IMMUNOCYTOCHEMICAL LOCALIZATION OF S-100 PROTEIN AND CALMODULIN IN C6 GLIOMA CELLS

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The localization of S-100, calmodulin and tubulin in the cultured C6 glioma cells was observed with the indirect immunoperoxidase method. The direct immunoperoxidase method for S-100 was also employed. The reaction products for S-100, calmodulin and tubulin were all found over filamentous structures in the interphase cells. Calmodulin and tubulin seemed to be localized in the cytoplasm, while S-100 was exhibited as intense granular reaction product in the nuclei.

In the mitotic cells, these proteins were shown to be present with characteristic localization in the mitotic apparatus. Calmodulin-specific staining was highly intense at the spindle poles.

In contrast, S-100 was shown to be concentrated on the chromosomes in all phases of mitotic cells. The direct method showed more accurate and defined localization of S-100 than the indirect method. This is the first demonstration of the possible interrelationship between S-100 protein and chromosome, although the biological significance of the fact is not known at present.

S-100 protein and calmodulin belong to the family of calcium-binding proteins which include homologous calcium-binding sites. Therefore, it was suggested that several Ca^{2+} -binding proteins have evolved from an ancestral precursor protein (7).

The S-100 protein unique to the nervous system is known to be primarily concentrated in the glial cells (5), although it has also been observed in other cells such as adipocytes (17), human chondrocytes (16) or folliculostellate cells of the pituitary gland (14). Biochemical studies have shown that S-100 is a mixture of two predominant components designated as S-100a and S-100b (8). These investigators have also shown that S-100a is a dimer of α - and β -subunit and S-100b is a dimer of two β -subunits. However, the biological role of S-100 remains to be clarified.

On the other hand, calmodulin is now generally believed to act as an intracellular receptor for many Ca^{2+} -mediating processes. Calmodulin was shown by immunofluorescence procedure to be localized in the mitotic apparatus in several types of mitotic cells, and to associate with the actin-containing microfilament

bundles in the interphase cells (4, 12, 19, 20). The tau factor, one of the microtubule-associated proteins which affect the assembly of microtubules *in vitro*, has been identified as calmodulin binding protein (15). These observations suggest that calmodulin might be involved in the regulation of the cyto-skeletal system.

Rat C6 glioma cells represented the first cloned cell line demonstrated to contain calmodulin (2), which has also been shown to be enriched in S-100 protein (11). Therefore, C6 glioma cell is an unique and favorable model system for studying both S-100 and calmodulin within a single cell. However, no detailed study for subcellular localization of S-100 in cultured cells has been made.

In the present study, we demonstrate the immunocytochemical localization of S-100 together with calmodulin and tubulin in C6 glioma cells.

MATERIALS AND METHODS

Cell culture

The rat glioma cell line C6 BU-1, obtained from Dr. Higashida, University of Kanazawa, was cultured at 37°C in 95% Dulbecco's modified Eagles medium with 5% fetal bovine serum without antibiotics in a humidified atmosphere of 95% air and 5% CO₂.

Cells were subcultured in log phase growth at least 24 hr prior to experiments in glass dishes containing 18×18-mm glass cover slips.

Antibodies

The production and isolation of rabbit antibodies to bovine brain native calmodulin and bovine brain S-100 β -subunit were performed as described elsewhere (9, 10). The specific immunoglobulin fractions were purified by appropriate antigen-conjugated affinity columns. The immunological properties of the antibodies for these proteins have also been described in detail (9, 10). Anti-S-100 β -subunit is able to react with S-100b and also S-100a, since both of these proteins include β -subunit. The monoclonal antibody to bovine brain α -tubulin was purchased from Amersham.

Immunocytochemical procedures

Cells on cover slips were fixed in 3.7% buffered formalin for 90 min at room temperature, post fixed for 7 min in acetone at -20°C, then incubated with a drop (100 μ l) of 10% goat serum and 3% BSA in Tris-Saline (10 mM Tris-HCl, 0.9% NaCl, pH 7.4) for 30 min. Subsequently, cells were incubated with 50 μ l of first antibodies (rabbit anti-calmodulin IgG, 8 μ g/ml, rabbit anti-S-100 IgG, 8 μ g/ml or mouse anti-tubulin IgG, 5 μ g/ml) in Tris-Saline with 3% BSA for 60 min. After 5 washes for 30 min each in Tris-Saline, cells were incubated with 50 μ l of appropriate peroxidase-labeled 2nd antibody (1 : 100 diluted goat anti-rabbit IgG or rabbit anti-mouse IgG, both purchased from Miles-Yeda LTD) in Tris-Saline with 3% BSA for 60 min.

Cells were washed with Tris-Saline for 30 min as described and finally incubated for 10 min in 3,3'-diaminobenzidine (0.2 mg/ml) and hydrogen peroxide at a final concentration of 0.03% with 10 mM Tris-HCl buffer (pH 7.4). The stained cells

on cover slips were rinsed in 10 mM NaN_3 solution and mounted on glass slides with glycerol-jelly.

For the direct immunoperoxidase procedure of S-100, S-100 β -specific Fab' was prepared and was conjugated with horseradish peroxidase [Hydrogen peroxide oxidoreductase E.C. 1,11,1,7] as described by Yoshitake *et al.* (21). As the control, the labeled anti-S-100 antibody was pretreated with 8-fold excess bovine brain S-100 and then was used for the incubation of specimens. Pretreatment of the antibody with calmodulin was also applied.

RESULTS

Immunoperoxidase staining of S-100 and calmodulin.

Indirect immunoperoxidase staining with specific antibodies against S-100 or calmodulin produced a distinctive and reproducible pattern in the fixed C6 glioma cells (Fig. 1). Under the same conditions, preimmune rabbit IgG did not exhibit any specific staining in the cells.

The direct immunoperoxidase method for S-100 was also tested as shown in Fig. 2. When cells were incubated with the labeled S-100-specific Fab' pretreated with 8-fold excess purified bovine brain S-100 protein, no reaction product was found. But no interference of the staining for S-100 was observed by pretreatment of the labeled antibody with the same amount of purified bovine brain calmodulin (Fig. 2b).

Subsequent close examination were performed by the direct method for S-100, because it produced a similar but clearer staining pattern to those from the indirect method.

Localization of S-100, calmodulin and tubulin in interphase cells.

The reaction product for S-100, calmodulin and tubulin were all found over

FIGS. 1a-c. Specificity of the indirect immunoperoxidase staining of S-100 and calmodulin is shown. The cells incubated with preimmune rabbit IgG (a) exhibit no reaction product. The cells incubated with anti-calmodulin IgG (b) or with anti-S-100 IgG (c) exhibit specific localization of these proteins. $\times 200$

FIG. 2.

FIG. 3.

FIG. 4.

filamentous structures in interphase cells (Fig. 3). The observed distribution of the reaction product for calmodulin and tubulin were similar, both of which were almost negative on the nuclei in interphase cells (Figs. 3a-d).

In contrast, S-100 was exhibited as intense granular reaction product in the nuclei, although it was also found in a lower intensity in the cytoplasm (Figs. 3e,f).

Localization of S-100, calmodulin and tubulin during mitosis.

In the mitotic cells, tubulin, calmodulin and S-100 were all shown to be present in each characteristic localization of mitotic apparatus (Fig. 4). The reaction product for tubulin was observed over the spindle fibers of metaphase and teleophase (Figs. 4a-e). In contrast, calmodulin-specific staining was highly intense at the spindle poles in all phases of mitotic cells (Figs. 4f-j). The half spindles between the poles and chromosomes were also slightly stained (Figs. 4f, g).

On the other hand, the heavy deposit specific for S-100 was exactly demonstrated on the chromosomes in all phases of mitotic cells (Figs. 4k-o). As the cells entered prophase, S-100 specific reaction product emerged as a number of dense particles which seemed to be a formation of chromosomes (Fig. 4k).

DISCUSSION

Recent publications of indirect immunofluorescence studies have shown that calmodulin might associate with fibrous elements in interphase cells (4, 12, 20). Observations of mitotic cells revealed that calmodulin was concentrated in the spindle poles and the half spindles between the poles and chromosomes (4, 12, 19, 20), suggesting that calmodulin might be involved in the regulation of the movement of chromosomes to the poles (4).

Our present study represented the similar localization of calmodulin in interphase cells and also in mitotic cells of cultured C6 glioma.

The S-100 and calmodulin have homologous Ca^{2+} -binding domains which could form EF-hand (18), and this fact suggests that they belong to the Ca^{2+} -binding protein family also including troponin C or parvalbumin. Calmodulin can be considered to be a Ca^{2+} receptor in non muscle cells, and troponin C may serve the same function in the skeletal muscle cells. The S-100 may play an analogous role to calmodulin in glial cells. In fact, the S-100 protein is indicated to induce disassembly of microtubules with a high efficiency than calmodulin *in vitro*, although the physiological significance in this study is unclear (1).

In the present results, S-100 appears to be distributed on fibrous or granular

FIGS. 2a-c. Specificity of the direct immunoperoxidase method for S-100 is shown. The specimens were incubated with peroxidase-labeled anti-S-100 Fab'. The characteristic staining (c) did not occur when anti-S-100 Fab' was pretreated with 8-fold excess purified bovine brain S-100 for 2 hr at 30°C (a), but did not alter after pretreatment of the antibody with calmodulin (b). $\times 200$

FIGS. 3a-f. Staining with the indirect method for tubulin (a, b), calmodulin (c, d) and with the direct method for S-100 (e, f) is shown in interphase cells. $\times 1,000$

FIGS. 4a-o. Staining with the indirect method for tubulin (a-e), calmodulin (f-j) and with the direct method for S-100 (k-o). The characteristic localization of each protein in mitotic cells is shown; tubulin over spindle fibers, calmodulin at the spindle poles and slightly at the half spindles between the poles and chromosomes, and S-100 on the chromosomes. $\times 1,000$

structures predominantly in the nuclei of interphase cells, differing from the staining pattern of calmodulin. Further, we have obtained strikingly different localization of S-100 from calmodulin in mitotic cells.

Nuclear localization of S-100 has been reported in several studies (3, 13), but our present result is of the first demonstration of a specialized chromosomal localization of S-100. The possibility that this acidic protein may interact with the nuclear basic proteins during the immunocytochemical procedures is ruled out since another acidic protein, calmodulin was demonstrated at a different location in the same cells.

Recently, Ishikawa *et al.* (6) reported that S-100 protein stimulates the release of prolactin from prolactin-secreting clonal cells *in vitro*.

Suzuki *et al.* (18) reported the enhancement of adipose S-100 protein release by catecholamine. According to our knowledge, these are the only works implying the physiological role of this protein.

Our present immunocytochemical study of S-100 in mitotic C6 glioma cells could afford additional clues for understanding the biological role of this protein.

Biochemical determination of S-100 in the isolated chromatin fraction, and the electron microscopic examination of S-100 in the chromosomes are now in progress.

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