ISOLATION AND IDENTIFICATION OF CELL SURFACE NUCLEIC ACIDS-BINDING PROTEINS

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Resume

Motivation: Investigation of endogenic extracellular nucleic acids (exNAs) demonstrate that exNAs are not intact molecules, but could influence functioning of cells (Garcia-Olmo et al., 2000). To realize their potencies nucleic acids should interact with cell surface receptors or penetrate into cells. Binding and penetration of nucleic acids into cells was shown to depend from interaction with cell surface proteins (Laktionov et al., 1999). Thus these interactions are important in respect to functioning of extracellular nucleic acids. In practical aspects, these interactions determinate delivery and the efficiency of antisense oligonucleotides (ODN) and efficiency of transfection.

Results: It was shown that similar cell surface proteins bind single and double stranded DNA, few of these proteins were separated and identified by MS/MS sequencing.

Introduction

Penetration of nucleic acids and oligonucleotides into cells and proteins involved in this process were extensively investigated. Few protein candidates that can be involved in penetration of ODNs were described (Diesbach et al., 2000; Griffoni et al., 2001; Siess et al., 2000; Geselowitz, Neckers, 1995; Benimetscaya et al., 1997; Kimura et al., 1994). Keratinocytes are known as the only one cell type readily absorbing oligonucleotides and accumulating compounds in the nuclei (Noonberg et al., 1993), but the proteins that participate in binding and penetration of NAs into keratinocites are not identified.

Earlier we have investigated interaction of different ODNs with keratinocytes by affinity modification of proteins of cell surface with 32P-labeled reactive oligonucleotide conjugates. Binding of ODNs with proteins does not depend from sequence of oligonucleotide whereas patterns of affinity labeled oligonucleotide-binding proteins and the extent of modification of individual proteins depend on the type of reactive group of the conjugate. It was clearly demonstrated that 68 kDa oligonucleotide-binding proteins are exposed out of the cells and participate in binding of oligonucleotides.

The objectives of this work were investigation of affinity modification of cell surface proteins with ss- and ds-deoxyribooligonucleotides, development of the method of separation of nucleic acids binding proteins, separation and identification of the proteins responsible for binding of nucleic acids with cell surface.

Materials and Methods

Oligonucleotide pCAGTAATATCTAGGA (p(N)16) was synthesized in a synthesizer ASM-700 (BioSet, Russia) by the phosphorimidite method. Oligonucleotide pCAGTAATATCTAGGA-deg-rU (p(N)16degU) was synthesized as referred (Duran et al., 1990). The 5’ end was labeled with 32P using T4 polynucleotide kinase. Oligonucleotide p(N)16 was conjugated to alkylating reagent 4-[N-2-chloroethyl-N-methyl)amino]benzylamine (ClR-) through its 5’-phosphate (Mishenina et al., 1979). Fluorescein isothiocyanate was conjugated with ODN-rU after modification of 5’-phosphate with diaminopentane (DAP) in 0,1 M Na 2CO 3 for 4 hours at room temperature as described earlier (Haralambidis et al., 1987).

Cells of human epidermoid carcinoma cell line A431 were grown in DMEM medium with 10% heat inactivated fetal bovine serum at 37°C, 5% CO 2. In affinity modification experiments cells were incubated with 1 µM reactive oligonucleotides for 1h at 37°C, separated into membrane cytosole (MC), cytosole (CF) and nuclear fractions (NF) and analyzed by SDS-PAGE as described earlier (Laktionov et al., 1999).

The modified proteins from A431 cells were affinity purified on Ultrogel A2−antifluorescein antibodies. The ODN-binding proteins eluted with Glycin/HCl pH 2.5 were separated by SDS-PAGE. The protein 68 kDa band was identified by autoradiography, Coomassie staining of the gel and immunochemistry staining of the nitrocellulose blot and sequenced by MS/MS sequencing.
Results and Discussion

Interaction of cell surface proteins with ss- and ds deoxyribonucleic acids were investigated by affinity modification of cellular proteins with reactive ss- and ds ODNs (Fig. 1). It was shown that reactive ds ODNs (lines 1, 2) modified similar sets of cell surface proteins as the ss ODNs (lines 3-5). Proteins with molecular masses about 68 kDa that were shown earlier to be responsible for binding of oligonucleotides with cell surface bind ss- and ds ODNs and seems to be universal for binding of ss and ds nucleic acids.

In order to work out method of separation of these proteins we investigate affinity modification of A431 cells and cellular fractions of A431 cells with $[^{32}P]CIRp(N)_{16}$. In contrast to living cells, incubation of cytosolic, membrane-cytosolic or nuclear fractions with the affinity reagents resulted in labelling of a great number of proteins. The 68 kDa proteins were present in this mixture as minor components (Fig. 2). The data obtained demonstrate that affinity modification of cell surface proteins of living cells with ODN bearing specific ligands in contrast to other approaches enable to reveal and separate the proteins that are important for ODN binding with living cells.

5'-Fluorescein-labeled oligonucleotide conjugate with uridine bound to the 3'-end through an diethylene glycol (deg) linker was used as the affinity reagent for isolation of 68 kDa cell surface proteins after oxidation of the 3’ ribose moiety with sodium periodate. The modified with $^{32}P$-labeled Flu-DAP-p(N)$_{16}$degU proteins from $1x10^9$ A431 cells were affinity
purified on Ultrogel A2-antifluorescin antibodies. The ODN-binding proteins eluted with Glycin/HCl pH 2.5 were concentrated with ultrafiltration and separated by SDS-PAGE. Specific protein bands were revealed after immunostaining with anti-Flu antibodies and silver staining of NC blot, autoradiography of the gel (Fig. 3.) and with Coomassie staining.

Proteins were sequenced by MS/MS sequencing and identified with data bank search. It was found that 68 kDa band contains few ODN-binding proteins: albumin and keratin 1 among them. Cytokeratin 1 was shown earlier to be express onto cell surface and revealed with antibodies on a surface of living cells (Mahdi et al., 2001). This data confirm possibility of this protein to bind extracellular nucleic acids and its importance for interactions of nucleic acids with cells.

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References