Regulation of the Synthesis of S-100 Protein in Rat Glial Cells*

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The influence of serum on the synthesis of the nervous system specific S-100 protein has been investigated in the rat glial cell clone C61a. In sparse cells, where S-100 synthesis is low, removal of serum leads to a 2- to 3-fold increase of the rate of S-100 synthesis relative to total soluble cell protein. Measurements of S-100 content by radioimmunoassay showed that sparse cells are accumulating S-100 much more rapidly in the absence of serum.

Our findings suggest that serum components inhibit the expression of S-100 synthesis in sparse cells by a concentration-dependent mechanism. This effect does not correlate directly with the reduced cellular growth rate in low serum or serum-free medium. Lipid-free medium, which has little growth-promoting activity, maintains the capacity to lower the rate of S-100 synthesis. A partially purified extract of platelets can substitute for serum in repressing the synthesis of the S-100 protein.

S-100 is an acidic, soluble protein that is specific for neural tissue and located primarily in glial cells (see Ref. 1 for review). The term S-100 protein designates a group of acidic polypeptides defined immunologically. Whether one or more of these chains is represented in C6 cells is not known. A detailed discussion of these polypeptides is presented in Ref. 1. Several glial cell lines have been established that synthesize S-100 in tissue culture (2, 3). The rat glioma line C6 was reported to accumulate S-100 in the stationary phase. Detailed investigations by Pfeiffer et al. (4, 5) showed that the S-100 content increased at the end of the logarithmic growth phase and suggested that homologous cell-cell contacts may be involved in the increase of the S-100 protein. The increased content of S-100 in dense cells was found to be regulated at the level of protein synthesis with no apparent change in the rate of degradation (6, 7).

The induction of S-100 in C6 cells may serve as a model system for the study of some aspects of the differentiation of neural cells. Although no specific function has so far been attributed to S-100, its widespread occurrence in brain of vertebrate species, and the expression during neural maturation, has attracted considerable interest (1). Little is known so far about the molecular mechanism of S-100 regulation in C6 cells. Although the original observations by Pfeiffer et al. (4) support the hypothesis of cell-cell contacts triggering a signal process, we have found that increased S-100 synthesis, no components of such a system have yet been identified. Recent experiments by Marks and Labourdette (8) showed that drugs which disrupt microtubules inhibit the synthesis of S-100 in stationary cultures. In addition, succinyl concanavalin A stimulated the synthesis of S-100 in logarithmically growing cells. They suggested that binding of the lectin to one or more glycoproteins on the cell surface may trigger a signal that is then transmitted to the sites of transcription or translation by a process that involves elements of the cytoskeleton.

The present report shows that serum components, possibly in part derived from platelets, also play a role in regulating the synthesis of S-100 in C6 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The rat glioma clone C61A was obtained from Dr. S. E. Pfeiffer, University of Connecticut, Farmington, Conn., and grown in minimal essential medium supplemented with nonessential amino acids (0.2 mM) and glutamine (2 mM) (9), and 10% fetal calf serum (Pacific Biological). Stock cultures were maintained in T-flasks of 75 cm² surface (Falcon) in 15 ml of medium. For subcultures, 5 × 10⁶ cells/2 ml were transferred at intervals of 6 to 8 days and grown at 37°C in a humidified incubator with the flow of air and CO₂ adjusted to maintain the medium between pH 7.2 and 7.4. For most experiments, cells were plated in tissue culture dishes of 6-cm diameter (Falcon) in 5 ml of medium. Fresh medium was added in 2-day intervals.

**Cell Counts**—For growth curves, cell monolayers were washed twice with NaCl/P,1 and detached with 0.04% trypsin and 0.04% EDTA in NaCl/P. Cell numbers were determined with an electronic particle counter (Coulter), calibrated with a hemacytometer.

**Labeling Conditions, Preparation of Soluble Extracts**—To determine the rate of S-100 synthesis as a fraction of the rate of total soluble protein synthesis, cells in a 6-cm dish were washed three times with 2 ml of supplemented medium with reduced leucine content (20 μM) and incubated for 3 h with 80 μCi/ml of [4,5-H]leucine (New England Nuclear, 51.6 Ci/mmol) in 2.5 ml of the same medium. At the end of the pulse, the cells were washed three times with 2 ml of NaCl/P, containing 0.01% leucine. VT buffer (0.5 ml) was added and the dishes were put on dry ice. The cells were broken by five cycles of freezing and thawing, then scraped with a rubber policeman and transferred to a centrifuge tube in a final volume of 0.7 ml. Supernatants were prepared by centrifugation at 30,000 rpm for 1 h in a SW 50.1 rotor in 0.7-ml tubes in nylan adapters. All samples were frozen and stored at −70°C until assayed.

**Rate of Synthesis of S-100**—The method used are described in

1 The abbreviations used are: NaCl/P, (phosphate-buffered saline), 0.14 M NaCl, 10 mM sodium phosphate, pH 7.2; VT buffer, 50 mM sodium veronal, pH 8.0, 0.1% Triton X-100, 80 mM NaCl, 0.1 mM EDTA, and 0.02% NaN₃; PFG, fibroblast growth factor; IGF, epidermal growth factor.
RESULTS AND DISCUSSION

The synthesis of S-100 in C6 cells has been reported to be induced when the cells are in close contact at the stationary phase of growth (3-7). During work that was initiated to study the molecular basis of this phenomenon, it was discovered that the rate of S-100 synthesis was increased when the cells were incubated without serum. This is contrary to earlier observations from other laboratories (4, 8). Possible reasons for this discrepancy will be discussed below.

When serum is removed from logarithmically-growing C61a cells (Fig. 2), the relative rate of S-100 synthesis increases linearly during the first 3 days. After this initial phase, the synthesis rate continues to increase at a slower rate and is about five times higher after 8 days without serum. The effect was observed not only with C6 cells obtained from Dr. R. T. Proffitt, S. Feldman, B. P. Schimmer (University of Toronto) and used in the experiments of Labourdette and Marks (7) (Fig. 3) as well as with cells obtained from Dr. Augusti Tocci, Laboratorio de Embriologia, Naples, Italy (data not shown). Cells proliferate very slowly in serum-free medium (Fig. 2). We used delipidated serum to rule out the possibility of a simple correlation between growth rate and S-100 synthesis. Serum lipids were extracted under conditions that do not denature proteins (13). Within 2 days after addition of delipidated serum, cells grow

Portions of this paper (including "Materials and Methods," additional Figs. 1 to 4, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda Md. 20014. Request Document No. 79M-1688, cite authors and include a check or money order for $1.80 per set of photocopies.

mental design is that described under “Experimental Procedures,” using C6 cells obtained from Dr. S. Pfeiffer (C6P) or C6 cells obtained from Dr. B. Schimmer (C6S), which were grown as described in the legend to Fig. 2 and, where indicated, were switched to serum-free medium 2 days prior to labeling with [3H]leucine. Panels A, A’, etc., represent duplicate gels. Input counts represent total radioactivity in protein slices No. 33 is the dye front.

Logarithmic growth is fully restored by this lipid resupplementation.

Fig. 5 shows the relative rate of S-100 synthesis in sparse cells that have been incubated for 2 days with different concentrations of normal and delipidated serum. It can be seen that the modulation of S-100 synthesis is gradual and occurs over a wide range of serum concentration. The effect of delipidated serum on S-100 synthesis is similar to that of normal serum. It can therefore be concluded that it is not sufficient to inhibit cellular division in order to induce the synthesis of S-100 protein. Additional evidence for the distinction between cell growth and serum-regulated synthesis was obtained by inhibiting cellular division with hydroxyurea, which did not significantly affect the synthesis of S-100 in the presence of serum (data not shown), as had been reported previously by Pfeiffer et al. (4).

An induction in the rate of S-100 synthesis is expected to cause the protein to accumulate intracellularly, unless there also occurs a proportional change in the rate of degradation or the protein is secreted. To distinguish between these possibilities, the S-100 content of C6 cells was determined by radioimmunoassay. Sparse cells, 5 days after serum removal, contain 5 times more S-100 than parallel cultures that were grown in serum (Table I). Therefore, the increased rate of S-100 synthesis in serum-free medium leads to accumulation of S-100 in the cells. The alternate and more direct approach to the question would be to measure the rate of turnover of S-100 directly. In attempts to do such experiments, we found that the high levels of radioactivity required to label subconfluent cells were toxic when these cells were grown in the absence of serum. Therefore, direct measurements of the rate of S-100 degradation could not be carried out.

The data in Fig. 2 were obtained under conditions where cell density of the cells in 10% serum and of the cells in serum-free medium was not identical and was changing during the experiment. It appears important to know to what extent cell density effects are superimposed on the effects of serum starvation. The data in Fig. 6 were obtained by plating cells at different densities such that, at the time at which the rate of S-100 synthesis was measured, the cell density of all cultures was essentially identical (approximately 1 x 10^5 cells/cm^2). Under these conditions, removal of serum results in a time-dependent increase in the rate of S-100 synthesis in cells maintained in the absence of serum, while the rate of S-100 synthesis in sparse cells in different serum supplements. C6 cells were plated as described in the legend to Fig. 2 and then incubated without serum (A) or with 10% dialyzed serum that had been delipidated (B), delipidated and resupplemented with a mixture of fatty acids and cholesterol (C), and with untreated control serum (D). Protein concentrations were the same in delipidated and control serum. The total radioactivity in protein/10^6 cells was 3.53 x 10^6 cpm (no serum), 3.77 x 10^6 cpm in 10% delipidated serum, and 4.37 x 10^6 cpm in 10% fetal calf serum.

Fig. 4. Growth of C6 cells in different serum supplements. C6 cells were plated as described in the legend to Fig. 2 and then incubated without serum (A) or with 10% dialyzed serum that had been delipidated (B), delipidated and resupplemented with a mixture of fatty acids and cholesterol (C), and with untreated control serum (D). Protein concentrations were the same in delipidated and control serum. The total radioactivity in protein/10^6 cells was 3.53 x 10^6 cpm (no serum), 3.77 x 10^6 cpm in 10% delipidated serum, and 4.37 x 10^6 cpm in 10% fetal calf serum.
that at least two mechanisms exist for controlling the synthesis of S-100 cells. Cell cultures were grown in T-25 flasks with 1.2 × 10^5 cells (for 10% serum control) or 7.5 × 10^4 cells (no serum samples). After 2 days in 10% serum, the cells in the flask were washed three times with serum-free medium and incubated with or without serum supplement. Fresh medium was added 1 and 4 days later. On the 5th day, the cells were rinsed with warm NaCl/P, and harvested by scraping with a rubber policeman. The suspended cells were washed two more times with NaCl/P, by centrifugation at 150 × g for 5 min. The cell pellets, containing 2.7 to 3.7 × 10^4 cells from 4 flasks, were freeze-thawed in V7 buffer and cell supernatants (1.4 ml, final volume) were prepared as described under "Experimental Procedures." Aliquots were removed for determinations of total protein content (12). The S-100 protein content was determined in duplicate dishes as described (1).

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Relative S-100 content as per cent of soluble protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Serum</td>
<td>10% Serum</td>
</tr>
<tr>
<td>1</td>
<td>0.234</td>
</tr>
<tr>
<td>2</td>
<td>0.324</td>
</tr>
</tbody>
</table>

**Fig. 6.** Time course of the rate of S-100 synthesis after serum removal in sparse, density-matched cells. Cells were incubated at different densities. After 2 days in 10% serum, the cells were washed and further incubated in serum-free medium (Δ) or in 10% serum (□) for the indicated time. The relative rate of S-100 synthesis was determined in duplicate dishes as described. In order to obtain comparable cell densities at the time of labeling with [3H]leucine, the different growth rates and length of incubation were compensated by appropriately varying the number of cells plated. All densities were in the range 2.8 to 3.5 × 10^4/dish and, for 10% serum, 2.1 to 4.2 × 10^4/dish.

**TABLE II**

<table>
<thead>
<tr>
<th>Serum concentration</th>
<th>Rate of S-100 synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% soluble protein</td>
</tr>
<tr>
<td>10</td>
<td>0.021</td>
</tr>
<tr>
<td>3</td>
<td>0.034</td>
</tr>
<tr>
<td>1</td>
<td>0.044</td>
</tr>
<tr>
<td>0.1</td>
<td>0.048</td>
</tr>
<tr>
<td>0</td>
<td>0.062</td>
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</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Total soluble protein</th>
<th>S-100 synthesis rate in per cent of soluble protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10% Serum</td>
<td>4.54</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td>I</td>
<td>Insulin + transferrin</td>
<td>4.88</td>
<td>0.067 ± 0.007</td>
</tr>
<tr>
<td>I</td>
<td>FGF</td>
<td>5.24</td>
<td>0.063 ± 0.007</td>
</tr>
<tr>
<td>I</td>
<td>Insulin + transferrin</td>
<td>4.54</td>
<td>0.064 ± 0.003</td>
</tr>
<tr>
<td>I</td>
<td>EGF</td>
<td>4.98</td>
<td>0.023 ± 0.001</td>
</tr>
<tr>
<td>I</td>
<td>Platelet factor</td>
<td>4.16</td>
<td>0.067 ± 0.004</td>
</tr>
<tr>
<td>II</td>
<td>10% Serum</td>
<td>4.55</td>
<td>0.023 ± 0.002</td>
</tr>
<tr>
<td>II</td>
<td>Platelet factor</td>
<td>5.50</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>II</td>
<td>No addition</td>
<td>3.92</td>
<td>0.058 ± 0.002</td>
</tr>
</tbody>
</table>

C6. This medium contains insulin, transferrin, FGF, and a submaxillary gland extract. We have tested some of these components as well as a platelet extract for their ability to repress the synthesis of S-100 protein. The data in Table III show that neither insulin, transferrin plus EGF (16), nor insulin, transferrin plus FGF (17), inhibit the rate of S-100 synthesis, but addition of a partially purified platelet extract inhibits S-100 synthesis. The active component in the platelet extract does not increase the growth rate of C6 cells under these conditions. It seems unlikely that the active component is the platelet-derived growth factor (18) alone because, in preliminary experiments, platelet extracts purified beyond CM-Sephadex chromatography with hydroxylapatite lose the ability to repress S-100 protein synthesis but retain growth-stimulatory activity for 3T3 cells. It clearly will be of interest to purify the material present in platelets that is responsible for the repression of S-100 synthesis.

There is a discrepancy between our results and those reported by Pfeiffer et al. (4) and Labourdette and Marks (7) in that neither of these investigators found an increase in S-100 synthesis in the absence of serum. One possibility is that the cell line used by us has changed from the original cells. We cannot entirely rule out this possibility. However, as shown...
above (Fig. 3), we have tested, in addition to the cells originally obtained from Dr. Pfeiffer, cells supplied by Dr. B. P. Schimmer, as well as cells supplied by Dr. Augusti Tocco (data not shown), and find that they behave identically under our growth conditions. One possible difference between our results and those reported previously is that under our growth conditions, as well as those of Bottenstein et al. (15), C6 cells will grow, although slowly, in the absence of serum, while this was not true under the growth conditions originally employed by Pfeiffer et al. (4). Whether this represents a significant difference remains to be determined.

It would appear from the data presented that two separate mechanisms exist for controlling the rate of S-100 protein synthesis in C6 cells. The first is a repression effect possibly due to binding of a hormone-like molecule to the cell surface, which is present in serum and may be derived in part from platelets. Secondly, there is an induction effect elicited by cell-to-cell contact as originally described by Pfeiffer et al. (4). Either of these effects may be influenced by concanavalin A binding to the cell surface and may be responsible for the induction of S-100 synthesis by this lectin (6).

The function of S-100 is unknown, but one would presume that it serves an important function for glial cells, since it is present in serum and may be derived in part from platelets. Secondly, there is an induction effect elicited by cell-to-cell contact as originally described by Pfeiffer et al. (4). The induction of S-100 protein like S-100 may be controlled, not only by a positive signal (induction) at the cell surface, but also by negative signal (repression) which, in the case of C6 cells, is controlled by binding of a hormone-like molecule to the cell surface and may be responsible for the induction of S-100 synthesis by this lectin (6).

Acknowledgment—We are grateful to Dr. R. Munson for advice and the generous gift of C6 cells, to Dr. M. Lieberman for a gift of epidermal growth factor, and to Drs. S. Pfeiffer, B. P. Schimmer, and Augusti Tocco for cultures of C6 cells.

REFERENCES


Additional references are found on p. 1520.

Supplemental Material to:

Regulation of the Synthesis of S-100 Protein in Rat C6 Glial Cells

By N. Greer, B. Moore, K. T. Proftit, J. W. Over, K. Caldwell, and L. Cimino

Previous premises for the immunoprecipitation of S-100 protein using double antibody precipitation combined with polyacrylamide gel electrophoresis (PAGE) have been described by Froata and Laskoski (2). A new procedure for the isolation of the protein is described in this paper. The isolation was performed by using a column of Sepharose 6B. The protein was then precipitated with polyethylene glycol (PEG) and then electrophoresed on a SDS-PAGE gel. The gel was stained with Coomassie Blue and silver nitrate. The gel was then scanned in a densitometer and the band was excised. The band was then electrophoresed on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then stained with Coomassie Blue and silver nitrate. The membrane was then cut into the desired size and the band was excised. The band was then electrophoresed on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then cut into the desired size and the band was excised. The band was then electrophoresed on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then cut into the desired size and the band was excised. The band was then electrophoresed on a SDS-PAGE gel and transferred to a nitrocellulose membrane.
S-100 Synthesis in Glial Cells

Figure 1. Comparison of S-100 precipitation with second antibody and 1520 in Glial Cells S-100 synthesis. a) 100 pmol of 1520-125I (2500 cpm) in 0.5 ml normal buffer were incubated for 30 min at 0°C followed by 30 min at 4°C. 10% and 20% of the supernatant dilution (prediluted five times) were applied to 3% or 4% polyacrylamide gels (12 cm x 5 cm). After 60 min of electrophoresis, the gels were stained with 0.1% Coomassie blue R-250 in 40% ethanol and washed for 72 h in 4°C. Gel slices were then cut and counted for 5 h in toluene-4HCl. The immunoreactivities were precipitated with 50% PEG and 0.5 M NaCl buffer without removing the precipitate. The gel slices were then boiled for 2 min in 1 M NaOH and 23% mercaptoethanol and counted with 3M NaOH scintillation fluid. The specific precipitation with precipitation was 0.1% EMH fraction was 2.5% for both precipitants. 

C6 cells in high rod density synthesized S-100 proteins (1,2). Panel A of Fig. 2 shows that S-100 precipitation and a soluble extract of confluent C6 cells, followed by polyacrylamide gel electrophoresis, yields a protein with the same electrophoretic characteristics as 1520. Panel B, below. This procedure is shown when the immune precipitable is carried out with 100 pmol of 1520, 230 pmol of 1520, and 230 pmol of 1520.

GEL SLICE NUMBER

Figure 2. Quantitative determination of the relative size of S-100 precursors. Varying amounts of 1520-125I were precipitated and the amount of radiolabeled 1520 precipitated after gel electrophoresis. Extracts were from stationary (S) or logarithmic (G) cultures as in Fig. 3. Sera were analyzed for phosphatase activity in various fractions and were precipitated with 50% PEG and 0.5 M NaCl buffer without removing the precipitate. The gel slices were then boiled for 2 min in 1 M NaOH and 23% mercaptoethanol and counted with 3M NaOH scintillation fluid. The specific precipitation with precipitation was 0.1% EMH fraction was 2.5% for both precipitants. 

GEL SLICE NUMBER

Figure 3.诱导剂对非特异性背景的影响以及与其沉淀的特异性。使用S-100和S-25标记的S-100合成产物被提取并根据沉淀的亲和力差异进行沉淀。图中的数据是通过在不同的条件下对沉淀物进行沉淀来获得的。
Regulation of the synthesis of S-100 protein in rat glial cells.
R Gysin, B W Moore, R T Proffitt, T F Deuel, K Caldwell and L Glaser


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