some central nervous system neurons (19).

These studies demonstrate that immunoreactive TRF is found in significant amounts in two clonal cell lines of central nervous system origin. The results from the successive purification procedures indicate that the immunoreactive material found in these cells is identical with the tripeptide pGlu-His-Pro-NH_2, the structure which has been assigned to TRF by the laboratories of Shally and Guillemin (20, 21).

The origin of the intracellular TRF is most likely to be found within the cells themselves. It is unlikely that traces of TRF present in the

incubation medium, due to TRF present in fetal calf serum or the horse serum, were accumulated by these cells because: (1) no TRF was detected in samples of horse serum and fetal calf serum, (2) no uptake of [^{3}H]TRF by these cells should be detected under a wide variety of conditions in preliminary experiments, and (3) when cells were incubated with [^{3}H]proline and then subjected to a 5-step purification, there remained radioactive material which corresponds to TRF, indicating that these cells are capable of synthesizing TRF.

These observations suggest that these cells lines will be useful models for the study of several aspects of TRF metabolism.

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