Identification of Proteins Released by Mammalian Cells That Mediate DNA Internalization through Proteoglycan-dependent Macropinocytosis*

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Naked DNA plasmid represents the simplest vehicle for gene therapy and DNA-based vaccination purposes; however, the molecular mechanisms of DNA uptake in mammalian cells are poorly understood. Here, we show that naked DNA uptake occurs via proteoglycan-dependent macropinocytosis, thus challenging the concept of a specific DNA-internalizing receptor. Cells genetically deficient in proteoglycans, which constitute a major source of cell-surface polyanions, exhibited substantially decreased uptake of likewise polyanionic DNA. The apparent paradox was explained by the action of DNA-transporting proteins present in conditioned medium. Complexes between these proteins and DNA require proteoglycans for cellular entry. Mass spectrometry analysis of cell medium preparations identified several proteins previously shown to associate with DNA and to participate in membrane transport of macromolecular cargo. The major pathway for proteoglycan-dependent DNA uptake was macropinocytosis, whereas caveolae-dependent and clathrin-dependent pathways were not involved, as determined by using caveolin-1 knock-out cells, dominant-negative constructs for dynamin and Eps15, and macropinocytosis-disruptive drugs, as well as confocal fluorescence co-localization studies. Importantly, a significant fraction of internalized DNA was translocated to the nucleus for expression. Our results provide novel insights into the mechanism of DNA uptake by mammalian cells and extend the emerging role of proteoglycans in macromolecular transport.

Cells have developed efficient barriers to ensure genetic diversity and for protection from disadvantageous non-self genes. Gene therapy and cellular protein delivery strategies rely on the entry of large, polyvalent molecules across these barriers. The introduction of gene-based therapies into the clinic is hampered by a limited understanding of macromolecular transport in mammalian cells. Mechanistic studies are thus required for a more rational design of DNA and protein delivery vehicles (1, 2).

The most straightforward strategy for gene delivery is represented by free (or “naked”) DNA (3). Intravenous administration of DNA in mice results in significant uptake and expression in the liver, whereas other tissues generally show limited expression levels (4, 5). Several studies have shown significant expression of DNA injected intradermally or intramuscularly (6–9). Accordingly, DNA uptake has been demonstrated in primary liver sinusoidal liver cells (10) and keratinocytes (11) in vitro, but also in primary macrophages (12).

Early work suggested association of exogenous DNA with mammalian cells (13, 14), and several DNA- and oligonucleotide-binding proteins have been isolated from cell membrane preparations (11, 15–17). A functional role in DNA uptake has not been convincingly shown for any of these membrane proteins. Initial studies had suggested an involvement of class A scavenger receptor in DNA uptake by macrophages (18); however, studies with class A scavenger receptor knock-out mice failed to confirm this notion (19). The mechanism of DNA uptake by mammalian cells thus remains largely unknown. We set out to study the uptake of DNA and found an unexpected role for heparan sulfate proteoglycan.

EXPERIMENTAL PROCEDURES

Materials—Fluorophores, labeled markers, secondary antibodies, and DNase I were from Invitrogen. Labeling of DNA and heparan sulfate (HS)² was performed as recommended by the manufacturer. pGL3 luciferase plasmid was provided by Dr. Å. Oldberg (Lund University, Lund, Sweden), and the luciferase assay kit was from Promega. pEX_EF1_Cav-1-YFP, pcDNA3.1(-)-HA-Dyn2 WT/K44A plasmids were from ATCC (Manassas, VA). The GFP-Eps15-EΔ95/295 construct was a kind gift from Dr. A. Dautry-Varsat (Institut Pasteur, Paris, France); HS-2, HS-6, and chondroitin sulfate (CS) preparations were the same as described (20). HIV-Tat peptide (GRKKRRQRRRPPQC-amide) was synthesized by

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2 The abbreviations used are: CM, conditioned medium; HS, heparan sulfate; CS, chondroitin sulfate; HA, hyaluronic acid; GAG, glycosaminoglycan; TRITC, tetramethylrhodamine isothiocyanate; CHO, Chinese hamster ovary; PG, proteoglycan; FACS, fluorescence-activated cell sorter; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; HIV, human immunodeficiency virus; AF488, Alexa Fluor 488; AF647, Alexa Fluor 647.
Innovagen AB, Lund, Sweden. All chromatography columns were from Amersham Biosciences, and fine chemicals were from Sigma.

**Cell Culture and Conditioned Medium (CM) Production**—Wild-type Chinese hamster ovary cells (CHO-K1), PG-deficient CHO mutants (pgsA-745 and pgsB-618), wild-type and cav1(−/−) 3T3 mouse embryo fibroblast cells, and C6 rat glioma cells were obtained from the ATCC. T24 bladder carcinoma cells were a kind gift from Dr. I. Olsson (Lund University, Lund, Sweden), and human umbilical vein endothelial cells were purchased from Cambrex (Walkersville, MD). CHO cells were routinely cultured as described (21). 3T3 mouse embryo fibroblasts, C6 cells, and human umbilical vein endothelial cells were cultured as recommended by the supplier. T24 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. All cells were cultured in a humidified 5% CO₂, 37 °C incubator. For production of CM, cells were supplemented with 10% fetal calf serum. All cells were cultured in a humidified 5% CO₂, 37 °C incubator. For production of CM, cells were grown to confluence in 75- to 500-cm² flasks, extensively rinsed with serum-free medium, and then incubated in F12K medium as indicated under “Results” and in the figure legends. CM was loaded onto a 5-ml heparin affinity column (HiTrap Heparin HP; GE Healthcare) (flow-through was designated as “non-binding” fraction), followed by 2 column volumes F12K wash and 10 column volumes phosphate-buffered saline, pH 7.4, supplemented with NaCl to a final concentration of 0.2 M. Proteins bound to the matrix were eluted with 5 column volumes 2 M NaCl in phosphate-buffered saline, pH 7.4, and designated as “binding” fraction. Peak fractions were desalted on PD-10 desalting columns (GE Healthcare) using protocols as recommended by the manufacturer.

**DNA and HS Uptake Studies**—Uptake of fluorescein-labeled DNA oligonucleotides, DNA plasmid, or HS was determined by fluorescence-activated cell sorting analysis as described (21). Some cells were treated with DNase I from bovine pancreas (10 units/ml, 2 units/sample) for 15 min at 37 °C in 10 mM Tris, pH 7.6, 0.5 mM CaCl₂, 2.5 mM MgCl₂, and 150 mM NaCl. Luciferase reporter gene assay was performed as described (22). For competition studies, HS was depolymerized with HNO₂ at pH 3.9 (23) followed by size fractionation on a Bio-Gel P-6 column into three separate pools: intact chains (>40 disaccharides), oligosaccharides (dodeca- to octadecasaccharides), and disaccharides (24). In some cases, cells were treated with the cholesterol-depleting agent methyl-beta-cyclodextrin (1 mM), the macrophagocytosis inhibitor amiloride (2.5 mM), or the actin-disrupting agent latrunculin A (2.5 μM) for 30 min prior to incubation and during uptake experiments.

**Confocal Fluorescence Microscopy**—Cells were plated on chamber slides and allowed to grow for 48 h. For live cell microscopy, cells were incubated with AF488-labeled DNA oligonucleotides (5 μg/ml) and heparin binding CM components (20 μg/ml) for 3 h. Extracellular DNA was removed by three consecutive rinses with ice-cold phosphate-buffered saline supplemented with 100 μg/ml dextran sulfate, followed by immediate analysis. For the co-localization studies, labeled DNA oligonucleotide was allowed to form complexes with CM components for 30 min. Dextran-AF647 or transferrin-AF647 (final concentrations of 100 and 150 μg/ml, respectively) was added just prior to addition of the solutions to the cells. The cells were rinsed as above, fixed with 4% (w/v) paraformaldehyde, and mounted in Permafluor (Beckman Coulter).

In the studies using HA-dynamin-WT/K44A, GFP-Eps15-EΔ95/295, and Cav1-YFP construct, 2 × 10⁶ CHO-K1 cells were electroporated at 340 V in 700 μl of phosphate-buffered saline containing 50 μg of plasmid DNA using a BTX ECM399 (Genetronics) electroporator. Immediately after electroporation cells were plated in chamber slides and allowed to grow 24–48 h before further analysis. Cells were then incubated with DNA-AF647 (5 μg/ml) and heparin binding CM components (20 μg/ml) for 3 h or with transferrin-AF647 (150 μg/ml) for 30 min in F12K without serum. Extracellular DNA was removed as described above. Slides were then fixed in 4% (w/v) paraformaldehyde followed by permeabilization in 0.1% Triton X-100. Cells transfected with HA-tagged dynamin constructs were blocked overnight in 2% bovine serum albumin and stained using the 12CA5 anti-HA antibody (1:100) followed, after extensive rinsing, by AF488 goat anti-mouse IgG (1:500). Actin was counterstained using phalloidin-TRITC (1:500). Finally, cells were mounted in Permafluor.

For quantifications, in total more than 50 cells from at least five low magnification images were assigned to each of the expressing and non-expressing groups as determined by GFP or anti-HA staining. Ligand uptake was quantified using LCS software, and a ratio of the uptake between expressing and non-expressing cells was calculated for each image. The mean ratio ± S.D. is presented. All cells were analyzed using Leica confocal scanning equipment TCS SP2 II.

**Fluorescence Quenching and Gel Retardation**—For fluorescence quenching analysis, a mixture of DNA (5 μg/ml) and ethidium bromide (DNA:EtBr 20:1, w/w) in 1 mM Tris, 50 mM NaCl, and 1 mM EDTA (or 1 mM CaCl₂ and 0.5 mM MgCl₂ for samples with divalent cations) was supplemented with varying concentrations of heparin binding CM components (0–250 μg/ml) and/or highly sulfated HS (0–250 μg/ml). EtBr fluorescence was determined following a 30-min incubation at 20 °C using a FLUOstar OPTIMA (BMG) spectrophotometer (excitation 337 nm; emission 620 nm). For the gel retardation assay DNA was mixed with heparin binding CM components for 30 min at 20 °C in 150 mM NaCl, 5% glycerol, and 1 mM EDTA. DNA and CM mixtures were then separated on a 1% agarose gel containing ethidium bromide in TAE buffer (40 mM Tris-acetate 1 mM EDTA, pH 8.0), and DNA migration was visualized on a UV board.

**Mass Spectrometry Identification**—Heparin binding CM components were dissolved in LDS sample buffer (Invitrogen) and loaded on a 4–12% Bis-Tris NuPAGE gel. The gel was stained using Colloidal Blue (Invitrogen). Bands of interest were excised and analyzed by MALDI time-of-flight according to the method described (25). For MS/MS analysis the peptide extract was spotted directly onto a stainless steel MALDI target and was left to dry. Matrix solution containing 5 mg/ml α-cyano-4-hydroxy cinnamic acid, 50% acetonitrile, 0.1% trifluoroacetic acid, and 50 μM citric acid was added and allowed to dry, and MS/MS spectra were acquired using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, CA) in positive reflector mode. Protein identification was performed using the GPS Explorer software, with an in-house Mascot.
search engine (Matrix Science, London, UK) searching the NCBI non-redundant data base (taxa, Rodentia).

Statistical Analyses—Each data point in the figures is the mean ± S.D. In some cases the error bars were smaller than the drawn symbols.

RESULTS

Temperature-dependent Uptake and Expression of Naked DNA in Mammalian Cells—DNA uptake was studied by flow cytometry (Fig. 1A), demonstrating a time-dependent and linear increase up to 48 h, although there appeared to be a lag phase during the initial 2–4 h (Fig. 1B). Cells were detached by trypsin treatment and extensively rinsed prior to flow cytometry analysis; however, it could not be ruled out that fluorophore-labeled DNA was associated with the cell exterior, especially after short incubation periods. In Fig. 1B, however, it is evident that DNase treatment in addition to trypsin treatment does not influence the cell-associated fluorescence. Dose-response experiments showed saturable DNA uptake kinetics with maximal uptake at ~10 μg/ml (Fig. 1C). It has previously been shown that DNA oligonucleotides enter cells in a temperature-independent manner (17). We next incubated cells with DNA at 37 or 4 °C, demonstrating a strong temperature dependence. The same holds true for HS, i.e. another ubiquitous polyanion (Fig. 1D). Cells incubated with DNA plasmid encoding a luciferase reporter gene displayed dose-dependent luciferase activity (Fig. 1E). Together, these results demonstrate saturable and temperature-dependent uptake of naked DNA and that a significant fraction of internalized DNA reaches the nucleus for transcription.

DNA and glycosaminoglycans (GAGs), e.g. HS, CS, and hyaluronic acid (HA), constitute the dominating macromolecular polyanions in the cell interior and on the cell exterior, respectively, sharing some basic biophysical characteristics. We next asked whether DNA and various GAGs compete for uptake via common transport pathway/s. Highly sulfated, heparin-like HS (HS-6) as well as less sulfated HS (HS-2), exhibiting a lower charge density as compared with CS, were used. Both HS-6 and HS-2 showed dose-dependent inhibition of DNA uptake (~65–80% inhibition at 8 μg/ml HS); however, even at 80 μg/ml, CS and HA showed very limited or no inhibition of DNA uptake (Fig. 1F). Moreover, the inhibitory activity of HS was size-dependent (Fig. 1G); DNA uptake was reduced by ~80 and 35% by intact HS-6 chains (E) and HS-6 oligosaccharides (dodeca- to octadecasaccharides), respectively, whereas HS disaccharides were completely inactive even at the highest concentration tested (4 μg/ml). DNA efficiently inhibited HS-2 uptake, whereas inhibition of HS-6 uptake was less efficient (data not shown). The results indicate that the polyanions DNA and GAGs compete for cellular uptake in a structure-specific, size-dependent, and charge density-dependent manner.

Components in Conditioned Medium Mediate DNA and HS Uptake via Cell Surface PGs—As cell surface PGs provide a polyanionic envelope around the cell exterior and previously have been shown to promote the uptake of various polybasic ligands (26), we hypothesized that uptake of free DNA may be negatively regulated by PGs. To test this idea, we used CHO cell mutants (pgsA-745) deficient in xylosyl transferase that catalyzes the first step in GAG chain assembly onto the PG core protein, anticipating greater DNA uptake in these cells as compared with PG-expressing wild-type cells. Uptake of DNA over a period of 5 h was similar in wild-type (filled symbols) and
PG-deficient cells (open symbols) at all concentrations tested (Fig. 2A, squares). When cells were incubated for 24 h, DNA uptake was 2- to 3-fold greater in wild-type than in PG-deficient cells (Fig. 2A, circles). These results were unexpected and contrary to our predictions. PgsA-745 cells produce ~5% PG as compared with wild-type CHO s (27). We next included another PG-deficient CHO cell mutant (pgsB-618) that produces ~15% PG as compared with wild-type cells (28). The data demonstrate a direct correlation between DNA uptake efficiency and PG levels in recipient cells (Fig. 2B). Moreover, this was also valid for the HS preparations described in Fig. 1, which lends further support for a common uptake mechanism of DNA and HS. There was a clear tendency toward increased relative PG dependence with increasing polyanion charge density (HS-6 > HS-2 > DNA; Fig. 2B). These results, together with the initial lag phase in DNA uptake (Fig. 1B), made us speculate that naked DNA transport is mediated by secreted components that accumulate in the culture medium. To this end, serum-free naked DNA transport is mediated by secreted components that accumulate in the culture medium. To this end, serum-free

FIGURE 2. Cell medium components mediate DNA internalization via proteoglycans. A, wild-type, CHO-K1 cells (filled symbols, ■ and ○), and PG-deficient, pgsA-745 cells (open symbols, □ and △) were incubated with the indicated amounts of YOYO-1-DNA for 5 h (squares) or 20 h (circles). B, wild-type (CHO-K1) and PG-deficient (pgsB-618 and pgsA-745) CHO cells were incubated with YOYO-1-DNA (1 μg/ml, rhodamine Green-HS-2 (20 μg/ml), or rhodamine Green-HS-6 (20 μg/ml) for 24 h. C, wild-type and PG-deficient cells were incubated with YOYO-1-DNA (1 μg/ml) for 3 h either in fresh medium (Ctrl.) or in medium conditioned for 24 h with CHO-K1 cells (CM). Data are presented as the amount of uptake relative to cells in fresh medium (100% uptake). D, CHO-K1 cells were incubated with YOYO-1-DNA (1 μg/ml) in the time interval 15 min to 4 h either in fresh medium (Ctrl.) or in CM (10 μg/ml total protein). A–D, DNA and HS uptake was determined by FACS and presented as the mean ± S.D. (n = 5–8).

with fresh medium (Fig. 2C). Similar results were obtained irrespective of DNA labeling method (intercalation with YOYO-1 or covalent conjugation with AF488).

CM-mediated DNA transport increased in a time-dependent and saturable manner, suggesting an endocytic uptake mechanism (Fig. 2D), which was further supported by a vesicular staining pattern of internalized DNA (Fig. 3A). A significant fraction of internalized DNA was translocated to the nucleus for expression (Fig. 3B). Importantly, CM-mediated DNA uptake was not restricted to CHO cells, as it was also demonstrated in human bladder carcinoma cells, rat glioma cells, and primary human endothelial cells (Fig. 3C). To gain further insight into the mechanism of CM-mediated DNA uptake, CM was separated on a heparin (highly sulfated HS) affinity column into a non-binding and binding fraction. Non-bound CM material exhibited insignificant DNA transport activity (Fig. 3D). Heparin binding CM components stimulated DNA uptake in a dose-response-dependent manner, and at 10 μg/ml the effect was ~7-fold greater as compared with equivalent amounts of unfractionated CM (Fig. 3D) and 1.5-fold greater than the HIV-Tat transduction peptide that is known to bind to and mediate transport of DNA (21).

These results suggested that heparin binding CM components may directly bind to and form complexes with DNA. Consistent with this hypothesis, CM components induced a substantial and concentration-dependent mobility shift of plasmid DNA in a gel retardation assay (Fig. 3E). Complex formation between DNA and heparin binding CM components was further supported by CM-induced quenching of fluorescent DNA. In addition, highly sulfated HS almost completely abrogated CM-dependent quenching of DNA fluorescence (Fig. 3F). Together with the fact that HS had no effect on DNA fluorescence per se, either in the presence or absence of divalent cations (Fig. 3F, diamonds), our results indicate that HS efficiently competes with DNA for binding to CM components and that complex formation between DNA and HS is unlikely.

SDS-PAGE separation and mass spectrometry analysis (peptide mass fingerprint and MS/MS) of the heparin binding CM fraction identified 13 dominating protein bands (Fig. 3F and Table 1), several of which have previously been shown to associate with DNA and HS (see “Discussion”).

CM-mediated DNA Uptake Involves Membrane Raft-dependent Macropinocytosis—In recent years the vast complexity of endocytosis pathways has been realized, involving not only classical, clathrin-dependent but also non-classical, caveolin-dependent and caveolin-independent membrane raft-associated pathways (29). Ectopic expression of dominant-negative variants of dynamin and Eps15 has become widely used for specific inhibition of clathrin-dependent endocytosis (30, 31). Accordingly, we found significant inhibition of the uptake of transferrin, a well established ligand of clathrin-dependent endocytosis, in cells expressing dominant-negative constructs of dynamin and Eps15 (Fig. 4). However, CM-mediated DNA uptake was unaffected by either construct, and internalized DNA showed weak co-localization with transferrin, as determined by confocal fluorescence microscopy (Fig. 4C). Together, these results
TABLE 1

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Mass spectrometry identification of CM components

Here, we present evidence that DNA uptake by mammalian cells is mediated by secreted proteins via an endocytotic mechanism that strictly requires cell surface PGs. These results advance our understanding of DNA transport in mammalian cells and further strengthen accumulating evidence for the role of PGs in macromolecular transport. Although DNA uptake is induced severalfold by secreted components, a parallel and less

show that DNA uptake follows predominantly a non-clathrin-dependent route of entry.

Cholesterol- and sphingolipid-enriched membrane domains may exist either as separate “rafts” or, when associated with caveolin, form invaginations called caveolae, and these pathways seem to be partly overlapping (32). Methyl-β-cyclodextrin is a well established cholesterol-depleting agent employed for studying the involvement of rafts/caveolae in cellular transport (33, 34). Treatment of cells with this agent significantly reduced CM-mediated DNA uptake (Fig. 5A). Similar results were obtained with nystatin, which also disrupts lipid raft domains (results not shown). Lipid rafts have been associated with macropinocytosis that is characterized by actin membrane protrusions that pinch off into macropinosomes vesicles (35). Cells treated with amiloride that specifically inhibits the Na+/H+ exchange required for macropinocytosis showed ~55% reduction of CM-mediated DNA uptake, and treatment with latrunculin A that disrupts actin filaments resulted in ~45% reduction of uptake (Fig. 5A). These data suggest that CM-mediated DNA uptake requires intact lipid rafts and an intact actin cytoskeleton, as well as active Na+/H+ exchange, which is consistent with uptake through macropinocytosis. Accordingly, internalized DNA showed strong co-localization with dextran, which is a marker of macropinocytosis (Fig. 5B). Finally, we investigated the possible involvement of caveolae-dependent pathways. DNA uptake was unperturbed in caveolin-1-deficient mouse embryo fibroblast cells (Fig. 5C), and internalized DNA did not co-localize with caveolin-1-yellow fluorescent protein (Fig. 5D), providing evidence that caveolin-associated endocytosis has no major role in CM-mediated DNA uptake.

DISCUSSION

Here, we present evidence that DNA uptake by mammalian cells is mediated by secreted proteins via an endocytotic mechanism that strictly requires cell surface PGs. These results advance our understanding of DNA transport in mammalian cells and further strengthen accumulating evidence for the role of PGs in macromolecular transport. Although DNA uptake is induced severalfold by secreted components, a parallel and less

Uptake of Naked DNA by Mammalian Cells

FIGURE 3. Heparin binding CM components mediate DNA uptake and expression through endocytosis. A, CHO-K1 cells were incubated with DNA-AF488 (1 μg/ml) for 3 h in fresh medium supplemented with heparin binding CM components. Live cells were analyzed for DNA uptake by confocal fluorescence microscopy. B, CHO-K1 cells were incubated with 5 μg/ml luciferase-encoding DNA plasmid in fresh medium supplemented with heparin binding CM components at the indicated concentrations. Cells were analyzed for luciferase activity. C, CM/DNA uptake at the indicated ratios either in the presence (H9262 g/ml) for 3 h neither fresh medium (C6, C6 rat glioma cells, T24 human bladder carcinoma cells, and primary human umbilical vein endothelial cells (HUVEC) were incubated with DNA-AF488 (1 μg/ml) for 3 h in either fresh medium (Ctrl) or CM (10 μg/ml total protein). Data are normalized and presented as the amount of uptake relative to Ctrl. (100% uptake) as determined by FACS. D, CHO-K1 cells were incubated with DNA-AF488 (1 μg/ml) for 3 h in whole CM or CM fractions separated over a heparin affinity column yielding binding (Hep.-binding CM) and non-binding (Hep.-non-bind) components. As a comparison, DNA uptake by an equivalent amount of Hep-Tat is shown. DNA uptake was determined by FACS. E, F, complex formation between DNA and CM components. E, 0.2 μg of a 5-kb DNA plasmid was mixed with heparin binding CM components at the indicated ratios and separated on a 1% agarose gel. F, DNA, labeled with ethidium bromide (20:1, w/w), was mixed with heparin binding CM components or with highly sulfated HS at the indicated ratios either in the presence (△) or in the absence (○) of divalent cations. Alternatively, DNA and heparin binding CM components at a fixed ratio (1:25) were mixed with HS at the indicated HS:DNA ratios (△). Fluorescence was determined by spectrophotometry. All values are relative to the fluorescence of DNA/ethidium bromide only (100%). G, identification of CM-transporting DNA components. Heparin binding CM components were separated by SDS-PAGE, and major bands were excised and analyzed by mass spectrometry. The identities of CM proteins are shown in Table 1. Presented data are representative of at least three independent experiments. Quantitative data are the mean ± S.D. (n = 3–6).
Uptake of Naked DNA by Mammalian Cells

FIGURE 4. DNA uptake does not depend on clathrin-mediated endocytosis. A, CHO-K1 cells expressing either wild-type dynamin (Dyn-WT), dominant-negative dynamin (Dyn-K44A), or dominant-negative Eps15 (Eps15-mut) were incubated with AF647-labeled transferrin (Tfn) or DNA-AF647 (5 μg/ml) and heparin-binding CM components (20 μg/ml) (DNA/CM) and analyzed by confocal fluorescence microscopy as described under “Experimental Procedures.” Left columns, grayscale images visualizing ligand uptake. Right columns, composite images visualizing ligand uptake (blue), actin counter-stain (red), and dynamin/Eps15 expression (green). B, quantitative data of experiments shown in panel A from analysis of ~20 cells displaying no dynamin/Eps15 expression or significant expression of either dynamin construct or Eps15. Data are presented as the ratio (mean ± S.D., n = 5–6, see “Experimental Procedures”) of ligand uptake in expressing versus non-expressing cells in the same experiment. C, CHO-K1 cells were co-incubated for 3 h with transferrin-AF647 and DNA-AF488 (5 μg/ml) and analyzed by confocal microscopy. Results shown are representative of three independent experiments.

efficient pathway involving direct binding of DNA to a cell surface receptor molecule cannot be ruled out. However, considering the high number of potential DNA binding partners in the extracellular environment it is, in our view, highly unlikely that DNA is taken up as a single molecule in the in vivo situation.

We further demonstrate that DNA uptake predominantly involves a non-clathrin-dependent, non-caveolae-dependent macropinocytic pathway. We have previously shown that LL-37-mediated DNA uptake occurs via PGs and non-caveolar lipid raft-dependent endocytosis (22), and several groups have demonstrated a role for PGs in cationic gene delivery (36). Hengge and co-workers (11) suggested that primary keratinocytes internalize DNA by macropinocytosis, as both amiloride and its more potent derivative N,N-dimethylamiloride substantially reduced DNA uptake, which is consistent with our results.

The specificity of polyanion uptake is quite intriguing. Our observations are in line with previous in vivo studies showing efficient inhibition of hepatic DNA uptake by HS, but not by CS or HA (5). In another study, DNA plasmid was injected intra-muscularly, showing that heparin strongly inhibited DNA reporter gene expression (9). Conversely, Harris et al. (37) showed that uptake of HA via the HARE (HA receptor for endocytosis) pathway was unaffected by HS, whereas CS was a potent inhibitor of HA transport. The authors concluded that HARE may specifically recognize N-acetyl groups of amino sugars of HA and CS, but not N-sulfated glucosamine found in HS and heparin. The results presented here would suggest that DNA shares some structural requirements with HS not found in CS and HA, allowing binding to secreted components and subsequent uptake via the PG-dependent route. This notion is supported by the identification of moesin as one of the most abundant proteins in CM. Moesin has previously been identified as a cell surface DNA receptor (11) as well as an HS receptor (38), but with poor binding to CS and HA. The HS binding activity of moesin has been attributed to the presence of several stretches of the general structure (XBBXBBX)2 (XBBXBB, and (XBBXBB), i.e. consensus sequences for HS binding, in its C-terminal tail (39). The identification of moesin as a cell surface receptor is difficult to reconcile with its lack of a transmembrane region or obvious glycosylphosphatidylinositol attachment site. Intracellular moesin and the other ezrin–radixin–moesin (ERM) proteins have been shown to act as adapt-

3 B indicates a basic amino acid and X any amino acid.
tors between the actin cytoskeleton and transmembrane proteins (40). From our data, a model in which extracellular moesin similarly acts as an adaptor between an extracellular ligand (e.g. DNA) and an endocytosis receptor (PG) may be envisaged. This model would also explain several independent observations regarding the role of nucleolin, another major CM component, in cellular uptake of macromolecular cargos, such as the cytokine midkine (41), lactoferrin (42), and HIV-1 virus particles (43). Interestingly, nucleolin was recently shown to act as a cell surface receptor for acharan sulfate, a GAG structurally related to H2A, H2B, H3, and H4, i.e. the same histone variants identified in CM (Table 1). In the same report it was further shown that PGs mediate nucleosome binding to the cell surface. The authors proposed that the positively charged N termini of histones extending from the nucleosome core particles were available for binding to negatively charged PGs, a model that agrees well with the data presented in our study. Whether cellular exchange through the PG-dependent route of nucleic acids or other polyanions has a critical role in pathophysiology remains to be determined. Furthermore, the distribution of DNA-transporting proteins in various tissues and serum is another area of future research.

REFERENCES

Uptake of Naked DNA by Mammalian Cells

Identification of Proteins Released by Mammalian Cells That Mediate DNA Internalization through Proteoglycan-dependent Macropinocytosis
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