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Multimolecular Complexes Containing p65 Subunit of NF-κB and Cytoskeletal Proteins in A431 Cells[1]

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Abstract—The nuclear factor-kappaB (NF-κB) is a rapidly inducible transcription factor involved in cell responses to a number of external and internal stimuli. We have demonstrated previously that the p65 subunit of NF-κB associates with actin structures, stress-fibers, and focal contacts and that p65 colocalizes in cytoplasm and redistributes to the nucleus with α-actinin-4. To find out other components of multimolecular complexes containing p65 and α-actinin-4 existing in cytoplasm of A431 cells we isolated this complexes. Size-exclusion chromatography and immunoprecipitation experiments showed that actin-binding protein α-actinin-4 and p65 subunit of transcription factor NF-κB are found in cytoplasmic complexes not connected with cytoskeletal structures with molecular weight of about 600 kDa. Tubulin and tropomyosin are found in these complexes, too.

Key words: protein complexes, alpha-actinin-4, NF-κB

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It is currently thought that the cytoskeleton is involved in intracellular signal transduction and regulation of gene expression [1, 2] but the particular mechanisms of this phenomenon is still unclear. One of the possible mechanisms may be the participation of cytoskeletal proteins as a scaffold for formation multimolecular complexes including signaling molecules.

It was found previously that NF-κB, which is a rapidly inducible transcription factor [3], colocalizes with actin structures, stress-fibers, and focal contacts and interacts with fibrillar actin in vitro [4]; however, it remains unclear with what particular cytoskeletal proteins it may interact. It was shown that kinase MEKK1, one of the NF-κB activators, is able to interact with the actin-binding protein α-actinin [5]; further investigations demonstrated the possibility of the interactions between p65-subunit of NF-κB and α-actinin-4 in the cytoplasm. The methods of immunofluorescence and confocal microscopy showed that α-actinin-4 and p65 colocalized in the cytoplasm and co-redistributed together in the nucleus under influence of growth factors and cytochalasin D [6]. Moreover, both proteins were revealed in immunoprecipitates of total cell lysates, even in the course of the cytoskeleton reorganization under the influence of epidermal growth factor or cytochalasin D. Among proteins interacting with α-actinin during affinity chromatography, p65 and p50 subunits of NF-κB were detected [7]. At the same time, it remained unclear whether these proteins are components of a single protein complex.

In this connection, the aim of the present study was to identify the protein content of the complex and find out if p65 and α-actinin-4 are included in one and the same complex. Since interaction of cytoskeletal proteins with signaling molecules may not be sufficiently strong, complexes can disintegrate in the process of isolation. It was therefore decided to use pretreatment of cells with low concentrations of formaldehyde, which is applied widely for creation of linkages between interacting proteins [8].

EXPERIMENTAL

The cells of epidermoid carcinoma line A431 were obtained from the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences). The cells were grown at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cultures that had reached subconfluent state were used for the experiments. In one experiment, 6 culture dishes (9 cm in diameter, NUNC, USA) were used. Cells were pretreated for 10 min with a solution of 30 μM formaldehyde in serum-free medium. Effect of formaldehyde was stopped by changing the medium for 0.125 M solution of glycine in PBS for 5 min. Cells was lysed by SF-buffer (100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM K-phosphate buffer pH 7.0, 0.5 M sucrose, 1% Triton X-100, protease inhibitors). After 10-min lysis on ice,

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Triton-insoluble fraction was precipitated from the destroyed cells by centrifugation at 12000 g, then supernatant was collected and filtered through a 0.45-μm filter (Millipore, USA). A 400-μl aliquot of the lysate obtained was applied on the superose 6HR column (GE Healthcare, USA) in the FPLC Pharmacia System (Pharmacia, Sweden). Calibration of the column was carried out using a set of proteins (Amersham, Sweden): ribinuclease A (MW 13700), ovalbumin (MW 48100), albumin (MW 67000), aldolaze (MW 184000), catalase (MW 232000), ferritin (MW 401400), thyroglobulin (MW 669000), as well as blue dextran 2000 (MW 2000000). Elution was carried out with F-buffer (100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 10 mM K-phosphate buffer, pH 7.0) at a flow rate of 0.2 ml/min; 0.4-ml fractions were collected on ice. The relative amount of protein in the mobile phase at the outlet of the column was measured in dynamic mode with an optical detector UV-1 (Pharmacia) at a wavelength of 280 nm. After adding 0.05% sodium deoxycholate and 5% trichloracetic acid, fractions were cooled on ice and the forming protein pellets were collected by centrifugation at 15000 g. Proteins was analyzed by electrophoresis in polyacrylamide gel in denaturation conditions in the presence of SDS [9]; 12.5% gel was used. After electrophoresis gel was stained with Kumassi diamond blue or transferred to a Immobilon-P membrane (Millipore, USA) [10]. Transfer of proteins from gel to membrane was carried out in Tris-glycine buffer pH 8.3, with 10% methanol and 0.1% SDS. Immunoblotting was carried out in accordance with the ECL Western blotting protocols (Amersham). Membranes were washed with buffer TBST (25 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 20 min and subsequently blocked with 5% defatted dry milk diluted in TBST for 1 h. To enhance the immunoblotting signal, SuperSignal West Dura Extended Duration Substrate was used (Pierce, USA). Hemiluminiscence was registered by ChemiDoc (Bio-Rad, USA). We used mouse monoclonal antibodies against α-tubulin, high molecular weight isoforms of tropomyozin (Sigma, USA), and α-actinin-1 (Santa Cruz Biotechnology, USA) and polyclonal rabbit antibodies against α-actinin-4 (ImmunoGlobe, Germany) and p65-subunits of NF-κB (Abcam, UK). Rabbit antibodies against mouse immunoglobulins and goat antibodies against rabbit immunoglobulin conjugated with horseradish peroxidase (Sigma, USA) were used as the second antibodies in the immunoblotting. To prevent nonspecific binding during immunoprecipitation, 50 μl of a mixture of sepharose and protein G (Amersham, Sweden) was added to 1 ml of the fraction derived from gel filtration and incubated with gentle mixing for 1 h at 4°C. Then sepharose was centrifuged at 2000 g, supernatant was added to the antibody and incubated overnight at 4°C with gentle mixing. Then the mixture of sepharose with protein G was added and incubated for 4 h at 4°C with gentle mixing. The immunoprecipitates were centrifuged at 2000 *g*, washed 4 times in PBS and separated by electrophoresis with subsequent immunoblotting.

RESULTS

As a result of gel-filtration, 30 fractions were obtained that contained protein complexes and individual proteins with molecular weights ranging from 10 to 2000 kDa. Figure 1 presents the elution profile of the cell lyzate and protein electrophoresis patterns of the corresponding factions. It can be seen that both fractions containing individual proteins and fractions containing multimolecular complexes consist of many components . Analysis of the fractions by Western blotting has shown that p65 protein is present in fractions 12–17 in varying amounts and is most abundant in fractions 13, 15, and 16 (Fig. 2). α-Actinin-1 was detected in the area of about 800 kDa (fractions 11–14 in Fig. 2) and α-actinin-4 was mostly present in fraction 15. Since p65 interacts with both isoforms, such a dual distribution is understandable. In addition, other proteins were found, potentially able to interact with p65: it was an actin-binding protein tubulin and the main structural protein of microfilaments, tropomyosin. α-Tubulin was found in almost all fractions, starting from the area corresponding to 50 kDa. High-molecular weight isoforms of tropomyosin were present in fractions 13–16, corresponding to molecular weights of 600–800 kDa (Fig. 2). Thus, it was found that p65, α-actinin-1, α-actinin-4, tubulin, and tropomyosin can be present in the same fractions. Yet, α-actinin-1 was found in fractions with lower content of α-actinin-4, and α-actinin-4 was detected together with the p65 in the absence of α-actinin-1. Since different multimolecular complexes can be found in this area, we used the method of cross-immunoprecipitation in order to determine if these proteins belong to one and the same complex. For this, we pooled fractions 12–16 and analyzed the contained complexes linked with formaldehyde using immunoprecipitation with polyclonal antibodies against proteins p65 and α-actinin-4. As a result, complexes were identified that had similar electrophoretic profile as regards intensity and location of bands (Fig. 3). Immunoblotting showed that in immunoprecipitates obtained with antibodies against α-actinin-4 contained protein p65 but not tubulin or tropomyosin. α-Actinin-4 on the blot was represented by two bands corresponding to the full-length protein and, in a less amount, to its fragment of 75 kDa (Fig. 4). In immunoprecipitates from fractions with antibodies against p65, α-actinin-4 detected in the complex, was represented by the 75-kDa fragment but α-actinin-1 was not detected. Tropomyosin and tubulin are found in the same complex (Fig. 4).

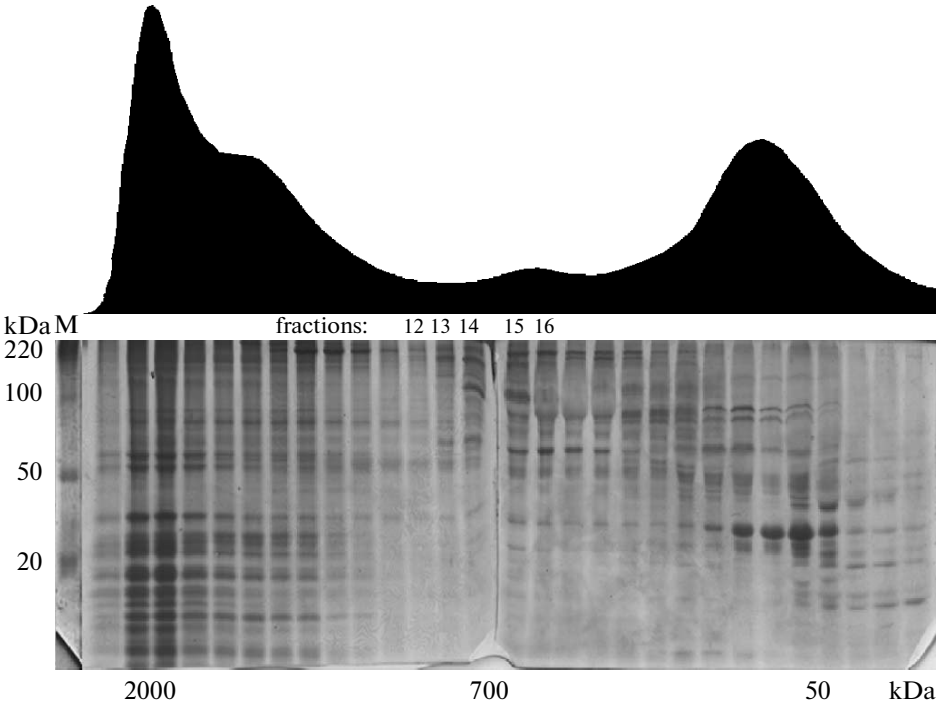

Fig.1. Elution profile obtained from separation of A431 cell lyzate and electrophoretic pattern of proteins. After electrophoresis, gel was stained with Coomassie diamond blue. Numbers denote fraction number.

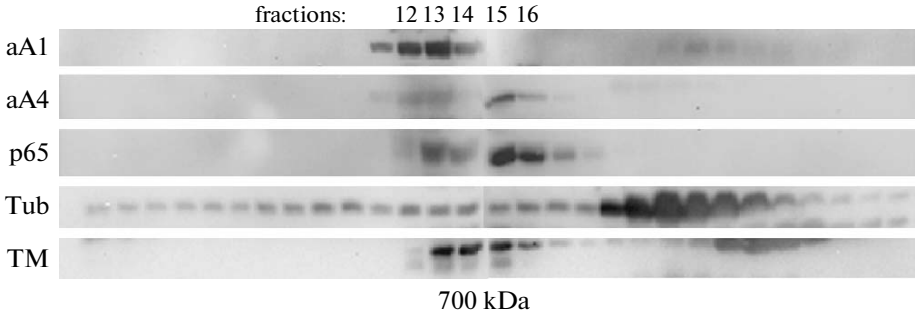

Fig.2. Western-blot analysis of cytosol fractions after gel chromatography on Superose 6. Numbers denote fractions containing α-actinin-1 (aA1), α-actinin-4 (aA4), p65-subunit of NF-κB (p65), tubulin (Tub) and tropomyosin (TM).

DISCUSSION

Considering that the SF-buffer, which does not alter the G- to F-actin ratio in lysates and preserves the structures of cytoskeleton [11], the obtained results indicate that α-actinin and p65, in addition to interaction with the structures of cytoskeleton, exist in the cytosol in the form of macromolecular complexes, much higher than their total mass. In addition, other proteins are also present in these complexes. Previously it was shown that tropomyosin, in addition to binding F-actin, locates in the cytoplasm in the form of big particles [12]. Therefore it was necessary to find out whether it is incorporated in the revealed complexes. It turned out that tropomyosin is indeed involved in the complex together with p65. In the same high-molecular weight fraction, the main structural protein of cytoskeleton, tubulin, was also found. In order to determine whether these proteins are in the same complex, cross-immunoprecipitation was carried out from the combined area of 600–900 kDa fractions. The results demonstrated that proteins p65 and α-actinin-4, as well as its 75-kDa fragment, are involved indeed in the same complex. Besides p65 and α-actinin-4, these complexes include tubulin and tropomyosin as well. Electrophoresis analysis showed that, in addition to the identified proteins, these complexes contain a number of other proteins that may be permanent structural elements of the analyzed complexes. In the future we plan to identify these proteins by methods of mass spectrometry.

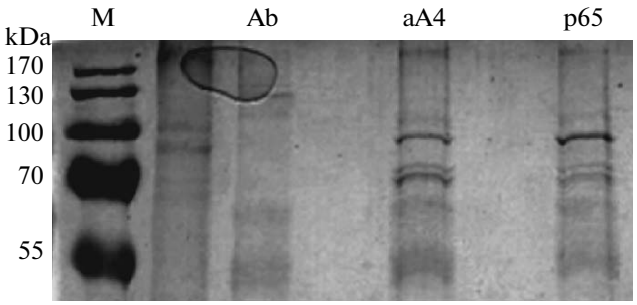

Fig.3. Electrophoretic analysis of immunoprecipitates from fractions 12–16. M, marker; Ab, negative control, polyclonal rabbit antibody against α-actinin-4; aA4 and p65, immunoprecipitates with antibodies against α-actinin-4 and p65-subunit of NF-κB. After electrophoresis gel was stained with Coomassie diamond blue.

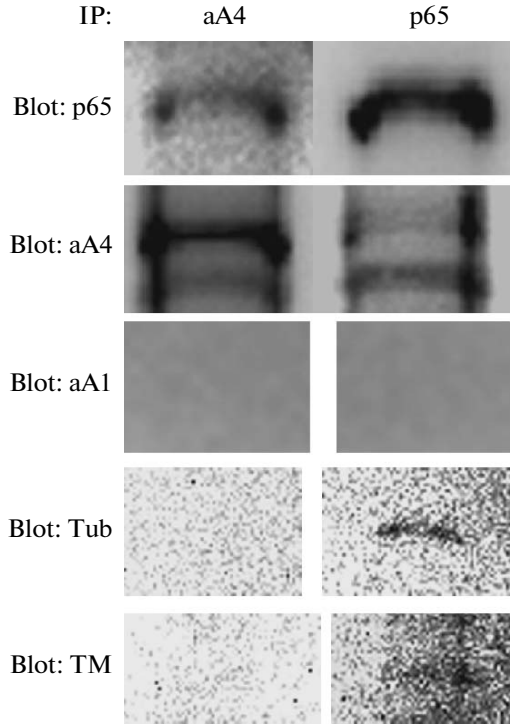

Fig.4. Western-blot analysis of immunoprecipitates with antibodies against α-actinin-4 (IP: aA4) and p65-subunit of NF-κB (IP: p65). aA4, α-actinin-4; Tub, tubulin; TM, tropomyosin; aA1, α-actinin-1 aA4, Tub, and TM are present in complex with p65, and aA4 is not.

Thus, the results of the present work suggest that the elements of both actin and tubulin cytoskeleton may participate in the organization of multimolecular complexes that include p65, and possibly other signaling molecules. Non-muscle isoformes of α-actinin are represented by two actinins: α-actinin-1 and α-actinin-4. Previously, 75-kDa fragments of both α-actinins were found in the cytoplasm [13]. The role of these fragments is unclear but our data suggest that the 75 kDa fragment is involved in the formation of the complex with the p65-subunit of NF-κB. From previous experiments we know that α-actinin-4 co-localizes in the cytoplasm and migrate to the nucleus together with p65 subunit of transcription factor NF-κB [6] and that the full-length α-actinin-4 and fragments of α-actinins 1 and 4 are found in the fractions of proteins tightly bound with chromatin [13]. Together with our data this suggests a potential role of α-actinins in the signal transduction.

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