Investigation of surface oligonucleotide-binding proteins of eucaryotic cells by affinity modification with reactive oligonucleotide derivatives

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Interactions between oligodeoxyribonucleotides (ODN) with different sequences and cell proteins were examined using the affinity modification by [^{32}P]-labeled reactive oligonucleotide derivatives. 3´-Terminal ribouridine oxidized with sodium periodate, 4-[(*N*-2-chloroethyl-*N*-methyl)amino]benzylamine, and the maleimide residue were used as reactive groups. All the compounds used are specific reagents. The set of the discovered nucleic acid-binding (NA-binding) proteins depends on the chemical properties of the affinity reagent. The presence of the hydrophobic group at the 5´-terminus of the ODN molecule is the key factor determining the variety of the discovered NA-binding proteins. The cells of different origin (A431, HeLa, KB, MCF-7, Hep-2, K562, Cos-7, NIH/3T3, human-lung primary epithelial cells, and porcine kidney primary cells) are characterized by the same set of NA-binding proteins whose affinity modifications depends on the conditions of incubation of oligonucleotides with the cells. Treatments of cells disturbing the integrity of the cellular membrane (scrapping, treatment with trypsin, or cell permeabilization with streptolysin O or saponin), disrupt interactions between NA-binding proteins from native cells and ODN.

Key words: oligonucleotides, oligonucleotide-binding proteins, nucleic acid receptors, affinity modification.

Inhibition of gene activity under the action of antisense oligonucleotides, immune-stimulating activity of nucleic acids (NA), and gene immunization are based on interactions between nucleic acids and cell biopolymers.[1—4] The penetration into cells is a step necessary for nucleic acids to realize their biological potential. Interactions of nucleic acids with surface proteins are apparently a necessary step of transport and, in addition, may be responsible for particular non-specific effects of NA. It was demonstrated that treatment of cells with trypsin essentially inhibits the penetration of ODN into cells.[5,6] Binding of NA to specific cell surface proteins is a necessary condition for the efficient transport of NA irrespective of whether NA penetrate into cells *via* receptor-mediated endocytosis[7,8] or through alternative mechanisms.[9]

The affinity modification by reactive oligonucleotide derivatives is widely used in investigations of NA-binding proteins. Cell NA-binding proteins were modified with the use of photoactivable ODN derivatives[10—12] or alkylating ODN derivatives[13—15] as affinity reagents and by treating ODN—protein complexes with cross-linking reagents.[16] The affinity modification offers a unique possibility to isolate and identify NA-binding proteins of living cells because this approach enables one to simulate interactions between the living cells and NA.

Because of the variety of the oligonucleotides, reactive groups, conditions of modifications, and cell models used in studies and the possible inaccuracy of the molecular weight determination, a large number of NA-binding proteins were described.[7—18] Althought some proteins have been identified,[9,17,18] it is still unclear whether NA-binding proteins are universal or, by contrast, different types of cells express different specific NA-binding proteins. It remains unknown which NA-binding proteins perform the ODN transport.

We examined the affinity modification of cell NA-binding proteins with the use of alkylating, aldehyde, and maleimide derivatives of ODN. It was demonstrated that all the compounds used are specific reagents, and the set of the discovered NA-binding proteins depends on the nature of the affinity reagent. Nucleic acid-binding proteins of different tissue origin were studied with the use of alkylating [^{32}P]-labeled oligonucleotide derivatives. The dependence of the affinity modification of NA-binding proteins on the conditions of in-

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cubation and the mode of treatment was examined. Our experiments showed that cells of different origin express the same set of NA-binding proteins whose detection depends on the conditions of the formation of ODN-protein complexes and the integrity of the cellular membrane.

Experimental

Oligonucleotides and synthesis of modified oligonucleotides. The oligonucleotides 5′-pCAGTAAATATCTAGGA ($p(N)_{16}$) and 5′-$p(T)_{16}$ were synthesized on an ASM-700 oligonucleotide synthesizer (BioSet, Novosibirsk, Russia) by the phosphoramidite method. The oligonucleotide $p(N)_{16}$-deg-rU was synthesized on an automated ASM-700 synthesizer with the use of (dimethoxytrityl)diethylene glycol β-(β-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, which was prepared from diethylene glycol (deg) (Fluka) according to a procedure described previously,[19] starting from dimethoxytrityl-rU, CPG-500.

The ^{32}P radioactive label was inserted by exchanging 5′-terminal phosphate with the use of T4 polynucleotide kinase. The alkylating group (ClR) was introduced into the oligonucleotides by the attachment of 4-[(*N*-2-chloroethyl-*N*-methyl)amino]benzylamine to 5′-terminal phosphate $[^{32}P]p(N)_{16}$ according to a procedure described previously.[20] The presence of active chlorine in the alkylating group was determined in the reaction with 0.5 *M* sodium thiosulfate by keeping the mixture at ~20 °C for 10 h.[21]

5′-Fluorescent-labeled ODN was designed by activation of 5′-terminal phosphate of ODN[20] followed by incubation with a 0.1 *M* solution of 1,5-diaminopentane (DAP) in DMSO. The ODN conjugate with DAP was separated from the components of the reaction mixture by gel filtration on Sephadex G-25 and incubated with fluorescein isothiocyanate in 0.1 *M* Na_2CO_3 at ~20 °C for 4 h.[22]

With the aim of attaching the maleimide residue (MaM) at the 5′-terminal phosphate of ODN, DAP was bound to activated phosphate as described previously. Modified ODN was isolated by gel filtration on Sephadex G-25 and incubated in 50% DMSO with a threefold molar excess of *N*-hydroxysuccinimide ester of 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid.[23] An excess of the reagent was removed by gel filtration on Sephadex G-25. The concentration of the maleimide groups was determined from a decrease in the optical density at 412 nm (ε = 13600 for 2-nitro-5-benzoate) in the reaction of Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) with 2-mercaptoethanol in the absence or presence of modified ODN.

The maleimide residue was attached at the 3′-terminus of ODN by oxidation of $p(N)_{16}$-deg-rU or Flu-$p(N)_{16}$-deg-rU with 0.01 *M* $NaIO_4$ at ~20 °C for 10 min followed by incubation with 0.1 *M* lysin (pH 10.0) at ~20 °C for 1 h. The resulting conjugate was freed from salts by gel filtration on Sephadex G-25 and incubated in 50% DMSO with a threefold molar excess of *N*-hydroxysuccinimide ester of 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid.[23] An excess of the reagent was removed by gel filtration on Sephadex G-25 and the concentration of the maleimide groups was determined as described above.

All the ODN derivatives thus designed, except for ClR-ODN, were purified by electrophoresis in 20% polyacrylamide gel (PAAG) containing 7 *M* urea followed by electroelution.

Cells and conditions of their cultivation. Cells of the lines A431 (human epidermal carcinoma), HeLa (carcinoma of the human cervix), KB (epithelial carcinoma of the human oral cavity), MCF-7 (carcinoma of the human breast), Hep-2 (carcinoma of the human larynx), LECh (human lung epithelial primary cells), K562 (human myelogenous leukemia), Cos-7 (African green monkey kidney cells), SPEV (porcine embryonic kidney cells), and NIH/3T3 (mouse fibroblasts) were cultered in a DMEM medium containing 10% FCS (fetal calf serum) and antibiotics (penicillin and streptomycin, 100 IU mL^{-1}). Prior to the experiments on the affinity modification, cells were seeded into 24-well plates and cultured for 2—3 days. The density of the cell monolayer cultures used were no higher than 70% of the monolayer, and the density of the cell suspension cultures were no higher than $0.8 \cdot 10^6$ cells mL^{-1}. The cell viability was determined by staining with Trypan Blue.

Affinity modification of oligonucleotide-binding proteins. Prior to the addition of the affinity reagents, the cells were twice washed with PBS (0.01 *M* sodium phosphate buffer, pH 7.4, 0.0027 *M* KCl, 0.137 *M* NaCl) (Sigma) or DMEM and then incubated with 1 μM reactive ODN in PBS (or in a DMEM medium) at 37 °C for 1 h under 5% CO_2. After incubation, the cells were washed three times with PBS (or a DMEM medium), scrapped, and precipitated by centrifugation at 3000 rpm for 3 min. Then the precipitate of the cells was resuspended in a 25 μL lysis buffer containing 0.6% Nonidet P-40, 50 *mM* Tris-HCl pH 7.5, 0.15 *M* NaCl, 5 *mM* NaF, 1 *mM* PMSF, 2 *mM* EDTA, and 1 *mM* Na_3MoO_4.[24] The cells were incubated on ice for 5 min and then centrifuged at 12000 rpm with the aim of separating the membrane-cytosolic (MC) and nuclear (N) fractions. The supernatant containing the membrane-cytosolic fraction was transferred into new test tubes and an equal volume of the application buffer was added. Then the mixture was heated at 100 °C for 5 min and centrifuged at 12000 rpm for 5 min. The precipitate of cell nuclei was resuspended in a lysis buffer (25 μL) and an equal volume of the application buffer was added. Then the mixture was heated at 100 °C for 5 min and centrifuged at 12000 rpm for 5 min. The specimens were prepared from equal amounts of the cells. The proteins were separated by disc electrophoresis in 10—20% polyacrylamide gradient gel containing sodium dodecyl sulfate (SDS). After drying of the gel, the proteins modified by radioactive-labeled oligonucleotide derivatives were visualized by autoradiography. Taking into account that the modification of the proteins by oligonucleotides led to a change in protein mobility, proteins with known molecular weights (lactoferrin, lysozyme, and IgG) modified by the same oligonucleotide reagent were used as molecular weight markers.[25]

Study of the specificity of the affinity modification of proteins. To examine the specificity of binding of proteins to the reactive ODN derivatives, the cells were incubated with the 1 μM $[^{32}P]$-labeled reactive ODN derivative in the presence of a 10- or 50-fold molar excess of unlabeled $p(N)_{16}$, a 50-fold molar excess of free fluorescein, and a 50-fold molar excess of plasmid DNA. The fractions were prepared and the proteins were separated by electrophoresis as described above.

Study of cellular localization of NA-binding proteins. The cells modified by $[^{32}P]ClRp(N)_{16}$ were treated with 0.25% trypsin at ~20 °C for 3 min and separated into the cytosolic,

membrane-cytosolic, and nuclear fractions by treatment with saponin. With the aim of permeabilizing the cellular membrane, the cells were twice washed with PBS and incubated with a solution of saponin (40 μg mL^{-1}) or streptolysin O (10000 IU mL^{-1}) at ~20 °C for 20 min. Under the conditions used, these reagents form pores in the plasmatic membrane.[26] After the treatment, the cells were precipitated by centrifugation and the supernatant was collected as the cytosolic fraction. The cells were resuspended in PBS and separated into the membrane-cytosolic and nuclear fractions. The proteins were analyzed as described above. The specimens were prepared from the same amount of the cells. The amount of the protein was estimated from the color of the nitrocellulose blot obtained under the action of colloidal silver. With the aim of studying membrane localization of the NA-binding proteins, the cells were incubated with $[^{32}P]ClRp(N)_{16}$ and then the cell surface proteins were hydrolyzed by treatment with a 0.125% solution of trypsin at ~20 °C for 5 min. The trypsin was inhibited by the addition of fetal calf serum, the cells were washed off from the serum with the use of PBS, and cell fractions were prepared. The NA-binding proteins were analyzed as described above.

Influence of the integrity of the cellular membrabe on the modification of NA-binding proteins and intracellular transport of ODN. With the aim of examining the influence of the integrity of the cellular membrabe on the affinity modification of NA-binding proteins by the reactive derivatives $[^{32}P]ClRp(N)_{16}$, the cells were treated with saponin and removed from the surface either with the use of a scraper or by treatment with a 0.25% solution of trypsin containing 0.53 *mM* EDTA. The cells thus removed were washed three times with PBS, resuspended in PBS (10^7 cells mL^{-1}), and incubated with 1 *μM* $[^{32}P]ClRp(N)_{16}$ as described above.

With the aim of examining the influence of the integrity of the surface proteins on the affinity modification of oligonucleotide-binding proteins by $[^{32}P]ClRp(N)_{16}$, the cells were removed by a 0.25% solution of trypsin containing 0.53 *mM* EDTA. After neutralization of the trypsin with FCS, the cells were washed with an excess of PBS. Then the cells ($(0.5—1)\cdot 10^7$ cells mL^{-1}) were incubated with 1 *μM* $[^{32}P]ClRp(N)_{16}$ in PBS at 37 °C for 1 h.

Results and Discussion

We synthesized different reactive ODN derivatives and then used them as affinity reagents in the study of NA-binding proteins. In preliminary experiments, we showed that the oligonucleotide 5′-pCAGTAAATATCTAGGAU can also be used for the affinity modification of proteins after periodate oxidation of ribose. However, the attachment of uridine through the diethylene glycol linker leads to a substantial increase in the extent of modification of oligonucleotide-binding proteins.

Previously, it has been demonstrated[3,4,13,14,25] that oligonucleotides bearing the alkylating reactive group, *viz.*, 4-[(*N*-2-chloroethyl-*N*-methyl)amino]benzylamine, and terminal ribouridine, can be used for the affinity modification of proteins and nucleic acids. The affinity modification of cell proteins was performed with the use of covalent conjugates of the reactive groups with two oligonucleotides, *viz.*, 16-mer homothymidylate and a 16-mer heterogeneous oligonucleotide. We chose $p(N)_{16}$ because this oligonucleotide possesses high affinity for the ODN-binding nuclear protein, which has been identified as glyceraldehyde-3-phosphate dehydrogenase.[27] Cells from the A431 line were used for the study of the affinity modification of surface proteins by different reactive derivatives of the oligonucleotide $p(N)_{16}$degU. It was shown that the set of the discovered membrane proteins depends substantially on the nature and the position of the reactive group (Fig. 1). The oligonucleotide derivatives $p(N)_{16}$degU and $p(N)_{16}$degU-Lys-MaM containing free 5′-terminal phosphate modify a much wider range of proteins as compared to oligonucleotides whose 5′-terminal phosphate is modified by reactive groups or the fluorescein residue. It should be noted that both oligonucleotide derivatives react in a similar fashion with proteins with a molecular weight lower than 30 kDa and modify various high-molecular-weight proteins. Reactive oligonucleotide derivatives modified at 5′-terminal phosphate have different efficacy in the modification of proteins with the same molecular weight. 5′-Modified

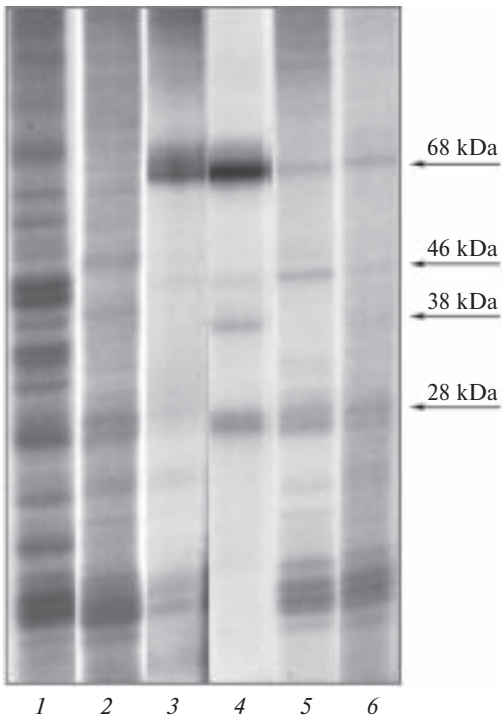

Fig. 1. Affinity modification of membrane-cytosolic NA-binding proteins of intact cells from the A431 line by different derivatives of the oligonucleotide $p(N)_{16}$degU: *1*, $p(N)_{16}$degU; *2*, $p(N)_{16}$degU-Lys-MaM; *3*, MaM-DAP-$p(N)_{16}$degU; *4*, Flu-DAP-$p(N)_{16}$degU; *5*, Flu-DAP-$p(N)_{16}$degU-Lys-MaM; *6*, ClR-$p(N)_{16}$degU. The cells were incubated with the oligonucleotide derivatives (1 μmol L^{-1}) in DMEM at 37 °C for 1 h. The proteins were analyzed by SDS disc electrophoresis in gradient (10—20%) PAAG followed by autoradiography.

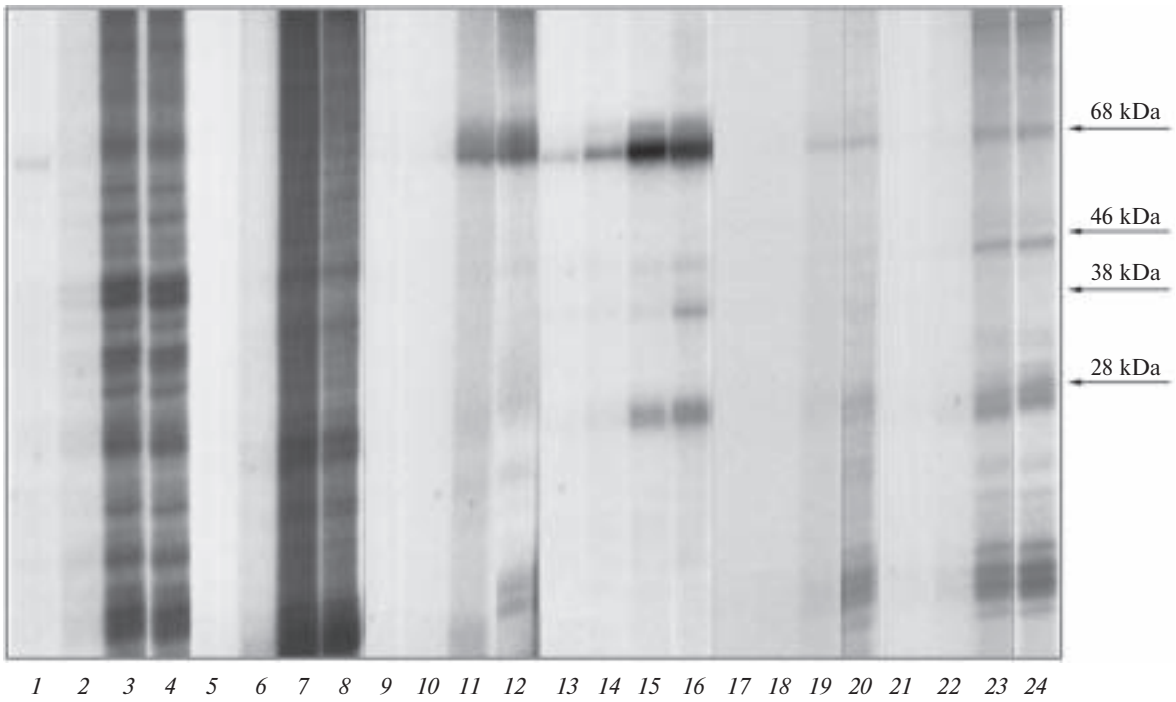

Fig. 2. Specificity of binding of reactive derivatives of the oligonucleotide $p(N)_{16}$degU to cell proteins: *1—4*, $p(N)_{16}$degU; *5—8*, $p(N)_{16}$degU-Lys-MaM; *9—12*, MaM-DAP-$p(N)_{16}$degU; *13—16*, Flu-DAP-$p(N)_{16}$degU; *17—20*, Flu-DAP-$p(N)_{16}$degU-Lys-MaM; *20—24*, ClR-$p(N)_{16}$degU. Cells from the A431 line were incubated with the oligonucleotide derivatives (1 μmol L^{-1}) in DMEM at 37 °C for 1 h in the presence of potential competitors: 150 μg mL^{-1} of plasmid DNA (*1*, *5*, *9*, *13*, *17*, *21*), 50 μmol L^{-1} of $p(N)_{16}$degU (*2*, *6*, *10*, *14*, *18*, *22*), or 50 μmol L^{-1} of Flu (*3*, *7*, *11*, *15*, *19*, *23*); and in the absence of the competitors (*4*, *8*, *12*, *16*, *20*, *24*). The proteins were analyzed by SDS disc electrophoresis in gradient (10—20%) PAAG followed by autoradiography.

oligonucleotide derivatives bind surface proteins more selectively and the proteins detected with the use of these derivatives are much fewer in number. Hence, the modification of proteins by reactive oligonucleotide derivatives depends not only on the properties of the reactive group but also on the modifications at the 5′-terminus of the oligonucleotide molecule.

To examine the specificity of binding of oligonucleotides to cell proteins, the cells were incubated with reactive oligonucleotide derivatives in the presence of a nonmodified oligonucleotide and double-stranded DNA (ds-DNA) (Fig. 2). The nonmodified oligonucleotide and DNA efficiently inhibit the affinity modification of NA-binding proteins by reactive oligonucleotide derivatives. Free fluorescein (Flu) has no effect on the affinity modification of proteins. Hence, the modification of proteins by reactive oligonucleotide derivatives proceeds specifically, and binding to proteins is determined by the oligonucleotide component of the affinity reagent.

Experiments on the affinity modification of cells of different tissue origin by derivatives of the oligonucleotides $p(N)_{16}$ and $p(T)_{16}$ with 4-[(*N*-2-chloroethyl-*N*-methyl)amino]benzylamine demonstrated that the molecular weights of main NA-binding proteins found in the membrane-cytosolic fraction are identical with those found in the nuclear fractions. Nucleic acid-binding proteins with molecular weights of 68, 38, and 28 kDa were revealed upon incubation of the cells with $ClRp(N)_{16}$ in PBS (Fig. 3, *a*). Incubation in a DMEM medium led to an increase in the extent of modification of all proteins, except for the 38-kDa protein whose degree of modification remained unchanged. The affinity modifications of yet another protein with a molecular weight of 46 kDa and a series of low-molecular-weight minor proteins were observed (Fig. 3, *b*). Upon incubation of cells with $ClRp(N)_{16}$ in PBS in the presence of 1.5 *mM* Ca^{2+}, the same proteins underwent the affinity modification as those modified upon incubation of the cells with $ClRp(N)_{16}$ in a DMEM medium. Hence, the Ca^{2+} ions are necessary for the interaction of ODN with low-molecular-weight proteins and with the 46-kDa protein. The addition of the Mg^{2+} ions or ATP to PBS or the addition of fetal calf serum to a DMEM medium has no effect on the affinity modification of NA-binding proteins (data are not presented).

Binding of oligonucleotides to the revealed proteins showed no pronounced dependence on the nucleotide sequence. Alkylating oligonucleotide derivatives with different sequences modify proteins with different efficacy.

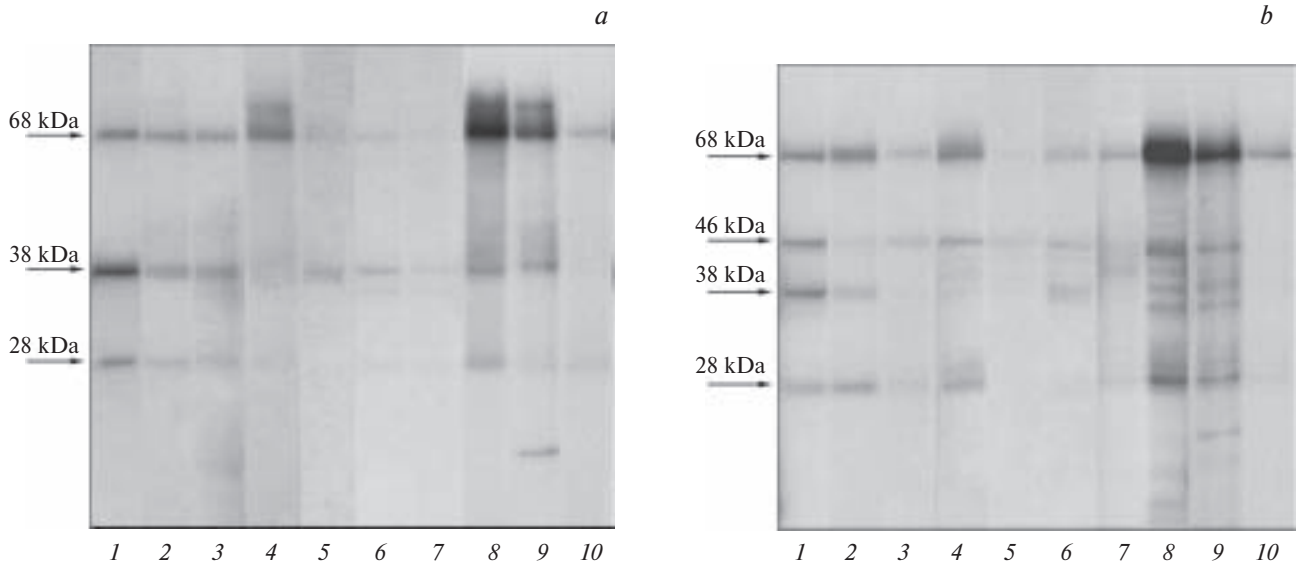

Fig. 3. Affinity modification of membrane-cytosolic NA-binding proteins of intact cells from different lines by the alkylating oligonucleotide derivative [^{32}P]ClRp(N)$_{16}$ in PBS (*a*) and in a DMEM medium (*b*): *1*, A431; *2*, HeLa; *3*, KB; *4*, MCF-7; *5*, HEp-2; *6*, LECh; *7*, K562; *8*, Cos-7; *9*, SPEV; *10*, NIH/3T3. The cells were incubated with [^{32}P]ClRp(N)$_{16}$ (1 μmol L^{-1}) in PBS at 37 °C for 1 h. The proteins were analyzed by SDS disc electrophoresis in gradient (10—20%) PAAG followed by autoradiography.

However, the set of NA-binding proteins remains unchanged (data not presented).

Nucleic acid-binding proteins were found in the membrane-cytosolic fraction, whereas they were present in a lesser amount in the nuclear fraction, and were virtually absent in the cytosolic fraction (except for the 38-kDa protein). Previously, we have identified the 38-kDa NA-binding protein, which was found in the cytosolic fraction of permeabilized cells (Fig. 4), as glyceraldehyde-3-phosphate dehydrogenase.[27] The fact that treatment of the cells, which were preincubated with [^{32}P]-labeled ClRp(N)$_{16}$, with trypsin led to hydrolysis of the 68-kDa protein indicates that this protein is exposed on the outer cell surface (Fig. 5). Treatment of the cells with trypsin and destruction of the integrity of the cellular membrane, which takes place upon treatment with saponin or removal of the cells with a scraper prior to incubation with an affinity reagent, leads to rejection of the affinity modification of proteins by ClRp(N)$_{16}$ (Fig. 5) and to a substantial decrease in accumulation of ODN in the cells. The results obtained in the present study provide evidence that these proteins can be involved in NA binding to cells and transport of NA into cells.

We showed that inhibitors of active transport (sodium azide, chloroquine, and monesin) and ATP have no effect on the affinity modification of NA-binding proteins in PBS or a DMEM medium and accumulation of ODN in cells (data not presented).

Incubation of ClRp(N)$_{16}$ with the membrane-cytosolic, cytosolic, and nuclear fractions led to affinity labeling of a large number of proteins, NA-binding proteins from living cells being also found but only as minor components (data not presented). Hence, cell proteins capable of NA binding do not all participate in recognition and transport of NA in living cells.

Various NA-binding proteins were revealed in cells of different tissue origin. In the promyelocytic leukemia cells HL-60, monocytic leukemia cells U937, and T lymphoblastic leukemia cells CEM, membrane-cytosolic proteins with molecular weights of 75 and 28 kDa were revealed by the photoaffinity modification by reactive ODN derivatives in PBS.[10] Upon incubation of cells from the T15, HeLa, Hep-2, HepG-2, and Caco-2 lines with 1 *μM* phosphorothioate ODN in a Hanks solution, a 46-kDa protein was revealed. After separation of proteins from the membrane-cytosolic fraction of the cells by electrophoresis followed by electrobloting and incubation with radioactively labeled phosphorothioate ODN, proteins with molecular weights of 21, 30, 35, 46, 72, 80, and 110 kDa were found.[28] It should be noted that the concentration of the Ca^{2+} ions (1.3 mmol L^{-1}) in the Hanks solution is close to the physiological concentration of Ca^{2+} (1.86 mmol L^{-1}) in a DMEM medium, which, apparently, facilitates binding of ODN to the 46-kDa protein. In addition, it is known that phosphorothioate analogs of ODN exhibit a substantially higher affinity for proteins as compared to phosphodiester ODN.[29,30] The predominant detection of the 46-kDa protein is attributable to high affinity of this protein for phosphorothioate ODN and also to the fact that researchers did not stabilize oligonucleotide-protein com-

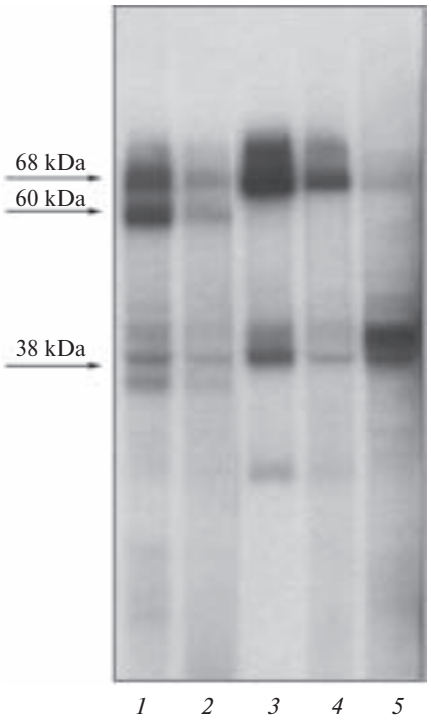

Fig. 4. Study of cellular localization of NA-binding proteins: ODN-binding proteins from cells treated with trypsin (*1*, *2*) or saponin (*3*—*5*): the membrane-cytosolic (*1*, *3*), nuclear (*2*, *4*), and cytosolic (*5*) fractions. The cells from the A431 line modified by $[^{32}P]ClRp(N)_{16}$ were treated with trypsin (0.25%, 5 min) or saponin (40 μg mL^{-1}, 20 min). After treatment of the cells with saponin, the supernatant was collected as the cytosolic fraction and the remaining cells were separated into the membrane-cytosolic and nuclear fractions (see Experimental). The proteins were analyzed by SDS disc electrophoresis in gradient (10—20%) PAAG followed by autoradiography.

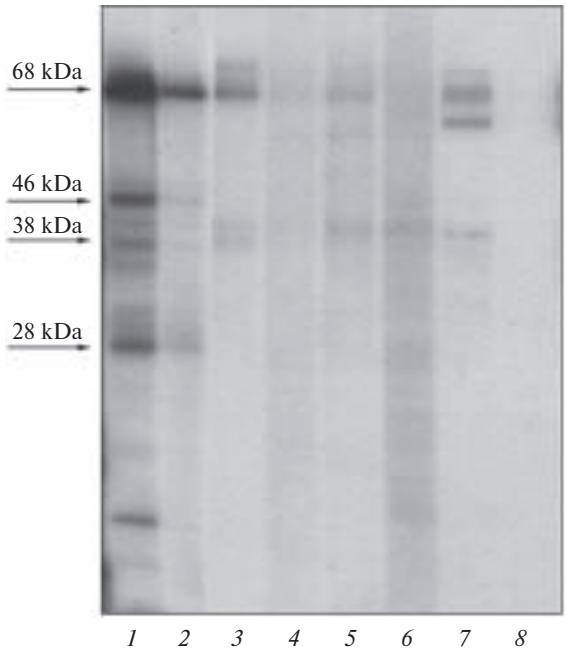

Fig. 5. Influence of the factors disturbing the integrity of the cellular membrane on the modification of NA-binding proteins by the oligonucleotide derivative $[^{32}P]ClRp(N)_{16}$: NA-binding proteins of intact cells from the A431 line (*1*, *2*), cells from the A431 line pretreated with saponin (40 μg mL^{-1}, 20 min) (*3*, *4*); pretreated with trypsin (0.25%, 5 min) (*7*, *8*), and removed with a scraper (*5*, *6*): the membrane-cytosolic (*1*, *3*, *5*, *7*) and nuclear (*2*, *4*, *6*, *8*) fractions, respectively. The intact cells, trypsinized cells, and cells, which were treated with saponin or removed from the support with a scraper, were incubated with 1 μM $[^{32}P]ClRp(N)_{16}$ in a DMEM medium at 37 °C for 1 h. The proteins were analyzed by SDS disc electrophoresis on gradient (10—20%) PAAG followed by autoradiography.

plexes by modifying ODN with the use of reactive groups or cross-linking reagents and, hence, revealed the most stable complexes. Later on, the authors of the cited studies described proteins with molecular weights of 28— 30, 46, 67, 70—90, and 115 kDa using the same experimental approaches and cell lines.[31] In another study,[16] it was demonstrated that surface proteins with molecular weights of 20—22, 29—32, 43—46, 79—85, 137—147 kDa underwent the affinity modification upon incubation of the cells from the K562 line with phosphorothioate ODN in PBS followed by treatment with a cross-linking reagent, *viz.*, bis(sulfosuccinimidyl) suberate. A 66-kDa cell protein was isolated from hepatocytes of the HepG-2 line using the photoaffinity modification by the biotinylated ODN conjugate with benzophenone.[11] We revealed proteins with molecular weights of 18, 23, 30, 35, 40—42.5, 67—72, 78—82, and 145 kDa using the affinity modification of mouse splenocytes by alkylating reactive ODN derivatives in a RPMI medium.[4] We found proteins with molecular weights of 35 and 61—63 kDa upon incubation of the cells of keratinocyte origin (A431, HaCaT) with the same reagent in PBS.[13] It should be noted that in the cited study, we used the same alkylating oligonucleotide derivative ($[^{32}P]ClRp(N)_{16}$) and the cells from the same lines as those used in the present study. The difference in the molecular weights of the described proteins is, apparently, associated with the inaccuracy of the molecular weight determination because our earlier investigation was carried out with the use of molecular weight markers, which were insufficiently characterized. In the present study, we demonstrated that the set of NA-binding proteins that are revealed depends on the type and mode of the attachment of the reactive group to the oligonucleotide, on the presence of additional modifications in the affinity oligonucleotide reagent, and on the conditions of incubation of oligonucleotides with cells. Incubation of cells with affinity reagents in a DMEM medium leads to the affinity modification of a larger number of proteins as compared to those obtained with the use of PBS. Binding of ODN to the discovered pro-

teins showed no pronounced dependence on the sequence of the oligonucleotide used, which is in agreement with the earlier data.[12,13] However, reactive oligonucleotide derivatives with different sequences have different efficacy in the modification of proteins and, hence, particular NA-binding proteins may be either revealed or not revealed depending on the sensitivity of procedures used for the detection of oligonucleotide-protein complexes. The modification of 5′-terminal phosphate enhances specificity of interactions between oligonucleotides and cell proteins. The fact that from one to two NA-binding proteins were revealed in a number of studies[18,29] results, apparently, from the use of particular ODN, reaction groups, conditions of formation, and procedures for the detection of ODN-protein complexes.

We showed that incubation of cells of different tissue origin with an alkylating oligonucleotide reagent performed under the same conditions leads to the affinity modification of proteins with equal molecular weights (see Figs. 2 and 3). Protein bands in an autoradiograph differ in intensity depending on the cell line, *i.e.*, although the same set of NA-binding proteins are expressed by cells from different lines, different amounts of these proteins are produced. The different level of expression of NA-binding proteins may be responsible for the efficiency of penetration of nucleic acids into cells of different lines. It is known that nucleic acids are generally less efficiently captured by primary cells and cells of lymphocyte origin than by cells of keratinocyte origin.[33]

One of the major paths of the transport of ODN into the cells from the K562, MOLT-4, HL60, and DA1 lines involves receptor-mediated endocytosis.[7,8] Inhibitors of endocytosis and the formation of lysosomes have no effect on the transport of ODN into cells.[5,6] It was suggested[9] that NA can be transported also through a membrane channel formed with the participation of the 45-kDa protein. We found that destruction of the cellular membrane hinders the affinity modification of surface NA-binding proteins, whereas inhibitors of active transport (sodium azide, chloroquine, and monesin) and ATP have no effect on the affinity modification of NA-binding proteins in PBS or DMEM and accumulation of ODN in cells. Previously, we have demonstrated[13] that pretreatment of cells with trypsin arrests the transport of fluorescent-labeled ODN into cells of the A431 line. Hence, the revealed proteins are of importance not only for binding of NA to cells but also for the transport of NA into cells.

It should be noted that the Ca^{2+} ions serve as the major component of the DMEM medium that provide binding of proteins to NA. Actually, it was shown that penetration of ODN into cells depends on the presence of the Ca^{2+} ions in the medium.[6,34] It is also known that the Ca^{2+} ions are the major component ensuring the integrity and regeneration of the cellular membrane after destruction.[35] The addition of the Ca^{2+} ions to PBS leads, apparently, to regeneration of the normal state of the cellular membrane, which facilitates interactions of ODN with additional proteins.

The results of the present study provide evidence that cells of different tissue origin express the same NA-binding proteins, whereas the variety of NA-binding proteins that are revealed depends on the reactive affinity reagent used, the presence of modification at 5′-terminal phosphate, the conditions of the formation, and the procedures used for the detection of NA—protein complexes. The integrity of the cellular membrane is a key factor responsible for binding of NA to proteins of living cells.

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