Heparin Inhibits Retrovirus Binding to Fibronectin as Well as Retrovirus Gene Transfer on Fibronectin Fragments

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Fibronectin fragments have been shown to improve retrovirus gene transfer efficiency by binding retrovirus and target cells. Using a novel virus adhesion assay, we confirmed binding of type C oncoretrovirus vectors to the heparin II domain of fibronectin and demonstrated inhibition of viral binding and gene transfer by heparin.

Fibronectin (FN) fragments have been shown to improve retrovirus gene transfer into primitive hematopoietic cells, including murine (9, 20) and primate (10) long-term repopulating stem cells or human cells repopulating immunodeficient NOD-SCID or BNX mice (7, 12). The technology has also been applied successfully to clinical studies (1, 5). The underlying biological mechanism has at least partly been resolved, as it was demonstrated that FN binds not only hematopoietic target cells (26, 27) but also recombinant retrovirus particles (9, 18). For this reason, colocalization of retrovirus and target cells on the FN fragments has been proposed as the mechanism responsible for improved retrovirus transduction of hematopoietic cells in the presence of FN (9, 18). The putative site for retrovirus binding is localized within type III repeats 12 to 14 of FN, containing the high-affinity heparin II (Hep-II) domain (Fig. 1) (11, 29). FN fragments combining the Hep-II domain with domains allowing integrin-mediated cell binding, such as the central cell binding domain or the type III repeat-connecting segment region (Fig. 1), are most active in enhancing retrovirus gene transfer efficiency (9).

Given the importance of the Hep-II-domain in this colocalization model, we were interested in investigating heparin as a potential inhibitor of retrovirus binding and, consequently, retrovirus transduction on FN fragments. Inhibitory effects of heparin and other sulfated polysaccharides on infection have been reported for other enveloped viruses, such as human immunodeficiency virus type 1 (6), cytomegalovirus (2), and Rous sarcoma virus (25). During lentivirus infection, heparin seems to interfere with intracellular events necessary for viral replication (6), whereas in the case of Rous sarcoma virus, negatively charged sulfated proteoglycans inhibit infection most likely through electrostatic inhibition of viral adsorption to the cell surface (25).

So far, most studies investigating the binding of retrovirus to FN have applied indirect methods using transduction efficiency for NIH 3T3 fibroblasts, which were added to FN-bound virus,

as the readout (9, 18). More-direct evidence of retrovirus-binding to FN was provided by studies demonstrating binding of the retrovirus envelope protein gp70 using enzyme-linked immunosorbent assay technology (14). For the studies presented here, a radioactive virus binding assay, that allows direct and quantitative detection of virus bound to FN and is independent of downstream steps of infection was developed. This seems warranted, as viral infection is a multistep process and competitors of viral binding might also interfere with other downstream events in the viral infectious cycle.

Sucrose density centrifugation and purification of retrovirus vectors. To generate radioactively labeled retrovirus for binding studies, purification of retrovirus is required. We addressed this requirement by applying a method previously described for the purification of vesicular stomatitis virus (17) that uses ultracentrifugation and a sucrose-step gradient to purify viral particles within the interphase of the gradient. First, localization of infectious retrovirus in the different fractions of the gradient after centrifugation was analyzed. These experiments were performed using two well-characterized, stable retrovirus producer clones: (i) a GP+envAM12 (15) amphotropic producer clone for the retrovirus vector N2/Zip-TKNEO (TKNEO; infectious titer of approximately 10^5 /ml [19]) expressing the marker neomycin phosphotransferase (NEO) and (ii) a GP+E86 (16) ecotropic producer clone for the retrovirus vector MSCV-MGMT^{P140K}-IRES-GFP (MGMT-GFP; infectious titer of approximately 10^6 /ml [21]) expressing the marker green fluorescent protein (GFP), (Fig. 2A and B). Supernatant from the stable producer clones was filtered through a 0.45- μ m-pore-size filter and loaded upon a sucrose gradient of 7 ml of 20% sucrose and 3 ml of 55% sucrose in 50 mM Tris, pH 7.4, with 150 mM NaCl and 0.5 mM EDTA. This gradient was centrifuged for 90 min at 25,000 rpm on a Beckman SW27 centrifuge at 4°C. After ultracentrifugation, 1-ml fractions starting from the bottom of the tube were quantitatively analyzed for the presence of infectious virus. Titration of infectious particles was performed by determining the number of cells expressing NEO (resistance to 0.75 mg/ml of G418 per ml; Gibco, Eggenstein, Germany) or, GFP (flow cytometric analysis; FacsCalibur with CellQuest software [Becton Dickinson]) following exposure of NIH 3T3 cells to serial dilutions of the different gradient fractions.

These initial experiments demonstrated that after centrifuga-

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FIG. 1. Schematic diagram of the FN molecule. Depicted are the A and B chains of the plasma FN molecule, including binding sites for macromolecules and the locations of the chymotryptic fragments FN 30/35 and FN 42 (22, 23) as well as the recombinant fragment CH 296 (11) used in the experiments. CBD, cell binding domain, ligand for $\alpha_5\beta_1$ integrins; CS-1, connecting segment 1, ligand for $\alpha_4\beta_1$ integrins; III-CS, type III repeat-connecting segment, alternately spliced; heparin-II (Hep-II), C-terminal high-affinity heparin binding site. Symbols: narrow shaded bars, type I repeats (45 amino acids [aa]); shaded ovals, type II repeats (60 aa); wide shaded bars, type III repeats (90 aa).

gation, most infectious retrovirus was present around the interphase within the third and fourth milliliters (Table 1), and viral titers recovered in these interphase fractions (2×10^3 to 5×10^3 infectious particles per ml for TKNEO and approximately 3×10^6 infectious particles per ml for MGMT-GFP) roughly equalled the viral titers present in the supernatant before centrifugation, with a maximal difference of 1 log. Above and below the interphase, titers dropped significantly, and in the supernatant above the gradient very little virus could be recovered. These data indicate the feasibility of recovering biologically active retrovirus after ultracentrifugation around the interphase of the gradient.

Radioactive labeling of retrovirus with [^{35}S]methionine. As in preliminary studies, [^3H]uridine labeling of viral RNA was too inefficient to allow binding studies; subsequent experiments were performed utilizing ^{35}S -protein labeling. For the

initial experiments, two high-titer retrovirus producer clones were used: the ecotropic MGMT-GFP producer clone described above and a GP+envAM12 amphotropic producer clone for the retrovirus vector PGKmADA (Fig. 2C) (infectious titer of approximately $10^7/\text{ml}$ [4]). Cells were starved for 2 h in Dulbecco modified Eagle medium without methionine before $100 \mu\text{Ci}$ of [^{35}S]methionine per ml (specific activity, $>1,000 \text{ Ci/mmol}$; Amersham, Braunschweig, Germany) was added. Following overnight culture, supernatant was filtered through a $0.45\text{-}\mu\text{m}$ -pore-size filter and sucrose gradient centrifugation was performed as described above. One-milliliter fractions were harvested from the bottom of the tube, diluted 1:20 with phosphate-buffered saline (PBS), and incubated on 35-mm dishes (Falcon, Heidelberg, Germany) coated with $4 \mu\text{g}$ of FN 30/35 fragment per cm^2 or bovine serum albumin (BSA) (Sigma, Deisenhofen, Germany), as described earlier (8, 18). After

FIG. 2. Schematic diagram of retrovirus-vectors. (A) The N2/Zip backbone of the TKNEO vector (19) and (C) the Zip backbone of the PGKmADA vector (4) are both based on the Moloney murine leukemia virus (MMLV). (B) The MSCV backbone of the MGMT-GFP vector is based on the murine embryonic sarcoma virus (MESV) (21, 28). In the TKNEO vector, the NEO reporter gene is transcribed from an internal herpes simplex virus thymidine kinase promoter (HSV-TKpr); in the PGKmADA vector, the murine adenosine deaminase (mADA) gene is transcribed from an internal human phosphoglycerate kinase promoter (PGKpr). In the MGMT-GFP vector, O^6 -methylguanine methyltransferase (MGMT) and the marker GFP are transcribed from the MESV promoter, 5' and 3' of an encephalomyocarditis virus internal ribosome entry site (IRES).

TABLE 1. Recovery of retrovirus after ultracentrifugation^a

Fraction	MGMT-GFP titer (% GFP-positive cells per ml) ^b	TKNEO titer (G418 ^r colonies per ml)	
		Expt 1	Expt 2
1st ml	$4 \times 10^4 \pm 6 \times 10^4$	$<10^2$	10^2
2nd ml	$2 \times 10^5 \pm 5 \times 10^5$	3×10^2	2×10^2
3rd ml	$3 \times 10^6 \pm 3 \times 10^6$	4×10^3	5×10^3
4th ml	$3 \times 10^6 \pm 3 \times 10^6$	4×10^3	2×10^3
5th ml	$4 \times 10^5 \pm 3 \times 10^5$	ND ^c	ND
6th ml	$4 \times 10^5 \pm 3 \times 10^5$	ND	ND
7th ml	$7 \times 10^4 \pm 7 \times 10^4$	ND	ND
Supernatant above gradient	$4 \times 10^4 \pm 5 \times 10^4$	<10	10
Supernatant before centrifugation	$8 \times 10^5 \pm 7 \times 10^5$	3×10^4	3×10^3

^a Infectious titer of different fractions recovered after ultracentrifugation of retrovirus on a two-step sucrose gradient. Interphase fractions 3 and 4 are in boldface.

^b Mean values $\pm$ standard deviations for four independent experiments.

^c ND, not done.

incubation for 30 min at 37°C, dishes were washed three times with PBS. Radioactive proteins remaining on the dishes thereafter (bound radioactivity) were recovered by extensive trypsin digestion (Trypsin-EDTA; Gibco) and measured in a scintillation counter (LS 6500; Beckman, Palo Alto, Calif.). To prove viral specificity of ³⁵S-labeled proteins adhering to FN fragments, producer cell lines were compared with the parental, non-virus-producing cell line NIH 3T3. For these cell lines, all labeled proteins, except viral proteins, should be identical; therefore, radioactivity detected from supernatant of the producer cell lines only should indicate retrovirus origin.

As shown in Table 2, a 10- to 20-fold increase of bound radioactivity was noted in the virus-containing interphase fractions 3 and 4 for supernatant of virus producer cell lines but not of NIH 3T3 cells. Also, no bound radioactivity above background was recovered from the interphase fractions of PGKmADA supernatant after incubation on non-virus-binding BSA control plates. These lines of evidence suggested that bound radioactivity recovered from FN-coated plates corresponded to ³⁵S-labeled retrovirus proteins.

Adhesion of ecotropic and amphotropic retrovirus to FN fragments. For these and further experiments, after ³⁵S labeling and centrifugation, fractions 3 and 4 were pooled and diluted 1:20 in PBS and radioactively labeled viral supernatant

TABLE 2. Binding of radioactively labeled supernatant from retrovirus producer cells to FN fragments

Fraction	Adherent radioactivity (cpm) ^a				
	Expt 1			Expt 2	
	PGKmADA on FN 30/35	PGKmADA on BSA	NIH 3T3 on FN 30/35	MGMT-GFP on CH 296	NIH 3T3 on CH 296
1st ml	58	55	105	48	25
2nd ml	55	54	85	41	37
3rd ml	633	58	135	180	31
4th ml	933	62	210	1,190	64
5th ml	ND	ND	ND	56	25
6th ml	ND	ND	ND	96	21

^a Adherent radioactivity on FN or BSA after [³⁵S]methionine labeling, ultracentrifugation, and incubation for different gradient fractions of two virus producer lines and the respective parental cell line NIH 3T3. Interphase fractions 3 and 4 are in boldface. ND, not done.

TABLE 3. Adhesion of radioactively labeled retrovirus to FN fragments

Fragment or control	Concn (μ g/cm ²)	Adherent radioactivity (cpm) (mean $\pm$ SD) ^a		
		PGKmADA (n = 3)	TKNEO (n = 3)	MGMT-GFP (n = 6)
FN 30/35	4.0	$3,810 \pm 228^*$	$1,580 \pm 574^*$	ND ^b
	0.5	ND	469 ± 454	ND
FN 42	4.0	ND	$1,500 \pm 712^*$	ND
	0.5	ND	$833 \pm 519^*$	ND
CH 296	4.0	ND	ND	$2,680 \pm 332^*$
BSA		546 ± 185	410 ± 176	843 ± 345

^a Adherent viral radioactivity on FN or BSA after [³⁵S]methionine labeling, ultracentrifugation, and incubation of interphase fractions for three viral cell lines. *, significantly different from control (BSA) values ($P < 0.05$ [two-tailed *t* test]).

^b ND, not done.

was tested for adherence to dishes coated with FN fragments, as described above. Adhesion of retrovirus to FN fragments was investigated for amphotropic TKNEO and PGKmADA and ecotropic MGMT-GFP retroviruses. As demonstrated in Table 3, all three retroviruses showed significant binding to dishes coated with 4 μ g of FN 30/35, FN 42, or CH 296 fragments per cm² compared to BSA controls. As demonstrated for the TKNEO vector, binding was substantially reduced on dishes coated with 0.5 μ g of FN fragments per cm². Next, the correlation between retrovirus titer and binding to FN fragments was investigated. Various dilutions of [³⁵S]methionine-labeled TKNEO supernatant were incubated on dishes coated with 4 μ g of commercially available FN per cm² (Boehringer Mannheim, Mannheim, Germany), comprising both chains of FN including the Hep-II domain. Increasing the concentration of retrovirus by lowering the dilution of the interphase fractions from 1:20 (397 cpm) via 1:10 (798 cpm) to 1:5 (1,051 cpm) led to a progressive increase in retrovirus binding in this experiment.

Heparin inhibits retrovirus binding to FN. As FN binds heparin with high affinity within the Hep-II domain (3, 29) and the viral binding site is localized within this domain (9), we hypothesized that heparin might inhibit retrovirus binding to FN. To prove this hypothesis, unfractionated heparin isolated from porcine mucosa (Sigma) was added during incubation of labeled MGMT-GFP or TKNEO retrovirus on FN fragments. As shown in Table 4, bound radioactivity was significantly reduced in the presence of 5 to 500 μ g of heparin per ml. This was observed with both viral vectors and for all FN fragments investigated, indicating that heparin at these concentrations substantially inhibits retrovirus binding to FN. For the MGMT-GFP vector, the inhibition of viral binding to CH 296 by heparin was highly significant ($P = 0.0001$ to 0.0004 ; two-tailed, paired *t* test) for all concentrations tested. For this vector, some reduction of bound radioactivity in the presence of heparin was also observed on BSA for heparin concentrations of 10 to 500 μ g/ml ($P = 0.02$ to 0.05). This finding indicated a certain degree of nonspecific, heparin-sensitive binding of retrovirus to BSA-coated plastic dishes, especially for higher heparin concentrations. However, this effect ac-

TABLE 4. Inhibition of retrovirus binding by heparin

Retrovirus	Fragment or control	Adherent radioactivity (cpm) at indicated heparin concn ($\mu\text{g/ml}$) ^a					
		0	5	10	50	100	500
MGMT-GFP	CH 296	2,680 $\pm$ 332*	775 $\pm$ 385‡	701 $\pm$ 394‡	651 $\pm$ 294‡	665 $\pm$ 336‡	502 $\pm$ 280‡
	BSA	843 $\pm$ 345	656 $\pm$ 440	566 $\pm$ 255†	551 $\pm$ 283†	558 $\pm$ 283†	425 $\pm$ 241†
TKNEO	FN 30/35	1,150				764	286
	FN 42	1,770				293	221
	BSA	350					
TKNEO	FN 30/35	2,640				278	360
	FN 42	3,840				182	661
	BSA	562					

^a Adherent viral radioactivity on FN or BSA after [³⁵S]methionine labeling, ultracentrifugation, and incubation of interphase fractions for three viral cell lines. Data for MGMT-GFP are means $\pm$ standard deviations for four experiments; other data are from single experiments. *, significantly different from control (BSA) values ($P < 0.05$ [two-tailed t test]); †, significantly different from values obtained in the absence of heparin ($P < 0.05$ [two-tailed t test]); ‡, significantly different from values obtained in the absence of heparin ($P < 0.01$ [two-tailed t test]).

counted for only approximately 20 to 25% of the changes in viral binding detected on CH 296, indicating that most of the inhibition of retrovirus binding to FN was due to a specific effect of heparin. Inhibition of retrovirus binding by heparin was also demonstrated for a commercially available 40-kDa FN fragment spanning the Hep-II domain (Boehringer Mannheim) (data not shown).

Heparin inhibits enhanced retrovirus transduction of primary CD34⁺ cells on FN fragments. We next investigated the effect of heparin on retrovirus transduction efficiency for primary human CD34⁺ hematopoietic cells in the presence of FN fragments. For these experiments, mobilized peripheral blood progenitor cells from patients were collected according to a protocol approved by the local ethics committee. CD34⁺ cells were selected twice on an MS+ separation column (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity of CD34⁺ cells as assessed by flow cytometry was higher than 90%. Cells were prestimulated for 48 h with 100 ng of recombinant human stem cell factor (Amgen, Munich, Germany) per ml and 100 U of recombinant human interleukin-3 (Novartis, Nuremberg, Germany) per ml. Thereafter, cells were incubated for 24 h in TKNEO supernatant containing the same cytokines and various concentrations of unfractionated heparin on dishes coated with 4 μg of CH 296 per cm^2 or BSA as a control substrate. Cells were harvested through vigorous washing with cold medium and centrifuged, and growth of hematopoietic progenitor cells was assessed using standard methylcellulose-based clonogenic assays (24). Half of the cells were grown with the addition of 1.5 mg of G418 per ml (dry powder, adjusted to pH 7.4; Gibco) to define the percentage of transduced clonogenic cells. Control colonies infected with GP+envAM12 supernatant (mock) consistently demonstrated <1% background colony growth after exposure to 1.5 mg of G418 per ml.

As expected, there was a clear increase in the percentage of G418-resistant clonogenic progenitor cells after infection on CH 296, in comparison to BSA, and this effect was completely lost when heparin (500 $\mu\text{g/ml}$, equivalent to 70 IU/ml) was added during the retrovirus transduction procedure. The percentages of resistant clonogenic progenitor cells (means $\pm$ standard errors of the means of four experiments) were 16.4% $\pm$ 6.8% and 2.4% $\pm$ 4.5% for cells transduced on FN 30/35 and

BSA, respectively, in the absence of heparin and 4.5% $\pm$ 4.7% ($P < 0.05$ compared with the value obtained in the absence of heparin; two-tailed t test) and 3.0% $\pm$ 6.0% for cells transduced on FN 30/35 and BSA, respectively, in the presence of 500 μg of heparin per ml. Thus, heparin completely abolished enhanced transduction efficiency on CH 296. No effect of heparin on the transduction rate for clonogenic progenitor cells was noted on BSA-coated control dishes, arguing for a distinct inhibitory effect of heparin on FN-enhanced retrovirus gene transfer. Reduced transduction of CD34⁺ cells was not due to reduced binding of target cells to FN in the presence of heparin, as heparin at the concentration used in our studies slightly enhances binding of hematopoietic progenitor cells to FN (D. Carstanjen, unpublished observations).

Heparin production by producer cell lines. As it has been demonstrated that retrovirus producer cell lines can secrete proteoglycans (13), we tested several amphotropic retrovirus producer cell lines based on the GP+envAM12 producer cell line for the presence of heparin. All cell lines tested negative at a threshold level for detection of 0.02 U of heparin per ml, equivalent to approximately 2.8 μg of heparin per ml. Nevertheless, a negative effect on transduction by low concentrations of heparin present within the supernatants of these cell lines cannot definitively be excluded, as we have shown that heparin concentrations as low as 2 $\mu\text{g/ml}$ can inhibit the enhancing effect of FN on retrovirus transduction of hematopoietic cells (25a).

Conclusion. In this report, we demonstrate a novel technology to study the interaction of retrovirus vectors with matrix molecules directly and thus independently of events downstream in the retrovirus infectious cycle. We introduce a method to generate ³⁵S-labeled retrovirus and use the radioactively labeled virus to investigate the binding to FN fragments containing the C-terminal high-affinity heparin-binding domain Hep II. We demonstrate binding of virus to dishes coated with recombinant FN fragments expressed in *Escherichia coli* (11), chymotryptic fragments isolated from human plasma (22, 23), and commercially available plasma FN, all of these preparations containing the Hep-II domain. The data are in agreement with previously published observations that the main adhesive domain for retrovirus lies within the Hep-II domain of FN (9). Binding was dose dependent for coating

concentrations of FN fragments, as well as for viral load in the supernatant.

According to the colocalization model of FN-enhanced retrovirus gene transfer, inhibition of retrovirus binding to FN should reduce viral presentation to target cells and thus gene transfer efficiency. Here, we introduce heparin as an inhibitor of retrovirus binding to FN and as a potent inhibitor of FN-enhanced retrovirus gene transfer. Binding of ecotropic or amphotropic retrovirus vectors was inhibited by heparin, as was FN-enhanced gene transfer into hematopoietic cells, thus further supporting the colocalization model of FN-enhanced retrovirus gene transfer.

As extracellular matrix-based gene transfer is an elegant method of improving ex vivo retrovirus gene transfer efficiency, the radioactive viral binding assay presented here might be useful for identifying novel enhancer molecules by screening other matrix proteins for viral binding. These studies might include retrovirus vectors pseudotyped with different surface proteins. In addition, the assay might prove helpful in defining the critical amino acid sequences required for viral adhesion to matrix molecules.

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