Hyaluronan must be exported from its site of synthesis, the inner side of plasma membrane, to the extracellular matrix. Here, we identified the multidrug-associated protein MRP5 as the principle hyaluronan exporter from fibroblasts. The expression of the MRP5 (ABC-C5) transporter was silenced in fibroblasts using RNA interference, and a dose-dependent inhibition of hyaluronan export was observed. Hyaluronan oligosaccharides introduced into the cytosol competed with the export of endogenously labeled hyaluronan and the MRP5 substrate fluoroescin. Because cGMP is a physiological substrate of MRP5, the intracellular concentrations of cGMP were modulated by the drugs 3-isobutyl-1-methylxanthine, propentofyllin, l-NMMA, zaprinast, and bromo-cGMP, and the effects on hyaluronan export were analyzed. Increasing the cGMP levels inhibited hyaluronan export and decreasing it afforded higher concentrations of zaprinast to inhibit the export. Thus, cGMP may be a physiological regulator of hyaluronan export at the level of the export MRP5.

Hyaluronan is a large glycosaminoglycan abundantly present in the extracellular matrix. Hyaluronan biosynthesis proceeds by alternate transfer of UDP-GlcA and UDP-GlcNac at the reducing end at the inner face of the plasma membrane, from where the growing hyaluronan chain is exported into the extracellular matrix (1–4). Recently we discovered that hyaluronan is exported through the protoblast membrane of Streptococci by an ABC transporter (5). The streptococcal hyaluronan transporter had structural and functional homology to human multidrug resistance transporters; accordingly, inhibitors of these transporters prevented hyaluronan export from human fibroblasts. Thus, two distinct processes are required for hyaluronan deposition in the extracellular matrix, namely hyaluronan synthesis catalyzed by the hyaluronan synthase within the cytosol and hyaluronan export mediated by a member of the ABC transporter family, this most likely being the MRP5 transporter (6). The in vitro achieved inhibition of hyaluronan export also worked in vivo in an animal model of osteoarthritis, where inhibition of hyaluronan export prevented proteoglycan loss and collagen degradation in osteoarthritic cartilage (7). Like most ABC transporters, MRP5 can export a variety of substrates (8–10), and cGMP appears to be the only physiological relevant one up to now (11).

In the extracellular matrix, hyaluronan acts primarily as a viscous space-filling agent or lubricant in the synovial fluid of joints or the vitreous body of the eye. The largest amounts occur in the skin, particularly around capillaries (12). Its rapid turnover rates with a half-life <6 h (13) indicate that it is not merely an inert matrix component but may fulfill pivotal physiological functions, including random motility of cells, their invasion, and proliferation to name but a few (14). Under conditions of ischemia-induced inflammation, hyaluronan is over-produced and can lead to generalized edema formation (15), but particularly so in the edema of the lung (16), in the myocardial tissue after infarction (17), in stroke (18), and in the ischemic inflamed renal cortex of diabetic rats (19).

However, as the MRP5 has only been identified as the hyaluronan exporter by inhibition studies (6), it is necessary to further ascertain the role of the MRP5 transporter in hyaluronan export as this may have important functional and pathophysiological consequences, particularly so with respect to the role of cGMP in regulating vasodilation and tissue hydration.

EXPERIMENTAL PROCEDURES

Materials—MRP5 and control siRNA2 were obtained from Ambion, Cambridgeshire, UK. Monoclonal rat anti-MRP5 antibodies were from Chemicon International. Medical grade hyaluronan (Hylumed Medical) was a gift from Genzyme, Cambridge, MA. Other chemicals were from Sigma.

Cells and Cell Culture—MRP5-overexpressing human embryonic kidney (HEK) 293 cells were kindly provided by Prof. Piet Borst, Amsterdam, The Netherlands, and grown in RPMI medium and 10% fetal calf serum. Mouse fibroblasts were grown in serum-free Quantum 333 (PAA Kölbe). All cultures were supplemented with streptomycin/penicillin (100 units of each/ml).

General Methods—The determination of hyaluronan production has been described (7). Knock down of endogenous MRP5 by RNA interference was performed according to the manufacturer’s protocol and purification of hyaluronan oligosaccharides and fluorescent labeling as described by Seyfried et al. (20). The sizes of the purified oligosaccharides were deter-

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2 The abbreviations used are: siRNA, small interfering RNA; HEK, human embryonic kidney; l-NMMA, Nω-nitro-l-arginine methyl ester; NO, nitric oxide.
mined by mass spectrometry. Fractions containing tetra-, hexa-, and octasaccharides were collected and used for the following experiments.

Inhibition of Endogenously Synthesized Hyaluronan Export by Hyaluronan Oligosaccharides—Human fibroblasts were seeded at 50% confluency and grown for 24 h. The medium was withdrawn and the cells were incubated in Dulbecco’s medium containing 1 M sucrose, 10% polyethylene glycol 600 in the absence or presence of hyaluronan, hyaluronan oligosaccharides, a mixture of N-acetylgalactosamine, and glucuronic acid at concentrations of 100 μg/ml for 15 min at 37 °C. The cells were suspended in a mixture of Dulbecco’s medium/H2O 1:2 (v/v) for 5 min to lyse the pinocytotic vesicles. The loading and lysis procedure was repeated once. Afterward the cells were washed three times with 10 mg of Pronase and incubated at 37 °C overnight. The supernatant was determined by mass spectrometry. Fractions containing tetra-, hexa-, and octasaccharides were isolated. Hyaluronan was sedimented for 15 min at 4000 × g and centrifuged at 37 °C, and the precipitated hyaluronan was determined.

Identification of the Hyaluronan Exporter in Mouse Fibroblasts—To assess the role of MRP5 in hyaluronan export, mouse fibroblasts were transfected with mixtures of the siRNA numbers 1–3 based on the MRP5 sequence and non-targeting control siRNA sequences. Fig. 1A and Tables 1 and 2 show that the siRNA mixtures 1 and 3 specifically deleted MRP5 mRNA as compared with the β-actin control.

When the siRNAs were used separately, only siRNA number 3 was effective, and this effect was enhanced by combination with siRNA numbers 1 or 2. The combination of siRNA numbers 1 and 2 had no effect. Therefore, the effect of MRP5 knock down was measured with the mixtures of siRNA numbers 2 and 3 and 1 and 3. Fig. 1, C and D, shows the reduction of hyaluronan export at 48 and 72 h after transfection by a mixture of the MRP5-specific siRNA numbers 1 and 3 in one experiment and siRNA numbers 2 and 3 in the other. The reduced efficiency of the siRNAs at higher concentrations may arise from a disproportionate of siRNA and transfection reagent (21). In conclusion, hyaluronan export was most effectively reduced by siRNA numbers 1 and 3 at 48 h after transfection. These results indicated that MRP5 is involved in hyaluronan export from mouse fibroblasts.

Hyaluronan Oligosaccharides in the Cytosol Compete with Endogenous Hyaluronan Export—This experiment was performed to show that hyaluronan synthesis and export are two different and independent processes. Hyaluronan oligosaccharides were prepared by digestion of hyaluronan with hyaluronidase, and tetra- to octasaccharides were isolated. Hyaluronan of high molecular weight, hyaluronan oligosaccharides, or a mixture of the monosaccharide building blocks of hyaluronan glucuronic acid and N-acetylgalactosamine were introduced at concentrations of 100 μg/ml into the cytosol of hyaluronan-synthesizing mouse fibroblasts by osmotic lysis of pinocytotic vesicles (22). The osmotic lysis of pinocytotic vesicles results in a fluid uptake of 0.1% of the cellular volume, and thus the intracellular concentrations of the hyaluronan oligosaccharides could have been diluted to subphysiological levels.
FIGURE 1. Inhibition of hyaluronan export by MRP5-siRNA. Fibroblasts were plated in a 12-well plate and incubated overnight. Cells at 50–60% confluency were transfected with a mixture of siRNA numbers 1 and 3, as compared with a control siRNA. MRP5-specific and non-sense siRNA were used at concentrations of 20 nM following the manufacturer’s protocol. A, specific inhibition of MRP5 mRNA expression. Cells were lysed 48 h after transfection, and the RNA for β-actin and MRP5 was detected by reverse transcription PCR. B, specific inhibition of protein expression. The relative intensities of the MRP5 protein at 190 kDa were determined by Western blotting with an MRP5-specific monoclonal antibody. C, inhibition of hyaluronan export after transfection with a mixture of siRNA numbers 1 and 3. D, inhibition of hyaluronan export after transfection with a mixture of siRNA numbers 2 and 3. The supernatant was withdrawn after 48 (open bars) and 72 h (hatched bars) for determination of hyaluronan concentration. The degree of inhibition was related to the control as 100% with 8.8 μg/ml of hyaluronan after 48 h and 23 μg/ml after 72 h, respectively. The error bars indicate the S.D. of three determinations.
be estimated to be \(~100 \text{ ng/ml}\) (23). The cells were then incubated with \(^{3}\text{H}\)glucosamine to label endogenously synthesized hyaluronan. After 24 h, the amount of radioactive hyaluronan that was released into the culture supernatant was determined. Fig. 2 shows that hyaluronan oligosaccharides interfered with the export of endogenous hyaluronan, whereas high molecular weight hyaluronan and a mixture of glucuronic acid and N-acetylglucosamine had no effect.

**MRP5 Inhibitors Reduce Hyaluronan Oligosaccharide Export**

In a previous report we have shown that a variety of inhibitors interfered with hyaluronan export from fibroblasts (6). Fluorescent hyaluronan oligosaccharides were introduced into the cytosol by osmotic lysis of pinocytotic vesicles. Hyaluronan was then metabolically labeled by incubation with \(^{3}\text{H}\)glucosamine, and the radioactivity of exported \(^{3}\text{H}\)hyaluronan in the culture supernatant was determined, indicating that hyaluronan oligosaccharides in the cytosol inhibited endogenous hyaluronan export. The error bars indicate the range of two determinations.

**Cytosolic Hyaluronan Oligosaccharides Interfere with the Export of the MRP5 Substrate Fluorescein Diacetate**—Hyaluronan oligosaccharides were introduced into the cytosol of MRP5-overexpressing HEK293 cells by osmotic lysis of pinocytotic vesicles. The cells were then incubated with the non-fluorescent fluorescein diacetate, which can freely diffuse into the cell where it is cleaved by an esterase to the fluorescent MRP5 substrate fluorescein. The appearance of fluorescent fluorescein diacetate, which diffuses into the cells where it is cleaved into the fluorescent MRP5 substrate fluorescein. The appearance of fluorescence in the culture supernatant was recorded. The error bars indicate the S.D. of four determinations. The differences in export inhibition by hyaluronan oligosaccharides between MRP5-overexpressing HEK293 cells and wild type HEK293 cells were statistically significant (p < 0.01).

measured the effects of altered intracellular cGMP concentrations on hyaluronan export (Fig. 5). Propentofylline is a general phosphodiesterase and nucleoside transport inhibitor (24). 3-Isobutyl-1-methylxanthin is a PDE5 and PDE4 inhibitor and raises the intracellular cGMP and cAMP levels, but it does not inhibit MRP5 (25). Propentofylline and 3-isobutyl-1-methylxanthin inhibited hyaluronan export in a concentration-dependent manner. Bromo-cGMP is a membrane-permeable cGMP analog that competes with the transport of cGMP through MRP5 (8). It also inhibited hyaluronan export.

The influence of NO that is produced by nitric oxide synthase and activates the guanylate cyclase (26) was also analyzed. When the NO synthase was inhibited by 1-NAME, hyaluronan export was slightly enhanced (Fig. 5B). Such an enhance-
ment by L-NAME was also observed in the presence of zaprinast, which inhibits MRP5 transport at micromolar concentrations (27). When NO was exogenously supplied by increasing concentrations of sodium nitroprusside in the presence of L-NAME, hyaluronan export was again inhibited (Fig. 5C). In conclusion, Fig. 5 shows that all inhibitors that raised the intracellular cGMP levels decreased hyaluronan export in a concentration-dependent manner.

**DISCUSSION**

In a previous study, we demonstrated that inhibitors of the ABC transporter family reduced hyaluronan export by human fibroblasts. However, as these inhibitors act on several members of the ABC transporter family, our evidence for MRP5 as a hyaluronan exporter was only indirect (6). Hence the aim of this study was to identify the principle hyaluronan exporter definitively. Thus, RNA interference was used to post-transcriptionally suppress MRP5 RNA in a sequence-specific manner (28). Mouse fibroblasts transfected with MPR5-specific siRNA showed a dose-dependent decrease in hyaluronan export as compared with the control. These results considerably strengthen our initial finding that MRP5 is the principle hyaluronan exporter from mouse fibroblasts (6).

This conclusion is supported by our results on the export inhibition of endogenously synthesized hyaluronan and of the MRP5 substrate fluorescein by cytosolic hyaluronan oligosaccharides. The oligosaccharides had to be introduced into the cytosol by osmotic lysis of pinocytotic vesicles. This procedure has been shown to maintain excellent cell viability (29, 30), plating efficiency, and minimal damage to the cells (31). Nonspecific effects of the method were confined to transient decrease of protein and DNA synthesis (32). Further nonspecific effects were controlled by introducing high molecular weight hyaluronan or a mixture of glucuronic acid and N-acetylglucosamine. However, we cannot exclude the possibility that particularly the intracellular hyaluronan oligosaccharides exert unknown signaling effects on hyaluronan secretion. But a likely explanation for the difference of hyaluronan oligosaccharides and high molecular weight hyaluronan on export inhibition of endogenously synthesized hyaluronan is a competition of non-reducing hyaluronan terminus for entry into the MRP5 channel.

It has been postulated that MRP5 controls the intracellular concentration of cGMP as an overflow pump to protect the biochemical signal cascade from overshooting cGMP concentrations that could arise from high production or low degradation (11, 25). MRP5 has a particular high affinity for cGMP ($K_m = 2 \mu M$) as compared with cAMP ($K_m = 400 \mu M$) (8). cGMP is synthesized in an NO-dependent manner and degraded by phosphodiesterases, particularly PDE5. Consequently, inhibition of the NO synthase decreases the intracellular cGMP level and results in higher concentrations of the MRP5 export inhibitor zaprinast to inhibit hyaluronan export (Fig. 5B).

Export of polysaccharides through an ABC transporter had previously only been documented for bacterial polysaccharides (33), and thus MRP5 appears to be the first identified member of a eukaryotic polysaccharide plasma membrane transporter. Because of the promiscuous nature of the members of the ABC
transporter family (34), it would not be surprising to find other members of multidrug-resistant transporters as hyaluronan exporters in other tissues apart from fibroblasts, because these transporters are often expressed in a tissue-specific pattern.

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