Biochemistry (Moscow), Vol. 68, No. 11, 2003, pp. 1239-1246. Translated from Biokhimiya, Vol. 68, No. 11, 2003, pp. 1540-1549.

Interaction of Keratin K1 with Nucleic Acids on the Cell Surface

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Received October 18, 2002
Revised version January 22, 2003

Abstract—The interaction of surface proteins from A431 cells and cellular extracts with nucleic acids was investigated using affinity modification with ^{32}P-labeled reactive oligonucleotide derivatives. Proteins with molecular weights of 68, 46, 38, and 28 kD as well as several low molecular weight proteins capable of binding to nucleic acids were found on the surface of intact cells. It was demonstrated that a protein with molecular weight of 68 kD is exposed at the cell surface, since the treatment of cells with trypsin results in the cleavage of this protein. Disruption of the integrity of the cell membrane (scrapping, treatment with trypsin, or permeabilization of the cell membrane with streptolysin O or saponin) disrupts the interaction of the reactive oligonucleotides with the cell surface proteins. Affinity modification of the cytosolic and membrane–cytosolic cell fractions with labeled oligonucleotides results in the modification of a large number of proteins, where proteins with molecular weights of 68, 46, 38, and 28 kD can be found as minor components. Surface oligonucleotide-binding proteins with molecular weight of ~68 kD were isolated by affinity chromatography after the modification of intact A431 cells with a reactive oligonucleotide derivative. The isolated surface oligonucleotide-binding proteins from A431 cells were sequenced, and one of the proteins was identified as keratin K1.

Key words: oligonucleotides, oligonucleotide binding proteins, nucleic acid receptors, affinity modification, keratin K1

Experiments with the use of antisense oligodeoxyribonucleotides (ODN) revealed their ability to penetrate into cells, to be accumulated in cellular compartments, and to interact with cellular nucleic acids [1]. There are data indicating that one of the oligonucleotide intracellular transport mechanisms is a receptor-mediated endocytosis, or liquid phase endocytosis at increased concentrations (more than 1-2 μM) [2]. Indeed, it was shown that oligonucleotides can be detected in the clathrin-coated vesicles as well as in lysosome- and endosome-like vesicular structures [3-5]. It was demonstrated by other investigations that inhibitors of endocytosis and formation of lysosomes slightly decrease the oligonucleotide transport into the cell and, apparently, oligonucleotides can permeate across the plasma membrane by another mechanism that is different from endocytosis [6, 7]. Cell treatment with trypsin dramatically suppresses oligonucleotide transport into the cell [8]. These data suggest that regardless of the mechanism of oligonucleotide transport into the cell, their interaction with the cell surface proteins is essential. The following techniques were used to isolate cell surface proteins that bind to the nucleic acids (NA): affinity modification of the cellular proteins by various reactive derivatives of phosphodiester and phosphorothioate oligonucleotides [9-11]; affinity chromatography [12, 13]; native gel electrophoresis [14]. Some of these proteins have been identified [15-17], but it is still unknown which of the discovered NA-binding proteins carry out oligonucleotide transport into the cell.

We have shown previously that cells of different origin express the same combination of NA-binding proteins, whose detection is dependent on the type of oligonucleotide reagent, conditions for the formation and detection of ODN–protein complexes, and on the integrity of the cell membrane [18]. We have studied NA-binding proteins in A431 cells using affinity modification by reactive ^{32}P-labeled oligonucleotides. To develop the

Abbreviations: NA) nucleic acids; ODN) oligodeoxyribonucleotide; FITC) fluorescein isothiocyanate; Flu) fluorescein residue; PBS) Phosphate Buffered Saline; DMEM) Dulbecco's modified Eagle's medium; DAP) diaminopentane; DEG) diethylene glycol; U_o) oxidized uridine.

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0006-2979/03/6811-1239$25.00 ©2003 MAIK "Nauka/Interperiodica"

isolation strategy for surface oligonucleotide-binding proteins, we have studied the influence of incubation conditions as well as different cell treatment on the affinity modification of NA-binding proteins. Surface oligonucleotide-binding proteins with molecular weight of ~68 kD were isolated by an affinity chromatography technique. Isolated oligonucleotide-binding proteins from A431 cells were sequenced, and one of them was identified as keratin K1.

MATERIALS AND METHODS

Oligonucleotides and synthesis of modified oligonucleotides. Oligonucleotide 5′-pCAGTAAATATCTAGGA ($p(N)_{16}$) was synthesized using automatic DNA-synthesizer ASM-700 (Biosset, Novosibirsk, Russia) according to the standard phosphitamide protocol. [2-(2-Dimethoxytrityloxyethoxy)ethyl]-(2-cyanoethyl)-(N,N-diisopropyl)amidophosphite obtained according to previously described protocol [19] was used for the synthesis of $p(N)_{16}$-deg-U oligonucleotide. 2′-OMe-U-RNA-CPG (Glen Research, USA) was used as a carrier support for the synthesis of $p(N)_{16}$-deg-U.

Radioactive label (^{32}P) was introduced by exchange of a 5′-terminal phosphate using T4-polynucleotide kinase. The alkylating group (ClR) was introduced in the oligonucleotides by the attachment of 4-[(N-2-chloroethyl-N-methyl)amino]benzylamine to a 5′-terminal phosphate of the $[^{32}P]p(N)_{16}$ according to a previously described technique [20]. The presence of active chlorine in the alkylating group was determined by the reaction with 0.5 M sodium thiosulfate for 10 h at room temperature [21].

To obtain 5′-fluorescein-labeled ODN (Flu-ODN), 5′-terminal phosphate was activated [20] and ODN was incubated with 0.1 M diaminopentane (DAP) solution in dimethylsulfoxide for 15 min. The conjugate of ODN with diaminopentane was isolated from the reaction mixture by gel filtration on Sephadex G-25 and incubated with fluorescein isothiocyanate (FITC) in 0.2 M Na_2CO_3 for 4 h at room temperature [22]. The obtained Flu-DAP-$p(N)_{16}$-deg-U derivative was purified by electrophoresis in 20% polyacrylamide gel in the presence of 7 M urea followed by electroelution.

To obtain the reactive dialdehyde group, the ribose moiety of 3′-terminal uridine in $p(N)_{16}$-deg-U or Flu-$p(N)_{16}$-deg-U was oxidized by 0.01 M $NaIO_4$ for 10 min immediately before use. After the modification of cell proteins by the obtained derivatives, $p(N)_{16}$-deg-U_o or Flu-$p(N)_{16}$-deg-U_o (U_o, oxidized uridine), the resulting conjugates (Schiff bases) were reduced by a 0.4 mg/ml solution of $NaBH_4$ for 10 min.

Cells and cultivation conditions. A431 cells were cultivated in DMEM containing 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 U/ml). For affinity modification, the cells were cultivated in the wells of a 24-well plate for 2-3 days before the experiment. Viability of the cells was estimated by staining with trypan blue.

Affinity modification of NA-binding proteins. The ability of surface proteins from A431 cells to interact with oligonucleotides was studied by affinity modification with the reactive oligonucleotide derivatives. The cells were rinsed twice with PBS or DMEM and incubated with 1 μM solution of oligonucleotide derivative in PBS (or DMEM) for 1 h at 37°C. After the incubation, the cells were rinsed with PBS (or DMEM) three times, scraped, and precipitated by centrifugation for 3 min at 3000 rpm. The cell precipitate was resuspended in 25 μl of lytic buffer containing 0.6% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 1 mM Na_2MoO_4 [23]. The cells were incubated on ice for 5 min and centrifuged for 5 min at 12,000 rpm for the separation of membrane–cytosolic and nuclear fractions. The supernatant, containing membrane–cytosolic fraction was transferred in other tubes, then an equal volume of the buffer for the application to the gel was added, heated for 5 min at 100°C, and centrifuged for 5 min at 12,000 rpm. The nuclear precipitate was resuspended in 25 μl of lytic buffer, and then an equal volume of the buffer for the application to the gel was added, heated for 5 min at 100°C, and centrifuged for 5 min at 12,000 rpm. Samples were prepared using equal amounts of cells. The proteins were analyzed using SDS-PAGE in a 10-20% gradient gel. After the gel was dried, the proteins modified by radioactive labeled oligonucleotide derivative were visualized by radioautography. Lactoferrin, lysozyme, and mouse IgG1, modified by the same oligonucleotide reagent, were used as protein markers [24], since the protein modification by oligonucleotides changes the protein mobility.

Investigation of NA-binding protein localization. To study the cellular localization of NA-binding proteins, the cytosolic, membrane–cytosolic, and nuclear fractions were isolated from the cells modified with $[^{32}P]ClRp(N)_{16}$ by treatment with saponin. To permeabilize the plasma membrane, the cells were washed twice with PBS and incubated for 20 min with a solution of saponin (40 μg/ml) or streptolysin O (10,000 U/ml) at room temperature. Under these conditions, the reagents produce pores in the plasma membrane [25]. After the treatment the cells were precipitated by centrifugation; the supernatant was collected as the cytosolic fraction, while the cells were resuspended in PBS, separated into membrane–cytosolic and nuclear fractions, and the proteins were analyzed as described earlier. The samples were prepared using equal amounts of cells. The amount of protein was estimated by staining the nitrocellulose blot with colloidal silver.

To investigate the membrane localization of NA-binding proteins, the cells were first incubated with

[^{32}P]ClRp(N)$_{16}$, and after that the cell surface proteins were hydrolyzed by treatment with 0.125% trypsin for 5 min at room temperature. Trypsin was neutralized by the addition of FCS, the cells were rinsed with PBS, and cellular fractions were separated. NA-Binding proteins were analyzed as described earlier.

Effect of the cell membrane integrity on the modification of NA-binding proteins and intracellular oligonucleotide transport. To study the effect of plasma membrane integrity on affinity modification of NA-binding proteins by the reactive [^{32}P]ClRp(N)$_{16}$ derivative, the cells were treated with saponin, scraped, or detached from the surface by 0.25% trypsin solution containing 0.53 mM EDTA. The collected cells were three times washed with PBS, resuspended in PBS (10^7 cells per 1 ml) and incubated with 1 μM [^{32}P]ClRp(N)$_{16}$ as described earlier.

Production and characterization of antibodies against fluorescein. To obtain the specific antibodies against fluorescein, FITC was conjugated to bovine serum albumin and used as an antigen [26]. To isolate the antibodies against fluorescein (Flu), the affinity sorbent was prepared from Ultrogel A2 activated by BrCN [27]. The latter was incubated with 1 M lysine solution at pH 8 for 2 h. The remaining reaction sites were blocked by the treatment with 1 M glycine solution, pH 9.6, for 30 min. The obtained sorbent was washed with 20-fold volume excess PBS, 20-fold volume of 0.2 M glycine buffer (pH 2.5), 20-fold volume of 0.25 M borate buffer (pH 8.7), and equilibrated with 10-fold volume of PBS. After that, a 4 mg/ml FITC solution in 1 M $NaHCO_3$, pH 8, was added to the resin modified with a lysine spacer (0.1 ml of the reagent per 1 g of resin) and incubated for 10 h at room temperature. The sorbent was washed in 20-fold volume of PBS, 20-fold volume of 0.2 M glycine buffer (pH 2.5), 20-fold volume of 0.25 M borate buffer (pH 8.7), and equilibrated with 20-fold volume of PBS.

To perform the affinity chromatography, total immunoglobulin fraction was isolated from rabbit antiserum and applied on the chromatographic column during 2 h. The column was washed with 10-fold volume of PBS containing 0.05% Tween-20 and with 10-fold volume of PBS. The specific antibodies were eluted with 0.2 M glycine buffer (pH 2.5) and then with 0.25 M borate buffer (pH 8.7). The amount of protein was determined spectrophotometrically at 280 nm. Specificity of the obtained antibodies towards fluorescein was investigated by dot immunoassay [26]. To check the ability of the antibodies to bind the oligonucleotides, ^{32}P-labeled Flu-DAP-p(N)$_{16}$-deg-U derivative was applied to a column containing immobilized anti-fluorescein antibodies. Radioactivity of the eluate and sorbent was measured to determine the extent of antibody binding to Flu-DAP-p(N)$_{16}$-deg-U.

Affinity chromatography of oligonucleotide-binding proteins, modified by Flu-DAP-p(N)$_{16}$-deg-U$_o$, on Ultrogel A2 with immobilized anti-Flu-antibodies. The affinity sorbent was prepared by the incubation of cyanogen bromide activated Ultrogel A2 in 0.01 M borate buffer, pH 8.7, with rabbit antibodies against fluorescein (5 mg of antibodies per 1 ml of resin). The remaining reactive sites were blocked by 0.1 M glycine solution, pH 8.0, and the resin was rinsed with 15 ml of 0.15 M NaCl.

Membrane–cytosolic fraction, obtained from 10^9 A431 cells modified by Flu-DAP-p(N)$_{16}$-deg-U$_o$ as described earlier, was applied to the column containing anti-Flu-antibodies immobilized on a resin (Ultrogel A2) and incubated for 15 min. After the incubation the affinity sorbent was washed with 20-fold volume of PBS containing 0.1% SDS, and oligonucleotide-binding proteins were eluted with 0.2 M glycine buffer, pH 2.5. The eluate was concentrated using a Centricon-50 membrane (Millipore, USA) with a molecular weight cut off 50 kD. Concentrated eluate was diluted with an equal volume of the buffer for application, then heated for 5 min at 100°C, and centrifuged for 5 min at 14,000 rpm. The proteins were separated by SDS-PAGE in a gradient gel (10-20%).

Series of control samples were analyzed in duplicate. The proteins were applied on a nitrocellulose membrane (0.45 μm) by electroelution, one lane was stained with colloidal silver, and another with anti-Flu-antibodies. After the staining, the proteins modified by radioactively labeled oligonucleotide derivative were detected by radioautography. Gel containing the proteins isolated from 10^9 cells was stained after electrophoresis with Coomassie G-250 solution. The gel lane corresponding to the proteins with molecular weight of ~68 kD was collected for subsequent sequencing.

RESULTS AND DISCUSSION

To perform affinity modification of the cell proteins, conjugates of 4-[(N-2-chloroethyl-N-methyl)amino]-benzylamine with 16-mer heterogeneous oligonucleotide pCAGTAAATATCTAGGA (p(N)$_{16}$) were used. As we demonstrated earlier, oligonucleotide p(N)$_{16}$ exhibits an increased affinity to a nuclear NA-binding protein [28]. Our experiments on the affinity modification of various tissue cells by reactive oligonucleotides revealed that the number of detected proteins has a minor dependency on the oligonucleotide sequence; however, the degree of NA-binding protein modification is higher when using ClRp(N)$_{16}$ than ClRp(T)$_{16}$ [18]. When the cells were incubated with ClRp(N)$_{16}$ in PBS, the proteins with molecular weights of 68, 38, and 28 kD are the main targets for affinity modification (Fig. 1, lanes *3*, *4*). Incubation in DMEM increases the degree of modification of all these proteins with exception of a 38 kD protein (whose modification degree does not change) and also results in the emergence of several new proteins, the

major one having molecular weight of 46 kD (Fig. 1, lanes *1*, *2*). Modification of the same proteins is observed in DMEM containing 10% fetal serum, and in the medium without serum (data not presented). NA-Binding proteins were found in the membrane–cytosolic cell fraction. The nuclear fractions contained smaller amount of the proteins, and cytosolic practically none except for the 38 kD protein. NA-Binding protein with molecular weight of 38 kD from the cytosolic fraction of permeabilized cells (Fig. 1, lane *9*) was earlier identified by us as glyceraldehyde-3-phosphate dehydrogenase [28]. Trypsin treatment of cells previously incubated with $[^{32}P]ClRp(N)_{16}$, results in hydrolysis of the 68 kD protein, which indicates its exposition on the outer cell surface (Fig. 1, lane *5*). The presence of this protein in the membrane–cytosolic and nuclear fractions supports the idea that the protein is not only involved in binding, but also in intracellular oligonucleotide transport.

It has been shown that one of the mechanisms for oligonucleotide transport into K562, MOLT-4, HL60, and DA1 cells is receptor-mediated endocytosis [3, 4]. However, is it possible that there are other ways for the oligonucleotides to penetrate into a cell, since the inhibitors of endocytosis and lysosome formation inhibit the oligonucleotide transport only to a minor extent [6, 7] or do not affect oligonucleotide transportation at all (as demonstrated in the case of keratinocytes [29]). One of these transport mechanisms for nucleic acids can be transport across a membrane channel that is formed with the presence of a 45-kD NA-binding protein [13].

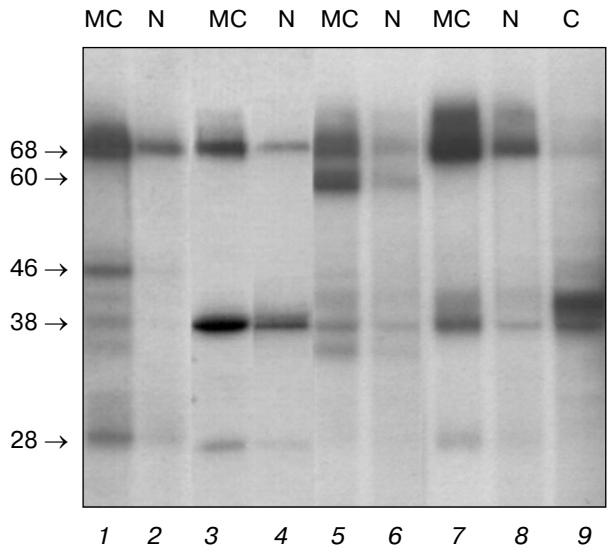

Fig. 1. Study of the cellular localization of NA-binding proteins. A431 cells were incubated with 1 μM $[^{32}P]ClRp(N)_{16}$ in PBS (*1*, *2*) or DMEM (*3*, *4*). Cells modified in PBS were treated with trypsin (0.25%, 5 min) (*5*, *6*) or saponin (40 μg/ml, 20 min) (*7-9*). After cell treatment with saponin, cytosolic (C) fraction was collected as a supernatant. Membrane–cytosolic (MC) and nuclear (N) fractions were isolated from the remaining cells as described in "Materials and Methods". Proteins were analyzed by SDS-PAGE in a gradient polyacrylamide gel (10-20%) with subsequent radioautography. Molecular weights (kD) of the proteins are indicated on the left.

We have demonstrated that inhibitors of active transport (sodium azide, chloroquine, and monensin) and ATP have no influence either on the affinity modification of NA-binding proteins in PBS and in DMEM or on the accumulation of the oligonucleotides in the cell (data not presented). Hence, these proteins probably bind to the oligonucleotides on the cell surface and are not involved in the endocytosis, whereas the oligonucleotide transport is accomplished according to another mechanism that is different from endocytosis. The treatment of cells with trypsin as well as disruption of cell membrane integrity by treatment with saponin and scraping results in cancellation of protein affinity modification with $ClRp(N)_{16}$ (Fig. 2), and in a dramatic decrease in oligonucleotide content in the cells. Partial disruption of the cell surface by scraping, which provides the intracellular delivery of macromolecules, was proposed by McNeil [30], and applied for delivery of morpholine oligonucleotides into the cell [31]. However, the same report demonstrated that morpholine oligonucleotides penetrate into the cells only during the first minute after the scraping, while there is no oligonucleotide accumulation in trypsin-treated cells. It is known that the disruption and renaturation of the plasma membrane is a continuously ongoing process. For instance, 5-30% of muscle cells are damaged, and gaps in the cell membrane up to 1 μm in diameter are not lethal for mammalian cells [32]. Regeneration of the damaged plasma membrane requires Ca^{2+} and the complete process takes between a few seconds and up to 1 min [30]. Regeneration of all plasma membrane functions including the reconstruction of the cytoskeleton and cytoplasm contacts can take a longer time. It should be noted that the main component of DMEM that is responsible for protein binding to nucleic acids is Ca^{2+}. Indeed, it was shown that oligonucleotide transport into the cell depends on the presence of Ca^{2+} in the medium [7, 33]. Since Ca^{2+} is essential for integrity and renaturation of damaged cell membrane [30], the addition of Ca^{2+} into PBS obviously maintains the normal state of the cell membrane and allows oligonucleotides to interact with the cell surface proteins.

As we reported earlier, cell pre-treatment with trypsin disrupts not only the affinity modification of proteins with $ClRp(N)_{16}$, but also the transportation of fluorescein-labeled oligonucleotides into the cell [8]. Hence, it can be assumed that the detected proteins are important not only for nucleic acid binding to the cell, but also for their transport.

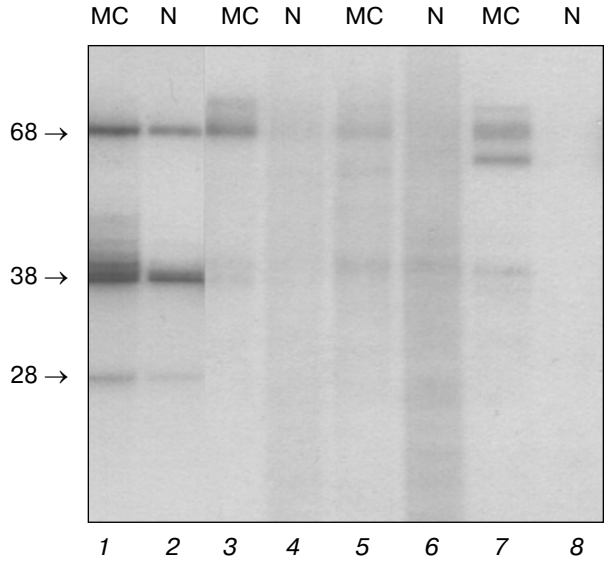

Fig. 2. Effect of cell membrane disruption on the modification of NA-binding proteins by the [^{32}P]ClRp(N)$_{16}$ oligonucleotide derivative. Intact A431 cells (*1*, *2*), cells which were pre-treated with saponin (40 μg/ml, 20 min) (*3*, *4*) or trypsin (0.25%, 5 min) (*7*, *8*), and cells scraped from the surface (*5*, *6*) were incubated with 1 μM [^{32}P]ClRp(N)$_{16}$ for 1 h at 37°C in PBS. Membrane–cytosolic (MC) and nuclear (N) fractions were isolated as described in "Materials and Methods". Proteins were analyzed by SDS-PAGE in a gradient polyacrylamide gel (10-20%) with subsequent radioautography. Molecular weights (kD) of the proteins are indicted on the left.

Treatment of membrane–cytosolic, cytosolic, and nuclear fractions with ClRp(N)$_{16}$ results in the affinity labeling of a large number of proteins, where NA-binding proteins of the intact cell are also found as minor components (Fig. 3). Therefore, not all proteins that are able to bind nucleic acids are involved in the recognition and transportation of the nucleic acids in the intact cell. This data suggest that the most acceptable technique for the isolation of surface oligonucleotide-binding proteins is affinity modification of the intact cells by a reactive oligonucleotide derivative. The following isolation of covalent affinity complex can be performed by making use of binding to a specific ligand introduced in ODN with an affinity sorbent.

Flu-DAP-p(N)$_{16}$-deg-U$_o$ was chosen as an affinity reagent containing a ligand for the following isolation of covalent complex. It was proved that Flu-DAP-p(N)$_{16}$-deg-U$_o$ modifies the same proteins as ClRp(N)$_{16}$ (Fig. 4). Moreover, the same proteins are labeled after the modification of cell surface proteins by the reactive double-stranded and single-stranded oligonucleotides. (Fig. 4). Non-modified oligonucleotides and DNA efficiently inhibit the affinity modification of NA-binding proteins by the reactive single- and double-stranded oligonucleotide derivatives, while free fluorescein does not affect the affinity modification of proteins (data not shown). Therefore, the chosen affinity reagent can be used for the isolation of surface oligonucleotide-binding proteins.

A431 cells (10^9) were modified in DMEM by the Flu-DAP-p(N)$_{16}$-deg-U$_o$ oligonucleotide reagent; the modified proteins were extracted from the membrane–cytosolic cell fraction on the affinity sorbent containing anti-fluorescein antibodies. To prove the fact that by using affinity chromatography the desired proteins were extracted, an aliquot of a specific eluate after SDS-PAGE was transferred to a nitrocellulose membrane (0.45 μm) by electroelution, and one trial was stained with colloidal silver, while another was stained with anti-Flu-antibodies. It was demonstrated that rabbit anti-Flu-antibodies specifically bound the same ODN–protein complexes as those detected by radioautography.

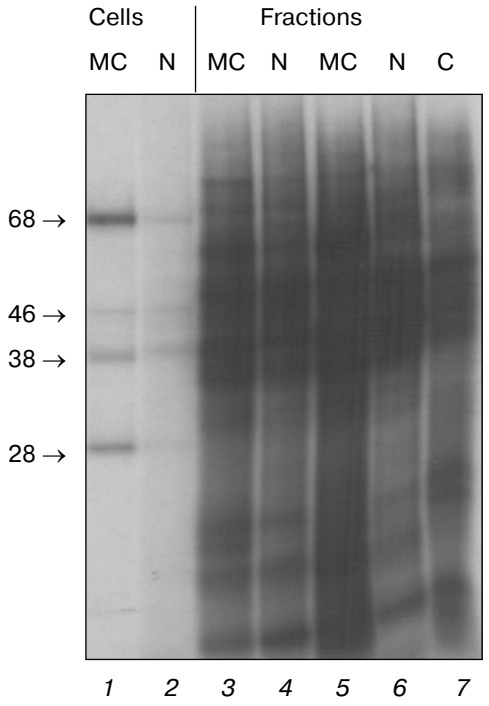

Fig. 3. Affinity modification of the cell fractions and intact A431 cells with [^{32}P]ClRp(N)$_{16}$. Cells and cell fractions were incubated for 1 h with 1 μM [^{32}P]ClRp(N)$_{16}$. Affinity modification of NA-binding proteins in DMEM: intact A431 cells (*1*, *2*), membrane–cytosolic (MC) and nuclear (N) fractions (*3*, *4*), and modification of membrane–cytosolic (MC), nuclear (N), and cytosolic (C) fractions treated with saponin (40 μg/ml, 20 min) (*5-7*). Proteins were analyzed by SDS-PAGE in a gradient gel (10-20%) with subsequent radioautography. Molecular weights (kD) of the proteins are indicated on the left.

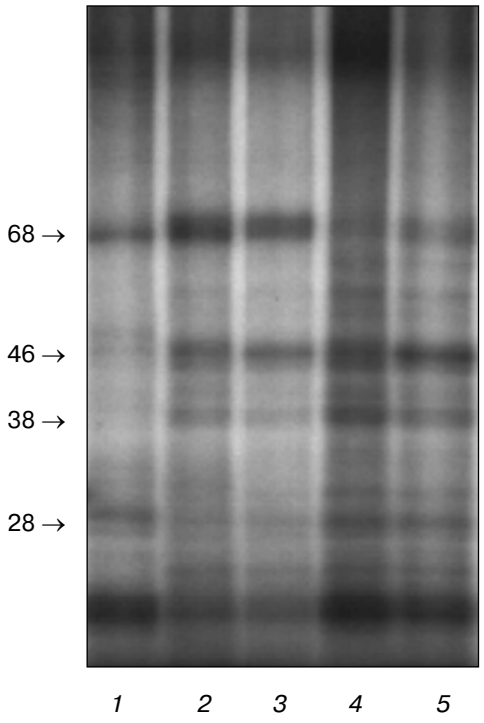

Fig. 4. Affinity modification of membrane–cytosolic NA-binding proteins of intact A431 cells by different derivatives of the single- and double-stranded $p(N)_{16}$-deg-U_o oligonucleotide: $p(N)_{16}$-deg-U_o/$p(N)_{16}$ (*1*), ClR-$p(N)_{16}$-deg-U_o/$p(N)_{16}$ (*2*), Flu-DAP-$p(N)_{16}$-deg-U_o (*3*), $p(N)_{16}$-deg-U_o (*4*), ClR-$p(N)_{16}$-deg-U_o (*5*). Cells were incubated for 1 h with 1 μM oligonucleotide derivative in DMEM at 37°C. Proteins were analyzed by SDS-PAGE in a gradient gel (10-20%) with subsequent radioautography. Molecular weights (kD) of the proteins are indicated on the left.

Localization of proteins after the staining of nitrocellulose filter with colloidal silver also corresponded to the lanes on the radioautograph (Fig. 5). It is important to note that the modification with oligonucleotide derivative increases the protein molecular weight by ~5 kD and thus decreases its electrophoretic mobility. Hence, the modified proteins are located on electrophoregram above the same proteins that were not modified by an affinity reagent.

Affinity chromatography was already used for the isolation of NA-binding protein in the previous investigations. Various affinity sorbents were applied to extraction of the above mentioned proteins. For instance, Loke et al. isolated an oligonucleotide-binding protein with a molecular weight of 80 kD using oligo(dT)-cellulose [3]. Hanss et al. used biotinylated heterogeneous 20-mer phosphorothioate oligonucleotide immobilized on streptavidin-agarose to isolate a 45 kD protein that is involved in the formation of a membrane channel for nucleic acid transport [13]. A sorbent with immobilized streptavidin was used for the extraction of a 66 kD protein, modified by biotinylated conjugate of oligonucleotide with benzophenone [15]. However, it should be noted that avidin is characterized by unspecific binding with proteins and nucleic acids due to the presence of strong basic domains in the protein structure. Unspecific binding significantly complicates the use of avidin for affinity chromatography. For this reason, we could not isolate the individual proteins in the preliminary experiments for the isolation of affinity complexes with biotinylated ODN on avidin-Sepharose. Rabbit anti-Flu-antibodies used as an affinity sorbent result in highly specific binding and extraction of proteins modified with fluorescein-labeled oligonucleotide reagent by means of affinity chromatography technique.

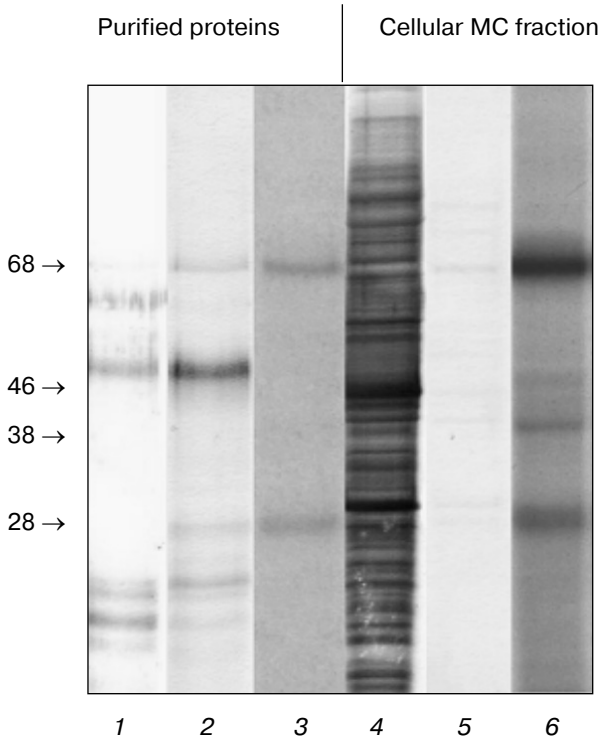

Fig. 5. Isolation of surface ODN-binding proteins using affinity modification of A431 cells by [^{32}P]Flu-DAP-$p(N)_{16}$-deg-U_o oligonucleotide derivative with subsequent affinity chromatography on a sorbent with immobilized antibodies against fluorescein. *1*, *2*, *3*) Oligonucleotide-binding proteins extracted using affinity modification with subsequent affinity chromatography: protein staining with colloidal silver (*1*), immunoperoxidase detection by using anti-fluorescein antibodies (*2*), radioautograph (*3*). *4*, *5*, *6*) Proteins of A431 cell lysate affinity modified by [^{32}P]Flu-DAP-$p(N)_{16}$-deg-U_o: protein staining with colloidal silver (*4*), immunoperoxidase detection by using anti-fluorescein antibodies (*5*), radioautograph (*6*). Proteins were analyzed by SDS-PAGE in a gradient gel (10-20%) with subsequent radioautography. Molecular weights (kD) of the proteins are indicated on the left.

Amino acid sequencing of the isolated proteins with molecular weight of ~68 kD was performed by mass-spectrometry. The results identified one of the extracted proteins as keratin K1.

The presence of cytokeratins on the cell membrane was reliably proved in several investigations within recent years [34-36]. For instance, Mahdi et al. determined cellular localization of keratin K1 using specific antibodies against epitopes encoded by the 1st and 2nd exons of the keratin K1 gene. Using these antibodies in combination with immunoperoxidase staining, immunofluorescence, and transmission microscopy techniques, it was shown that at least two of these keratin K1 epitopes are exposed on the outer membrane surface of non-permeabilized human umbilical vein endothelial cells (HUVECs). Flow cytometry confirmed the presence of keratin K1 on the surface of HUVEC cells. Furthermore, the number of specific membrane sites for keratin K1 exposed on the cell surface was determined to be equal to $7.2 \cdot 10^4$ molecules per cell [34]. These data correlate with the amount of oligonucleotide-binding proteins with a molecular weight of 68 kD on the surface of epithelial A413-line cells ($6.2 \cdot 10^4$ binding sites per cell) [8]. Hence, keratin K1 is the most likely candidate for the primary recognition and binding to the nucleic acids on the cell surface. The ability of keratin K1 to bind extracellular ligands is not unique: recent investigations demonstrated keratin K8 is a plasminogen receptor in the endothelial cells, hepatocytes and breast cancer cells [37-40], while cytokeratin K18 binds to the complex of thrombin with antithrombin [41]. Moreover, the interaction of keratin K18 with the DNA was reported [42].

Until recently it was commonly thought that cytokeratin K1 (belonging to the protein family of intermediate filaments) is involved only in the formation of cellular cytoskeleton. It was shown within recent years that keratin K1 is localized on the membranes of endothelial cells along with a urokinase plasminogen activating receptor [34], and therefore is involved in a multi-protein receptor complex. Within this multi-protein complex, keratin K1 is exposed on the outer surface of HUVEC cells and is involved in the binding of kininogens [43] and factor XII [44]. It was shown that keratin K1 contributes in the regulation of kallikrein–kinin system [45], and the ability of keratin K1 to be phosphorylated by cellular kinases suggests that it is involved in the induction of intracellular signaling systems [46].

The crucial point in the activation of kallikrein–kinin system is a conversion of prekallikrein into kallikrein. This conversion takes place on the endothelial cell surface according to a poorly understood mechanism, only in the case if high-molecular-weight kininogens are also bound to the endothelial cells. It was shown that antibodies against keratin K1 prevent the binding of prekallikrein with the cells and therefore suppress its activation [34]. Hence, the binding of kininogens to the cells is essential for the normal function of the kallikrein–kinin system.

It is known that thiophosphate oligonucleotides with an increased affinity to proteins have an influence on arterial pressure, cardiac output, and heart contraction [47]. One of the mechanisms of nonspecific action of nucleic acids action can be competitive binding with keratin K1, which results in the inhibition of the kallikrein–kinin system.

This work was supported by the Russian Foundation for Basic Research (RFBR, project No. 01-04-49862), Federal Program No. 43.073.1.1.2503 (project "Gene-directed biologically active compounds as anti-tumor and anti-virus drugs"), Civil Research and Development Foundation (CDRF, project No. REC 008), and INTAS (project No. 991-630).

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