Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10384–10388, October 1994
Physiology

The prolactin gene is expressed in the hypothalamic–neurohypophyseal system and the protein is processed into a 14-kDa fragment with activity like 16-kDa prolactin

CARMEN CLAPP*†, LUZ TORNER*, GABRIEL GUTIÉRREZ-OSPINA*, EVA ALCÁNTARA*, FRANCISCO J. LÓPEZ-GÓMEZ*, MAKOTO NAGANO‡, PAUL A. KELLY‡, SALVADOR MEJÍA*, MIGUEL A. MORALES*, AND GONZALO MARTÍNEZ DE LA ESCALERA*

*Centro de Neurobiología e Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, 04510 México DF, México; and ‡Unité 344, Endocrinologie Moléculaire, Institut National de la Santé et de la Recherche Médicale, Faculté de Médicine Necker, 75730 Paris Cedex 15, France

Communicated by Howard A. Bern, June 27, 1994

ABSTRACT **The 23-kDa form of prolactin (PRL) has been proposed to function as both a mature hormone and a prohormone precursor for different uniquely bioactive forms of the molecule. We have shown that the 16-kDa N-terminal fragment of PRL (16K PRL) inhibits angiogenesis via a specific receptor. In addition, 16K PRL stimulates natriuresis and diuresis in the rat, and kidney membranes contain high-affinity specific binding sites for this PRL fragment. 16K PRL can be derived from an enzymatically cleaved form of PRL (cleaved PRL). With the use of a specific 16K PRL antiserum, we have localized a 14-kDa immunoreactive protein in the paraventricular and supraoptic nuclei of the hypothalamus and in the neurohypophysis. Reverse transcription–polymerase chain reaction of RNA from isolated paraventricular nuclei showed the expression of the full-length PRL mRNA. The neurohypophysis was found to contain the enzymes that produce cleaved PRL, small amounts of PRL, and cleaved PRL. Medium conditioned by neurohypophyseal cultures, enriched with the 14-kDa immunoreactive protein, has antiangiogenic effects that are blocked by the 16K PRL antiserum. These results are consistent with the expression of PRL in the hypothalamic–neurohypophyseal system, and the preferential processing of the protein into a 14-kDa fragment with biological and immunological properties of 16K PRL.**

Prolactin (PRL) is a multifunctional protein hormone involved in the control of a wide variety of physiological processes in vertebrates, including osmoregulation, reproduction, immune responses, growth, and development (1, 2). PRL exists in several molecular forms that result from posttranslational modifications of the predominant form, which is a single polypeptide chain with three disulfide loops and a molecular mass of 23 kDa (23K PRL; ref. 3). Molecular heterogeneity of PRL occurs not only in the anterior hypophysis (3) but also in the immune system (4) and the brain (5). These findings have led to the suggestion that PRL can be a prohormone that becomes processed to biologically active peptides capable of mediating unique functions (3).

We have shown that the 16-kDa N-terminal fragment of PRL (16K PRL), while retaining some PRL-like bioactivities (6), has specific effects not shared with the parent PRL molecule. 16K PRL, obtained after the proteolysis of rat PRL with mammary gland enzymes, and recombinant human 16K PRL, but not rat or human PRL, inhibit angiogenesis—i.e., the formation of new capillary blood vessels (7, 8). Furthermore, this antiangiogenic effect appears to be mediated by a unique 16K PRL receptor (9). Besides angiogenesis, other functions appear to be affected by 16K PRL. We have recently found that 16K PRL, but not PRL, stimulates natriuresis and diuresis in the anesthetized rat (C.C. and W. K. Samson, unpublished observations). Moreover, kidney membranes contain high-affinity specific binding sites for 16K PRL (10). Therefore PRL-derived molecules may be specifically involved in the regulation of angiogenesis, natriuresis, and possibly other functions.

16K PRL can be derived from the reduction of the intermediate disulfide bond that holds together a form of PRL with a cleavage in the large disulfide loop (11). In the rat, the known site of cleavage occurs at Tyr-145, leaving an N-terminal polypeptide with a molecular mass of 16,364 Da (12, 13). Cleaved PRL, the immediate precursor of 16K PRL, has been identified in the anterior pituitary gland and in the circulation (3, 12), and the cleaving enzymes have been found in several target tissues of PRL (3, 5, 11, 14). Detection of endogenous 16K PRL has been limited by its low crossreactivity with the PRL antibody (6).

Here we report findings consistent with the expression of PRL in the hypothalamic–neurohypophyseal system and its posttranslational processing into a 14-kDa fragment with biological and immunological properties of 16K PRL.

MATERIALS AND METHODS

16K PRL. 16K PRL was isolated after the enzymatic proteolysis of 23K PRL [rat B-6; National Hormone and Pituitary Program (NHPP)] by a particulate fraction from rat mammary gland homogenates, disulfide-bond reduction, and gel filtration as reported (11).

16K PRL RIA. An RIA was established, using an antiserum raised in rabbits against the 16K PRL preparation, at a final dilution of 1:20,000. The specificity of the 16K PRL antiserum was tested by competition with 16K PRL and 23K PRL (rat B-6; NHPP). No crossreactivity was observed with rat growth hormone (NHPP), vasopressin, oxytocin, angiotensin II, and neurophysins I and II (Sigma), β-lipotropin (NHPP), β-endorphin (Pierce), [Met]enkephalin (Peninsula Laboratories), and adrenocorticotropin (residues 1–39; Bachem).

Animals. Female Wistar rats (200–250 g) were used in the day of estrus, as determined by daily examination of vaginal smears for two or three consecutive cycles.

Immunocytochemistry. Rats were anesthetized with sodium pentobarbital and perfused with phosphate-buffered

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PRL, prolactin; 23K and 16K PRL, 23- and 16-kDa PRL; NHPP, National Hormone and Pituitary Program; bFGF, basic fibroblast growth factor; PVN, paraventricular nucleus; SON, supraoptic nucleus; RT, reverse transcriptase.

†To whom reprint requests should be addressed at: Centro de Neurobiología, Universidad Nacional Autónoma de México, Apartado Postal 70228, 04510 México DF, México.

saline (PBS; pH 7.4) for 5 min followed by neutral buffered 4% paraformaldehyde for 45 min. Brain and hypophyses were fixed for 24 h at 4°C, then transferred to 30% (wt/vol) sucrose until they sank. Tissues were sectioned in a cryostat and subjected to immunocytochemistry using the ABC kit from Vector Laboratories. Positive staining with the 16K PRL antiserum was visible in the anterior and neurohypophyses, starting at 1:10,000 and 1:1000 dilutions, respectively. Most results were obtained with a 1:500 dilution of either anti-16K PRL or anti-PRL (rat S-9; NHPP) antisera.

Western Blot Analysis. Neurohypophyseal lobes were sonicated [1 mg/50 μl of 0.01 M Tris·HCl/0.14 M NaCl/1% Triton X-100 (pH 8) with aprotinin at 0.2 unit/ml and 1 mM phenylmethylsulfonyl fluoride] and subjected to SDS/PAGE (11). Proteins were blotted onto nitrocellulose membranes, probed with the 16K PRL antiserum (1:500), and developed by using the alkaline phosphatase second antibody kit (Bio-Rad).

Incubation of Neurohypophyses. Neurohypophyseal lobes were washed in Ca^{2+}-deficient Krebs–Ringer solution for 10 min at 37°C and cultured in 20 μl of Krebs–Ringer solution for 1 h at 37°C. The resulting conditioned media were pooled and stored at −70°C.

Endothelial Cell Growth Bioassay. Bovine brain capillary endothelial (BBCE) cells were isolated and grown and the proliferation assay was performed as previously reported (8). The effect of 4 days of incubation with 16K PRL, 23K PRL, or neurohypophyseal-conditioned medium was compared with the stimulation induced by basic fibroblast growth factor (bFGF) (50 pM; GIBCO/BRL) in the presence or absence of the 16K PRL antiserum (1:1000 dilution).

RNA Isolation. Hypothalamic paraventricular nuclei (PVNs) were isolated from frozen brain slices by the micropunching technique (15). Total RNA was extracted from the PVN, cerebral cortex, and anterior hypophysis according to the guanidinium thiocyanate/phenol procedure (16).

Primers for PCR and Southern Blot Analysis. Four different primers specific for rat (r) PRL cDNA were synthesized. Two of them were forward primers: rPRL-A (5′-TGTTCTGGTGGCGACTGCCAGACACCT-3′) from exon 2 and rPRL-B (5′-ACTTCTTCCCTAGCTACTCCTGAAGAC-3′) from exon 3. The two downstream primers were rPRL-C (5′-TATCTTTTCGATCCCTTCGAGAAGCCG-3′) from exon 4 and rPRL-D (5′-GCAGTTGTTTTTATGGACAATTTGGCA-3′) from exon 5. For the full-length PRL mRNA, the combinations rPRL-A and -C and rPRL-A and -D should generate products of 388 and 586 bp, respectively, whereas rPRL-B and -C and rPRL-B and -D generate products of 220 and 418 bp, respectively. Primers for the amplification of rat cyclophilin were rCyc-1 (5′-CGACATCACGGCTGATGGCGAGCCC-3′) and rCyc-2 (5′-TTACAGGGTATTGCGAGCAGATGGGG-3′).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR). Five micrograms of total RNA was reverse transcribed for 1 h at 37°C in 30 μl containing 20 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM each dNTP, 1 μg of oligo(dT) primer, 1 μg of random primer, 25 units of RNasin (Promega), and 200 units of RT (Moloney murine leukemia virus). After incubation, the RT mixture was heated at 96°C for 5 min and chilled on ice. Six microliters of the RT reaction mixture was added to the PCR mixture in 50 μl containing 20 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM each dNTP, 50 pmol of each primer, and 1 unit of *Taq* DNA polymerase. After denaturation at 95°C for 1 min and 30 sec, amplification was performed for 30 cycles (94°C for 30 sec, 65°C for 1 min, and 72°C for 1 min and 30 sec), followed by 72°C for 10 min at the end of the amplification.

Southern Blot Analysis. Five to 10 μl of the RT-PCR reaction mixture was loaded on a 1.8% agarose gel and blotted onto Zeta-Probe GT membranes (Bio-Rad). Membranes were hybridized with primer rPRL-B at 42°C for 16 h in a solution containing 5× SSC (1× = 150 mM NaCl/15 mM sodium citrate, pH 7.0), 0.02 M NaH_2PO_4, 7% SDS, 10× Denhardt's solution, and salmon sperm DNA at 100 μg/ml, washed at 50°C, and exposed to Kodak XAR-5 film at −80°C.

Analysis of PRL-Cleaving Activity. A 14,000 × *g* particulate fraction from isolated PVN or neurohypophyseal lobe homogenates was prepared and incubated with rat PRL (B-6; NHPP) as described (11). The PRL cleavage products were analyzed by Western blots probed with the 16K PRL antiserum.

RESULTS

Specificity of the 16K PRL Antiserum. The 16K PRL antiserum was characterized in a 16K PRL RIA (Fig. 1*A*). Increasing concentrations of 16K PRL displaced radioiodinated 16K PRL from the 16K PRL antiserum with an EC_{50} 1/100 that of 23K PRL (0.2 vs. 20 nM, respectively). Western blots probed with the 16K PRL antiserum stained the 23K and 16K PRL standards with similar intensities (Fig. 1*B*).

Immunocytochemical Detection of a 16K PRL-Like Antigen in the Hypothalamic–Neurohypophyseal System. Immunocytochemistry performed with the 16K PRL antiserum showed a positive signal throughout the anterior hypophysis (Fig. 2*B*), indistinguishable from that obtained with the 23K PRL antiserum (Fig. 2*A*). In the neurohypophysis the 16K PRL antiserum showed positive staining homogeneously distributed throughout the section (Fig. 2*B*), whereas no positive immunoreaction was observed when the 23K PRL antiserum was used (Fig. 2*A*). Neither antiserum stained the intermediate hypophyseal lobe (Fig. 2 *A* and *B*). 16K PRL immunostaining was eliminated from both the anterior and neurohypophyses by preabsorption with 16K PRL (1 μM; Fig. 2*C*) and 23K PRL (10 μM; not shown). However, the immunoreaction was not modified by preabsorption with vasopressin (200 μM), oxytocin (200 μM), neurophysins I or II (20 μM; not shown). Cells that corresponded in size to magnocellular neurons were heavily stained with the 16K PRL antiserum in both the hypothalamic PVN and the SON (Fig. 2 *D* and *E*). Consistent with the location of the immunoreactive perikarya in the above nuclei, nerve fibers were labeled in the internal lamina of the median eminence (Fig. 2*F*), the pathway of magnocellular neuronal projections from both nuclei towards the neurohypophysis (17).

Western Blot Detection of 16K PRL-Like Antigens in the Hypothalamic–Neurohypophyseal System. Neurohypophyseal extracts and the medium from incubated neurohypophyses were analyzed by Western blots probed with the 16K PRL antiserum (Fig. 3). In neurohypophyseal extracts, the immunoreactivity was associated with a predominant protein band with a migration rate corresponding to an apparent molecular mass of 14 kDa and with less-abundant proteins with appar-

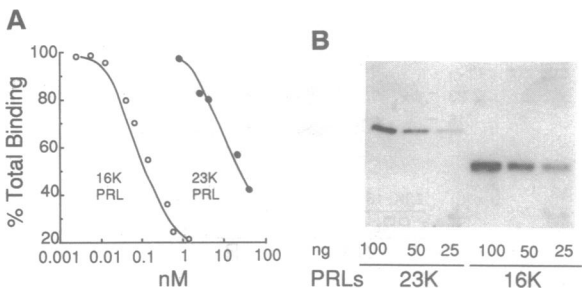

FIG. 1. (*A*) Displacement of ^{125}I-16K PRL from the 16K PRL antiserum (1:20,000) with increasing concentrations of 16K and 23K PRL standards. Values are means of triplicate determinations. (*B*) Different concentrations of the 23K and 16K PRL standards separated by SDS/PAGE, blotted into a nitrocellulose membrane, and probed with the 16K PRL antiserum (1:500).

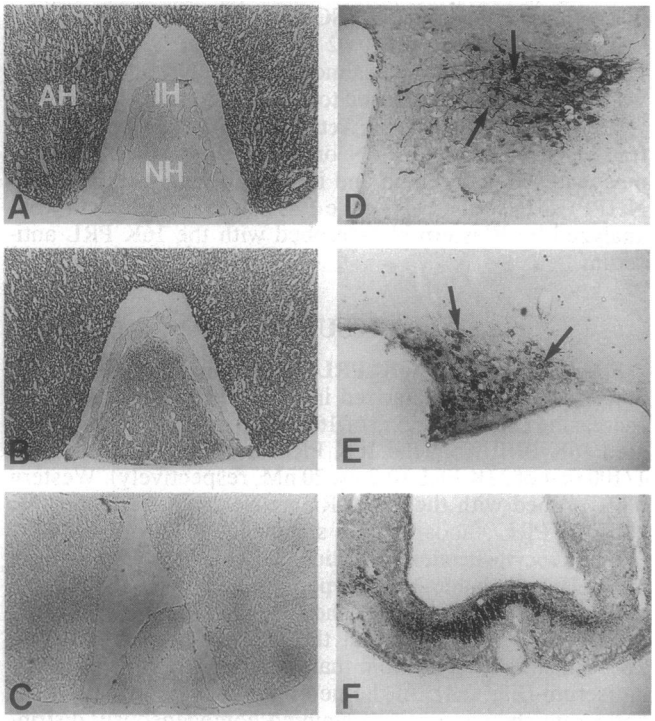

FIG. 2. (*A–C*) Hypophyseal horizontal sections showing the anterior (AH), intermediate (IH), and neural (NH) lobes: immunoreaction with 23K PRL (*A*) or 16K PRL (*B* and *C*) antisera (1:500). In *C*, the 16K PRL antiserum was preabsorbed with 16K PRL (1 μM). (*D–F*) Immunoreaction with 16K PRL antiserum (1:500) in the hypothalamic PVN (*D*) and supraoptic nucleus (SON) (*E*) and in the internal lamina of the median eminence (*F*). Labeled magnocellular neurons (arrows) are indicated. Results are representative of 10 independent experiments. (×10.)

ent molecular masses of 23 and 25 kDa. The 14- and 23-kDa immunoreactive proteins were present both under nonreducing conditions and after reduction of the proteins with 5% (vol/vol) 2-mercaptoethanol, whereas the 25-kDa protein disappeared under reducing conditions. Medium conditioned by neurohypophyseal cultures, while being devoid of many of the proteins in the neurohypophyseal extract, consistently showed the presence of the 14- and the 23-kDa immunoreactive proteins. A 5-fold higher concentration of the 14-kDa over the 23-kDa immunoreactive protein was found (15 and 3 ng/μl, respectively) in the conditioned medium by densitometric analysis calibrated with different concentrations of the 23K PRL standard. No 25-kDa immunoreactive protein was detected in the conditioned medium by nonreducing

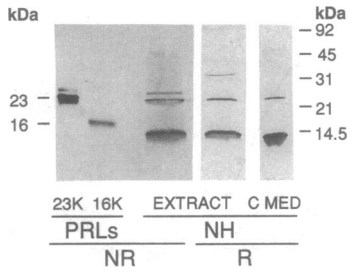

FIG. 3. Blots probed with the antiserum to 16K PRL (1:500) from a neurohypophyseal (NH) extract and medium conditioned (C Med) by incubated neurohypophyses (1 h in Krebs–Ringer solution at 37°C). Proteins were separated by SDS/PAGE under nonreducing (NR) or reducing (R) conditions. The migration positions of 23K and 16K PRL standards and molecular mass markers are indicated at left and right, respectively.

Western blots (not shown). When an equal amount of the 16K PRL standard was added to either the neurohypophyseal extract or the conditioned medium before Western blot analysis, two bands that comigrated with the 16K PRL standard and the 14-kDa immunoreactive protein were observed in both mixtures (not shown).

Inhibition of Endothelial Cell Proliferation by the Conditioned Medium of Incubated Neurohypophyses. At 12.5 nM, 16K PRL inhibited the bFGF-stimulated proliferation of BBCE cells, while the same concentration of 23K PRL had no effect (Fig. 4*A*). The neurohypophyseal conditioned medium (1:75) inhibited the endothelial cell proliferation stimulated by bFGF, whereas the nonconditioned medium was inactive (Fig. 4*B*). The inhibition of bFGF-stimulated BBCE cell proliferation by the above concentrations of 16K PRL and the neurohypophyseal conditioned medium was blocked by coincubation with the 16K PRL antiserum (1:1000) (Fig. 4).

Detection of PRL mRNA in the PVN. Total RNA prepared from isolated PVNs was reverse transcribed and amplified by using primers A, B, C, and D complementary to nucleotides in exons 2, 3, 4, and 5 of the rat PRL gene, respectively. Southern blot analysis of the PCR products after an amplification of 30 cycles is shown in Fig. 5. Amplification of the PVN samples with the PRL primer combinations A/C, A/D, B/C, and B/D yielded fragments of 388, 586, 220, and 418 bp, respectively. The sizes of the amplified products were con-

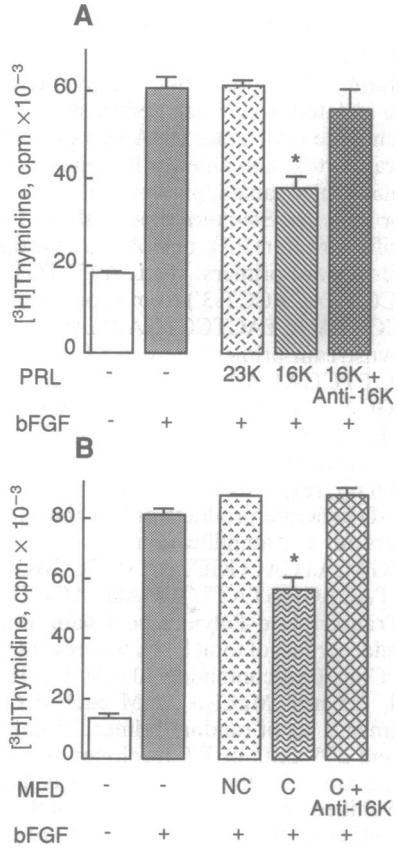

FIG. 4. [3H]Thymidine incorporation into BBCE cells was measured in response to the following conditions: (*A*) bFGF (50 pM) alone or in combination with 12.5 nM 23K PRL, 16K PRL, or 16K PRL together with the 16K PRL antiserum (1:1000 dilution). (*B*) bFGF (50 pM) alone or in combination with nonconditioned (NC) or conditioned (C) media (1:75) or conditioned medium together with the 16K PRL antiserum (1:1000 dilution). Conditioned medium contained the 14-kDa immunoreactive protein at 12.5 nM in the final concentration tested. Values are means of triplicate determinations. *, $P < 0.05$ vs. corresponding controls.

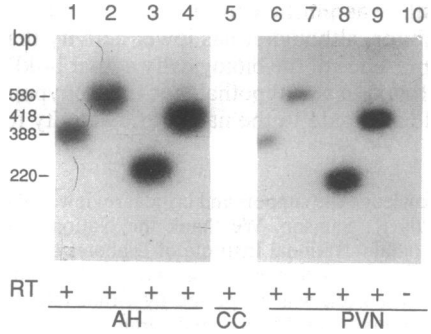

FIG. 5. Southern blot analysis of PCR fragments from reverse-transcribed total RNA from the anterior hypophysis (AH), cerebral cortex (CC), and PVN, amplified (30 cycles) with different rat PRL cDNA primer combinations. Lanes 1 and 6, amplified fragment with primer A and C (388 bp); lanes 2 and 7, primers A and D (586 bp); lanes 3 and 8, primers B and C (220 bp); lanes 4, 5, 9, and 10, primers B and D (418 bp); lane 10, without RT (negative control with RNA). Hybridization was with primer B. The size of the fragments is indicated. Autoradiographs for PVN and CC were exposed for 16 h with an enhancing screen and the autoradiograph for AH, 1 h without screen.

sistent with the predicted sizes for the full-length 23K PRL mRNA and identical to those amplified by the same primer combinations in the anterior hypophysis-positive control. No positive signal was observed in cerebral cortex, where no immunostaining was detected (not shown). Similarly, no signal was detected in the absence of RT (negative controls). The cyclophilin gene was amplified in the PVN and anterior hypophysis (not shown). No smaller products were detected that could relate to an alternative spliced transcript even after a 60-cycle amplification (not shown).

PRL-Cleaving Enzymes in the Hypothalamic–Neurohypophyseal System. The incubation of 23K PRL with acidified membrane extracts from the PVN or neurohypophysis resulted in the proteolysis of added PRL to yield cleaved PRL, as evidenced by Western blots probed with the 16K PRL antiserum carried out under nonreducing and reducing conditions. A 25-kDa immunoreactive protein was generated (not shown), which upon reduction dissociated into 16K PRL (Fig. 6). The PRL-cleaving enzymatic activity of the PVN was less potent than that of the neurohypophysis, since some 23K PRL remained after incubation with the PVN extract but not after the incubation with the neurohypophyseal extract.

DISCUSSION

This report demonstrates the presence of PRL mRNA in the hypothalamic–neurohypophyseal system of the rat and indicates at this site not only the synthesis of PRL but also its preferential posttranslational processing into a lower molecular weight form. In addition, we have found a case in which a small molecular weight form of PRL, a 14-kDa fragment, predominates over the full-length 23K PRL. We also show that this 14-kDa molecular form shares immunological and biological properties with 16K PRL, a fragment of PRL with well-characterized antiangiogenic properties and recently discovered natriuretic effects.

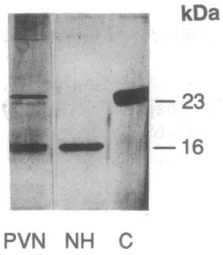

FIG. 6. Reducing Western blot probed with the 16K PRL antiserum showing the proteolysis of PRL (2 μg) after incubation with membranes (3 μg of protein) from the PVN, the neurohypophysis (NH), or in the control incubated without tissue (C). Results are representative of three independent experiments.

PRL synthesis has been reported in several extrahypophyseal tissues, including the brain. Production of brain PRL has been supported by immunocytochemical, RIA, and bioassay data from normal and hypophysectomized animals (18–20). Using RT-PCR, Emanuele and colleagues (21) demonstrated the presence of PRL mRNA in whole hypothalamus and in several brain extrahypothalamic regions, indicating the expression of PRL in the brain. Evidence for the molecular heterogeneity of brain PRL has been provided. Different antigenic determinants have been proposed to exist in brain and pituitary PRLs (22), and Western blot analysis has indicated the presence of cleaved PRL in homogenates of whole hypothalamus (5). Moreover, the brain was shown to contain not only the 23K PRL mRNA but also a deletion mutant that encodes a protein with a predicted molecular mass of 16 kDa (21).

In this study we have used polyclonal antibodies directed against the 16-kDa N-terminal fragment of PRL coupled with immunocytochemical techniques to investigate the endogenous localization of 16K PRL-like proteins. The antiserum is specific for 16K PRL with low potency to react with 23K PRL in solution and has no crossreactivity with several major peptides from the hypothalamic–neurohypophyseal system. Immunocytochemical images are consistent with the localization of 16K PRL-like antigens in the hypothalamic–neurohypophyseal system of the rat. The immunoreaction was found in magnocellular neurons within the PVN and SON, in their neuronal projections towards the neurohypophysis (internal lamina of the median eminence), and in the neurohypophysis itself.

The finding of PRL in the hypothalamic–neurohypophyseal system is somehow surprising. Using antisera raised against 23K PRL, most reports failed to detect immunoreactive PRL in the PVN and in the neurohypophysis, although they did indicate PRL-immunopositive cells in the SON, but only after the inhibition of axonal transport by colchicine treatment (19). Considering that PRL fragments such as 16K PRL have low immunoreactivity with 23K PRL antibodies (6), we propose that the failure to detect PRL immunoreactivity in this system by prior studies was due to the predominance of small fragments of the protein.

To further characterize the 16K PRL-like antigens found in the hypothalamic–neurohypophyseal system, we analyzed neurohypophyseal extracts by Western blots probed with the 16K PRL antiserum. The immunoreactivity found in the neurohypophysis was associated with a protein of an apparent molecular mass of 14 kDa, and with less abundant 23- and 25-kDa proteins, indicating that the 14-kDa molecule is the main protein recognized by the 16K PRL antiserum in the immunocytochemical studies. In support of the 14-kDa protein as a PRL fragment, we have observed that the discrepancy of ≈2 kDa between this protein and 16K PRL does not appear to involve an artefactual protein mobility due to interference with material from the neurohypophyseal samples. 16K PRL standard added to neurohypophyseal extracts or conditioned media migrates to a 16-kDa position as a band distinct from that of the 14-kDa immunoreactive protein. The 14-kDa protein could correspond to a proteolytically processed product of 16K PRL or to an independent product of PRL proteolysis. During recombinant synthesis of primate PRL, Cole and colleagues (23) observed a cleavage site at Ile-133 that could generate a PRL fragment of approximately 14 kDa. Two other immunoreactive proteins were detected in the neurohypophysis that comigrated with 23K PRL and cleaved PRL, respectively. The fact that the 25-kDa protein disappeared under reducing conditions favors its nature as cleaved PRL. However, the 25-kDa protein did not resolve into a 16-kDa band under reducing conditions as would have

been expected for cleaved PRL, albeit a slight contribution of the 25-kDa protein to the 14-kDa band cannot be ruled out. The relatively low amounts of 23K PRL found are in agreement with its lack of detection by immunocytochemical studies.

We found an additional 16K PRL-like property of the 14-kDa immunoreactive protein by analyzing the antiangiogenic bioactivity of the conditioned media of neurohypophyseal cultures. The *in vitro* inhibition of endothelial cell proliferation is a specific effect of 16K PRL not shared with 23K PRL (7, 8). The conditioned medium inhibited BBCE cell proliferation, and this inhibition was blocked by the 16K PRL antiserum. Accordingly, inhibition of endothelial cell proliferation by the conditioned medium involves immunoreactive 16K PRL-like molecules. We have shown that this medium contains two such molecules, a 14-kDa and a 23-kDa protein. The inhibition is not likely to involve the 23-kDa protein, since, as previously reported, and confirmed in this study, 23K PRL is incapable of inhibiting growth of endothelial cells. On the other hand, the 14-kDa immunoreactive protein is present in conditioned medium in a higher concentration (5-fold) than 23K PRL. In fact, the tested dose of the conditioned medium contained the 14-kDa protein at 12.5 nM and displayed an inhibition of magnitude (40%) similar to that produced by 12.5 nM 16K PRL. These results support the idea that the 14-kDa protein could correspond to an endogenous form of PRL with 16K PRL-like actions. Definitive identification of the 14-kDa protein awaits determination of its primary amino acid sequence.

We have investigated whether these members of the PRL family are synthesized locally and whether the 14-kDa form could result from an alternatively spliced PRL message or the posttranslational modification of 23K PRL. In isolated PVNs, RT-PCR amplification and Southern analysis revealed products with the same size of those predicted for the full-length 23K PRL mRNA, and thus, identical to those amplified in the anterior hypophysis. Emanuele and colleagues (21) detected a deletion mutant mRNA in the rat hypothalamus, which lacks exon 4. We failed to detect such a fragment, even when a probe specific to exon 3 was used. These results indicate that the full-length PRL mRNA is expressed in the PVN, and thus support the conclusion that PRLs found in the hypothalamic–neurohypophyseal system are produced locally. Furthermore, the results show that the 14-kDa immunoreactive protein is generated not by an alternative splicing but by the proteolysis and reduction of the 23K PRL molecule. These conclusions are substantiated by the finding of small amounts of 23K PRL and cleaved PRL, together with the PRL-cleaving enzymes in the PVN and the neurohypophysis.

The functional implication of an antiangiogenic factor produced in the hypothalamic–neurohypophyseal system is not clear. Of interest is the fact that magnocellular neurons in both the PVN and SON express high levels of vascular endothelial growth factor (VEGF) mRNA (24). A more direct implication for the localization of a 16K PRL-like bioactive molecule at this site is derived from the observation that systemically administered 16K PRL, but not 23K PRL, stimulates sodium and water excretion in the anesthetized rat (C.C. and W. K. Samson, unpublished observations). This finding is consistent with the well-known role of the hypothalamic–neurohypophyseal system in the regulation of fluid and electrolyte homeostasis (25). In this regard it has long been claimed that the neurohypophysis secretes a natriuretic peptide that acts as a Na^+,K^+-ATPase inhibitor (26). Recently the 2.5-kDa joining peptide of the proopiomelanocortin molecule was purified from neurohypophysis as a Na^+ pump inhibitor, although it has lower activity than expected (27). The presence of the biologically active 14-kDa PRL-like molecule found in the hypothalamic–neurohypophyseal system could contribute to the natriuretic activity found at this site.

We acknowledge the support and critical review of the manuscript by Dr. Willis K. Samson. We thank the National Hormone and Pituitary Program–National Institute of Diabetes and Digestive and Kidney Diseases for their generous gifts of rat PRL and anti-rat PRL antiserum. This work was supported by Grant IN201992 from the National University of Mexico and Grant 1893-N9212 from the National Council of Science and Technology of Mexico.

1. Nicoll, C. S. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 2563–2566.
2. Russell, D. H. (1989) *Trends Pharmacol. Sci.* **10**, 40–44.
3. Sinha, Y. N. (1992) *Trends Endocrinol. Metab.* **3**, 100–106.
4. Montgomery, D. W., Shen, G. K., Ulrich, E. D., Steiner, L. L., Parrish, P. R. & Zukoski, C. F. (1992) *Endocrinology* **131**, 3019–3026.
5. DeVito, W. J. (1988) *Biochem. Biophys. Res. Commun.* **150**, 599–604.
6. Clapp, C., Sears, P., Russell, D., Richards, J., Levay-Young, B. & Nicoll, C. S. (1988) *Endocrinology* **122**, 2892–2898.
7. Ferrara, N., Clapp, C. & Weiner, R. I. (1991) *Endocrinology* **129**, 896–900.
8. Clapp, C., Martial, J. A., Guzman, R. C., Rentier-Delrue, F. & Weiner, R. I. (1993) *Endocrinology* **133**, 1292–1299.
9. Clapp, C. & Weiner, R. I. (1992) *Endocrinology* **130**, 1380–1386.
10. Clapp, C., Sears, P. S. & Nicoll, C. S. (1989) *Endocrinology* **125**, 1054–1059.
11. Clapp, C. (1987) *Endocrinology* **121**, 2055–2064.
12. Andries, M., Tilemans, D. & Denef, C. (1992) *Biochem. J.* **281**, 393–400.
13. Baldocchi, R. A., Tan, L., King, D. S. & Nicoll, C. S. (1993) *Endocrinology* **133**, 935–938.
14. Compton, M. M. & Witorsch, R. J. (1984) *Endocrinology* **115**, 476–484.
15. Palkovits, M. (1983) *Methods Enzymol.* **103**, 368–376.
16. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
17. Swanson, L. W. & Sawchenko, P. E. (1983) *Annu. Rev. Neurosci.* **6**, 269–324.
18. Toubeau, G., Desclin, J., Parmentier, M. & Pasteels, J. L. (1979) *J. Endocrinol.* **83**, 261–266.
19. Harlan, R. E., Shivers, B. D., Fox, S. R., Kaplove, K. A., Schachter, B. S. & Pfaff, D. W. (1989) *Neuroendocrinology* **49**, 7–22.
20. Emanuele, N. V., Metcalfe, L., Wallock, L., Tentler, J., Hagen, T. C., Beer, C. T., Martinson, D., Gout, P. W., Kirsteins, L. & Lawrence, A. M. (1986) *Neuroendocrinology* **44**, 217–221.
21. Emanuele, N. V., Jurgens, J. K., Halloran, M. M., Tentler, J. J., Lawrence, A. M. & Kelley, M. R. (1992) *Mol. Endocrinol.* **6**, 35–42.
22. Harlan, R. E. & Scammel, J. G. (1991) *J. Histochem. Cytochem.* **39**, 221–224.
23. Cole, E. S., Nichols, E. H., Lauziere, K., Edmunds, T. & McPherson, J. M. (1991) *Endocrinology* **129**, 2639–2646.
24. Ferrara, N., Houck, K., Jakeman, L. & Leung, D. W. (1993) *Endocr. Rev.* **13**, 18–32.
25. Van Wimersma Greidanus, T. B. & Hellebrekers, L. J. (1991) in *Brain Endocrinology*, ed. Motta, M. (Raven, New York), pp. 377–401.
26. De Wardener, H. E. & Clarkson, E. M. (1985) *Physiol. Rev.* **65**, 658–759.
27. Hamakubo, T., Furuta, H., Ichimura, M., Appalsamy, M., Mosqueda-Garcia, R., Robertson, D. & Inagami, T. (1992) *Biochem. Biophys. Res. Commun.* **189**, 691–696.