Modulation of Tyrosine Phosphorylation of p36 and Other Substrates by the S-100 Protein*

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The inhibitory effects of Ca<sup>2+</sup>-binding proteins on tyrosine phosphorylation of p36 protein isolated from bovine intestinal epithelium by immunoprecipitated p130<sub>mm</sub> were investigated. S-100 protein dose dependently inhibited the p36 phosphorylation, and calmodulin weakly depressed the phosphorylation, whereas parvalbumin and tropinin C had no significant effects.

The S-100 preparation purified from bovine brain did not contain phosphatase activity or ATPase activity. The concentration of ATP did not affect the S-100-mediated inhibition of phosphorylation but the substrate protein, p36, reversed the inhibition. S-100 similarly inhibited the tyrosine phosphorylation of p36 by p60<sup>++</sup> but did not affect the p36 phosphorylation by protein kinase C. S-100 inhibited the tyrosine kinase activity of p130<sub>mm</sub> using the other substrates tested as well. These results suggest that S-100 interacts with the substrate binding site of retroviral tyrosine-specific protein kinases and may play a regulatory role in the tyrosine phosphorylation.

A number of oncogenic viruses and growth factor receptors are known to encode or be associated with a tyrosine protein kinase activity (1, 2). A 36-kDa protein called p36 has been identified as the major substrate phosphorylated on tyrosine in Rous sarcoma virus-infected chicken embryo fibroblast cells (3–6). Gerke and Weber (7) isolated the 36-kDa protein from porcine intestinal epithelium, and intestinal p36 was shown to be comprised of a tetramer of M, 36,000 and 10,000 (p10) as a native complex of M, 85,000, termed calpactin 1, that can bind calcium, phospholipid, and actin. Sequence analysis of p10 demonstrated a striking homology with brain S-100 protein, a calcium-binding protein with functions yet to be demonstrated (7, 8).

S-100 is a low molecular weight acidic protein fraction which was first isolated by Moore (9) and termed S-100 to signify its partial solubility in saturated ammonium sulfate at neutral pH. We purified S-100 protein from bovine brain (10) or adipose tissue (11) using W-7-coupled Sepharose and detected it immunologically in T lymphocytes; therefore, the protein is not specific to brain tissue (12).

Little is known about the function of S-100 other than its calcium binding properties. Recent studies have shown that S-100 is a conserved protein fraction among mammals (13, 14), suggesting a potentially important role in cells. Although several in vitro activities of S-100 have been described (15–19), the distinct molecular targets of S-100 have not been identified. Recently calmodulin was found to enhance tyrosine phosphorylation of brain membrane proteins (20), 17-estra- diol receptor (21), and insulin receptor (22). However, little is known about the molecular mechanisms of the stimulation.

In this report the effects of calcium binding proteins including S-100 and calmodulin on tyrosine phosphorylation of p36 by retroviral tyrosine-specific protein kinases were investigated in purified assay systems. S-100 protein specifically inhibited the tyrosine phosphorylation of p36 and other protein substrates in vitro.

EXPERIMENTAL PROCEDURES

Materials—S-100 protein from bovine brain was prepared by Ca<sup>2+</sup>-dependent affinity chromatography on W-7 epoxy-activated Sepharose as described (10). p36 was isolated by the method of Gerke and Weber (23). The Ca<sup>2+</sup>-binding proteins calmodulin, parvalbumin, and tropinin C were purified to homogeneity as determined by SDS-PAGE according to the methods of Yazawa et al. (24), Moews and Krebsinger (25), and Collins (26), respectively. [γ-<sup>32</sup>P]ATP was obtained from Amersham, England. Mouse monoclonal antibody to p60<sup>++</sup> was obtained from Oncor, Inc., and anti-p130<sub>mm</sub> monoclonal antibody was prepared as described (27). Protein kinase C was purified from rabbit brain as described (28). Myosin light chain was prepared from chicken gizzard and rabbit skeletal muscle by the methods of Hathaway and Haeberle (29) and Perry (30). All other chemicals were of reagent grade or the best commercially available.

Phosphorylation Assays—Phosphorylation of p36 by retroviral gene products was carried out using p130<sub>mm</sub> and p60<sup>++</sup> immunoprecipitated from Fujinami or Rous sarcoma virus-infected chick embryo fibroblasts. Cell cultures grown in 100-mm culture plates were placed immediately on ice. All subsequent operations were carried out at 0 °C. Each plate received 2 ml of modified RIPA buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mM EDTA, 1% Trasylol. Cells were scraped from the plates with a rubber policeman and transferred to a 1.5-ml Eppendorf micro test tube. After the samples were vortexed vigorously for 30–45 s, they were centrifuged at 10,000 × g for 5 min. 100-μl aliquots of cell extract were incubated with 2 μl of the indicated antiserum for 60 min on ice. Five serum volumes of Protein A-Sepharose CL-4B (Sigma) (a 50% (v/v) slurry in modified RIPA buffer) were added and mixed for 30 min at 4 °C to absorb immune complexes, according to Kessler (31). The Sepharose pellet was washed 5 times with modified RIPA buffer and twice with 50 mM Tris-HCl (pH 7.4). The packed pellet of immune complex-Protein A-Sepharose was resuspended in 50 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.4), 10 mM MnCl<sub>2</sub>, and 2-10 μg of substrate proteins. The reaction was initiated with the addition of 10<sup>−5</sup> to 10<sup>−7</sup> M [γ-<sup>32</sup>P]ATP (6 × 10<sup>6</sup> cpm) and terminated by the addition of 20 μl of electrophoresis buffer containing 10% SDS. Assays were performed for 5, 10, 15, and 20 min at 30 °C and in all cases demonstrated a linear incorporation of [γ-<sup>32</sup>P]Phosphate into the substrate over the

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* The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation; EGTA, /[(ethylenebis(oxyethylenenitrilo)tetraacetic acid.}
Modulation of Tyrosine Phosphorylation of p36 and Other Substrates

Fig. 1. A, the effect of Ca\(^{2+}\)-binding proteins on p36 phosphorylation by p130\(^{ps}\). Immunoprecipitated p130\(^{ps}\) and 100 \(\mu\)g/ml p36 were incubated with or without (a) 20 \(\mu\)g each of troponin C (b), S-100 (c), parvalbumin (d), and calmodulin (e) in the assay mixture described under "Experimental Procedures." B, the results of subsequent phosphoamino acid analysis of p36 phosphorylated by p130\(^{ps}\) with (c) or without (a) 20 \(\mu\)g of S-100 protein. C, Coomassie staining of Ca\(^{2+}\)-binding proteins. Two \(\mu\)g each of M. marker proteins (a), troponin C (b), S-100 (c), parvalbumin (d), and calmodulin (e) were applied to the SDS-polyacrylamide (20%) gel.

Fig. 2. Dose-dependent inhibition of p36 phosphorylation by Ca\(^{2+}\)-binding proteins. p36 was phosphorylated by p130\(^{ps}\) in the presence of the indicated amount of S-100 (○), calmodulin (●), troponin C (X), and parvalbumin (□).

20-min assay. Electrophoresis was carried out in 10 or 18% polyacrylamide slab gels in the presence of 0.1% sodium dodecyl sulfate at 15 mA. The gel was stained with Coomassie Blue and, after destaining, exposed to Kodak X-Omat AR x-ray film, or \(^{32}\)P radioactivity in gel bands was measured by a liquid scintillation counter. Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 50 \(\mu\)l, 50 mM Tris-HCl (pH 7.0), 10 mM MgCl\(_2\), 0.5 mM CaCl\(_2\) or 1 mM EGTA, 25 \(\mu\)g/ml phosphatidylserine, 10\(^{-3}\) M [\(\gamma^{32}\)P] ATP (2 \(\times\) 10\(^5\) cpm), 2–10 \(\mu\)g of p36 as a substrate, and 0.05 \(\mu\)g of the enzyme (specific activity 0.12 \(\mu\)mol/min/mg) (28). The incubation was carried out at 30 °C for 5 min. The phosphorylation reaction was linear for at least 15 min.

Phosphoamino Acids Analysis—\(^{32}\)P-Labeled protein eluted from gels after tryptic digestion (32) was hydrolyzed in \(\text{HCl}\) for 90 min at 110 °C in 6 N HCl. Phosphoamino acids were separated by electrophoresis at pH 3.5 (1 kV, 80 min) on thin layer cellulose plates. Marker phosphoamino acids (2 \(\mu\)g each) were identified by spraying the plates with a ninhydrin stain and gentle heating, and the labeled phosphoamino acids were detected by autoradiography.

ATPase Assay—The myosin ATPase assay was carried out at 25 °C in a volume of 0.5 ml containing: 50 mM Tris-HCl (pH 7.0), 500 mM KCl, 5 mM EDTA, 0.1 mg/ml myosin, 0.2–1.0 mM ATP, and S-100 (100 \(\mu\)g/ml) for myosin Ca\(^{2+}\)-ATPase; 50 mM Tris-HCl (pH 7.0), 500 mM KCl, 10 mM CaCl\(_2\), 0.1 mg/ml myosin, 0.2–1.0 mM ATP, and S-100 (100 \(\mu\)g/ml) for myosin Ca\(^{2+}\)-ATPase. Assays were performed for 1, 3, and 5 min at 25 °C and in all cases demonstrated linear production of inorganic phosphate over the 5-min period. The reaction was started by the addition of ATP and terminated by the addition of 1 ml of 20% trichloroacetic acid. The preparations were filtered, and inorganic phosphate was measured according to the method of Martin and Dotty (33).

RESULTS

p36 protein isolated from bovine intestinal epithelium and immunoprecipitated p130\(^{ps}\) were incubated with [\(\gamma^{32}\)P]ATP
that phosphorylation of p36 was markedly suppressed by S-

The aliquots (25 pl) of assay mixture were removed at the indicated time to separate immunoprecipitated enzyme and the phosphorylation reaction was halted. 25 pl of 2 mg/ml S-100 was added to the reaction mixture and further incubated at 30 °C. The subsequent reaction was terminated by the addition of 20 µl of electrophoresis buffer containing 10% SDS at the indicated time. Electrophoresis was carried out in 18% polyacrylamide slab gels, and the subsequent autoradiogram was shown in the inset.

**TABLE I**

The effect of S-100 on myosin ATPase activity

<table>
<thead>
<tr>
<th>Without S-100 (100 µg/ml)</th>
<th>With S-100 (100 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td>0.0</td>
</tr>
<tr>
<td>Ca²⁺-ATPase</td>
<td>2.5</td>
</tr>
<tr>
<td>(K⁺-EDTA)-ATPase</td>
<td>3.6</td>
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**FIG. 4.** The effect of S-100 on the dephosphorylation of p36. The aliquots (25 pl) of assay mixture were removed at the indicated time to separate immunoprecipitated enzyme and the phosphorylation reaction was halted. 25 pl of 2 mg/ml S-100 was added to the reaction mixture and further incubated at 30 °C. The subsequent reaction was terminated by the addition of 20 µl of electrophoresis buffer containing 10% SDS at the indicated time. Electrophoresis was carried out in 18% polyacrylamide slab gels, and the subsequent autoradiogram was shown in the inset.

**FIG. 5.** Inhibition pattern of p36 phosphorylation by S-100. Reciprocal velocity versus 1/p36 with or without 100 µg of S-100 was plotted. All other conditions are as described under “Experimental Procedures.” The lines are those fit to the data point by using the equation of simple linear competitive inhibition.

in the presence or absence of calcium-binding proteins such as troponin C, S-100, parvalbumin, and calmodulin. Autoradiography as shown in Fig. 1 following SDS-PAGE revealed that phosphorylation of p36 was markedly suppressed by S-100 purified from bovine brain, and calmodulin weakly inhibited the p36 phosphorylation. With 30 µg/ml S-100 or calmodulin 49 or 10% of the phosphorylation was depressed, respectively, whereas the same concentrations of troponin C or parvalbumin did not affect the kinase activity. The phosphoamino acid analysis of phosphorylated p36 indicated that S-100 inhibited tyrosine phosphorylation of p36 by p130³⁰⁷ (Fig. 1B).

The data presented in Fig. 2 show the dose-dependent effect of Ca²⁺-binding proteins on the p36 phosphorylation by p130³⁰⁷. S-100 inhibited the p130³⁰⁷ tyrosine protein kinase activity in a dose-dependent manner with apparent Kᵢ value of 0.21 µM (4.2 µg/ml). This inhibition was not affected by the addition of 1.0 mM CaCl₂ or 0.5 mM EGTA (Fig. 3). Troponin C or parvalbumin did not affect the phosphorylation up to 100 µg/ml (Fig. 2). To rule out the possibility that our S-100 preparation contained phosphatase activity, dephosphorylation of phosphorylated p36 was examined in the presence (C) or absence of S-100 (○) (Fig. 4). ³²P incorporation to p36 was time-dependent and saturated at 60 min. Incubation of phosphorylated p36 with 1 mg/ml S-100 did not induce dephosphorylation for at least 30 min. The S-100 preparation did not contain ATPase activity, and no stimulatory effect on ATPase activity was observed (Table I). As shown in Fig. 5, the higher concentration of p36 reversed S-100-mediated inhibition of the phosphorylation; however, ATP did not alter the inhibitory effect of S-100 on the phosphorylation (data not shown).

Although p36 is a major substrate of several tyrosine protein kinases, recently Khanna et al. (34) and Gould et al. (35) reported that protein kinase C also phosphorylated p36 in vitro and in vivo. The inhibitory effect of S-100 on p36 phosphorylation by protein kinase C and p60⁶⁵⁷tyrosine protein kinase was examined. S-100 (100 µg/ml) did not significantly affect the protein kinase C activity, whereas 86% of p60⁶⁵⁷ tyrosine kinase activity was depressed (Table II).

To determine whether S-100 had a preferential inhibitory effect on the tyrosine phosphorylation of p36, other representative substrate proteins such as skeletal and smooth
muscle myosin light chain, and α-casein were incubated with immunoprecipitated p130⁰⁰ in the presence or absence of 0.1 mg/ml S-100. As shown in Table III, S-100 potently inhibited the phosphorylation of these substrate proteins to the same extent, suggesting that the inhibitory effect of S-100 on tyrosine phosphorylation was not affected by the difference of the substrate molecule.

**DISCUSSION**

The importance of the calcium ion as a general second messenger is well recognized in almost every biological field. Although the exact mechanism by which calcium ion exerts its influence remains to be investigated, its activity is mediated through a homologous class of calcium-binding proteins such as S-100 protein and calmodulin, which have a similar molecular structure. In this paper, we found that S-100 and Ca²⁺-binding proteins potently and directly inhibits its tyrosine phosphorylation of p36 or other substrate proteins.

Since higher concentration of the substrate protein reversed S-100-mediated inhibition of tyrosine kinase, S-100 may affect the interaction between tyrosine kinase molecules and the substrate protein. There are two possible mechanisms by which S-100 inhibits ⁴⁰⁰ incorporation into tyrosine residues: S-100 directly inhibits retroviral tyrosine kinase by binding to the substrate binding site; or S-100 alters the conformation of the substrate making it less susceptible to tyrosine residue phosphorylation. S-100 inhibits tyrosine phosphorylation of all tested substrate proteins to the same extent, and serine/threonine phosphorylation is not affected by S-100; therefore, it seems more likely that the first explanation is correct. As indicated in Fig. 1, S-100 itself cannot be the phosphate acceptor, although S-100 contains several tyrosine residues (S-100a, 3; S-100b, 2).

The S-100 protein used in this experiment is a mixture of S-100a and S-100b of bovine brain, but more than 80% of the protein was S-100b determined on a 20% polyacrylamide gel electrophoresis in the absence of SDS (data not shown). We previously measured the content of S-100b in human neuronal and non-neuronal tissues using a sandwich-type enzyme immunoassay system (36), and the levels of S-100b in the central nervous system (cerebellar cortex, 8.02 μg/mg protein; cerebellar medulla, 7.83 μg/mg protein) (37) were more than the Kᵢ value to inhibit tyrosine phosphorylation. Fig. 3 showed that the inhibitory effect of S-100 on tyrosine phosphorylation was Ca²⁺-independent. Previously we reported that S-100 could bind to microtubules in a Ca²⁺-dependent manner (18), suggesting the level of unbound S-100 protein is changeable. If so, tyrosine phosphorylation might be regulated by the Ca²⁺-dependent change of unbound S-100 concentration.

As shown in Fig. 3, it appears that S-100 inhibits exogenous substrate phosphorylation but does not depress autophosphorylation of p130⁰⁰. Weber et al. (38) reported that autophosphorylation of epidermal growth factor receptor is an intramolecular reaction. If so, this suggests that S-100 does not affect the intramolecular reaction of p130⁰⁰.

Michiel and Wang (20) reported that calmodulin stimulates tyrosine phosphorylation of 50- and 58-60-kDa peptides in rat brain membrane, and Patel and Marangos (39) demonstrated that S-100 protein inhibits the phosphorylation of several brain proteins in a calcium-dependent fashion, but phosphoamino acids of these proteins were not identified. The calcium-binding proteins, especially S-100, whose function has not yet been assigned may modulate tyrosine phosphorylation of brain proteins, although the mechanism of Ca²⁺-dependent regulation should be investigated further.

**REFERENCES**