BIOPHYSICS AND BIOCHEMISTRY

Interactions and Possible Functional Characteristics of Tag7-S100A4 Protein Complex

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 2, pp. 151-153, February, 2008
Original article submitted November 8, 2007

Peptidoglycane-recognizing protein Tag7 formed a complex with S100A4 (a representative of S100 protein family), the apparent dissociation constants in the absence and presence of Ca^{2+} were 2×10^{-8} M and 10^{-9} M, respectively. Analysis of fluorescence spectra of hydrophobic fluorescent probe 2-toluidinyl naphthalene-6-sulfonate in the presence of S100A4 and Tag7 proteins showed that extensive area or several sites are involved into the complex formation between these proteins. The formation of Tag7-S100A4 complex had virtually no effect on the role of S100A4 in the regulation of intracellular Ca^{2+} metabolism. Removal of not only Tag7, but also S100A4 from neutrophil conditioned medium reduced lysis of *E. coli* cell, while addition of the Tag7-S100A4 complex to the medium restored antibacterial activity.

Key Words: *S100A4; Tag7; neutrophils; calcium*

Tag7 protein belonging to a conservative family of pattern-recognizing proteins is present in insect and mammalian cells. Its functional activity is mediated by complex formation with various proteins. Participation of Tag7 in antitumor response is mediated by the formation of an active complex with Hsp70 heat shock protein [8]. Study of the antibacterial effect of Tag7 on gram-negative and gram-positive flora showed that the reaction of Tag7 with lysozyme was obligatory for the realization of the antibacterial effect towards *E. coli* [4]. Tag7 protein is present in neutrophil granules and is secreted during contact with microorganisms [10]. Protein S100A4 (metastasin) belongs to a family of small Ca-binding S100 proteins. Two aspects of functional activity of S100A4 protein were analyzed: its role in the progress and metastatic activity of tumor cells and its relationships with cell mobility, determined by interactions with cytoskeleton proteins actin, nonmuscular myosin, and tropomyosin) [6]. Protein S100A4 is expressed in the spleen, bone marrow, T lymphocytes, macrophages, and neutrophils [9]. We previously showed that S100A4 was present in the neutrophil secretion [3].

Here we studied the possibility of Tag7 and S100A4 complex formation and evaluated the functional significance of this interaction.

MATERIALS AND METHODS

Antibacterial activity of neutrophil conditioned medium was evaluated by lysis of *E. coli* over 10 min at ambient temperature as described previously [4]. Fresh *E. coli* cells were suspended in 10 mM so-

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0007-4888/08/1452-0191

dium phosphate buffer (pH 7.4), aliquots (100 μl) were transferred into wells of a microtitrattion plate, neutrophil conditioned medium was added (100 μl), and the decrease in optical density was measured at λ=600 nm. Control samples contained *E. coli* cells with culture medium. Neutrophils and conditioned medium were obtained as described previously [3]. Conditioned medium was collected after 24-h incubation, applied onto anti-S100A4 Sepharose and anti-Tag7 Sepharose, washed thoroughly with 10 mM sodium phosphate buffer with 0.5 M NaCl (pH 7.4), and bound proteins were eluted with 0.25 M triethylamine solution (pH 12.5). Protein S100A4 was isolated from CSML-100 cells as described previously [5]. Tag7 was isolated from CSML-0 conditioned medium using anti-Tag7 Sepharose according to the same protocol [8]. Recombinant S100A4 was obtained as described previously [5]. Recombinant Tag7 was obtained using pET-28 plasmid containing a fragment encoding for Tag7 human protein 22-196 amino acid residues (this plasmid was a kind gift from Dr. Andreas Bracher from Max Planck Institute of Biochemistry, Germany). The protein was expressed as inclusion bodies in *E. coli* BL21 (DE3) cells. Protein purification and refolding were carried out as described previously [7].

Plasma membrane (PM) vesicles closed with the cytoplasmic side out were obtained by differential centrifugation. Evaluation of ATP-dependent Ca^{2+} transport in PM fraction and Ca-ATPase activity [1], registration of 2-toluidinyl naphthalene-6-sulfonate (TNS) fluorescence [5], measurement of S100A4 protein by enzyme immunoassay [3], and visualization of S100A4 protein by immunoblotting [8] were described previously. The results were statistically processed using Student's test.

RESULTS

Protein S100A4 was detected in the eluate after chromatography of neutrophil conditioned medium on anti-S1004 Sepharose and anti-Tag7 Sepharose. The eluate from anti-S100A4 Sepharose contained 1.2 ng S100A4, eluate from anti-Tag7 Sepharose contained 0.17 ng protein. These data suggest that the Tag-7—S100A4 protein complex is present in the neutrophil conditioned medium and that the content of Tag7-bound protein is by one order of magnitude lower than the content of free S100A4. A possible explanation of differences in the content of S100A4 protein is the effect of Ca ions on protein-protein interaction [6].

In order to verify this hypothesis, recombinant S100A4 and Tag7 were preincubated for 1 h with

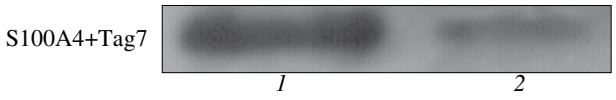

Fig. 1. Interaction of recombinant S100A4 and Tag7 proteins in the presence of 1 mM Ca^{2+} (*1*) and 1 mM EDTA (*2*).

1 mM EDTA or 1 mM Ca^{2+} and layered onto anti-Tag7 Sepharose, the Sepharose was thoroughly washed, bound proteins were eluted, separated in PAAG, and visualized using antibodies to S100A4. Protein S100A4 was present on rows in both cases, but the band with 1 mM Ca^{2+} was several-fold wider than that with 1 mM EDTA (Fig. 1).

Using anti-Tag7 Sepharose for separation of bound and free S100A4, we measured free S100A4 by enzyme immunoassay and from these data estimated the apparent dissociation constant for the complex, which was 2×10^{-8} M without Ca^{2+} and 10^{-9} M in the presence of 1 mM Ca^{2+}.

The nature of S100A4—Tag7 complex formation was studied using TNS hydrophobic fluorescent probe used for evaluation of protein-protein interactions [5]. The CSML-100 cell lysate served as the source of S100A4 protein and Tag7 protein was isolated from CSML-0 conditioned medium. The intensity of fluorescence in the S100A4+Tag7+Ca^{2+} mixture was significantly lower than the sum of individual fluorescence of proteins (Fig. 2). This attenuation of fluorescence was due to competition between Tag7 and TNS for the same hydrophobic binding sites on S100A4 protein molecule [5], which indicates pronounced protein-protein interaction. These results suggest that the formation of the complex between Tag7 and S100A4 proteins involves extensive area or several sites on these proteins.

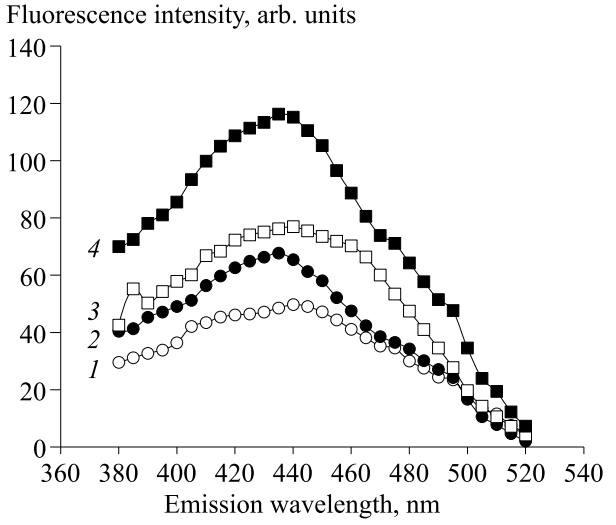

Fig. 2. Effects of Tag7 (*1*), S100A4 (*2*), and Tag7—S100A4 complex (*3*) on the TNS probe fluorescence spectra. *4*) summary protein spectrum.

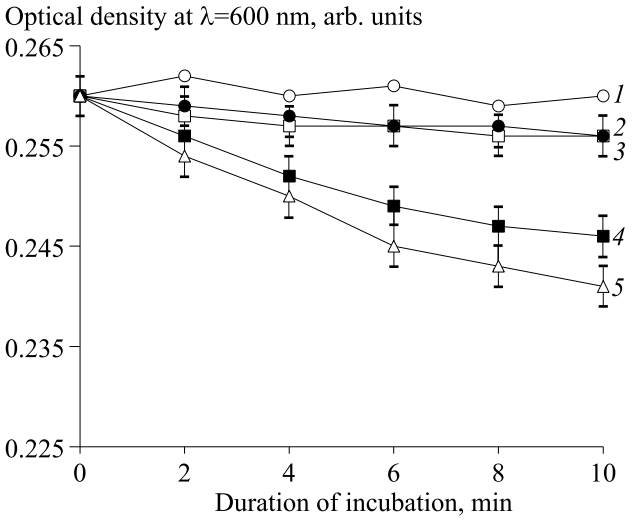

Fig. 3. Participation of S100A4 and Tag7 proteins in antibacterial activity of neutrophils (data of 3-5 independent experiments). Incubation of *E. coli* in culture medium (*1*); in neutrophil conditioned medium without S100A4 (*2*); in neutrophil conditioned medium without Tag7 (3); in neutrophil conditioned medium (*4*); in neutrophil conditioned medium without S100A4 and Tag7 with subsequent addition of the Tag7-S100A4 complex (*5*).

We previously found that S100A4 protein was involved in the regulation of intracellular level of calcium ions via activation of Ca-ATPase [2]. Here we studied the effect of Tag7 on changes in Ca-ATPase activity induced by S100A4 and evaluated ATP-dependent transport of Ca^{2+} ions in the plasmalemma fraction of CSML-100 cells in the presence of the studied proteins. Activity of Ca-ATPase (μmol Pi/mg) significantly increased (p=0.02) after addition of S100A4: from 2.1±0.3 (n=9) to 5.8±0.6 (n=4). Subsequent addition of Tag7 protein into the samples did not significantly change enzyme activity (6.3±0.5; p=0.24). Tag7 did not modify initial enzyme activity (2.5±0.2).

In series II, another method was used for evaluation of ATP-dependent transport of Ca^{2+} ions in the cytoplasmic membrane fraction under the effect of Tag7 and S100A4 protein mixture. The cytoplasmic membrane vesicles were loaded with Fluo-3 (fluorescent indicator of Ca^{2+}) to a final concentration of 5-7 μM in intravesicular environment. The content of Ca^{2+} ions accumulated in vesicles was calculated by measuring Fluo-3 fluorescence.

The concentration of Ca^{2+} ions in the plasma membrane fraction reached 29.4±2.5 μM (n=4) in the presence of S100A4 protein and 32.8±1.7 μM (n=4) after addition of Tag7-S100A4 complex; hence, no appreciable changes were recorded in the presence of Tag7 protein.

Hence, activation of Ca^{2+}-ATPase by S100A4 protein does not depend on the presence of Tag7 protein in the medium. Therefore, the complex formed by Tag7 and S100A4 proteins is virtually inessential for S100A4 participation in the regulation of intracellular Ca^{2+} exchange.

The possible biological role of Tag7 and S100A4 protein interactions was studied using a test system based on evaluation of antibacterial activity of Tag7 towards *E. coli* (Fig. 3). Removal of Tag7 or S100A4 from the medium led to reduction of antibacterial activity; removal of these proteins one by one produced similar effect. On the other hand, addition of the complex of recombinant Tag7 and S100A4 to step-by-step exhausted medium restored the rate of *E. coli* lysis to the initial level (Fig. 3). These data indicate a possible mechanism of realization of Tag7 antibacterial activity with participation of the Tag7—S100A4 complex.

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