Cellular Localization of Puromycin-Sensitive Aminopeptidase Isozymes

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We developed monoclonal antibodies (mAbs) against two isozymes of a cytosolic puromycin-sensitive aminopeptidase (PSA-I and PSA-II) purified from chicken brain. The isozymes could be distinguished using Ouchterlony double-immunodiffusion and Western immunoblot. Their distribution in neuronal and glial cells as visualized by indirect immunofluorescence with these mAbs was found to differ: PSA-I was confined mostly to glial lysosomes; PSA-II showed fibrillar distribution in both types of nerve cells, but in disparate patterns. These results and our findings of peptide structural differences suggest that the two PSA isozymes are expressed differently in the nervous system.

KEY WORDS: Aminopeptidase; cellular localization; enkephalinase; tissue culture; lysosomes.

INTRODUCTION

Puromycin-sensitive aminopeptidase (PSA) was first found in spleen lysosomes in 1941, but it has not been characterized well enough to be assigned an IUB enzyme number (1). It is the most abundant aminopeptidase in the brain, 80% of the activity being in the soluble and 20% in the membrane fraction (2). In vitro, it is active in formation and breakdown of neuropeptides, especially enkephalins (3). Cytosolic and membrane-associated PSAs have been purified and characterized from different vertebrates (4-8). Two forms of the cytosolic PSA were found in rat (9) and chicken brain (10). Recently, we analyzed the PSA-I and PSA-II purified to homogeneity from chicken brain by peptide mapping and sequencing, and found that their primary structures are related but not identical (11). The data indicated that they derive from translation of two mRNAs, whose genes most likely result from gene duplication. Since the isozymes are usually cell-specifically and/or temporally expressed, knowledge of their localizations in the nerve cells is important for shedding light on their cellular function. With anti-PSA mAbs, immunofluorescence showed the distribution of these isozymes to differ in primary chicken brain cultures.

EXPERIMENTAL PROCEDURE

Production of mAbs to PSA. The anti-PSA mAbs were produced by hybridoma techniques (12). PSA-I and PSA-II were purified as described elsewhere (11). BALB/C mice were immunized by biweekly intraperitoneal injections of 50 µg of each of the purified PSAs emulsified in Freund’s complete adjuvant for 6 weeks. Blood was collected from the tail veins and serum was analyzed for antibody production by Ouchterlony double-immunodiffusion (13). When an immunoprecipitin band was seen, spleen cells were harvested, incubated with 0.17 M ammonium chloride at 4°C for 10 min to lyse erythrocytes, and fused with a myeloma cell line (P3x63Ag8) at a ratio of 10:1 in 50% polyethylene glycol 1000 for 4 min. Cells were evenly suspended and distributed into 96-well microtiter plates (one drop per well). The next day an additional drop of double-strength HAT medium (hypoxanthine 1.36 mg/100 ml, aminopterin 0.036 mg/100 ml, thymidine 0.387 mg/100 ml) was added to each well. After one week, cells were fed with one drop of HT medium (hypoxanthine, thymidine; no aminopterin) to select for the growth of hybrids. About 17 days after cell fusion, antibody-producing hybrids were detected by an ELISA using...
pure PSA. Positive clones were subcloned by limiting dilution, re-screened with the pure antigens, and grown to confluency in 250-ml bottles. Clones yielding positive responses in an additional ELISA were then injected into the peritoneal cavity of immunosuppressed mice. Ascites fluid was collected 7-10 days later, retested in the ELISA, and stored at −80°C.

**Primary Neuronal and Glial Cell Cultures.** Primary neuronal cell culture from chicken embryonic telencephalon was established according to Pettman et al. (14). The neuronal cell cultures were prepared from 7- to 8-day embryonic chicken telencephalon (15). The absence of glial cells in this culture was due partly to late gliogenesis, which occurs after the 8th day in the in vivo development of chick telencephalon, and partly to suppression by polylysine substrate of the growth of the small number of glial cells contaminating this culture. To prepare pure glial cell culture, cells from 14-day-old embryonic telencephalon were kept for 3 weeks for segregating the neuronal cells.

**Western Immunoblot.** The Western blot transfer technique of Towbin et al. (16) was followed with some modifications. Briefly, 20 ug of protein of the partially purified PSA was applied to a 10% SDS slab gel and electrophoresed for 4 hr at 20 mA (17). Proteins were then transferred from SDS gel to a nitrocellulose sheet by electrophoretic transfer at 200 mA for 1 hr. The nitrocellulose sheet was then treated with 3% bovine serum albumin at room temperature for 2 hr. After rinsing in 0.01 M phosphate-saline buffer containing 0.2% bovine serum albumin and 0.05% Tween-20, the nitrocellulose strips were immersed in 0.02 mg/ml of anti-PSA mAb for 2 hr at room temperature. For the control experiments, the same amount of mAb isolated from preimmune serum was used instead of anti-PSA mAb. After brief rinsing with the same buffer, the strips were incubated with peroxidase-labeled goat anti-mouse IgG at 1:1000 dilution for 1 hr, followed by a brief incubation with peroxidase substrate.

**Immunofluorescence Studies of PSAs in Primary Cultures.** The cells in the dish were first washed with phosphate-buffered saline (PBS) then fixed with 4% paraformaldehyde and 0.01% glutaraldehyde, which were later washed out (18). The fixed cells were permeabilized with 0.05% saponin for 30 min and incubated with the primary antibodies (mAbs) for 2 hr at room temperature. The cells were then rinsed thoroughly with PBS containing 1% normal goat serum and incubated with labeled second antibodies (FITC-Goat anti-mouse IgG) for 1 hr. After being rinsed, they were examined under a fluorescent microscope equipped with relevant filters for FITC.

**RESULTS**

**Immunodiffusion.** Sera from the PSA-I and PSA-II immunized mice were used for Ouchterlony double-immunodiffusion. Precipitin lines were visualized either directly or after being stained with Coomassie blue. PSA-I formed one precipitin line with either of the antisera (Figure 1A). PSA-II formed two precipitin lines with its own antiserum (Figure 1B). PSA-II also cross-reacted with the PSA-I polyclonal antibodies, indicating that PSA-I and PSA-II share some common immunodeterminants.

**Immunospecificity of anti-PSA mAbs.** The cross-reactivity of the polyclonal antibodies prompted us to develop mAbs for PSA-I and PSA-II. They were selected by ELISA and Western immunoblot. Ten cell lines which produced mAbs for PSA-I and two for PSA-II were developed. MAb anti-I from hybridoma [2E11K] and anti-II from [B10-III] were selected for PSA-I and PSA-II respectively because of their selectivity and high titer. Anti-I reacted with the PSA-I 105-kDa protein (Figure 2), and Anti-II reacted specifically with the PSA-II 100-kDa protein.

**Indirect Immunofluorescence.** In immunofluorescence, PSA-II was distributed in all permeabilized neurons (Figure 3A) and glia (Figure 3B). Its fibrillar patterns indicated that it was cytoplasmic. Patches of PSA-II associated with the inner surface of the plasma membranes were found in the neuronal soma and the neurites. In most of the glia, PSA-II appeared as a hollow filamentous spindle engulfing the nucleus. It is not clear whether PSA-II is associated with other cytoskeletal proteins, or whether it establishes its own fibrous networks. PSA-II formed a highly structured matrix in the glial cytoplasm, and such structures were changeable (Figure 4). PSA-II was not evenly distributed. In some cells, it was mostly localized near the nucleus (Figure 4B); in others, near the plasma membrane (Figure 4D). In most of the glia, its network connected the nucleus and the plasma membrane (Figure 4A and 4C).

For PSA-I, anti-I labeled about 15% of the total glial cells. It was mostly localized in vesicles around glial nuclei (Figure 3C). These vesicles could be stained with acridine orange, an indicator for acidic organelles. Their morphology demonstrated that they were lysosomes. Not all the acidine-positive vesicles were labeled by anti-I, showing that PSA-I was localized only in certain populations or metabolic stages of lysosomes.