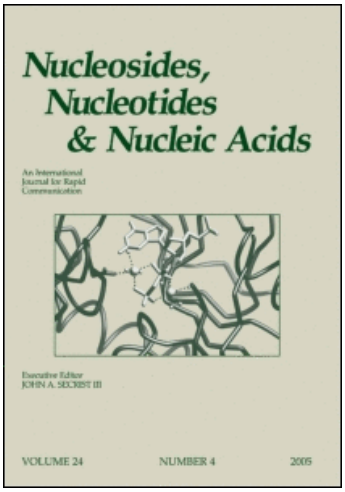

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Cell Surface Oligonucleotide-Binding Proteins of Human Squamous Carcinoma A431 Cells

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ABSTRACT

Affinity modified with Flu-DAP-p$(N)_{16}$degU oligonucleotide-binding proteins were isolated by affinity chromatography using Ultrogel A2 - anti fluorescein antibodies. After separation by SDS-PAGE the proteins with molecular masses about 68 kDa were MS/MS sequenced and identified as keratin K1, keratin K10, keratin K2e and albumin.

Key Words: Oligonucleotides; ODN-binding proteins; Nucleic acids receptor; Affinity modifications; Albumin; Keratin K1; Keratin K10; Keratin K2e.

Nucleic acids penetration into cells and the proteins involved in this process have been extensively investigated. Only few ODN-binding protein candidates that could be involved in penetration of ODNs were reported.[1,2] Keratinocytes are known to be unique cells readily absorbing oligonucleotides and accumulating them in the nuclei,[3] but the proteins that participate in binding and penetration of NAs into keratinocytes have not been identified yet.

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Earlier we have investigated interaction of different ODNs with keratinocytes using affinity modification of cell surface proteins with ^{32}P-labeled reactive oligonucleotide derivatives. ODNs binding with proteins do not depend on the sequence of the oligonucleotide whereas number and variety 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 at the cell surface and participate in accumulation of oligonucleotides.[4] The objectives of this work were development of the method of nucleic acids binding proteins separation, isolation and identification of the proteins responsible for binding of nucleic acids with cell surface.

Affinity modification of the A431 subcellular protein fractions with [^{32}P]CIRp(N)$_{16}$ demonstrate that in contrast to living cells modification, many additional proteins of cytosolic, membrane-cytosolic or nuclear fractions were affinity modified with [^{32}P]CIRp(N)$_{16}$. The 68 kDa proteins were present in this mixture as minor components (Fig. 1). The data obtained demonstrate that the affinity modification of cell surface proteins of living cells with ODN reactive derivatives in contrast to other approaches makes possible isolation of proteins that are important for ODN binding with living cells.

In order to select an efficient reactive oligonucleotide derivative for affinity modification of A431 cell proteins ODNs bearing different reactive groups were tested.

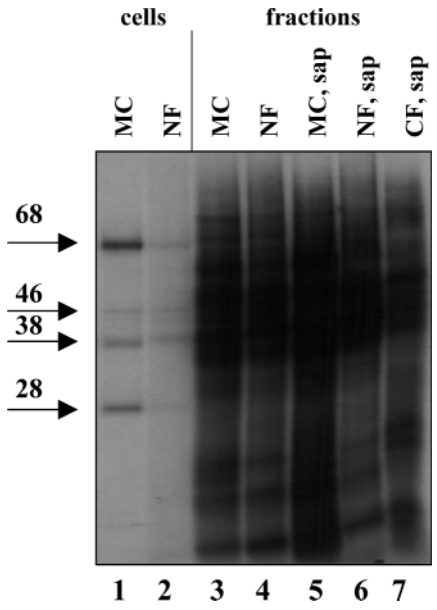

Figure 1. Affinity modification of cellular proteins with [^{32}P]CIRp(N)$_{16}$. Cells or cell fractions were incubated with 1 μM [^{32}P]CIRp(N)$_{16}$ at 37°C for 1 h. The modified proteins were analysed by 10–20% SDS-PAGE followed by autoradiography. 1, 2 – MC and NF of A431 cells modified with [^{32}P]CIRp(N)$_{16}$ in DMEM medium (control). 3, 4 – MC and NF of A431 cells modified with [^{32}P]CIRp(N)$_{16}$. 5, 6, 7 – modification of MC, NF, and CF of A431 cells treated with saponin.

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Reactive ODNs derivatives based on the 16-mer ODN pCAGTAAATATCTAGGA ($p(N)_{16}$degU) with uridine bound to the 3′-end through an diethylenglycol linker (deg) were synthesised. Diaminopentane or 4-[(N-2-chloroethyl-N-methyl) amino] benzylamine (ClR-) was bound at the 5′-end of the oligonucleotide after activation of phosphate with triphenylphosphine and dipyridyl disulfide. The 3′-end of $p(N)_{16}$degU was modified with lysine after oxidation of ribose with sodium periodate. The 5′- or 3′-amino groups were modified with 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid *N*-hydroxysuccineimide (MaM) or fluorescein isothiocyanate. The ribose moieties of $p(N)_{16}$degU and 5′-modified $p(N)_{16}$degU were oxidized with sodium periodate just before treatment of cells with ODN.

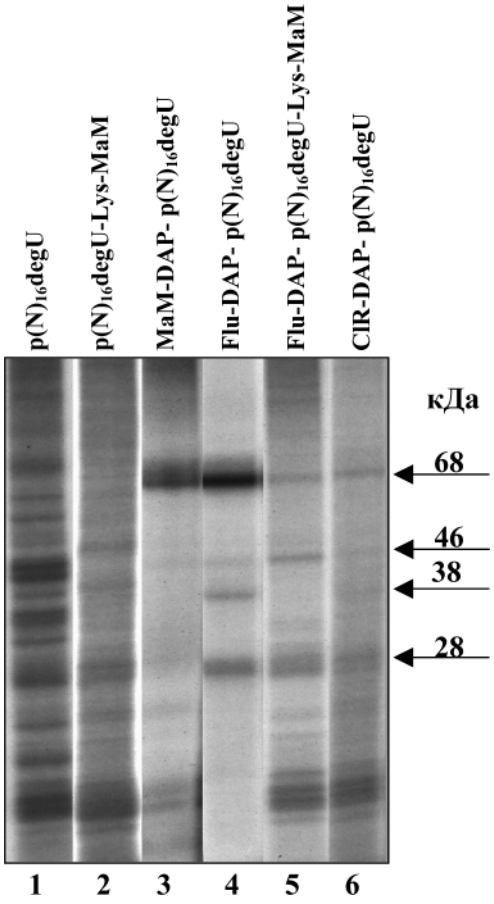

Figure 2. Affinity modification of MC proteins with reactive ODN conjugates in DMEM medium. A431 cells were incubated in DMEM medium with 1 μM [^{32}P] ODN derivatives at 37°C for 1 h. 1. $p(N)_{16}$degU 2. $p(N)_{16}$degU-Lys-MaM 3. MaM-DAP- $p(N)_{16}$degU 4. Flu-DAP- $p(N)_{16}$degU 5. Flu-DAP- $p(N)_{16}$degU-Lys-MaM 6. ClR- $p(N)_{16}$degU Modified proteins of MC-fraction of A431 cells were separated by 10–20% SDS-PAGE followed by autoradiography.

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It could be shown that a variety of membrane proteins detected depend substantially on the nature and the position of the reactive group (Fig. 2). 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. 5′-Fluorescein-labeled oligonucleotide derivative Flu-DAP-$p(N)_{16}$degU was shown to be the most efficient and suitable reagent for affinity isolation of 68 kDa cell surface proteins.

The ^{32}P-labeled Flu-DAP-$p(N)_{16}$degU proteins from 1×10^9 A431 cells were affinity purified on Ultrogel A2–antifluorescein 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.

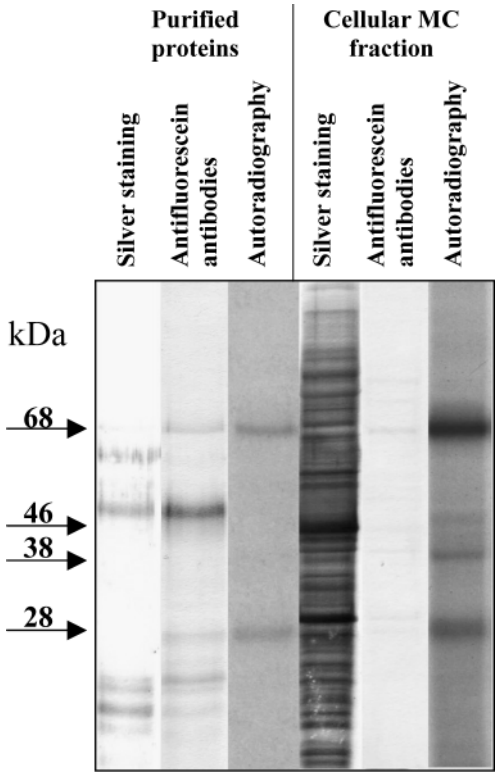

Figure 3. Separation of ODN-binding proteins of A431 cells. 10^9 cells were incubated with 1 μM [^{32}P] Flu-DAP- $p(N)_{16}$degU (oxidized with sodium periodate) in DMEM medium at 37°C, 5% CO_2 for 2 h. The MC fraction was prepared as described above and applied to the UltrogelA2-rabbit anti-fluorescein antibodies. After elution with Glycin/HCl pH 2.5 and subsequent electrophoretic separation, the ODN-binding proteins were transferred to nitrocellulose and autoradiographed or stained with colloidal silver or anti-fluorescein antibodies.

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Proteins were sequenced by MS/MS sequencing and identified with data bank search. It was found that 68 kDa band contains four ODN-binding proteins: albumin, keratin K1, keratin K10 and keratin K2e. Cytokeratin 1 was shown earlier to be exposed at the cell surface and was revealed with specific antibodies at the surface of living cells.[5] This data confirmed the possibility what this protein bind extracellular nucleic acids and is important for nucleic acids interactions with cells.

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