DNase X Is a Glycosylphosphatidylinositol-anchored Membrane Enzyme That Provides a Barrier to Endocytosis-mediated Transfer of a Foreign Gene

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DNase X is the first mammalian DNase to be isolated that is homologous to DNase I. In this study, we have examined its function using a novel monoclonal antibody and showed it to be expressed on the cell surface as a glycosylphosphatidylinositol-anchored membrane protein. High level expression was observed in human muscular tissues and in myotubes obtained in vitro from RD rhabdomyosarcoma cells. We observed that RD myotubes incorporated a foreign gene, lacZ, by endocytosis but that expression of the encoded coding product, β-galactosidase, was strongly inhibited. Overexpression of DNase X inhibited endocytosis-mediated gene transfer, whereas knockdown of DNase X with small interfering RNA had the opposite effect. These results reveal that DNase X provides a cell surface barrier to endocytosis-mediated gene transfer.

DNase X is a secretory endonuclease that hydrolyzes DNA to 3'-hydroxy oligonucleotides in the presence of divalent metal ions, such as Ca2+, Mg2+, and Mn2+ (1–3). Its physiological significance in body fluids has long been unclear; however, a recent study demonstrates that targeted disruption of murine dnasel increases the generation of anti-DNA antibodies and the development of a systemic lupus erythematosus-like syndrome (4). These findings indicate that DNase I plays an important role in eliminating extracellular DNA that can cause autoimmune diseases in animals.

In early investigations, most divalent cation-dependent DNase activities were regarded as being carried out by DNase I. However, more recent studies revealed the existence of several DNase I-like DNases, DNase X/Xib, DNase γ/DNASL3, and DNASEX is located at q28 of the human X chromosome and was the first gene to be found that encoded a protein homologous to DNase I (6–8). DNase X has an extra hydrophobic stretch at its C terminus, which is regarded as its most outstanding structural feature (5). This hydrophobic domain is conserved among mammalian DNase X proteins, suggesting that it has functional importance (9) and that DNase X might play a unique physiological role.

In the current study, we have generated a monoclonal antibody (mAb)3 specific for human DNase X and used it to characterize the molecule. We have presented evidence that DNase X is a glycosylphosphatidylinositol (GPI)-anchored membrane enzyme located on the cell surface and on early endocytic vesicles. Furthermore, we have demonstrated that DNase X hydrolyzes endocytosed extracellular DNA, thereby protecting cells from invasion by foreign genes.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa S3, COS-7, and CHO-K1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Human embryonic rhabdomyosarcoma RD cells, obtained from the Health Science Research Resources Bank of Japan, were maintained in growth medium (GM) (Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum, 100 units/ml penicillin, and mg/ml streptomycin). To induce myogenic differentiation, subconfluent cultures grown on collagen type I-coated dishes (Iwaki Scitech) were shifted to differentiation medium (DM) (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin) and cultured for the indicated number of days with daily medium replacement.

Expression Vectors—All expression vectors were constructed using pcDNA3.1 myc-His B (Invitrogen) by subcloning PCR-generated cDNAs. Unless otherwise noted, PCR products were inserted into the EcoRV site in-frame with the C-terminal Myc

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3 The abbreviations used are: mAb, monoclonal antibody; DPBS, Dulbecco’s modified phosphate-buffered saline; GPP, glycyolphosphatidylinositol; PI-PLC, phosphoinositide-specific phospholipase C; SERCA-1, sarcoplasmic reticulum Ca2+-ATPase 1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling; ncRNA, negative control RNA; GM, growth medium; DM, differentiation medium; FITC, fluorescein isothiocyanate; siRNA, small interfering RNA; ER, endoplasmic reticulum.
and His6 tags. cDNA fragments for phDNaseXΔC and pmFasΔC were amplified using the oligonucleotide primers phDNaseXΔC, 5′-CCACATGACATACCACTACTG-3′ (sense) and 5′-CTGCGTCTAGTCCATGCCA-3′ (antisense); pmFasΔC, 5′-CCACATGCTGGATCTGGGCTGTTC-3′ (sense) and 5′-TTCTGTATGCAGTTCCTTCAGC-3′ (antisense). The primers used were subcloning cDNA fragments for human DNase I and the extra C-terminal stretch of DNase X (amino acid 256–284) containing the hydrophobic domain simultaneously. The primers used were DNase I, 5′-GGGAGCCGGGGAGATCTTTCCCCC-3′ (sense) and 5′-CCTTCAGCATCACCTCCACTGG-3′ (antisense) and hydrophobic domain (HD), 5′-CTGAGCCAGGGCGACAGCGTC-3′ (sense) and 5′-GCCAGCGCGACGCCGCTGGG-3′ (antisense). phDNase X H130A was generated by site-directed mutagenesis using an LA PCR in vitro mutagenesis kit (Takara Bio) according to the manufacturer’s protocol. The expression vectors for human DNase I (phDNase I-myc-His), DNase X (phDNase X-myc-His), DNase γ (phDNase γ-myc-His), and DNASIL2 (phDNASIL2-myc-His) were generated in a previous study (5). pcDNA 3.1/myc-His/ lacZ (placZ), an expression vector for β-galactosidase, was purchased from Invitrogen.

**Transfection and Endocytosis Assays**—Transfection of expression vectors for the indicated DNases and mFasΔC was performed using FuGENE 6 reagent (Roche Applied Science) as described previously (9). placZ and a fluorescein isothiocyanate (FITC)-labeled lacZ, cDNA fragment (FITC-lacZ) were delivered into the cells using an α-helical cationic peptide, LLKLLKLWKKLLKLK (Transome IV, Wako Pure Chemical). FITC-lacZ was PCR-generated from placZ using FITC-labeled primers 5′-FITC-GACGGATCGGGAGATCTTTCCCCC-3′ and 5′-FITC-CGAAAAGGGCGGGGCTAGG-3′. The efficiency of endocytosis-mediated gene transfer was evaluated by measuring the β-galactosidase activity expressed from placZ. Cells in 60-mm culture dishes were loaded with 10 μg of placZ precomplexed with Transome IV. Control experiments were also performed using an empty vector to subtract the basal β-galactosidase activity. After 24 h, cells were harvested and β-galactosidase activity was assayed using the Betagro assay system (Promega) according to the manufacturer’s recommendations. Total protein in cell lysates was determined with a protein assay solution (BioChain), were incubated with anti-DNase X mAb at a concentration of 3 μg/ml overnight at 4 °C. The sections were counterstained with Contrast BLUE solution (KPL), and images were captured using a CK40 light microscope system (Olympus). The specificity of each immunostaining procedure was confirmed by a blocking experiment performed in the presence of an ~10-fold excess (200 ng/ml) of the immunizing DNase X peptide.

**Indirect Immunofluorescence**—Indirect immunofluorescence analyses were performed as described previously with some modifications (9). In brief, cells grown on a coverslip were fixed with 3.5% formaldehyde and permeabilized by incubating in a Cyto-nin solution (R & D Systems) at room temperature for 30 min. When detecting cell surface targets, the incubation with Cyto-nin was omitted. The first antibody reactions were performed in a humid sealed chamber overnight at 4 °C. The sections were counterstained with Contrast BLUE solution (KPL), and images were captured using a CK40 light microscope system (Olympus). The specificity of each immunostaining procedure was confirmed by a blocking experiment performed in the presence of an ~10-fold excess (200 ng/ml) of the immunizing DNase X peptide.
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...containing 5 units/ml Hoechst 33342, and observed with a fluorescence microscope. The cells were washed in DPBS, counterstained with 100 nM Alexa 488 and 594, respectively, and the antibody reaction was performed for 90 min at room temperature. The cells were rinsed twice with DPBS and fixed with 3.7% formaldehyde. Cell surface DNase X, mFasΔC, and cadherin were detected by indirect immunofluorescence as described above. The attached cells were rinsed with DPBS, collected in a 1.5-ml tube, and incubated in 100 μl of 5 units/ml PI-PLC at 37 °C for 20 min. They were then centrifuged at 2000 × g for 10 min, and the supernatant was collected in a separate tube (Sup fraction). The cell pellet was washed twice with DPBS by repeated suspension and centrifugation and resuspended in 500 μl of Leibovitz medium (cell fraction). Identical aliquots of the two fractions were subjected to Western blotting.

PI-PLC Treatment—Cells grown on coverslips were rinsed twice with DPBS and incubated in Leibovitz culture medium containing 5 units/ml Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma) at 37 °C for 30 min. The cells were rinsed twice with DPBS and fixed with 3.7% formaldehyde. Cell surface DNase X, mFasΔC, and cadherin were detected by indirect immunofluorescence as described above. The attached cells were rinsed with DPBS, collected in a 1.5-ml tube, and incubated in 100 μl of 5 units/ml PI-PLC at 37 °C for 20 min. They were then centrifuged at 2000 × g for 10 min, and the supernatant was collected in a separate tube (Sup fraction). The cell pellet was washed twice with DPBS by repeated suspension and centrifugation and resuspended in 500 μl of Leibovitz medium (cell fraction). Identical aliquots of the two fractions were subjected to Western blotting.

TUNEL Assay—Cells grown on coverslips were cultured for 30 min in the presence of placZ-Transome IV complex as described above. They were washed twice with DPBS, fixed with 3.7% formaldehyde in DPBS at room temperature for 10 min, and the 3'-OH ends produced in the endocytosed DNA were counterstained with 3.7% formaldehyde. Cell surface DNase X, mFasΔC, and cadherin were detected by indirect immunofluorescence as described above. The attached cells were rinsed with DPBS, collected in a 1.5-ml tube, and incubated in 100 μl of 5 units/ml PI-PLC at 37 °C for 20 min. They were then centrifuged at 2000 × g for 10 min, and the supernatant was collected in a separate tube (Sup fraction). The cell pellet was washed twice with DPBS by repeated suspension and centrifugation and resuspended in 500 μl of Leibovitz medium (cell fraction). Identical aliquots of the two fractions were subjected to Western blotting.

Capture of DNA Images in Fluorescence Microscopy—Nuclear and/or endocytosed DNAs were counterstained with Hoechst 33342 (a cell-permeable fluorescent dye) as described in each section. Observations of the DNA images were performed as follows unless otherwise noted. Images of nuclear DNA were taken through two neutral density filters (U-ND6 and U-ND25, Olympus) because of their strong fluorescence, whereas no neutral density filters were used for observing endocytosed DNA.

RNA Interference—Stealth RNA interference for human DNase X and a negative control RNA (ncRNA) duplex were obtained from Invitrogen. The small interfering RNA (siRNA) was directed against the target sequence 5’-CCGTTGCGAGAACATCTGATT-TT-3’. Subconfluent cultures of RD cells, seeded in 60-mm dishes, were shifted to DM and transfected with 120 pmol of siRNA using Lipofectamine 2000 transfection reagent (Invitrogen). The resulting culture was maintained in DM for 4 days with daily medium replacement. We observed no induction of 2’′,5’′-oligoadenylate synthetase 2 or signal transducer and activator of transcription 1b mRNA in siRNA-transfected cells, indicating the absence of detectable interferon responses (data not shown).

RESULTS

DNase X Is Expressed during Myogenic Differentiation of RD Cells—To examine DNase X expression at the protein level, we performed an immunohistochemical analysis of several human tissues using the newly developed anti-DNase X mAb B1 (supplemental Fig. S1). As shown in Fig. 1A, both cardiac muscle cells and skeletal muscle fibers were highly immunoreactive for DNase X, whereas there was little positive staining in the brain. These results are consistent with the gene expression profile determined previously (5–9) and confirm the expression of the DNase X protein in muscle cells.

The human rhabdomyosarcoma cell line RD forms myotubes when it is grown under low mitogen conditions (Fig. 1B). To monitor the changes in DNase X expression associated with myogenic differentiation, we performed Western blots and showed that DNase X expression began to increase on day 3 after induction of differentiation, reaching a maximum on day 5 (Fig. 1C, top panel). The induction of a muscle marker protein, sarcoplasmic reticulum Ca²⁺-ATPase 1 (SERCA-1), confirmed the occurrence of myogenic differentiation (Fig. 1C, middle panel).

DNase X Is Targeted to the Secretory Pathway and Delivered to the Cell Surface—We analyzed the intracellular distribution of DNase X by confocal laser scanning microscopy (Fig. 1D). Colocalization of DNase X with calreticulin and SERCA-1 revealed its association with the endoplasmic reticulum and sarcoplasmic reticulum, respectively. DNase X immunofluorescence also paralleled that of GM130, a marker of the Golgi apparatus. These results indicate that newly synthesized DNase X is directed to the secretory pathway of the cell. To determine its final destination, we immunostained non-permeabilized cells and showed that the surface of multinucleated RD myotubes was strongly positive (Fig. 1E), whereas the surface of undifferentiated cells was only weakly stained (Fig. 1E, white arrowheads). The membrane expression of DNase X was also confirmed by confocal microscopy (Fig. 1F). These results demonstrate that newly synthesized DNase X is targeted to the secretory pathway and delivered to the cell surface in RD myotubes.

The C-terminal Hydrophobic Domain Is Essential for Cell Surface Localization of DNase X—To determine whether membrane association is a specific hallmark of DNase X, COS-7 cells were transfected with expression vectors for DNase X and DNase I, and the membrane distribution of each was analyzed by immunostaining. To locate DNase I, we generated an anti-DNase I mAb, 10A2. A mutant form of the murine Fas antigen (mFasΔC), which is expressed on the cell surface as a transmembrane protein, was co-expressed with the DNases, and its membrane expression was monitored as a marker of transfection (Fig. 2A and B, right panels).

Indirect immunofluorescence revealed cell surface localization of the ectopic DNase X (Fig. 2A, upper panels). In contrast,
Membrane Binding of DNase X Is Mediated by a GPI Anchor—Eukaryotic proteins become membrane-anchored by several mechanisms, through being transmembrane proteins, through protein-protein interaction, or through binding to a GPI anchor (11, 12). Significantly, attachment to a GPI anchor requires a hydrophobic signal sequence located at the C termini of proteins (12). Given that DNase X has a C-terminal hydrophobic domain, we hypothesized that GPI anchoring might account for its membrane association. To evaluate this possibility, we tested the effect of phosphoinositide-specific phospholipase C (PI-PLC), an enzyme that releases GPI-anchored proteins, on DNase X membrane association. Indirect immunofluorescence revealed that PI-PLC treatment greatly reduced the signal intensity of an exogenous DNase X without affecting that of the co-transfected transmembrane protein mFasΔC (Fig. 3A).

The PI-PLC-mediated release of DNase X was further analyzed by Western blotting. As shown in Fig. 3B, DNase X (but not mFasΔC) was released into the supernatant by PI-PLC treatment. We obtained essentially the same results with the endogenous DNase X of RD myotubes (Fig. 3C); PI-PLC effected the release of DNase X without solubilizing cadherin, a membrane-integrated protein responsible for stable contact between cells (13). It is of note that an anti-Myc antibody failed to detect the ectopic DNase X released by PI-PLC treatment (data not shown), suggesting that the C-terminal hydrophobic domain there was no sign of DNase I membrane expression (Fig. 2A, lower panels).

DNase X is unique among family members in having a conserved hydrophobic domain at its C terminus; this suggested to us that the hydrophobic domain might play a role in the membrane attachment of DNase X. To evaluate this possibility, we constructed two mutant DNase derivatives and observed their cell surface localization. DNase X with a deleted hydrophobic domain (DNase XΔC) and DNase I C-terminally fused to the hydrophobic domain of DNase X (DNase I-HD). As shown in Fig. 2B, DNase XΔC failed to attach to the plasma membrane, whereas DNase I-HD acquired a membrane location. These results show that cell surface localization is a specific feature of DNase X and that the C-terminal hydrophobic domain is necessary and sufficient for this localization.

Endocytosis-mediated Gene Transfer Is Impaired in RD Myotubes—The endocytic activity of eukaryotic cells internalizes various cell surface and extracellular molecules, including foreign genes. Endocytosed genes are usually delivered to lysosomes and degraded by lysosomal acid DNases; however, certain cationic peptides are known to promote the escape of incorporated genes from endosomal degradation, thereby permitting transfer of the incorporated genetic information into the cell (14–16). As an initial approach to unveiling the function of DNase X, we observed endocytosis-mediated gene transfer in RD myotubes that express DNase X on the cell surface at high levels. We employed an artificial α-helical cationic peptide to evaluate the efficiency of gene transfer by endocytosis (17, 18). RD myotubes incorporated a fluorophore-conju-
gated transgene, FITC-lacZ, more effectively than undifferentiated cells (Fig. 4A). However, expression of the encoded product, β-galactosidase, in the differentiated cells was <10% of that observed in the undifferentiated cells (Fig. 4B).

To explain this finding, we hypothesized that the high level of membrane DNase X expression was responsible for the poor transgene expression in differentiated cells. To test this idea, we examined the association of DNase X with early and late endocytic vesicles in RD myotubes. As shown in Fig. 4C, DNase X and mFasΔC retained in the cells (Cells) or released into the buffer (Sup) were detected by Western blotting.

FIGURE 3. PI-PLC-mediated release of DNase X from the cell surface. A and B, effect of PI-PLC on the membrane association of ectopic DNase X. COS-7 cells, seeded on a coverslip in a culture dish, were co-transfected with vectors for DNase X and mFasΔC. A, 24 h post-transfection, cells were incubated in a buffer containing 5 units/ml PI-PLC or buffer alone (Control), and membrane DNase X and mFasΔC were detected by indirect immunofluorescence. Scale bars represent 50 μm. B, attached cells were collected in microtubes and incubated in the presence (+) or absence (−) of 5 units/ml PI-PLC. Thereafter, DNase X and mFasΔC retained in the cells (Cells) or released into the buffer (Sup) were detected by Western blotting. C, effect of PI-PLC on the membrane association of endogenous DNase X. RD cells, differentiating in DM for 6 days, were collected in microtubes and treated with PI-PLC as described above. DNase X and cadherin, retained in the cells (Cells) or released from the cells (Sup), were detected by Western blotting.
breaks into the DNA, probably both at the cell surface and within early endocytic vesicles.

**Overexpression of the Membrane-bound Form of DNase X Suppresses Endocytosis-mediated Gene Transfer**—To further address the role of DNase X, we introduced several mutant forms of DNase X into cells possessing a low level of endogenous DNase X activity and observed their effects on endocytosis-mediated gene transfer. Indirect immunofluorescence revealed ectopic DNase X expression on the surface of both HeLa S3 and undifferentiated RD cells (Fig. 5A, DNase X), whereas a deletion mutant lacking the C-terminal hydrophobic domain displayed no specific membrane binding (Fig. 5A, ΔC). These cultures were then loaded with the placZ-cationic peptide complex, and expression of the reporter gene was measured. As shown in Fig. 5B, overexpression of DNase X inhibited β-galactosidase expression by >70% in both HeLa S3 and undifferentiated RD cells, and inhibition was greatly diminished by the C-terminal deletion (Fig. 5B, ΔC).

To establish the importance of DNase X activity, we expressed an inactive mutant in the cells and measured endocytosis-mediated gene transfer levels. DNase X H130A, in which one of the two catalytic His residues is replaced by Ala, was expressed normally on the cell surface (Fig. 5A, DNase X H130A); however, it did not inhibit transgene expression (Fig. 5B). The DNA strand breaks produced by the ectopic forms of DNase X were visualized using the TUNEL technique. As shown in Fig. 5C, wild type DNase X lacZ plasmids incorporated by HeLa S3 cells were TUNEL-positive, whereas lacZ plasmids ectopically expressing inactive DNase X were TUNEL-negative.

**siRNA-mediated Knockdown of DNase X Reverses Inhibition of Endocytosis-mediated Gene Transfer in RD Myotubes**—To establish whether endogenous DNase X acts as an inhibitor of gene transfer, we asked whether down-regulation of endogenous DNase X would reverse the impaired endocytosis-mediated gene transfer in RD myotubes. Fig. 6A shows that the intensity of the DNase X band was greatly diminished in cells treated with an siRNA specific for human DNase X, whereas no such down-regulation was observed with an ncRNA duplex. The suppression of DNase X did not affect SERCA-1 expression (Fig. 6A) or myotube formation (Fig. 6C), indicating that DNase X per se is not essential for myogenic differentiation of RD cells.

We next cultured siRNA-treated myotubes in the presence of the placZ-peptide complex and examined the effect of DNase X suppression on endocytosis-mediated gene transfer (Fig. 6B). The siRNA-mediated knockdown of DNase X stimu-
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A

|     | Control | DNase X | DNase XAC | DNase X H130A |
|-----|---------|---------|-----------|---------------|
| RD (GM) |         |         |           |               |
| HeLa S3 |         |         |           |               |

B

![Graph showing relative β-galactosidase activity](image)

C

![Images showing DNase X localization](image)

FIGURE 5. Effects of DNase X overexpression on endocytosis-mediated gene transfer. A, cell surface localization of the DNase X variants. Growing RD and HeLa S3 cells were transfected with expression vectors for DNase X (wild type), DNase XAC (deletion mutant lacking C-terminal hydrophobic domain), or DNase X H130A (inactive mutant). 24 h post-transfection, the cells were fixed and subjected to indirect immunofluorescence microscopy. The DNA images were taken through a neutral density filter (U-ND25). B, effects of ectopic DNase X on expression of lacZ. Growing RD and HeLa S3 cells were transfected with empty vector or expression vectors for DNase X H130A, DNase X (WT), or DNase XΔC (ΔC). 24 h post-transfection, the cells were washed extensively with fresh culture medium and loaded with placZ-peptide complex. After a further 24 h of culture, the cells were collected and β-galactosidase activities were assayed. The results are normalized to total protein and expressed relative to the activity of mock-transfected cells. Values are means ± S.D. (n = 6). *p < 0.001 versus WT (Student’s t test). C, generation of 3'-OH nick ends in endocytosed lacZ plasmids. HeLa S3 cells, transfected with DNase X or DNase X H130A, were cultured in the presence of placZ peptide complex for 1 h, fixed, and subjected to TUNEL assay. After counterstaining with 10 μM Hoechst dye, the 3’-OH ends of placZ (upper panels) and placZ plus nuclei (lower panels) were observed by fluorescence microscopy. The DNA images were taken through a neutral density filter (U-ND25). White arrowheads indicate placZ incorporated by the cells. Scale bar represents 50 μm.

In a previous study, we observed that ectopic DNase X was found exclusively in the endoplasmic reticulum, as determined by immunofluorescence of its C-terminal Myc tag (9). This contradictory observation is explained by the fact that the GPI anchor is attached within the endoplasmic reticulum lumen, and at the same time, the protein to be anchored is cleaved of its C-terminal propeptide. Thus, the membrane-bound form of DNase X loses its C-terminal tag.

Endocytosis-mediated Gene Transfer and DNase X—We observed that endocytosis-mediated gene transfer is strongly suppressed in cells expressing DNase X at high levels and that DNase X activity is responsible for TUNEL-positive strand breaks in the endocytosed DNA. Furthermore, siRNA-mediated knockdown of DNase X reverses the impairment of gene transfer in the RD myotubes. Our results thus demonstrate that DNase X protects cells from endocytosis-mediated gene transfer by inactivating the incorporated foreign genes.

GPI-anchored proteins are associated with lipid rafts, which are detergent-insoluble membrane microdomains enriched in cholesterol and glycosphingolipids (11, 12). The biological significance of lipid rafts is not yet fully understood, but they have been implicated in diverse cellular activities, including signal transduction, cholesterol trafficking and endocytosis (11, 12). To date, at least three types of endocytic pathway have been identified in eukaryotic cells, the classical clathrin-mediated pathway, caveolae/raft-dependent endocytosis, and constitutive fluid phase endocytosis, also known as pinocytosis (22, 23). The endocytic pathways by which cells take up foreign genes remain unclear. However, the caveolae/raft-dependent and the fluid phase pathways are the most likely candidates, because both mediate nonspecific internalization of micro- and macro-substances from the extracellular environment (24, 25). Importantly, GPI-anchored proteins are known to be internalized...
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A basal level of expression of DNase X is seen in several mammalian tissues (5, 9). We have performed immunohistochemistry on several non-muscular tissues and observed that epithelial cells are generally positive for DNase X (data not shown). This shows that the DNase X membrane barrier is not restricted to muscle cells.

A Possible Clinical Application of Breaking through the DNase X Barrier—Skeletal muscle is an attractive target for therapeutic gene transfer for several reasons; it is the most abundant tissue in the human body, it is accessible to many systems of gene delivery, and turnover of its cells is relatively slow so that the effects of transgene expression are long lasting (29). In addition, skeletal muscle is rare among tissues in incorporating naked DNA (30).

Naked DNA transfer is a method of therapeutic gene delivery in which plasmid vectors are simply injected into a target tissue (31, 32). Because of its safety, simplicity, and lack of size limitation, naked DNA transfer is now receiving attention as an attractive gene delivery method for future gene therapies. Naked gene transfer has been tested in mice and shown to deliver genes into skeletal muscle (30); however, it is still of little practical use in humans because of its low efficiency (29). Although the factors limiting naked DNA transfer in vivo are not fully understood, our data strongly suggest that DNase X is a major barrier. Based on the results of this study, we propose that artificial control of DNase X activity using a specific inhibitor may provide a way to improve the results of naked gene transfer in future gene therapies.

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physiological significance of the DNase X barrier—It is a known fact that animal cells have the ability to take up foreign genes and express their encoded products, and this has been exploited by the development of transfection procedures suitable for experimental and clinical applications. However, the occurrence of spontaneous gene transfer is apparently harmful to animals, because the frequent integration and unregulated expression of foreign genes disturbs cellular activity and increases the risk of malignant transformation. Thus, suppression of foreign gene transfer by DNase X is probably important in maintaining healthy cell communities in vivo.

into recycling and early endosomes via the caveolae/raft-dependent and the fluid phase endocytic pathways (26–28). These observations suggest a possible reason for DNase X to be a GPI-anchored protein; the GPI anchor directs it to those pathways that support nonspecific endocytosis, thereby making it possible to intercept genetic invaders not only at the cell surface but also within endocytic vesicles. This idea is supported by the observation that the DNA-LL-37 complex is internalized into cells by a raft-dependent endocytic activity (16).
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