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Calcium-induced localization of calcium-activated neutral proteinase on plasma membranes

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The location of calcium-activated neutral proteinase (CANP) was determined in human erythrocytes by crosslinking CANP to co-localizing proteins using a photolabeling bifunctional reagent, 4,4′-dithiobisphenylazide (DTBPA). The crosslinked products were selectively isolated by immunoprecipitation with a polyclonal anti-CANP antibody and analyzed by SDS-polyacrylamide gel electrophoresis after cleavage of the crosslinkage. In the calcium-free incubation medium the main proteins crosslinked with CANP were cytosolic proteins such as hemoglobin. In the presence of calcium ions, on the other hand, membrane skeletal proteins such as spectrin, band 4.1, 4.2 and 6 proteins as well as band 3 were crosslinked with CANP. Addition of calcium ionophore further increased the amount of crosslinked membrane proteins. These results suggest that in the absence of calcium ions CANP exists diffusely in the cytoplasm and is crosslinked with cytoplasmic hemoglobin nonspecifically while in the presence of calcium ions CANP associated with membrane where it is crosslinked specifically with the lining proteins. Thus it is demonstrated biochemically that the localization of CANP is dynamic depending on the presence of calcium ions.

Introduction

Calcium-activated neutral proteinases (CANP, EC 3.4.22.17) are widely distributed in the animal kingdom and are found in various tissues. Two types of CANP have been shown to exist, μCANP and mCANP, which require 1–10 μM and 200–300 μM calcium for half-maximal activity, respectively. Each CANP molecule consists of a catalytic subunit (about 80 kDa) and a regulatory subunit (about 30 kDa) [1–4]. Recent progress has revealed that most CANP preparations consist of inactive proenzymes [5,6], which are converted to active forms through limited calcium-dependent autoproteolysis. This limited autoproteolysis lowers the calcium requirement for its catalytic activity. It has been observed that the high calcium concentration required for autoactivation is reduced in the presence of some phospholipids [7,8] or substrates [9]. The promotion of CANP activation by phospholipids from plasma membranes has also been reported [10]. These phospholipid effects raise the possibility that CANP activation may occur on cytoplasmic membranes. This activation process would require CANP to be translocated on the plasma membrane for its activation. In order to confirm the location of CANP on plasma membranes, we tried to demonstrate in human erythrocytes the co-localization of CANP and membrane proteins by means of crosslinkage formation between these proteins followed by isolation of the resulting CANP-containing complexes by immunoprecipitation with an anti-CANP antibody. We chose human erythrocytes because they have a limited variety of proteins comprised mainly of cytosolic hemoglobin and plasma membrane proteins, all of which had been well characterized. Since erythrocytes in mammals contain only μCANP [11], the present study provides information only about μCANP.

Materials and Methods

Materials. 4,4′-Dithiobisphenylazide (DTBPA) was purchased from Pierce Chemical Co. Calcium ionophore A23187 was from Sigma Chemical Co. Ltd. All

Abbreviations: CANP, calcium-activated neutral proteinase; DTBPA, 4,4′-dithiobisphenylazide; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

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other reagents were of the highest purity commercially available. Human blood was provided by volunteers in this laboratory. Standard μCANP was prepared from human erythrocytes as described before [12]. Anti-chicken CANP antibody was raised in a rabbit and purified on a CANP-Sepharose column. Cross-reaction of μCANP from human erythrocytes was confirmed by immunoblotting analysis with anti-chicken CANP antibody, which showed a band representing the large CANP subunit with a molecular weight of 80 kDa.

Preparation of human erythrocytes. Blood samples were collected in tubes containing 5 mM EDTA and immediately centrifuged at 4°C to separate plasma and erythrocytes. After removal of the supernatant and the fluffy upper precipitate which contains leucocytes, the packed precipitate was washed twice with phosphate-buffered saline (PBS) containing 5 mM EDTA.

Treatment of human erythrocytes with DTBPA. The washed erythrocytes were transferred to small glass test tubes and suspended in Tris-buffered saline (TBS) at a concentration of $2 \cdot 10^7$ cells/ml. After addition of either 5 mM EDTA, 1 mM calcium chloride or 5 mM calcium chloride plus 10 μM calcium ionophore, these tubes were incubated on ice for 30 min to disperse the reagent uniformly. Then 1 mM DTBPA in absolute ethanol prepared under a red safe light [13] was added with stirring to the preincubated erythrocyte suspension to a final concentration of either of 10^{-5} or 10^{-6} M (the resulting ethanol concentration was 1 or 0.1%). This mixture was further incubated in the dark for 30 min on ice to allow the diffusion of the reagent into the cells, and then was irradiated with 365 nm UV light (150 W, Ultraviolet Products Inc., CA) for 30 min from a distance of 5 cm. An aluminum foil reflector was placed 3 cm behind the sample tubes. After the irradiation, the mixture was centrifuged to remove the excess reagent and the sedimented cells were lysed in 20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 1% Triton X-100 and further incubated at 37°C for 18 h to solubilize the membrane proteins. The whole erythrocyte lysate was centrifuged at 140000 × *g* for 1 h to remove the insoluble materials and the supernatant was subjected to immunoprecipitation.

Separation and identification of CANP-linked proteins. The rabbit antiserum against chicken CANP (100-fold dilution) was added to the erythrocyte lysate and the mixture was incubated at 37°C for 8 h to complete the immunoprecipitation. Then the reaction mixture was centrifuged at 60000 × *g* for 40 min and the resulting precipitate was rinsed with PBS. The washed precipitate was incubated at 37°C for 5 h in 1% SDS and 100 mM 2-mercaptoethanol in 20 mM Tris-HCl (pH 7.4). This treatment separates the crosslinked proteins by reductive cleavage of the crosslinkages. The separated proteins were analyzed by electrophoresis in a 10% polyacrylamide slab gel prepared according to Laemmli [14] and stained with Coomassie brilliant blue. The identification of erythrocyte membrane proteins was carried out according to Steck [15].

Results

Proteins crosslinked with CANP in the absence of calcium ions

The electrophoretic profiles of the proteins separated by immunoprecipitation after DTBPA treatment were compared with those of the membrane and cytosol proteins (Fig. 1). In the absence of calcium ions, hemoglobin, the major cytosolic protein, was identified as the major protein co-precipitated with anti-CANP antibody after treatment with DTBPA at both 10^{-6} M and 10^{-5} M. Membrane proteins were not detected in the immunoprecipitate except for a small amount of band 6 protein. The large subunit of CANP (80 kDa) was recovered in the immunoprecipitate. However, bands corresponding to the small subunit (30 kDa) were not detected.

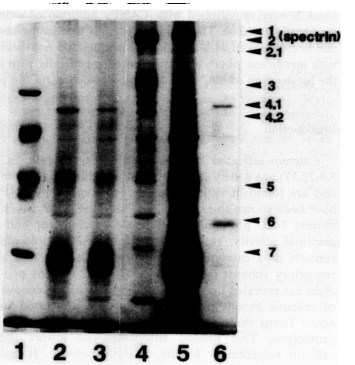

Fig. 1. Electrophoretic banding patterns of the proteins crosslinked with erythrocyte μCANP in the absence of calcium ions. The crosslinking reaction was performed with the indicated additions in TBS containing 5 mM EDTA. The proteins linked with CANP were isolated by immunoprecipitation and analyzed by electrophoresis in the presence of SDS on 10% polyacrylamide gels. Lane 1, molecular weight marker proteins; lane 2, 10^{-5} M DTBPA; lane 3, 10^{-6} M DTBPA; lane 4, control membrane proteins; lane 5, control cytosolic proteins; lane 6, purified μCANP from human erythrocytes. Marker proteins used were phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), and soybean trypsin inhibitor (M_r 20100). The membrane proteins, bands 1, 2, 2.1, 3, 4.1, 4.2, 5, 6 and 7 are marked (◂).

Proteins crosslinked with CANP in the presence of calcium ions

Crosslinkage formation in the presence of 1 mM calcium chloride is shown in Fig. 2. When cytosolic proteins in erythrocytes treated with DTBPA were subjected to electrophoretic analysis without prior immunoprecipitation, marked production of hemoglobin tetramer (M_r = 67 000) was observed (lane 8). This indicates that a sufficient amount of the DTBPA had penetrated into the cytosol. There were a few faint protein bands detected in the immunoprecipitate obtained after exposure to light in the absence of DTBPA (lane 2). This lane served as the negative control to confirm the validity of the present immunospecific separation of the proteins crosslinked with CANP. It is evident from lanes 3 and 4 that membrane proteins, especially lining proteins such as spectrin, band 6, band 4.1 and band 4.2 proteins, were immunoprecipitated at crosslinker concentrations of 10^{-6} M and 10^{-5} M. In addition to these proteins, band 3 protein, an integral membrane protein shown to be the anion channel, was also precipitated with the anti-CANP antibody. Cytosolic proteins were observed only slightly in the immunoprecipitate at crosslinker concentrations of either 10^{-6} M or 10^{-5} M.

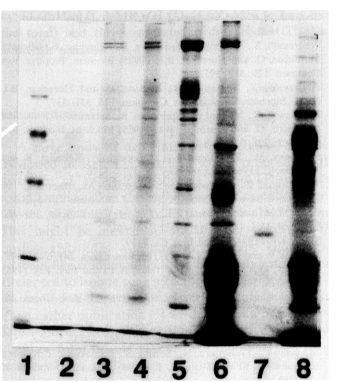

Fig. 2. Electrophoretic banding pattern of the proteins crosslinked with erythrocyte μCANP in the presence of calcium ions. The crosslinking reaction and analysis were performed as in Fig. 1 except that the reaction medium contained 1 mM calcium ions and no EDTA. Lane 1, molecular weight marker proteins; lane 2, without DTBPA; lane 3, 10^{-6} M DTBPA; lane 4, 10^{-5} M DTBPA; lane 5, control membrane proteins; lane 6, control cytosolic proteins; lane 7, purified μCANP; lane 8, 10^{-6} M DTBPA without immunoprecipitation and reduction with 2-mercaptoethanol. The membrane protein bands were identified as described in Fig. 1.

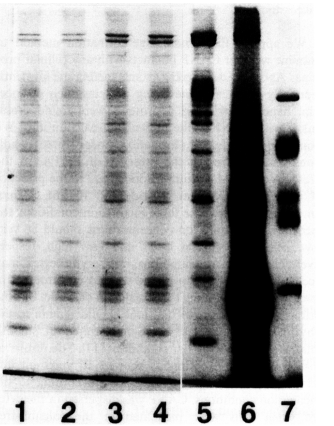

Fig. 3. The effect of calcium ionophore on crosslinkage formation involving CANP. The crosslinking reaction was performed as in Fig. 1 except that it was carried out in the presence of 5 mM calcium ions with or without 10^{-5} M calcium ionophore A23187. Electrophoresis was performed as in Fig. 1. Lane 1, 10^{-6} M DTBPA without calcium ionophore; lane 2, 10^{-5} M DTBPA without calcium ionophore; lane 3, 10^{-6} M DTBPA and calcium ionophore; lane 4, 10^{-5} M DTBPA plus calcium ionophore; lane 5, control membrane proteins; lane 6, control cytoplasmic proteins; lane 7, molecular weight marker proteins. The membrane protein bands were identified as described in Fig. 1.

Differently from the experiment in the absence of calcium ions, no protein band corresponding to intact CANP was detected on electrophoresis. This may be due to the autoproteolysis of CANP in the presence of calcium ions.

Effect of calcium ionophore. To increase the intracellular calcium concentration in erythrocytes, cells were incubated on ice with 10 μM calcium ionophore A23187, which has been shown to transport calcium ions across plasma membranes [16], and 5 mM calcium chloride for 30 min and then treated with the crosslinking reagent. Some new bands were detected upon analysis in addition to those observed in the presence of calcium ions alone (Fig. 3). The amounts of spectrin, ankyrin and band 5 protein were increased; the amount of band 6 protein remained unchanged, however. The faint bands below band 4.2 protein were not identified.

Discussion

We applied the crosslinkage formation method to examine whether CANP interacts with any cellular proteins. As we had expected, some membrane proteins, especially the membrane lining proteins such as spectrin, band 4.1, 4.2 and 6 proteins were found crosslinked with CANP in the presence of calcium ions. We examined crosslinkage formation under three conditions: calcium free in the presence of EDTA, in the presence of 1 mM calcium chloride, and in the presence of both 5 mM calcium chloride and 10 μM calcium ionophore. Under these three incubation conditions the intracellular calcium ion concentrations should be very low, near physiological levels and very high, respectively. Influx of calcium ions into erythrocytes may occur during incubation of the washed erythrocytes in the medium containing 1 mM calcium chloride, but the intracellular level of calcium ions should remain in the physiological range, as was demonstrated in smooth muscle cells under similar conditions [17]. The results of the crosslinkage experiments showed an apparent difference in the localization of CANP according to the incubation conditions. CANP was dispersed in the cytosol when cells were incubated in the calcium-free medium, while at least a portion of the CANP was attached to plasma membrane proteins in the presence of calcium ions. The calcium ionophore further increased the amount of membrane lining proteins crosslinked with CANP. This calcium-dependent crosslinkage formation suggests that CANP translocates onto membranes at increased levels of intracellular calcium ions. Accordingly, the crosslinkage formation of CANP and membrane proteins demonstrated in the present study is in good agreement with a recent report that the amount of CANP extracted in the cytosol fraction is decreased and more CANP is recovered in the membrane fraction in the presence of calcium [18]. Also recently, histological investigations have demonstrated the distribution of CANP around the plasma membrane: cytosol is stained diffusely with antibody against CANP [19,20], and the periphery of the cell is also evidently stained [21,22]. The present study clearly indicates a calcium-dependent localization of CANP on the plasma membrane lining proteins.

In conclusion, calcium affects the distribution of CANP: CANP disperses in cytosol in the absence of calcium ions and localizes on the plasma membrane in the presence of calcium ions. We, therefore, suggest the possibility that at locally high concentrations of intracellular calcium ions, CANP is transferred to the plasma membrane and converted to the active form. The physiological role of the active CANP molecule remains to be clarified.

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