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Characterisation of membrane oligonucleotide-binding proteins and oligonucleotide uptake in keratinocytes

Pavel P. Laktionov, Jean-Eudes Dazard[1], Eric Vives[1], Elena Y. Rykova, Jacques Piette[1], Valentin V. Vlassov and Bernard Lebleu[1,*]

Institute of Bioorganic Chemistry, Academy of Sciences Siberian Division, Novosibirsk 630090, Russia and [1]Institute of Molecular Genetics, UMR 5535, IFR 24, CNRS, 34293 Montpellier Cedex 5, France

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ABSTRACT

Inadequate cellular compartmentalisation of plasmid DNA and antisense oligodeoxynucleotides (ODNs) is generally considered as a major limitation in their use. In this study, an approach combining *in situ* visualisation of rhodamine-labelled ODNs and affinity modification of proteins by radiolabelled-alkylating ODN derivatives has been used to investigate the uptake of ODNs into keratinocytes. We confirm here that unmodified ODNs are efficiently taken up and accumulate in cell nuclei in primary keratinocytes as well as in HaCaT and A431 keratinocyte cell lines. Uptake is fast, irreversible, saturable and not significantly altered by incubation at low temperature. Affinity modification studies in keratinocyte cell lines has revealed two high-affinity, cell-specific interactions between ODNs and proteins of 61–63 kDa and 35 kDa. Trypsin pre-treatment of A431 cells and pre-incubation with polyanions, or with unlabelled nucleic acid competitors, inhibited the accumulation of rhodamine-labelled ODNs in nuclei as well as the affinity labelling of the 61–63 kDa doublet and 35 kDa ODN-binding proteins by reactive ODN derivatives. Finally, cell fractionation studies indicated that these ODN-binding proteins were essentially localised in the plasma membrane. Our results suggest that these ODN-binding proteins might be involved in the recognition and transport of ODNs into keratinocytes.

INTRODUCTION

Antisense and triplex technologies are promising strategies that may be widely used in the regulation of inappropriate expression of genes in pathological situations. However, several limitations to the wide-spread usage of these strategies in gene therapy remain. In particular, synthetic oligodeoxynucleotides (ODNs) and nucleic acids have been engineered to specifically inhibit the expression of their target, but poor uptake in most cell types and inadequate intracellular compartmentalisation remain major obstacles (1–4). It is therefore important to better understand the underlying mechanisms in order to improve the uptake of exogenous nucleic acids and ODNs into cells. One strategy relies on modification of the chemical structure of ODNs or on their association with delivery vehicles in order to increase their cellular uptake. Alternatively, a better understanding of the cellular transport mechanism could ultimately lead to the implementation of improved delivery strategies.

The uptake of ODNs by many cell types has already been investigated by several groups. It is generally observed that unmodified ODNs are poorly taken up and accumulate in endocytotic vesicles giving rise to a characteristic punctated perinuclear distribution (5–8). Uptake is strongly time-, sequence-, temperature- and energy-dependent and is affected by inhibitors of active transport (5,8–10). ODN internalisation in tissue-culture cells is thus considered to depend predominantly on adsorptive endocytosis and fluid-phase endocytotic (pinocytotic) processes.

Interestingly, in contrast to many other cells, *in vitro* cultured monolayers of primary keratinocytes and well-established keratinocyte cell lines readily take up unmodified synthetic ODNs which rapidly accumulate intranuclearly rather than being trapped in endosomal-like vesicles or bound to the cell surface (11,12). In addition, ODN-uptake and localisation were found to be not significantly altered by incubation at low temperature or by active transport inhibitors such as sodium azide, monensin and chloroquine (11). To our knowledge, the underlying mechanism of ODN uptake, and subsequent localisation, has not yet been investigated in these cells cultured as monolayers. It might involve a rapid escape from the endocytotic pathway as suggested for ODN–cationic lipid complexes (12,13) or a mechanism avoiding endocytosis. Moreover, the improved delivery of ODNs in keratinocytes has been questioned in a recent publication reporting that few keratinocytes when cultured as monolayers were found to accumulate ODNs in their nuclei with a punctate perinuclear staining as described for most cell lines. Conversely, the efficient uptake of ODNs in suprabasal keratinocytes of artificial skin appeared to be linked with late features of terminal stages of differentiation such as loss of membrane integrity (14).

The inconsistencies of these results in *in vitro* cultured monolayers of primary keratinocytes led us to check whether keratinocytes do indeed readily, and rapidly, take up ODNs that then localise in their nuclei, and ultimately to explore and better understand the underlying mechanisms. In this work we confirm initial data concerning the efficient uptake of ODNs in keratinocyte monolayers. We have also identified, in two keratinocyte cell

*To whom correspondence should be addressed. Tel: +33 4 67 61 36 62; Fax: +33 4 67 04 02 31; Email: lebleu@igm.cnrs-mop.fr

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

lines, a set of high-affinity membrane ODN-binding proteins, in possible relationship with the efficient ODN-uptake and preferential nuclear localisation of these compounds in these cells.

MATERIALS AND METHODS

ODNs and modified ODN synthesis

ODN 5′-pATAAGCAACAAGCCCTTC [p$(N)_{18}$] was synthesised on an ASM-102U DNA synthesiser (BioSet, Novosibirsk, Russia) by the phosphoramidite method. The 5′-end was radiolabelled with [^{32}P] using T4 polynucleotide kinase (Gibco, Grand Island, NY). The cetyltrimethylammonium salt of [^{32}P]p$(N)_{18}$ was 5′-labelled with the 4-[(N-2-chloroethyl-N-methyl)amino]benzylamine alkylating group after activation of its 5′-end phosphate with triphenylphosphine and dipyridyl disulfide in a non-aqueous solution (15), thus producing [^{32}P]ClRp$(N)_{18}$. Active chlorine was determined in the alkylating group by reaction with 0.5 M sodium thiosulfate for 10 h at room temperature. The yield of alkylating derivatives was >90%, as indicated by electrophoresis of the reaction mixture on 20% PAGE with urea, and specific radioactivity was ~50 Ci/mmol.

ODN-Rh 5′-pGGCTCCATTTCTTGCTCTC and its scrambled sequence 5′-pGGTCTTACTCTCCGTCTCT (Eurogentec, Seraing, Belgium) were modified with a hexamethylene-bridged pyridyldisulfide at the 5′ end and modified at their 3′-end with a 2-propanol-3-amino group (C7 amino-modifier CPG; Glen Research, Sterling, VA), which was reacted with 5-carboxytetramethylrhodamine N-hydroxy succinimidyl ester, (Interchim SA, Montluçon, France) in 500 mM $NaHCO_3/Na_2CO_3$, pH 9 and purified by HPLC as described previously (16).

ODN-Flu 5′-pGACCTCGCGCTCCTTG was assembled on Fluorescein CPG (Glen Research, Sterling, VA) using standard protocol for phosphoramidite chemistry and therefore labelled at its 3′-end. Its 5′-end was phosphorylated during automatic synthesis with Chemical Phosphorylation Reagent (Glen Research, Sterling, VA).

Isolation and culture of human epidermal primary keratinocytes

Human epidermal primary keratinocytes (HEPK) were freshly isolated from human neonatal foreskin specimens. At circumcision, foreskins were rinsed with a solution of PBS$^-$ (without Ca^{2+} and Mg^{2+}) containing gentamicin (Gibco, Grand Island, NY) at a concentration of 20 µg/ml for ~1 h. After incubation in dispase (25 U/ml) (Gibco, Grand Island, NY) during 18 h at 4°C, the epidermal layer of human keratinocytes was separated from the dermis. The tissue was then incubated at 37°C, 5% CO_2 in a solution of 0.05% trypsin containing 0.53 mM EDTA (Gibco, Grand Island, NY) during 15 min under mild agitation to favour cell dissociation. After trypsin neutralisation, the cell suspension was rinsed with a solution of PBS$^-$ containing 0.53 mM EDTA, then cells were gently resuspended in a low calcium Defined Keratinocyte-Serum Free Medium (DKSFM; Gibco, Grand Island, NY) and grown in Petri dishes. The medium was supplemented with antibiotics—penicillin and streptomycin at 100 U/ml (Gibco, Grand Island, NY)—and 15 ng/ml recombinant EGF of mouse submaxillary EGF (Sigma, St Louis, MO). Upon reaching 80% confluence, the culture medium was removed and the cell monolayer was rinsed twice with a solution of PBS$^-$ containing 0.53 mM EDTA. After detachment of the cells with a solution of 0.05% trypsin containing 0.53 mM EDTA for 2–3 min at 37°C, 5% CO_2, and inactivation with FCS, cells were rinsed twice in PBS$^-$, gently resuspended in DKSFM and re-seeded in Petri dishes at a density of 10^6 cells per 100 mm dish. The culture medium was changed every 2–3 days until the cells reach 80% confluence after which the cells could be further subcultured for a maximum of five passages.

Cell cultures

HeLa (human cervical carcinoma cell line), MCF-7 (human breast carcinoma cell line), A431 (human squamous carcinoma cell line; 17), passage 39 HaCaT (human immortalised keratinocyte cell line; 18), Saos-2 and U2-OS (human osteosarcoma cell lines) were grown in DMEM medium. Human dermal primary fibroblasts (HDPF) cells were grown in MEM medium. All media were supplemented with 10% heat-inactivated FCS and antibiotics—penicillin and streptomycin at 100 U/ml (Gibco, Grand Island, NY). Cell lines and primary cells were cultured on 12- or 24-well plates for the experiments with alkylating reagent and on 8- or 16-well LabTek chambers for microscopy (Nunc, Naperville, IL) and maintained at 37°C in 5% CO_2. Cell viability was assessed using the trypan-blue dye exclusion assay. Briefly, cell monolayers were incubated for 5 min in a 0.2 µm-filtered solution of 0.5% trypan-blue in PBS$^-$ and then rinsed twice with PBS$^-$ to discard the excess of reagent. This test has been used in parallel with ODN-uptake experiments.

ODN uptake

Upon reaching 30% confluence, living keratinocyte monolayer cultures were rinsed three times to remove the excess of FCS with the appropriate serum-free medium (DKSFM for HEPK and serum-free DMEM for the other cell lines) and incubated for 1 h at 37°C, 5% CO_2 with 1 µM ODN-Rhs or ODN-Flus or 5 µM free rhodamine as a negative control. Subsequently, cells were rinsed three times as described above. For experiments at 4°C, the protocol was the same except that the media used and the cell monolayers were cooled for 30 min at 4°C before all incubations at 4°C.

Cell processing for fluorescence microscopy

After incubation, cells were viewed by microscopy as either live or fixed specimens. Fixed specimens were prepared using 3.7% formaldehyde in PBS$^-$ for 10 min at room temperature, and rinsed twice with PBS$^-$. For each ODN-uptake experiment, nuclei were stained with 50 ng/ml of Hoechst 33258 (Sigma, St Louis, MO) in PBS$^-$ for 5 min at room temperature, and rinsed three times with PBS$^-$ before being mounted with H-1000 Vectashield® antifading mounting medium (Vector Laboratories, Burlingame, CA).

Fluorescence microscopy

The distribution of the fluorescence was analysed on a DM RX® fluorescence microscope (Leica, Wetzlar, Germany) equipped with a 100 W mercury lamp, plan Fluotar® oil immersion objectives (40/0.70–0.40; 100/1.30–0.60) (Leica, Wetzlar, Germany) and the following filter sets: excitation, BP340–380 nm; emission, LP430 nm for Hoechst staining; excitation, BP515–560 nm; emission, LP-590 nm for rhodamine (Leica, Wetzlar, Germany). Images were acquired with a slow scan charge-coupled device (CCD) camera KAPPA CF8/1 DX® interfaced to a PC computer using the KAPPA CF8/1 DX® v.3.1.5 camera control program (Kappa,

Gleischen, Germany). All images were further processed using the public domain Confocal Assistant program v.4.0.2 and AdobePhotoshop® v.3.0.5 software.

Identification of ODN-binding proteins

The ability of proteins from several cell lines to interact with ODNs was investigated by means of affinity modification studies with ^{32}P-labelled ODN, bearing a ClR-alkylating group that has already been extensively used (6,19,20). Briefly, the culture medium was discarded, cell monolayers were washed three times with PBS$^-$ and then incubated with 1 μM [^{32}P]ClRp(N)$_{18}$ in PBS$^-$ during 1 h at 37°C, 5% CO_2. After incubation, cells were washed three times with PBS$^-$, scraped from the plate, and centrifuged for 3 min at 3000 r.p.m. The supernatant was discarded and the pellet of cells was resuspended in 25 μl of lysis buffer with 1 mM PMSF, 10 μg/ml aprotinin and 150 μg/ml leupeptin (Sigma, St Louis, MO) containing 1% (v/v) Nonidet NP-40. For each sample, membrane-cytosolic (MC) and nuclear fractions (N) were separated by centrifugation at 14 000 r.p.m. for 3 min. After addition of an equal volume of SDS–PAGE sample buffer to the supernatant containing MC proteins, samples were heated at 100°C for 5 min and centrifuged at 14 000 r.p.m. for 3 min. The nuclear pellets were washed with 1 ml of PBS$^-$, centrifuged, resuspended in sample buffer and treated as described above. Proteins were fractionated by a 10–20% SDS–PAGE gradient. The gel was loaded with comparable amounts of proteins as determined by the equal number of cells treated in each condition. In order to visualise and quantify proteins modified by radiolabelled [^{32}P]ClRp(N)$_{18}$, gels were dried and exposed either to a BioMax MR® X-ray film (Kodak, Rochester, NY) or to a storage phosphor screen for >24 h. The storage phosphor screen was scanned in a PhoshorImager® 445 SI (Molecular Dynamics, Sunnyvale, CA). Treatment of the cells with 1 mM sodium azide as an inhibitor of active transport was performed by incubating cell monolayers as described above with [^{32}P]ClRp(N)$_{18}$.

Since modification of proteins with [^{32}P]ClRp(N)$_{18}$ changes their electrophoretic mobility, a set of purified proteins (IgG light and heavy chains, lactoferrin and lysozyme) modified with the same reagent ([^{32}P]ClRp(N)$_{18}$) were used as protein molecular weight markers as described (19).

ODN stability experiments

ODNs-Flu, synthesized as described in the above section, were radiolabelled with [^{32}P] using T4 polynucleotide kinase in exchange kinase buffer (Gibco, Grand Island, NY), then used for uptake and stability experiments. The uptake experiments conditions were as before except that incubations were performed during 30 min and 1 h. Aliquots from culture supernatants or from nuclear fractions (N), prepared as described above, were mixed with an equal volume of sample buffer (0.01 M Tris/boric acid pH 8.4 with 8 M urea) and applied on 20% (v/v) PAGE with 7 M urea (0.05 M TBE, pH 8.4). Equal levels of radioactivity from culture supernatant and nuclear fraction were applied on the gel.

Determination of dissociation constants and quantity of ODN-binding proteins per cell

For the estimation of the dissociation constant of ODN–protein complexes, cells were incubated with 0.005–10 μM [^{32}P]ClRp(N)$_{18}$, and equal amounts of proteins were fractionated by SDS–PAGE as described above. The concentration of alkylating ODN derivative was plotted as a function of gel band intensity, which was quantified using VideoTest® software (ClinBioTech, Moscow, Russia). The data fitted a single site binding model, and dissociation constants (Kd) of ODN–protein complexes could be determined from a double-reciprocal plot as previously described (21).

To estimate the quantity of protein molecules in A431 cell membranes, 0.77×10^6 cells were incubated with a saturating 10 μM concentration of [^{32}P]ClRp(N)$_{18}$, and the radioactivity of the membrane fraction was determined after washing of the cells. The number of protein molecules per cell was estimated from cell number, molar radioactivity of the [^{32}P]ClRp(N)$_{18}$ and radioactivity of the MC fraction.

Competition experiments

To study the specificity of the [^{32}P]ClRp(N)$_{18}$-protein binding, cells were incubated with 1 μM [^{32}P]ClRp(N)$_{18}$ in the presence of competitors—ssDNA (150 μg/ml), dsDNA (150 μg/ml), poly IC (50 μg/ml), ATP (100 μM), heparin (50 μg/ml), dextran sulphate (10 μg/ml), p(sN)$_{20}$ (10 μM) or p(N)$_{18}$ (50 μM). Preparation of MC fractions and electrophoresis were performed as described above in the section Identification of ODN-binding proteins. For *in situ* competition experiments, cell monolayers were pre-incubated for 5 min with the same competitors [ssDNA (50 μg/ml), dsDNA (50 μg/ml), ATP (100 μM), heparin (15 μg/ml), dextran sulphate (15 μg/ml), p(sN)$_{20}$ (10 μM), or p(N)$_{18}$ (50 μM)] prior to incubation either with 1 μM rhodamine-labelled ODNs or with 5 μM free rhodamine as a negative control. Cells were then rinsed three times as described in the ODN-uptake section.

Cell surface treatments and cell fractionation

For the affinity modification evaluation of ODN-binding proteins with [^{32}P]ClRp(N)$_{18}$, cells were either detached with a non-enzymatic cell dissociation solution (Sigma, St Louis, MO), or with a solution of 0.05% trypsin with 0.53 mM EDTA (Gibco, Grand Island, NY). After trypsin neutralisation with FCS, cells were washed with an excess of PBS$^-$. Then, $0.5–1 \times 10^7$ cells/ml in PBS$^-$ were incubated with 1 μM [^{32}P]ClRp(N)$_{18}$ as described earlier. For *in situ* uptake evaluation, cells were collected in the same way and incubated during 1 h at 37°C under continuous agitation with 1 μM rhodamine-labelled ODNs or with 5 μM free rhodamine as a negative control. Thereafter, cells were rinsed three times by centrifugation in DMEM medium, re-seeded in LabTek chambers as described in the cell cultures section and maintained at 37°C in 5% CO_2 for at least 18 h in order to allow them to attach before processing for microscopy.

To investigate the cellular localisation of ODN-binding proteins, cells were modified with 1 μM [^{32}P]ClRp(N)$_{18}$ and treated with 0.125% trypsin solution (Flow Laboratories, USA) for 5 min at room temperature. After trypsin neutralisation by addition of FCS, the cells were washed with PBS$^-$ and the MC fraction was prepared and analysed as described above. To permeabilize the cellular membranes, cells were scraped from the plate, washed with PBS$^-$ and incubated for 10 min on ice in 25 μl of 250 U streptolysin O (Sigma, St Louis, MO) or 40 μg/ml saponin solution (Sigma, St Louis, MO), in conditions known to cause pores in plasma membranes (22,23). The cells were centrifuged and the supernatants were collected as the cytosolic fraction and the pellet was washed

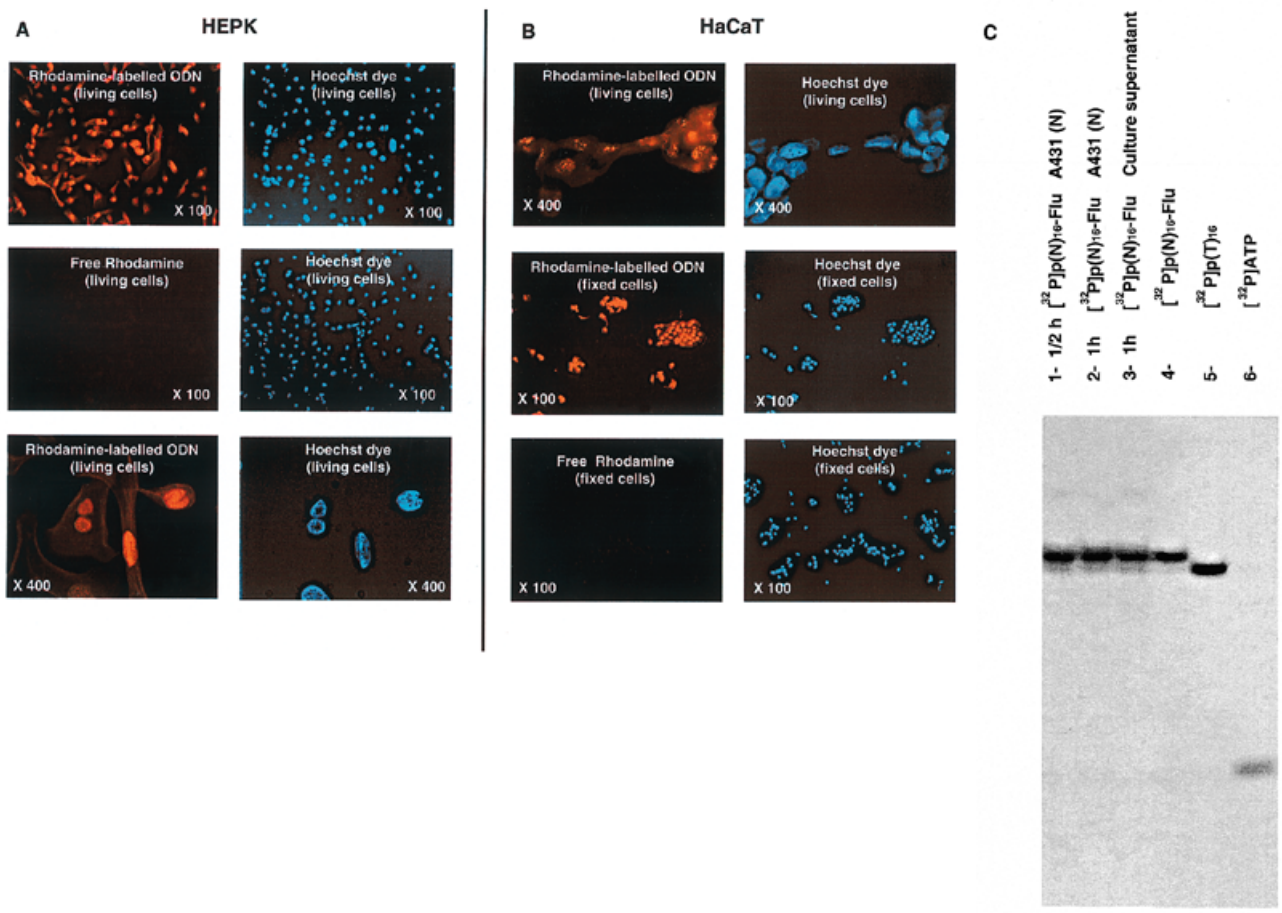

Figure 1. (**A**) Left panel, uptake of rhodamine-labelled ODNs (ODNs-Rh) by living HEPK. Top left panel, HEPK after 1 h incubation at 37°C, 5% CO_2 in standard culture medium (DKSFM) with 1 µM ODNs-Rh or 5 µM free rhodamine as a negative control. Simultaneous fluorescence and Hoechst dye staining images indicate uniform uptake in HEPK cultured at low confluence (~30%). Bottom left panel, actively dividing HEPK demonstrating full viability of these cells. Images were obtained from unfixed cells with a 400× magnification for fluorescence microscopy. (**B**) Right panel, uptake of ODNs-Rh in either live (top panel) or fixed (middle and lower panels) HaCaT. Highly subconfluent cultures (~30%) were incubated during 1 h at 37°C, 5% CO_2 in the presence of 1 µM rhodamine-labelled ODNs or 5 µM free rhodamine as a negative control (lower panel) in culture medium devoid of FCS (DMEM). (**C**) ODN stability in A431 cells. Radiolabelled ODNs-Flu ($[^{32}P]p(N)_{16}$-Flu) were incubated with living A431 cultures as in (B) or in A431 culture supernatant for the indicated time. Uptake conditions and nuclear preparations are described in Materials and Methods. 1 and 2, A431 nuclear (N) fractions after incubation with $[^{32}P]p(N)_{16}$-Flu; 3, A431 culture supernatant after 1 h incubation with $[^{32}P]p(N)_{16}$-Flu; 4, non-incubated control ODN $[^{32}P]p(N)_{16}$-Flu; 5, non-incubated fluorescein-uncoupled control ODN $[^{32}P]p(T)_{16}$; 6, non-incubated $[^{32}P]$ATP.

with 1 ml of PBS^{-}. The subsequent MC fraction was prepared as described earlier and collected as the membrane fraction. The gels were loaded with comparable amounts of proteins as determined by the equal number of cells treated in each condition and by colloidal silver staining of the blots.

RESULTS

Uptake of ODNs-Rh in primary keratinocytes and keratinocyte cell lines

The uptake of ODNs-Rh in either formaldehyde-fixed or living HEPK was studied. As shown in Figure 1A (left panel), living HEPK cultured in monolayers readily take up ODNs-Rh in standard incubation conditions (1 h, 1 µM ODN, 37°C, 5% CO_2). As confirmed by Exhaustive Photon Reassignment (EPR) deconvolution® system (Scanalytics, Billerica, MA) for high resolution fluorescence microscopy (24,25), ODNs-Rh mainly accumulate in the nuclei without apparent cytoplasmic staining of endosomes or lysosomes (data not shown) in keeping with published observations (11). Uptake is dependent upon cell confluence since below 50% confluence, up to 90% of HEPK were positive, whereas at 100% confluence, this proportion dropped to 30% with a majority of stained cells at cell population margins (data not shown). To avoid the possibility of permeation artefacts due to the fluorochrome itself, cells were incubated with an excess (5 µM) of free rhodamine. Under these conditions, no nuclear accumulation of fluorescence was observed (Fig. 1A, left panel). Moreover, as has already been shown in (11,12,14), similar uptake has been obtained with ODNs-Flu (data not shown) ruling out any possibility of rhodamine-related artefacts.

Increased uptake of ODNs could eventually be due to membrane permeabilisation or membrane damage in freshly dissociated tissue, or to changes occurring during terminal differentiation. In order to eliminate these possibilities, cell viability was assessed by trypan-blue exclusion and found to be ≥95% (data not shown). We also verified that the cells were

exponentially growing by flow cytometric profile analysis (data not shown) and by the presence of numerous cytokinesis figures harbouring ODN nuclear staining (Fig. 1A, left panel). Moreover, primary keratinocyte monolayers express very low levels of early differentiation markers such as involucrin (data not shown) or keratins 1 and 10 in keeping with previous data (26,27).

Dose-response experiments performed in living HEPK showed that nuclear accumulation could be detected at doses as low as 10 nM and saturated progressively between 200 and 500 nM. Fluorescence was seen in cell cytoplasm as well at these high concentrations (Fig. 1A, bottom left panel and data not shown). Furthermore, the nuclear uptake of ODNs-Rh is fast since kinetic studies indicate that all nuclei are labelled within 10 min (data not shown). Interestingly, the incubation of keratinocyte cell monolayers with ODNs-Rh at 4°C did not prevent their penetration (data not shown) in keeping with the data reported at 25°C or after treatment with active transport inhibitors (11), but in contrast to other cell types (5,8–10). At last, it should be noticed that none of these results depend on the ODN sequence since identical uptake and intracellular distribution have been obtained with ODNs of various sequences.

Similar uptake has been obtained with two human keratinocyte cell lines, namely HaCaT (Fig. 1B, right panel) and A431 (Fig. 5), in accordance with the observations of Nestle (12) for the A431 cell line, but in contrast to those of Noonberg *et al.* (11) for the HaCaT cell line. This latter discrepancy might be due to cell aging. Indeed, we noticed a decreased uptake of ODNs when increasing the number of cell passages (data not shown). In addition, we noticed that incubation of ODNs-Rh in the presence of FCS as described in the work of Noonberg *et al.* abolished the ODN-uptake (data not shown). This decrease might be due to the presence of FCS proteins as albumin, which is known to interact with many proteins and nucleic acids and therefore tends to drastically reduce adsorption (28). In striking contrast to keratinocytes, other primary cells (HDPF), epithelial cell lines (MCF-7, HeLa) or osteocarcinoma cell lines (U2-OS, Saos-2) fail to internalise ODNs-Rh under the same conditions (data not shown), as also shown in (5).

As shown in Figure 1C, ^{32}P-radiolabelled ODN-Flu conjugate migrates as a single band in a sequencing gel after incubation for various periods of time in A431 cells or in their cell culture supernatants. Uncoupling of the intact ODN from the fluorochrome or degradation of the ODN moeity should give rise to lower molecular weight bands which is clearly not observed over the time course of this experiment whether material in the cell supernantant or in the nuclear fraction is analysed.

The ODN-uptake by monolayers of HEPK and keratinocyte cell lines is thus saturable, fast and essentially irreversible. Altogether, these observations suggest cell-specific peculiarities in the mechanism of ODN-uptake which deserves further investigation.

Identification of ODN-binding proteins by affinity modification

The ability of proteins from several cell lines to interact with ODNs was investigated by means of affinity modification studies with an alkylating ODN derivative ([^{32}P]ClRp(N)$_{18}$). Several proteins were identified in A431 and HaCaT keratinocyte cell lines with apparent MW of 35, 43, 61–63 kDa doublet and 70 kDa (Fig. 2). However, by comparison with other cell lines (HeLa, HDPF and U2-OS), the 61–63 kDa doublet and the 35 kDa

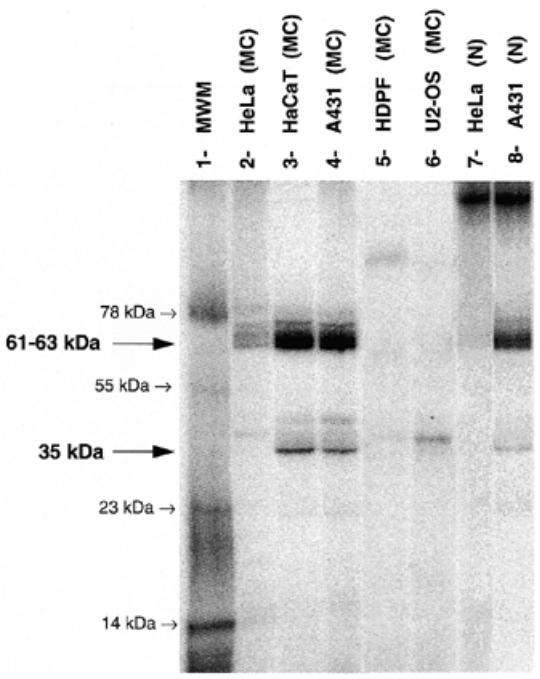

Figure 2. Affinity modification of keratinocyte proteins by a radiolabelled alkylating ODN derivative ([^{32}P]ClRp(N)$_{18}$). Membrane-cytosolic (MC) and nuclear (N) protein fractions were affinity-labelled by the alkylating ODN derivative. Cells were incubated in PBS$^-$ with 1 µM [^{32}P]ClRp(N)$_{18}$ for 1 h at 37°C, and further treated as described in Materials and Methods. Proteins corresponding to the MC and N fractions were fractionated by SDS–PAGE. Arrows indicate the MW of the major keratinocyte ODN-binding proteins. 1, molecular weight markers; 2, HeLa MC proteins; 3, HaCaT MC proteins; 4, A431 MC proteins; 5, HDPF MC proteins; 6, U2-OS cells; 7, HeLa N proteins; 8, A431 N proteins.

proteins appeared to be appreciably more abundant in keratinocytes and the major affinity modified proteins in these cells.

Interestingly, a similar set of ODN-binding proteins has been found in A431 nuclear extracts with an additional high MW protein (*vide infra*).

Identical experiments, with other reactive groups conjugated to the same ^{32}P-labelled ODN with an aldehyde or N-hydroxysuccinimide ester, were performed and gave identical affinity modifications of the 61–63 kDa doublet and 35 kDa proteins (data not shown).

Incubation of the cells in the presence of an inhibitor of active transport such as sodium azide did not interfere with the modification of the 61–63 kDa doublet and 35 kDa ODN-binding proteins by [^{32}P]ClRp(N)$_{18}$ (data not shown).

Characterisation of the 61–63 kDa doublet and 35 kDa ODN-binding proteins in A431 cells

The dissociation constants of ODN–protein complexes were estimated from the extent of modification of these proteins upon incubation with increasing concentration of the alkylating ODN derivative (21), and found to be 1 µM for the 61 and 63 kDa proteins and 2 µM for the 35 kDa one (Fig. 3).

The abundance of these ODN-binding proteins per cell was calculated from the specific radioactivity of the [^{32}P]ClRp(N)$_{18}$ probe, the number of cells, the radioactivity of the membrane fraction and the ratio of intensities of the 61, 63 and 35 kDa signals on autoradiography. It was estimated to be ~1.25 × 10^5

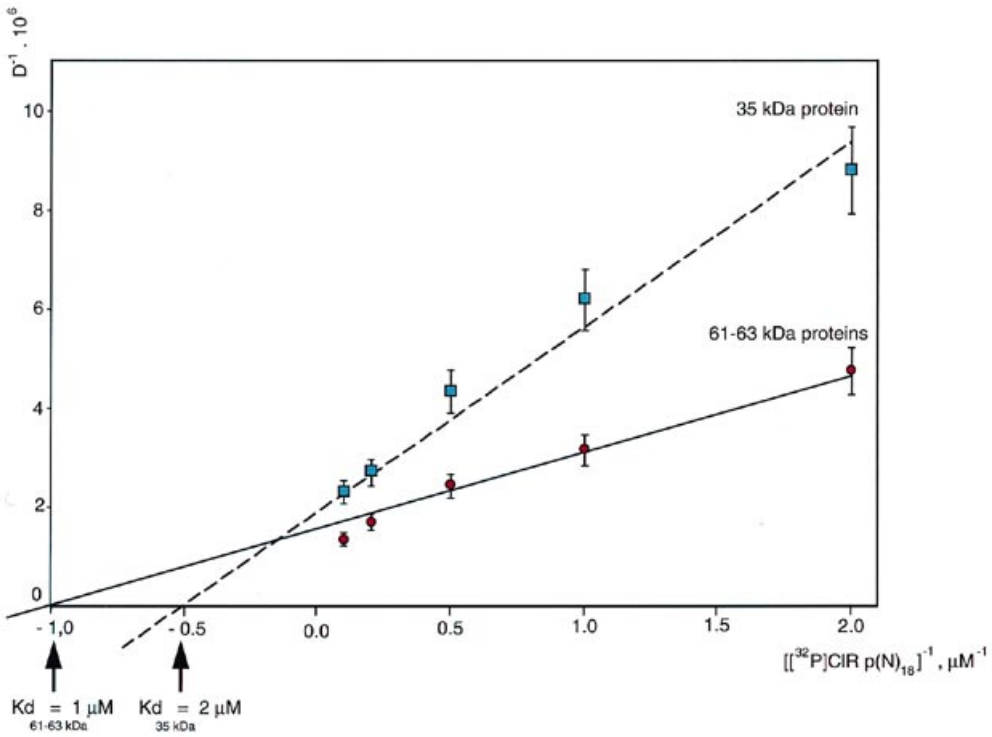

Figure 3. Estimation of the Kd of ODN–protein complexes. A431 cells were treated as in Figure 2 with increasing concentrations of the alkylating ODN derivative ($[^{32}P]ClRp(N)_{18}$) as described in Materials and Methods. The graph represents, in double-reciprocal units, the gel band intensity of specific radioactivity (arbitrary units D^{-1}) as a function of the concentration of the alkylating ODN derivative (μM^{-1}). Arrows indicate the Kds for the 61, 63 and 35 kDa protein–ODN complexes.

molecules per cell for each of the 61 and 63 kDa proteins and 0.5×10^5 molecules per cell for the 35 kDa protein.

Specificity of the interaction of ODNs with the 61–63 kDa doublet and 35 kDa ODN-binding proteins in A431 cells

In order to investigate the mechanism and specificity of binding of reactive ODN derivatives with proteins, A431 cells were incubated with alkylating ODN derivatives in the presence of potential competitors (Fig. 4). Unmodified phosphodiester [$p(N)_{18}$] or phosphorothioate [$p(sN)_{20}$] ODNs and to a lesser extent plasmid DNA (ssDNA, dsDNA) were the best competitors, while ATP did not inhibit the affinity modification of these two ODN-binding proteins, as expected. It should be noted that polyIC and heparin inhibited the affinity modification of the 61 kDa protein whereas dextran-sulfate preferentially inhibited that of the 63 kDa protein.

The same set of competitors was used in ODN-Rh-uptake experiments (Fig. 5). Consistently with our data on ODN-binding proteins, $p(N)_{18}$, $p(sN)_{20}$ and ssDNA inhibited the uptake of ODNs-Rh, while ATP had no effect.

Other polyanionic polymer competitors, such as heparin or dextran-sulphate, while inhibiting affinity modification of ODN-binding proteins with varying efficacy (Fig. 4), competed out ODN-Rh-uptake (Fig. 5).

pTn ODNs of lengths ranging from 2 to 16 bases were used as well; only those longer than 12 bases showed significant inhibition of affinity modification of the proteins (data not shown).

Cellular localisation of the 61–63 kDa doublet and 35 kDa ODN-binding proteins

Several cell surface pre-treatments and cellular extractions were undertaken to study the cellular localisation of these proteins.

As shown above, ODNs-Rh are efficiently taken up and accumulate in the nuclei in untreated cells (Fig. 1A and B). In contrast, trypsin pre-treatment of A431 or HaCaT cells abolished protein modification by alkylating ODN derivatives (Fig. 6A). Likewise, pre-treatment of the cells with trypsin completely inhibited the uptake of the ODNs-Rh into keratinocytes (Fig. 6B).

Cytosolic proteins of A431 cells were extracted by permeabilisation of cellular membranes with saponin or streptolysin O. Using both permeabilisation protocols, lower amounts of the affinity modified 61–63 kDa doublet and 35 kDa ODN-binding proteins were found in the cytosolic extracts than in the membrane fractions (Fig. 6C). It should be stressed here that the total protein concentration in the cytosolic fractions was higher than in the membrane fractions as seen from the colloidal silver staining of the blot (data not shown).

Interestingly, when a short treatment with trypsin was applied to A431 cells already modified with the alkylating ODN derivative, a partial proteolysis of the 61–63 kDa bands but not of the 35 kDa was observed (Fig. 6D). Whether this reflects a different accessibility of these two proteins in the plasma membrane or other reasons cannot be ascertained.

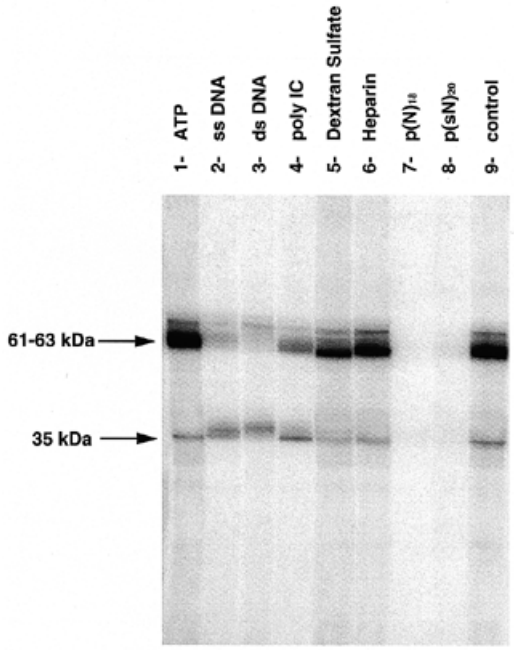

Figure 4. Specificity of the affinity modification of MC proteins in A431 cells. MC proteins from A431 cell line were affinity-labelled by the alkylating ODN derivative without any competitors (9), or in the presence of 100 µM ATP (1), 150 µg/ml ssDNA (2), 150 µg/ml dsDNA (3), 50 µg/ml poly IC (4), 10 µg/ml dextran sulphate (5), 50 µg/ml heparin (6) ; 50 µM $p(N)_{18}$ (7) or 10 µM $p(sN)_{20}$ (8). MC fraction preparation and SDS–PAGE were performed as described in Materials and Methods.

DISCUSSION

The design of improved methods for cellular delivery of ODNs and plasmid DNA is of great interest in the development of nucleic-acid-based therapeutic agents. However, the mechanism(s) of ODN uptake are poorly understood, and the proteins possibly involved in their recognition and internalisation have not been thoroughly characterised, except for Mac-1, a recently discovered ODN-binding protein, belonging to a family of leukocyte integrins (29).

In this study, we confirm that human keratinocytes are unique in their ODN-uptake ability, in keeping with previous data (11,12), but in contrast to most cell lines investigated so far. The interaction of ODNs with keratinocytes is characterised by a fast, saturable, irreversible and intense nuclear accumulation, at variance with kinetics of internalisation and localisation properties in other cell types including epithelial cell lines. Indeed, in most cases, incubation of cells with ODNs results in a perinuclear punctate pattern corresponding to ODN accumulation within vesicular compartments such as endosomes and/or lysosomes (5,7,8). These cells show no detectable nuclear accumulation when incubated with fluorescently labelled ODNs (7,30). As shown here, keratinocytes grown in monolayers rapidly accumulate ODNs in the nucleus with no apparent labelling of endocytotic vesicles even in short term experiments. In addition, the process is temperature independent and is not prevented by the addition of inhibitors of active transport as already reported (11).

Taken together, these results strongly support the hypothesis that uptake of unmodified ODNs in undifferentiated exponentially growing keratinocytes is not achieved by adsorptive, receptor-mediated or fluid-phase endocytosis as proposed for the uptake of ODNs in most cell types (6,11) and for the internaliz-ation of ODN–cationic lipid complexes (12,13). Alternatively, the present data could be explained by membrane receptor recognition, crossing through the plasma membrane through an unknown mechanism followed by rapid translocation from the cytoplasm to the nucleus as shown previously for ODNs microinjected into the cytoplasm (7,31,32). This mechanism of uptake is of interest with respect to antisense and antigene effects since sequestration of ODNs in endosomal/lysosomal compartments may constitute a sink for ODNs.

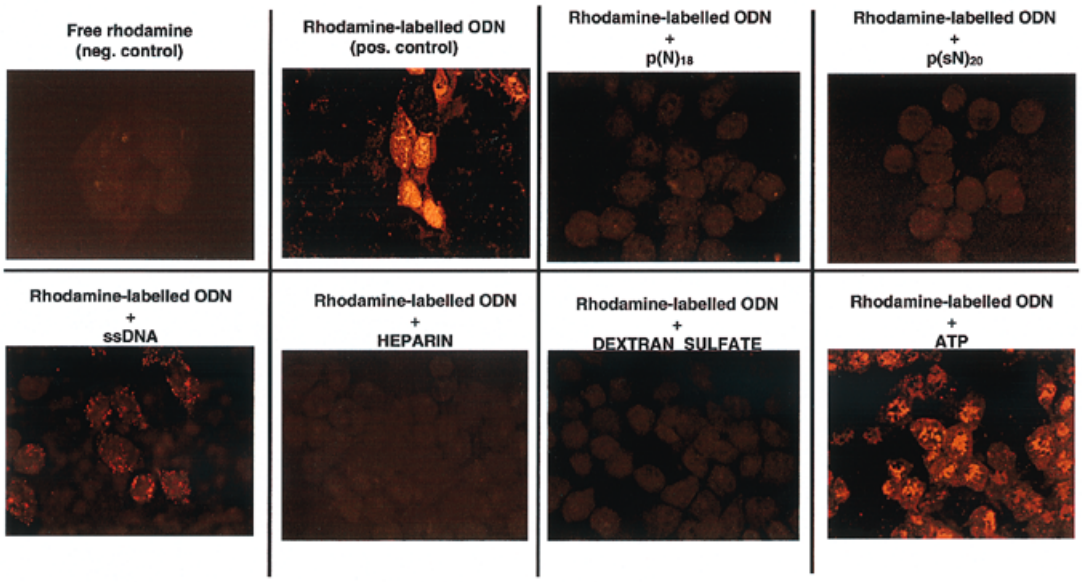

Figure 5. Effect of competitors on ODN-Rh-uptake in A431 cells. Cell monolayers were pre-incubated for 5 min in the presence of ATP (100 µM), ssDNA (50 µg/ml), heparin (15 µg/ml), dextran sulphate (15 µg/ml), $p(sN)_{20}$ (10 µM), or $p(N)_{18}$ (50 µM), and further incubated with ODNs-Rh as described in Materials and Methods.

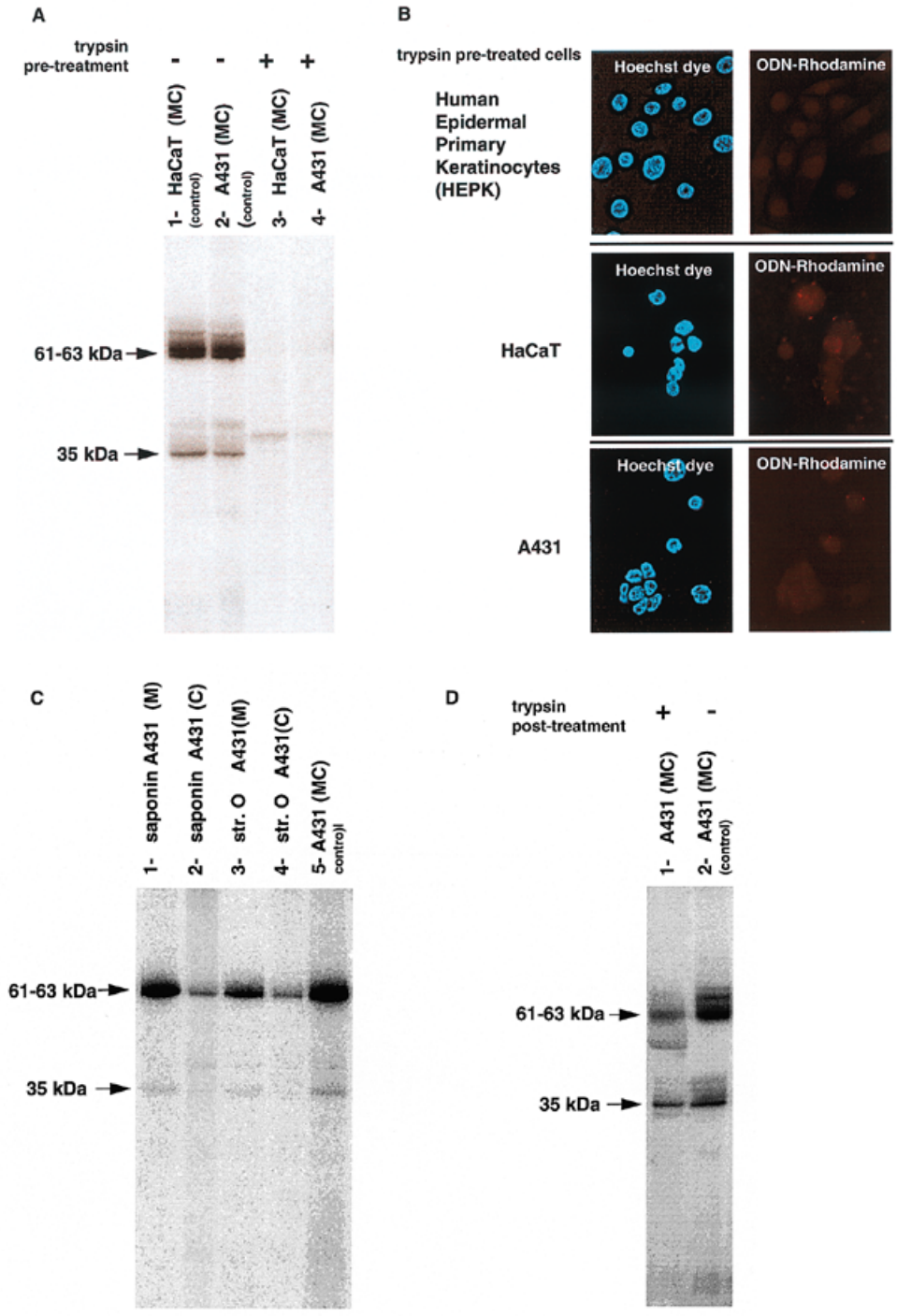

Figure 6. (**A**) Effect of trypsin pre-treatment on the affinity labelling of the cell surface proteins. A431 and HaCaT were pre-treated with trypsin prior to affinity modification of cells with the alkylating ODN derivative as in Figure 2. Cells were further treated as described in Materials and Methods and the proteins of the MC fractions were analysed by SDS–PAGE. 1, HaCaT affinity-modified proteins without pre-treatment with trypsin; 2, A431 affinity-modified proteins without pre-treatment with trypsin; 3, affinity modification of HaCaT proteins after treatment with trypsin; 4, affinity modification of A431 proteins after treatment with trypsin. (**B**) Effect of trypsin pre-treatment on the uptake of rhodamine-labelled ODNs in primary keratinocytes (HEPK), and A431 or HaCaT keratinocyte cell lines. Cells were pre-treated with trypsin prior to incubation with 1 µM ODNs-Rh, during 1 h at 37°C, 5% CO_2. (**C**) Extraction of the A431 ODN-binding proteins by permeabilization with streptolysin O or saponin. A431 cells were modified with the alkylating ODN derivative as in Figure 2, and treated with streptolysin O or saponin as described in Materials and Methods. The proteins corresponding to the cytosolic (C) and membrane (M) fractions were analysed by SDS–PAGE. The total protein concentration in the cytosolic fractions was higher than in the membrane fractions as seen from the colloidal silver staining of the blot (data not shown). 1, saponin, A431 membrane extraction (M); 2, saponin, A431 cytosolic extraction (C); 3, streptolysin O, A431 membrane extraction (M); 4, streptolysin O, A431 cytosolic extraction (C); 5, control. (**D**) Trypsin proteolysis of the A431 ODN-binding proteins pre-modified with the alkylating ODN derivative. A431 cells were modified with the alkylating ODN derivative as in Figure 2, submitted to a short term treatment with trypsin, and further processed as described in Materials and Methods. The proteins corresponding to the MC fractions were analysed by SDS–PAGE. 1, A431 affinity-modified proteins after treatment with trypsin; 2, A431 affinity-modified proteins without treatment with trypsin.

In order to work with young homogeneous keratinocyte cultures and to avoid the effects linked with replicative senescence (33), freshly isolated keratinocytes were passaged once before use in the selective DKSFM, and maintained in culture until the fifth passage. The use of this serum-free low calcium medium (<0.1 mM) allows the growth and selection of a highly enriched population of undifferentiated and non-stratified keratinocytes. These cells are considered as representative of *in vivo* basal-like keratinocytes as determined by the lack of ultrastructural, morphological features, and differentiation markers (26,27, 34–39; reviewed in 40). HEPK cultured in these standard clonogenic culture conditions are defined as *in vitro* cultured monolayers of keratinocytes.

A strict control of the cell population is important since facilitated uptake in keratinocytes has been questioned by Giachetti and Chin (14) who link efficient uptake of ODNs in suprabasal granular keratinocytes of artificial skin with the appearance of late features of terminal stages of differentiation and/or apoptosis such as loss of membrane integrity. Although there are clear parallels between apoptosis and epidermal terminal differentiation (41–44), it is unclear whether terminal differentiation of keratinocytes is a form of apoptosis (45). However this process has been shown to be triggered and restricted to the upper differentiated layers of the epidermis (14,46,47). Monolayers of keratinocytes grown *in vitro* in our standard culture conditions are thus not prone to differentiate and/or to feature early apoptotic markers although a small fraction (~3–5%) of these cells is still continuously and spontaneously undergoing terminal differentiation, expressing terminal differentiation markers and/or detaching from the solid matrix, thus mimicking the *in vivo* situation (our personal observations and 48,49). In addition, several keratinocyte cell lines, and in particular the A431 tumourigenic cell line, readily take up ODNs as well, but do not differentiate. Along the same line, we also verified that the cells were exponentially growing as attested by flow cytometric profile analysis and by the presence of numerous cytokinesis figures harbouring ODN nuclear staining. The process by which ODNs efficiently and rapidly accumulate in the nuclei of keratinocytes cultured *in vitro* as monolayers cannot therefore be explained by a change in viability and/or membrane permeability subsequent to terminal differentiation and/or apoptosis.

The affinity modification approach has already been successfully used for the investigation of ODN-binding proteins in several cell lines and tissues, and a large number of cell surface proteins have been claimed to bind ODNs, (5,6,10,19,20,50–55). In keratinocytes, we have identified specific interactions between ODNs and two major sets of proteins: a 61–63 kDa doublet and a 35 kDa protein. Whether they correspond or not with some of the ODN-binding proteins reported in previous studies cannot be ascertained. Interestingly, ODNs bind to a 63 kDa protein in HeLa cells as well, but the abundance of this protein band is much lower than in keratinocytes.

The dissociation constants of these ODN–protein complexes were estimated from the extent of modification of these proteins with respect to the concentration of alkylating ODN derivatives as described (21). Dissociation constants of 1 and 2 μM were calculated for the 61, 63 and 35 kDa protein–ODN complexes. These values are similar to the 3.5 μM value (or 2.2 μM for a phosphorothioate ODN derivative) described by Beltinger *et al.* (55) for high affinity ODN-binding proteins in K562 human leukemia cells and HS294T melanoma cells.

The quantity of 61, 63 and 35 kDa protein molecules per cell has been estimated as 1.25×10^5 for the 61 and 63 kDa proteins and 0.5×10^5 for the 35 kDa protein in keeping with previous data. As an example, the number of high-affinity ODN-binding sites on the surface of mouse fibroblast T15 cells was estimated by Scatchard analysis to be 1.8×10^5 sites per cell, although considerably larger numbers of low affinity sites were found (6.8×10^6 sites per cell) (10). Beltinger *et al.* (55) found 2×10^5 ODN-binding sites per cell in K562 leukemia cells, and Kitajima *et al.* (53) reported 3.4×10^5 ODN receptors in a mouse fibroblast tumour line.

The specificity of the interaction of the 61–63 kDa doublet and 35 kDa proteins with ODNs in A431 cells was monitored in competition experiments. $p(N)_{18}$, $p(sN)_{20}$ and plasmid DNA, but not ATP or very short ODNs, readily competed with ODNs-Rh for cellular uptake, and in a similar way for protein labelling with the alkylating ODN derivative in affinity modification experiments. Polyanionic polymer competitors, such as heparin or dextran-sulphate showed intermediate affinity modification efficacy of ODN-binding proteins whereas they competed out ODNs-Rh uptake. This apparent inconsistency between *in situ* observations and affinity modifications may be due to the differential affinity modification of the 61–63 kDa ODN-binding doublet; heparin completely inhibited affinity modification of the 61 kDa protein whereas dextran-sulfate preferentially inhibited that of 63 kDa (Fig. 4). A possible interpretation is that the interaction of ODNs with both 61 and 63 kDa ODN-binding proteins would be required for effective uptake. To summarise, specific inhibitory effects on ODN-uptake and consistent affinity modification data of the 61–63 kDa doublet and 35 kDa proteins are in keeping with the involvement of these ODN-binding proteins in the intracellular transport of ODNs into cells.

The 61–63 kDa doublet and 35 kDa ODN-binding proteins appear to be essentially localised in the plasma membrane. Indeed, the major part of these proteins is extracted in the membrane fraction upon permeabilisation with streptolysin O or saponin. Along the same lines, cell surface treatment by proteolytic enzymes confirmed the plasma membrane localisation of these proteins and emphasised the importance of membrane protein integrity in the ODN-uptake and protein affinity modification processes. Interestingly, these 61–63 kDa doublet and 35 kDa proteins in the membrane fraction were also found in the nuclear fraction, along with an additional high MW protein (Fig. 2). The presence of these ODN-binding proteins in the plasma membrane and in the nucleus as well could reflect their involvement in the extracellular recognition of ODNs, their transport across the plasma membrane and their intracellular routing to the nucleus, but no direct evidence is provided.

The purification of these proteins by affinity chromatography and the cloning of their cDNA will ultimately give tools for their characterisation and for an evaluation of their role in ODN-uptake and cell physiology. Moreover, a better understanding of the mechanism of uptake of ODNs in keratinocytes might ultimately be helpful to improve nucleic acid delivery strategies.

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