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Nucleotide and amino acid sequence of lymphocyte-derived corticotropin: Endotoxin induction of a truncated peptide

(protein microsequencing/immune neuroendocrine interactions)

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ABSTRACT **To determine the degree of similarity between pituitary and lymphocyte proopiomelanocortin, the lymphocyte mRNA was reverse transcribed, cloned, and sequenced. Murine lymphocyte mRNA was first purified by oligo(dT)-cellulose affinity chromatography and was reverse transcribed by using a selective 3′ antisense oligonucleotide primer directed at the boundary between the translated/nontranslated region on the 3′ end of exon 3. This cDNA was then amplified in a polymerase chain reaction with selective primers containing *Sal* I and *Kpn* I restriction endonuclease sites. Amplified cDNA was then directionally ligated into M13mp18 and M13mp19 bacteriophage and was sequenced. The nucleotide sequence encoding this peptide was identical to that of mouse pituitary corticotropin (ACTH). Elevated levels of lymphocyte immunoreactive ACTH were then induced with bacterial lipopolysaccharide and the peptide(s) was purified by antibody affinity chromatography and reverse-phase high-performance liquid chromatography. The predominant immunoreactive ACTH species was ≈3 kDa and its sequence was identical to pituitary ACTH(1–25). These results conclusively demonstrate that lymphocytes produce authentic ACTH and harbor its mRNA.**

The immune and neuroendocrine systems are now being viewed as having many similarities as well as the ability to communicate in a bidirectional manner (for review, see refs. 1 and 2). The hypothesis of bidirectional communication between these two systems is currently supported by studies showing that cells of the immune system synthesize and respond to a number of peptides that are related to pituitary hormones and peptide neurotransmitters (3, 4). Such molecules have principally been identified by immunologic and biologic techniques. In spite of marked similarities between the immune and neuroendocrine-derived substances, questions remain as to the degree of relatedness. To test for precise molecular identity versus biochemical similarity for a cortiocotropin (ACTH)-like peptide from lymphocytes (4–9), we determined the amino acid sequence of this molecule as well as the nucleotide sequence of its cDNA.¶

MATERIALS AND METHODS

Production and Purification of Lymphocyte-Derived ACTH. Spleens from 45 C3HeB/FeJ female mice were removed, dissociated, and resuspended at 1×10^7 cells per ml in culture medium in two equal batches. Culture medium was RPMI 1640 medium supplemented with 1% bovine serum albumin and 50 μg of lipopolysaccharide (LPS) per ml (*Escherichia coli* 0127:B8 Difco). After 24 hr of culture, ^{3}H-labeled L-amino acid mixture (2 μCi/ml; 1 Ci = 37 GBq) was added to the culture. After 48 hr of incubation, the culture supernatant fluid was collected by centrifugation. Then, 125 μl of ^{3}H-labeled immunoreactive ACTH (irACTH) purified by affinity chromatography was applied to a C18 reverse-phase HPLC column. A 0–60% acetonitrile linear gradient was applied to elute the column over a 45-min period at a flow rate of 1 ml/min. Aliquots of each 1-min fraction were assayed for ^{3}H by liquid scintillation spectroscopy.

Reverse Transcription and Amplification by Polymerase Chain Reaction (PCR) of the Lymphocyte-Derived Proopiomelanocortin (POMC) Transcript. Poly(A)-containing lymphocyte RNA (3 μg) was reverse transcribed with a cDNA synthesis system according to the manufacturer's instructions (Promega) using a selective antisense oligonucleotide primer directed toward the 3′ end of murine POMC exon 3 (bases 606–630; GenBank) (10). Target sequences from this cDNA (3 μl from a total reaction vol of 30 μl) as well as from a plasmid (1.0 ng) containing murine POMC (pMkSU16, kindly provided by M. Uhler; ref. 11) were amplified (30 cycles) in a PCR using the aforementioned antisense primer (200 ng) and a sense primer (250 ng) directed toward the 5′ end of murine POMC exon 3 (bases 101–122; GenBank) (10). PCR procedures were as described (12). Ten microliters of the PCR mixture (100 μl) was electrophoresed (constant voltage, 65 V) on an agarose gel (2%) that was stained with ethidium bromide (25 μg).

Cloning and Sequencing of Lymphocyte-Derived POMC cDNA. PCR reamplifications were performed with the aforementioned sense and antisense primers, which were modified to contain *Sal* I and *Kpn* I restriction sites, respectively. The lymphocyte and pituitary cDNAs were then directionally ligated into M13mp18 and M13mp19 bacteriophage and recombinants were selected on the *E. coli* host TG1 as described (12). Purified recombinant M13 single-stranded DNA was combined with a −40 M13 sequencing primer (15 bases; 0.5–1 pmol) and sequencing reactions were performed using a Sequenase reagent kit according to the manufacturer's protocol (United States Biochemical). Both orientations of the lymphocyte as well as pituitary cDNA inserts were sequenced manually on 4% and 6% polyacrylamide gels containing 8 M urea or with a Genesis 2000 automated DNA sequencer (DuPont) on 6% polyacrylamide gels with fluorescent dideoxynucleotide chain terminators.

Protein Microsequencing. The primary structure of the irACTH peptides was determined by the University of Texas Medical Branch Cancer Center Protein Chemistry Laboratory through microsequence analysis using an Applied Biosystems model 470A protein/peptide sequencer. All analyses

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Abbreviations: ACTH, corticotropin; irACTH, immunoreactive ACTH; LPS, lipopolysaccharide; PCR, polymerase chain reaction; POMC, proopiomelanocortin.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30489).

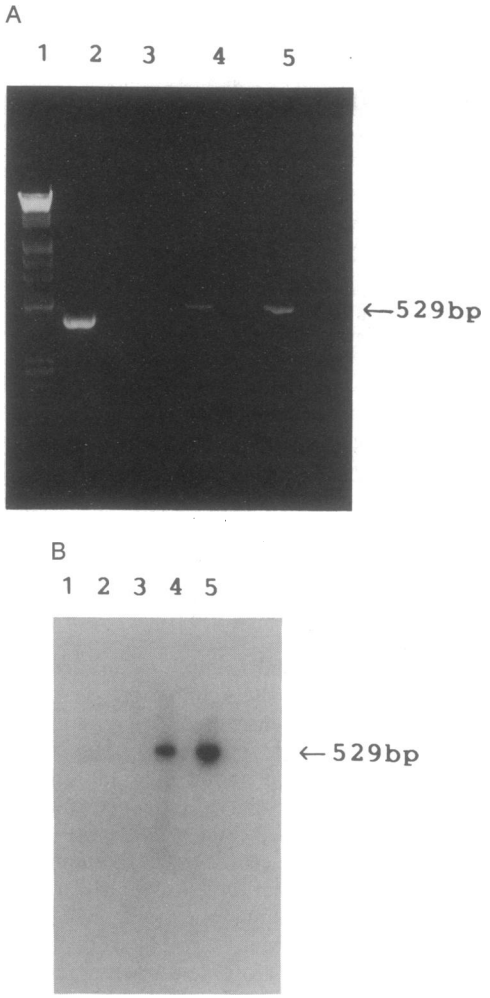

FIG. 1. PCR amplification of the ACTH-containing region of lymphocyte-derived POMC cDNA. (*A*) Ethidium bromide-stained agarose (2%) gel of PCR products. Lanes: 1, molecular mass markers; 2, Cetus control; 3, oligonucleotide primers without template; 4, lymphocyte POMC cDNA; 5, pituitary POMC cDNA. (*B*) Autoradiograph of *A* after alkaline transfer to a nylon membrane (GeneScreen*Plus*; DuPont) and hybridization with a nick-translated ([α-^{32}P]dCTP) cDNA probe (10^6 cpm; 3×10^7 cpm per μg of DNA) corresponding to exon 3 of murine pituitary POMC.

were repeated at least once and the repetitive yields were on the average 88%. The phenylthiohydantoin derivatives of the amino acids from the sequencer were identified and quantitated by HPLC using reverse phase chromatography with a C18 column as described (13).

RESULTS

Since recent reports have shown that an mRNA related to that of the ACTH precursor POMC is basally expressed in human lymphocytes (14, 15), we were interested in whether a similar situation existed in mice. To detect this putative transcript and at the same time ensure sufficient material for sequencing, we used the PCR. Lymphocyte mRNA from C3H/FeJ mice was used as a template for selective first-strand cDNA synthesis with an oligonucleotide primer complementary to the 3′ end of exon 3 of POMC mRNA. Fig. 1*A* shows that when this cDNA is introduced into a PCR with a second oligonucleotide primer representing the POMC sequence 173 base pairs (bp) 5′ to the ACTH-containing region of exon 3, there is amplification of the appropriate sized (529 bp) cDNA fragment. Selective amplification of a POMC-related target cDNA sequence was confirmed since this product hybridized with a nick-translated pituitary POMC probe (Fig. 1*B*). The authenticity of this cDNA was verified by nucleotide sequencing of the ACTH segment of this cDNA (Fig. 2). The pituitary and lymphocyte sequences were identical to each other as well as to published pituitary sequences (10). They differed from one published sequence (11) that apparently contains an erroneous AAC rather than AAG for lysine-15 of ACTH.

For purposes of purification and peptide microsequencing, elevated levels of lymphocyte irACTH were induced with bacterial LPS. This inducer was chosen because in contrast to induction by viruses or corticotropin-releasing factor, irACTH produced under LPS stimulation is specifically processed into a molecule that comigrates with ACTH(1–24) rather than with ACTH(1–39) (16–18). Thus, the analysis of this peptide would not only yield the amino acid sequence of lymphocyte ACTH but would also verify a new proteolytic cleavage site. To ensure that we were sequencing a newly synthesized ACTH, the polypeptide was intrinsically radiolabeled. For ACTH production, splenic lymphocytes from 45 mice were treated with LPS for 48 hr in the presence of ^{3}H-labeled amino acids as described (18). The culture supernates were harvested and the ACTH was purified by affinity chromatography on an anti-ACTH antibody-Sepharose column (19). Fig. 3 shows that the majority of the intrinsically radiolabeled irACTH eluted from reverse-phase HPLC at the same time as a synthetic ACTH(1–24) standard. It is important to note that this material was purified from the extracellular fluids and was therefore released. In addition, these results confirm earlier studies that LPS primarily induces truncated ACTH and this smaller form provides further evidence of *de novo* synthesis. Table 1 shows the results of amino acid sequence analyses (20) from 2 nmol of this truncated ACTH and shows that the sequence is identical to the amino-terminal end of pituitary ACTH. The signal di-

Residue 1 10

Murine NH_2-Ser Tyr Ser Met Glu His Phe Arg Trp Gly Lys Pro Val Gly Lys Lys Arg Arg Pro
Pituitary 5′-TCC TAC TCC ATG GAG CAC TTC CGC TGG GGC AAG CCG GTG GGC AAG AAA CGG CGC CCG

Murine
Lymphocyte 5′-TCC TAC TCC ATG GAG CAC TTC CGC TGG GGC AAG CCG GTG GGC AAG AAA CGG CGC CCG

20 30 39

Val Lys Val Tyr Pro Asn Val Ala Glu Asn Glu Ser Ala Glu Ala Phe Pro Leu Glu Phe-COOH
GTG AAG GTG TAC CCC AAC GTT GCT GAG AAC GAG TCG GCG GAG GCC TTT CCC CTA GAG TTC-3′

GTG AAG GTG TAC CCC AAC GTT GCT GAG AAC GAG TCG GCG GAG GCC TTT CCC CTA GAG TTC-3′

FIG. 2. Comparison of the nucleotide sequence of murine pituitary and lymphocyte ACTH.

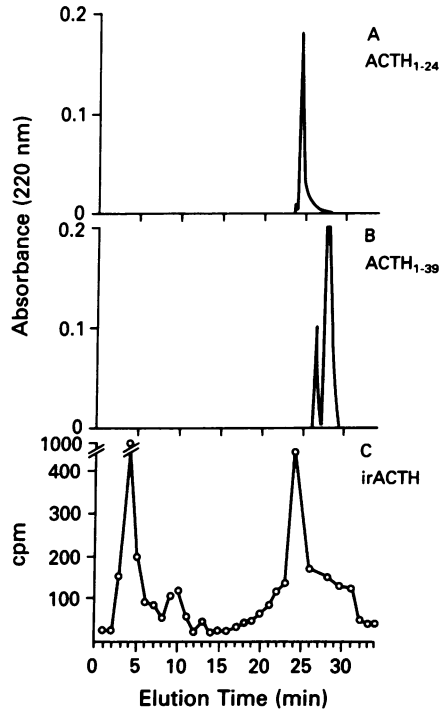

FIG. 3. Reverse-phase HPLC analysis of intrinsically radiolabeled irACTH from LPS-treated lymphocytes.

minished after 15 rounds of sequencing with several residues producing undetectable peaks on HPLC until the 25th cycle. After this cycle, no signal was observed through the remaining 14 cycles. To obtain more total material (6.4 nmol), a wider peak from the HPLC, which included the ACTH(1–39)-containing shoulder, was taken and sequenced. ACTH(1–39) represented <22% of the total ACTH synthesized, as determined by gel filtration of radiolabeled ACTH (data not shown) and analysis of the HPLC profile (Fig. 3). Since this sample contained some of the larger ACTH, we were able to sequence beyond residue 25. Table 1 shows that this sequence is identical to pituitary ACTH through the 28 cycles. It is important to note that residue 26 is a valine, which is consistent with murine (11), but not porcine or bovine (glycine in position 26), ACTH (12, 21). Thus, this species difference confirmed that the hormone was *de novo* synthesized by murine lymphocytes.

Table 1. Automated sequence analysis of irACTH

Edman cycle	Residue	Sample 1, pmol	Sample 2, pmol
0	—	2000*	6400*
1	Ser	174	2009
2	Tyr	148	5924
3	Ser	47	4140
4	Met	113	5488
5	Glu	96	4380
6	His	29	2113
7	Phe	61	2834
8	Arg	14	1770
9	Trp	15	1478
10	Gly	7	1929
11	Lys	8	2523
12	Pro	14	1617
13	Val	10	2605
14	Gly	4	621
15	Lys	5	632
16	Lys	—	1163
17	Arg	2	751
18	Arg	—	592
19	Pro	—	781
20	Val	1	490
21	Lys	—	606
22	Val	—	779
23	Tyr	1	392
24	Pro	—	608
25	Asn	1	126
26	Val	—	237
27	Ala	—	349
28	Glu	—	138

*Initial pmol applied to sequenator.

DISCUSSION

Collectively, these studies have conclusively demonstrated that lymphocytes express authentic POMC mRNA. Furthermore, this mRNA is translated and the resulting protein is processed and secreted as ACTH. One striking difference between the LPS-induced lymphocyte ACTH is its smaller size in comparison to that of the pituitary gland. The apparent cleavage of this peptide between residues 25 and 26 is to our knowledge the only example of such processing. Although this, of course, would not alter its steroidogenic activity, which resides in the first 24 amino acid residues, it might impact on those immunoregulatory properties of the hormone that involve the carboxyl-terminal end (22, 23). While the most conservative interpretation of the data is that the POMC gene is being expressed by lymphocytes, it is tempting to speculate about the function of this molecule. There are a number of studies that suggest that it does operate *in vivo*, at least in pathological situations. We have shown that lymphocytes can be induced to produce ACTH *in vivo* in hypophysectomized mice injected with Newcastle disease virus (24) and in children vaccinated with a typhoid vaccine (25). In addition, Dupont *et al.* (26) have documented a case in which normal lymphocytes in an inflammatory mass produced ACTH, which then induced adrenal steroidogenesis. Furthermore, in hypopituitary individuals with no pituitary ACTH response, Fehm *et al.* (27) showed that the administration of corticotropin-releasing factor resulted in an extrapituitary induction of ACTH and steroidogenesis, possibly by induction of lymphocyte ACTH. Therefore, at least in pathological situations, lymphoid-derived ACTH can act as a functional component of the neuroendocrine system.

It is also possible that lymphoid ACTH may play an important role as an endogenous regulator of the immune system. *In vitro*, ACTH variously inhibits antibody (22) and interferon γ production (23) while enhancing the B-lymphocyte functions (28). Lymphocytes possess ACTH receptors (29, 30) that in the microenvironment of a lymphoid organ might be the prime targets for ACTH produced by adjacent lymphocytes. Irrespective of the exact role it may play, these results clearly show that bona fide ACTH is being produced by the immune as well as neuroendocrine systems. This then provides one possible means for communication between these systems. Since ACTH is only one of many immunoreactive hormones produced by the immune system, it may serve as the prototype of a regulatory network based on signal molecules and receptors common to the immune and neuroendocrine systems.

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