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Localization of α-Inhibin-31 in Rodent Testis and Brain by Immunocytochemical Procedure and Western Blot Analysis

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Summary: Using an anti-serum against synthetic α-inhibin-31, we have studied its cellular locations in rodent testis and brain. Western blot analysis detected several molecular forms of the protein(s). A protein of 17 KDa in size was detected in testis of one month old mice. Two major bands of molecular weights 39 kDA and 21 kDA were detected in mature mouse testis. The 39 kDA protein was also present in male brain, female brain and ovary. No protein similar in size to α-inhibin-31 was detected with this antiserum, suggesting that the antibody recognized only the precursor of the α-IB-31 molecule. Immunocytochemical studies revealed that these molecules were located in the cytoplasm of the interstitial Leydig cells. The immunocyto-chemical reaction can be eliminated by pre-incubation with synthetic α-inhibin-31. Immunoreactive substances were also demonstrated at the blood-brain barrier on the external surface of the ependymal cells in the third ventricle of the rat brain. In the pituitary, similar pattern of localization was also observed in the cuboidal-columnar epithelial cells that lined the par tuberalis between the anterior and intermediate lobes. The immunoreaction to the neural cells cannot be completely abolished by competition with the synthetic peptide. These observations suggested that α-inhibins are located in the Leydig cells of the testis as well as in the brain, pituitary and ovary.

Biologically active peptides are important in regulating the endocrine functions of the hypothalamus-pituitary-gonadal axis. The gonadotropin releasing hormone (GnRH) (1,2) synthesized in the hypothalamus stimulates the biosyntheses of follicle stimulating hormone (FSH) and leuteinizing hormone (LH)

Abbreviations: GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; IB, inhibin; HRP, horse raddish peroxidase.

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in the anterior pituitary gland. Both FSH and LH are vital in the proper functions of the gonads (3,4). As a feedback mechanism, some water soluble gonadal peptides, termed inhibins (5-7), are secreted in the gonads to preferrentially down-regulate the biosynthesis of FSH in the pituitary gland. Several laboratories have isolated various forms of inhibins from both the ovary and the testis. The genes for some of the ovarian inhibins have also been isolated by recombinant DNA techniques (for a recent review, see 8).

We have isolated from human seminal plasma (9) an inhibin-like peptide containing 31 amino acids [ILP-31 or α-inhibin-31 (α-IB-31)] that subsequently was shown to be a segment of larger molecules with 52 (α-IB-52) and 92 (α-IB-92) amino acid residues (10). These large peptides also showed inhibin-like activities by in vitro assays. We have produced antisera in rabbits against synthetic peptides of these molecules (11,12). Anti-α-IB-31 sera (11) cross-reacted with α-IB-52 and α-IB-92 (10). In this report, we describe the immunocytochemisty and Western blot studies using the antiserum (11) against α-IB-31.

MATERIALS AND METHODS

Animals and tissue sections. Adult Sprague-Dawley rats and Bulb/C mice of different ages were obtained from Simonsen Laboratories (Gilroy, CA). Tissue sections were obtained by perfusion fixation with 4% formaldehyde and cryosectioned as described (13).

Immunocytochemistry. Freshly prepared cryosections were pretreated for 1 hr at 37°C with a blocking solution containing 20% fetal bovine serum in TBS buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl) and 0.05% (v/v) of Tween-20 (TBST). The slides were reacted with anti-α-IB-31 serum (11, 1:300 to 3,000 dilutions) in the same buffer for 2-3 hr at 37°C, washed extensively with TBST buffer, and then reacted similarly with the affinity-purified goat antibodies against rabbit IgG that had been conjugated with horse raddish peroxidase (HRP) (Pel-Freez Biologicals) for 2-3 hr at 37°C. After washing with 5 changes of TBS buffer for 20 min each, the slides were reacted with the substrate solution (0.5 mg/ml 4-chloro-1-napthol, 0.01 M imidazole, and 0.5% (v/v) hydrogen peroxide in TBS) for 30 min at room temperature. They were then washed with 3 changes of

TBS and counter-stained with either ethidium bromide or methylene blue. For ethidium bromide stained slides, they were mounted under coverglass with 50% glycerol in TBS and examined with both epiflourescence and dark field microscopy. The hematoxylin stained slides were mounted with Permount and examined with conventional light microscopy. All photomicroscopic analyses were performed with a Zeiss Photomicroscope III equipped with both UV epiflourescence and tungsten light sources and an automatic camera.

Western blot analysis. Mice of different ages were euthanasized by cervical dislocations. The various tissues were dissected out and homogenized immediately in TBS containing 0.5% Nonidet-40. The supernatants were adjusted to 2.3% sodium dodecyl sulfate (SDS), 10% glycerol and 0.1% bromophenol blue and analyzed with either 10% or 15% SDS-polyacrylamide gel electrophoresis. Commercial protein markers were included as molecular weight references (BRL). Separated proteins were transferred electrophoretically to nitrocellulose filter paper using a Bio-Rad Trans-blot cell. The filters were processed in a similar procedure as that described for cytochemistry. After blocking with fetal bovine serum, the filters were reacted with antiserum against α-IB-31 overnight under gentle agitation at room temperature. They were washed extensively and reacted with HRP-conjugated secondary antibody. The antibody binding was visualized by substrate reactions as before.

RESULTS AND DISCUSSION

Previously, we have reported α-IB-92 immunoreactivity in human sera, hypothalamus and pituitary gland (12). In order to identify the reactive proteins of the testis and brain in more detail, we have used the Western blot analysis to study tissues isolated from mice at different ages after birth. The antiserum against α-IB-31 reacted readily with the synthetic α-IB-31, but did not recognize any natural proteins of the same molecular weight (data not shown). This observation suggested that the antiserum against α-IB-31 detected probably the precursor molecules of this peptide. A protein species of 17 kDa molecular weight was detected in the testicular extracts of mice at one month after birth (Fig. 1, lane 1). Two major bands of molecular weights 39 and 21 kDa were detected in the mature testes of 3, 6, and 9 months old mice. Minor bands of 68 and 32 kDa were also observed in these testes (Fig. 1, lanes 2-4). The 39 kDa proteins were readily detectable in the brains of mice at 1, 3, 6, and 9 months of age (Fig. 1, lanes 5-8). The

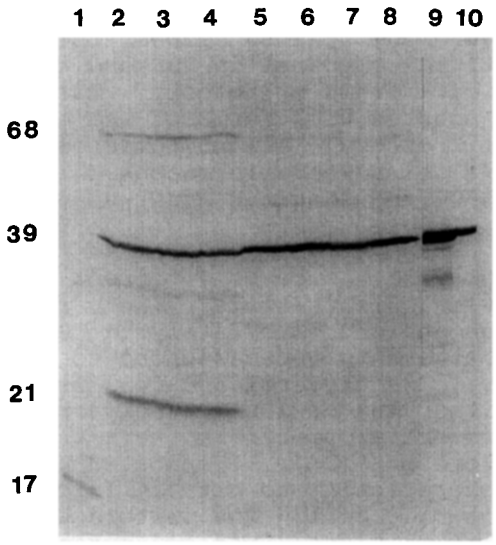

Figure 1. Western blot analysis of protein extracts from mouse tissues. Lanes 1, 2, 3, and 4: testicular extracts from 1, 3, 6, and 9 month old mice. Lanes 5, 6, 7, 8: brain extracts from 1, 3, 6, and 9 month old male mice. Lanes 9 and 10: protein extracts of ovary and brain from adult female mice. A major protein of 39 kDa is present in all tissues except the testicular extract from one month old mice. Other minor protein species of molecular weights 17, 21, and 68 kDa are also present in the testicular preparations.

present observations suggest the possibility that the 39 kDa precursor molecule for α-IB-31 is synthesized in both brain and testis of these mice. The onset of synthesis is somewhat earlier in the brain than in the testis. In addition, this 39 kDa protein was readily detected in the ovary and brain from adult female mice (Fig. 1, lanes 9, 10), suggesting that this protein may also be synthesized in these organs in the female.

In order to identify the cell types that contain the precursor molecules of α-IB-31, immunocytochemical reactions were performed with rat testis and brain sections. The interstitial Leydig cells in the testis were most intensely labelled with HRP substrate precipitates (Fig. 2A). Most precipitates were located within the cytoplasm of these cells. No significant labels were observed inside the seminerferous tubules. The antibody binding to these reactive materials in

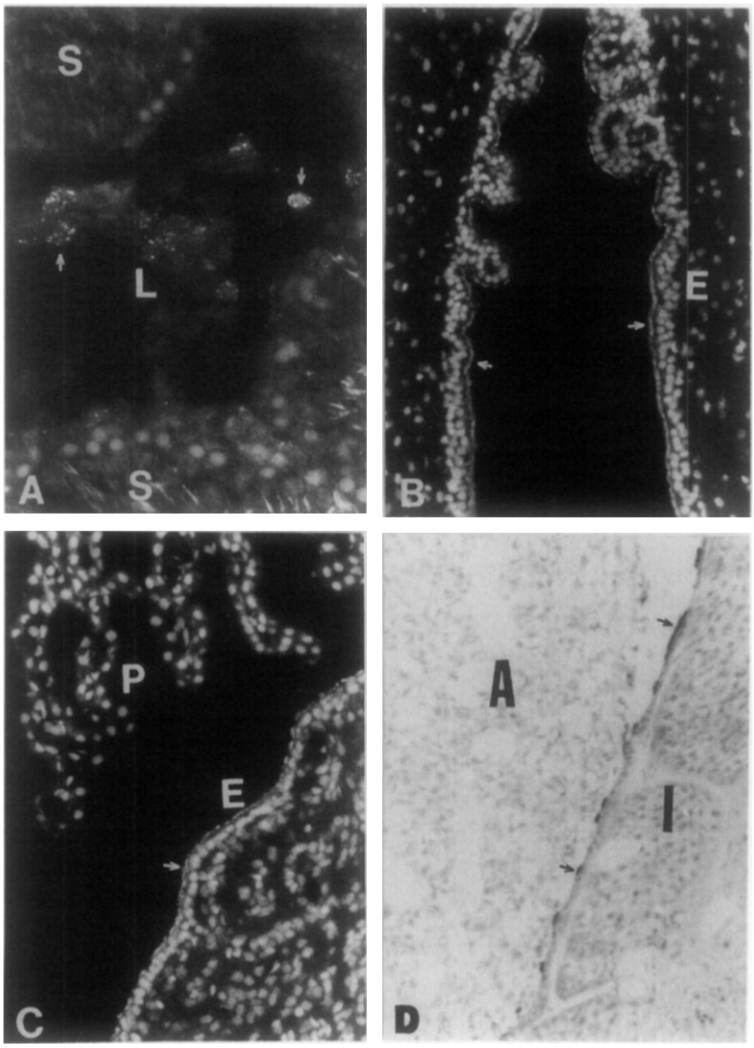

Figure 2. Cytological locations of the α-IB-31 precursor in rat testis and brain. **A. Testis.** The cytoplasm of the Leydig cells (L) are labelled with precipitates (arrows) from the immunoreaction. Little labels are detected in the seminerferous tubules (S). After the HRP substrate reaction, the cells were counter-stained with ethidium bromide. The section was photographed with combination of epiflourescence and dark filed optics. The immuno-precipitates were represented by bright reflective grains and the morphology of the cells were outlined by ethidium bromide flourescence. **B. Third ventricle of the brain.** The cuboidal ependymal cells (E) line the blood-brain barrier. Immunoprecipitates are located on the external lining of these cells within the ventricle (arrows). Same optics as in A. **C. Lateral ventricle of the brain.** Similar staining (arrows) of the ependymal cells (E) as in the third ventricle. Cells of the choroid plexus (P) are not labelled. Same optics as in A. **D. Pituitary.** Immuno-precipitates (arrows) are localized on the external surface of the cuboidal-columnar epithelial cells that line the par tuberalis (blood-brain barrier) between the anterior lobe (A) and intermediate lobe (I) of the pituitary gland. After the immunocytochemical reaction, the cells were counter-stained with methylene blue. Observations were recorded by bright field optics.

the Leydig cells was abolished by preincubation of the antiserum with the synthetic α-IB-31 (Fig. 3A, B, C, and D). Since the Sertoli cells have been postulated as the source of testicular inhibin (14,15), the localization of α-IB-31 precursors in the Leydig cells indicates that other biological peptides can also contain inhibin-like activities. Seidah et al. (16) had demonstrated a 94 amino acid peptide (β-inhibin) from human seminal plasma that had inhibin-like activity. Recent isolation and molecular characterization of its cDNA identified the human prostate as the synthetic site of β-inhibin (17).

Since previous RIA analyses had shown that α-IB-31, α-IB-52 and α-IB-92 were present in both the hypothalamus and the pituitary (12), we had focused our immunocytochemical studies of the α-IB-31 precursor in the brain on the third ventricular region and the pituitary gland. The major locations of the reactive materials to the antiserum are at the blood-brain barriers in both the third ventricle (Fig. 2B) and the lateral ventricle (Fig. 2C). Specifically, most of the labels were localized on the ventricular ciliates of the ependymal cells that line the blood-brain barrier. This observation suggests that the α-IB-31 materials are bound on the external membrane of these cells in the ventricular fluid. Interestingly, cells of the choroid plexus were not labelled with the immunocytochemical reactions (Fig. 2C). In the pituitary, most of the reactive materials were located on the cuboidal-columnar epithelial cells that line the par tuberalis between the anterior and intermediate (Fig. 2D). The morphology of these cells are very similar to that of ependymal cells of the third ventricle. Again, the labels were concentrated on the external membranes of these cells within the par tuberalis fluid. The immunoreactions of these materials in both the ependymal cells in the ventricles

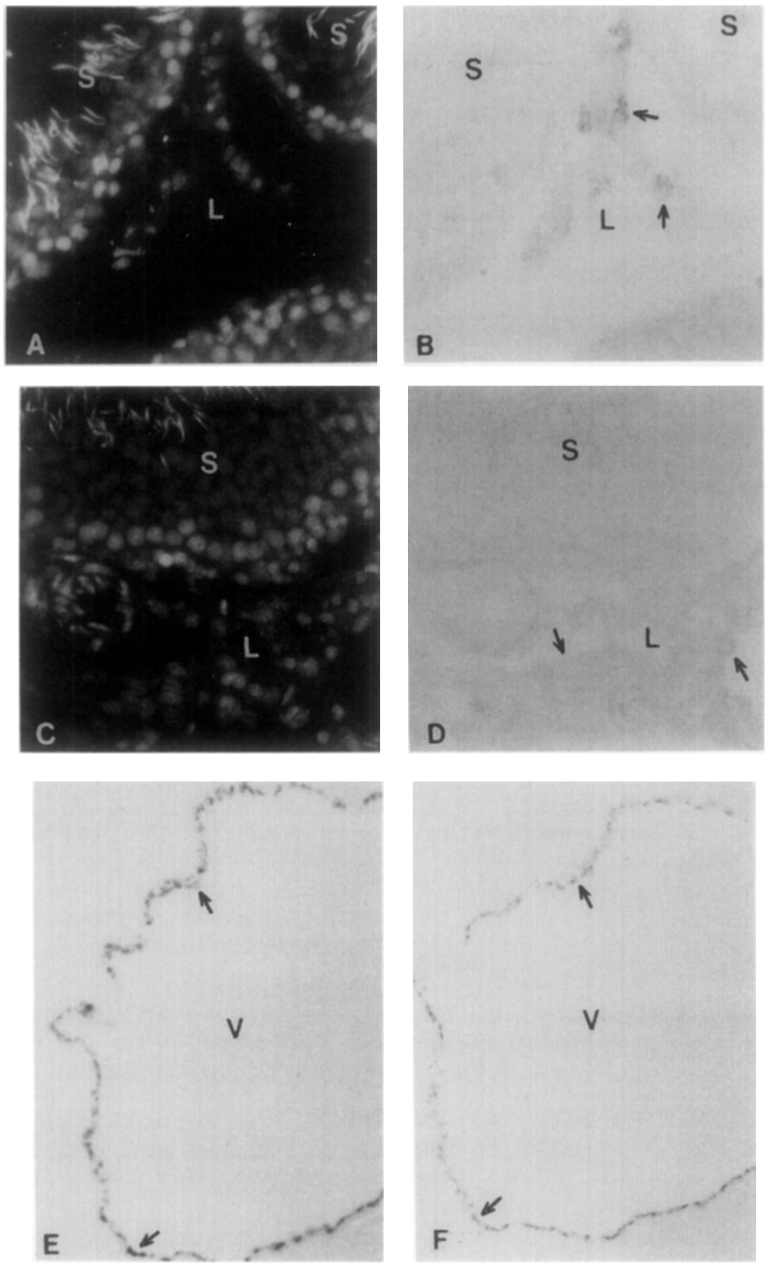

Figure 3. *Effects of pre-incubation of anti-α-IB-31 serum with synthetic α-IB-31 prior to immunocytochemical reaction.* **A.** Testicular section stained with ethidium bromide showing seminerferous tubules (S) and interstitial Leydig cells (L). **B.** Same section as in A. showing immuno-staining (arrows) of the Leydig cells by untreated anti-α-IB-31 serum. **C.** Testicular section stained with ethidium bromide showing seminerferous tubules (S) and Leydig cells (L). **D.** Same section as in C. showing a complete elimination of immuno-staining of the Leydig cells (arrows) by anti-α-IB-31 serum at 37°C for 1 h. **E.** Immuno-staining (arrows) of the ependymal cells of the third ventricle (V) in the brain with untreated anti-α-IB-31 serum. **F.** A serial section as in E. stained with anti-α-IB-31 serum preincubated with synthetic α-IB-31 as in D. Partial abolishment of the immunoreaction was observed.

and cuboidal-columnar epithelial cells to the antiserum were always stronger than those of the Leydig cells in the testis. Competition experiments showed that the immunoreaction could be reduced but not completely abolished by preincubation with synthetic α-IB-31 (Fig. 3E and F).

The present study has demonstrated the existence of reactive proteins to the anti-α-IB-31 serum in the testis, ovary and brain. However, the exact nature of the different molecular forms of α-IB-31 precursors in the testis is not clear. Significantly, only the 39 kDa proteins were present in the brain, ovary and testis.

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