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Uptake and intracellular fate of phage display vectors in mammalian cells

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Abstract

Receptor-mediated endocytosis is exploited in experimental systems for selective delivery of genes and drugs into specific cells. To improve targeting efficiency of delivery vectors, we have used phage display technology to isolate novel ligands for endocytosed receptors. We show here that phage vectors internalized by mammalian cells via integrin-mediated endocytosis can be rescued by cell lysis and quantitated by infection of bacteria. Immediately following uptake, phage enter an intracellular compartment where they remain intact, with phage titer unaffected by the addition of chloroquine. Phage are then translocated to a second intracellular compartment in which they are inactivated and their titer affected by chloroquine. Immunofluorescence microscopy showed an association of the second compartment with supranuclear organelles. The ability to recover internalized phage in an infectious form from two distinctive intracellular compartments provides a means to select novel ligands from phage libraries for targeted delivery of macromolecules into mammalian cells.

Keywords: Phage display; Phage internalization; Receptor-mediated endocytosis; Integrin

1. Introduction

Endocytotic pathways have been used to selectively deliver therapeutic agents to specific cells and to particular intracellular compartments [1,2]. In these systems, ligands to cell-specific receptors are either conjugated to macromolecules [3,4], liposomes [5,6], and synthetic gene complexes [7], or expressed on the surface of viral transfection vehicles [8,9]. The identity of the receptor and the mode of its interaction with the ligand-presenting vehicle determine the cell specificity of the delivery system and the intracellular localization of the transported molecules [1,10].

While selective drug delivery utilizing endocytotic

Abbreviations: BSA, bovine serum albumin; DOCNa, sodium deoxycholate; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HBSS(−), HBSS without Ca^{2+} and Mg^{2+}; IPTG, isopropyl β-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; pC89-phage, phage particles containing phagemid pC89 genome; PEP-1-phage, PEP-2-phage, and PEP-3-phage, phage particles containing recombinant pC89 genome and displaying integrin-binding peptides, PEP-1, PEP-2, and PEP-3, respectively; PMSF, phenylmethylsulfonyl fluoride

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pathways has proved effective in many in vitro models, the therapeutic application of these systems in vivo is limited by the ability to transfer sufficient quantities of these macromolecules to specific cells [2,11]. One approach to improve these characteristics is to identify novel cell-targeting ligands, which increase the rate and specificity for the transport of macromolecules. Phage-display technology offers large collections of potential ligands including short peptides, antibody fragments and randomly modified physiological ligands to cell receptors [12–15]. These systems have been effectively employed in studies of structural and functional aspects of receptor–ligand interactions using either purified receptors immobilized on a polymer surface (reviewed in [15]), or the receptors in their natural environment on the surface of living cells (for examples see [16–18]). To take advantage of phage display technology in targeting endocytosing receptors, phage vectors must fulfil the following criteria: they should be able to bind the receptors, undergo internalization, and be capable of being rescued from the cells and amplified for subsequent rounds of reselection.

In this study, we investigated the viability and intracellular pathway of phage display vectors that have been internalized by mammalian cells via receptor-mediated endocytosis. For model experiments, we chose integrin receptors and their ligands, integrin-binding peptides containing Arg-Gly-Asp (RGD) motif. Such peptides were previously reported to mediate binding and internalization of a variety of viral vectors [19–22], including filamentous bacteriophage fd-tet [23], by mammalian cells. While internalization of fd-tet phage was demonstrated by immunofluorescence microscopy [23], the intracellular fate of internalized phage and their capability of being recovered from cells in an intact form have not been studied. We expressed integrin-binding peptides on the surface of filamentous bacteriophage using a phagemid vector pC89 [24]. We hypothesized that two features of this vector, multivalent format of peptide display and a relatively small size, may be essential for phage uptake. In this vector, the peptides are displayed as fusions with the major coat protein (pVIII). Presentation of peptides in multiple copies on phage surface would maximize their interaction with cell receptors, and facilitate packaging of phage particles into the appropriate membrane vesicles for endocytosis. The small 3.5-kb genome of this vector determines particle length of 0.5 μm, compared to the fd-tet phage with a genome of 9.23 kb and estimated particle length of 1.3 μm [25]. We anticipated that the smaller phage particles would require less number of cell surface receptors for binding and packaging into endocytotic vesicles, thus promoting phage uptake. Using this system, we demonstrate that phage display vectors remain viable after internalization by mammalian cells, can be recovered from the cells, and subsequently amplified by infection of bacteria. This allowed us to perform a quantitative analysis of phage uptake by cultured human cells, and to identify two distinctive compartments along the intracellular pathway of internalized phage.

2. Materials and methods

2.1. Materials

Human HEp-2 laryngeal carcinoma and human ECV304 endothelial cell lines were obtained from the American Type Culture Collection. HEp-2 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% FBS (Sigma). ECV304 cells were propagated in Medium 199 (Life Technologies) supplemented with 10% of heat-inactivated FBS, and Antibiotic–Antimycotic cocktail (Life Technologies).

The bacteria strain *Escherichia coli* XLI-Blue was purchased from Stratagene. The phagemid pC89 was described previously [24], and the M13KO7 helper phage was obtained from Pharmacia Biotech.

Subtilisin (Protease Type XXVII; Nagarse) was obtained from Sigma. Integrin $\alpha_{IIb}\beta_3$ (platelet membrane glycoproteins, IIbIIIa) was purchased from Calbiochem. The synthetic peptides GRGDSP and GRGESP were obtained from Life Technologies.

2.2. Construction of gene VIII fusions

Three integrin-binding peptide sequences were expressed as fusions with the major coat protein VIII (Fig. 1). In order to prepare DNA cassettes encoding these peptides, the following oligonucleotides containing *Eco*RI and *Bam*HI restriction sites were syn-

thesized: PEP-1 (5′-CGGGTGAATTCGGTTGC-CGTGGCGATATGTTCGGTTGTGGGGATCCC-GCATG-3′); PEP-2 (5′-CGGGTGAATTCGGTG-CTTGTCGAGGTGATTGCTTAGGTGCGGATC-CCGCATG-3′); and PEP-3 (5′-CGGGTGAATT-CGGTGCTTGTCGTCGAGAAACGGCTTGGGC-TTGTGGTGCGGATCCCGCATG-3′). Oligonucleotides were annealed with a primer (5′-CAT-GCGGGATCC-3′), and the complementary strand synthesized by extension of the primer with Sequenase Version 2.0 (U.S. Biochemical). The resulting double-stranded DNA fragments were digested with *Eco*RI and *Bam*HI, purified by PAGE (16%) followed by extraction and ethanol precipitation using established protocols [29].

The phagemid pC89 vector DNA was purified on Qiagen columns (Qiagen), digested with *Eco*RI and *Bam*HI, and the peptide-encoding inserts cloned. The proper structure of the recombinant gene VIII was confirmed by sequencing using Sequenase Version 2.0 kit (U.S. Biochemical).

2.3. Phage growth, purification, and titering

Phage particles of the original phagemid pC89 vector and the recombinants encoding integrin-binding peptides were grown by superinfection of phagemid-bearing bacteria with a helper phage M13KO7 essentially as described [30]. Phage were precipitated with 5% PEG 8000/0.27 M NaCl at 4°C for 1.5 h. Phage were pelleted by centrifugation (11 500×*g*, 30 min) and resuspended in 0.015 supernatant volume of TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.5). Phage samples were incubated for 30 min at 70°C, and the contaminating debris removed by centrifugation (15 000×*g*, 5 min). Phage concentration was determined in ampicillin-transducing units (TU). Serial dilutions of phage samples were prepared in LB medium, and 10 μl of each dilution were added to 90 μl of *E. coli* XLI-Blue in late log phase of growth followed by incubation for 10 min at room temperature. Fifty microliters of each sample were spread on LB-Amp plates. Ampicillin-resistant colonies were counted after overnight incubation at 37°C.

2.4. Analysis of recombinant coat proteins

Serial dilutions of phage samples were subjected to SDS-PAGE (16%) in a Tricine buffer [31,32] using minislab gel apparatus (Bio-Rad), and the gels were stained with Coomassie blue.

Cyclic conformation of phage-displayed integrin-binding peptides was analyzed by estimating the disulfide bonds formed between two cysteine residues flanking the inserts. Phage proteins were subjected to electrophoresis under non-reducing conditions, electrotransferred onto Immobilon P^{SQ} PVDF membrane (Millipore), and the presence of disulfide bonds was tested using DIG Protein Labeling and Detection kit (Boehringer) according to the manufacturer's instructions. Since the wild-type pVIII lacks cysteine [33], all the disulfide groups detected in the recombinant pVIII were attributed to integrin-binding peptides.

2.5. Phage binding to immobilized $\alpha_{IIb}\beta_3$ integrin

Microtiter plates (96-well MaxiSorp Nunc-Immuno Plates, Nunc) were coated with $\alpha_{IIb}\beta_3$ integrin at 1 μg per well as described [34]. Before incubation with phage, wells were washed twice with 100 μl of Buffer A (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, pH 7.4). Non-specific binding was blocked by incubating the wells with 100 μl of Buffer A plus 30 mg/ml BSA for 2 h at room temperature followed by a wash with Buffer A containing 1 mg/ml BSA. Phage samples were diluted in Buffer A containing 1 mg/ml BSA and the competitor peptide GRGDSP or GRGESP was added when required. To perform phage binding in the presence of EDTA, 1 mM $CaCl_2$ in Buffer A was substituted for 2 mM of EDTA. Phage were added to the wells in 100-μl aliquots and incubated for 2 h at room temperature. Following the incubation, the wells were aspirated and washed ten times with 100 μl of Buffer A containing 0.5% Tween 20. The attached phage were eluted with 100 μl of 0.1 M glycine, pH 2.0 containing 1 mg/ml BSA and incubating for 15 min at room temperature. The eluates were taken from the wells, neutralized with 6 μl of 2 M Tris, and phage titers were determined as described above.

2.6. Phage internalization assay

HEp-2 and ECV304 cells were grown in 6-well tissue culture plates (Falcon) for 2 days to reach a

subconfluent monolayer. The cells were washed with the appropriate tissue culture medium containing 10% FBS, and 1 ml of the same medium was added to each well. After preincubation of the cells for 30 min at 37°C, phage diluted in the tissue culture medium were added into the wells and mixed by repeated tilting of the plates. In the experiments with chloroquine, the cells were preincubated with the tissue culture medium containing 10% FBS and 100 μM chloroquine for 30 min at 37°C before adding the phage. Following the incubation of the cells with phage at 37°C for required period of time, the internalization events were stopped by immersion of the plates in ice for 5 min. The following procedures, until the lysis of the cells, were performed either on ice or at 4°C. The cells were washed four times with 3 ml of HBSS (Life Technologies) and once with HBSS(−). Extracellular phage were inactivated by incubating the cells with 1 ml of subtilisin 3 mg/ml in Buffer B (HBSS(−), 20 mM Tris, 2 mM EDTA, pH 8.0) for 1 h. The cells were detached from the wells by gentle pipetting and collected into microcentrifuge tubes. Complete disaggregation of the cells into single-cell suspension was confirmed by microscopic inspection. To inactivate the subtilisin, cells were pelleted by centrifugation (1000×*g*, 1 min), resuspended in 0.7 ml of 1 mM PMSF in HBSS(−) containing 2 mM EDTA and incubated for 15 min. Cells were washed with Buffer B, pelleted again, and the pellets resuspended in 0.5 ml of lysis buffer (2% DOCNa (Sigma), 10 mM Tris, 2 mM EDTA, pH 8.0) at room temperature, vortexed two times for 10 s at maximum speed and incubated for 1 h at room temperature. The lysates were vortexed again for 10 s, and the serial dilutions were prepared in lysis buffer. Ten microliters of each dilution were mixed with *E. coli* XLI-Blue bacteria, and 2 μl of 30 mM $CaCl_2$ were added to each sample in order to neutralize EDTA in the lysis buffer. The samples were processed for quantitation of phage titer as described above.

2.7. Immunofluorescence microscopy

HEp-2 and ECV304 cells grown on 8-well Lab-Tek chamber glass slides (Nunc) were washed with the appropriate tissue culture medium supplemented with 10% FBS, and covered with 0.15 ml of the medium. Phage diluted in the same medium were added into wells and mixed by pipetting. Following incubation of the cells with phage at 37°C for 16 h, the wells were gently rinsed with PBS and the chambers were removed from the slides. The cells were washed with PBS and fixed in 3.7% formaldehyde (Fisher Scientific) in PBS for 10 min. Fixed cells were permeabilized with 0.1% saponin (Sigma) in PBS (Buffer C) for 15 min, incubated with blocking Buffer D (Buffer C plus 1% BSA and 0.025% NaN_3) for 15 min, and then incubated with rabbit anti-fd bacteriophage IgG (Accurate Chemical and Scientific Corporation) diluted 1:500 in Buffer D for 1 h. The cells were washed with Buffer C and incubated with FITC-conjugated swine anti-rabbit immunoglobulins (Dako) diluted 1:30 in Buffer D for 1 h. After washing with PBS, the gaskets were detached from the slides, and the cells were mounted in Antifade (Oncor). The cells were examined using a Zeiss Axiovert microscope with a 63× oil immersion objective. Photographs were taken with TMAX 400 film (Kodak).

3. Results

3.1. Conformation and accessibility of phage displayed peptides

In order to direct phage particles to bind integrin receptors and be internalized, we displayed integrin-binding peptides in the pVIII coat protein of the

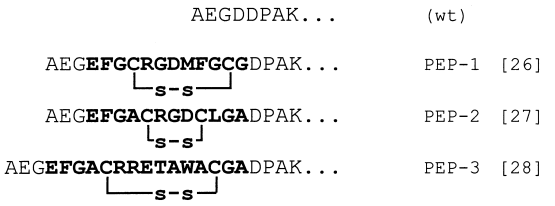

Fig. 1. Amino acid sequence of the N-terminal portion of the wild-type major coat protein pVIII and three integrin-binding peptides expressed as fusions with pVIII [26–28]. The peptides have been isolated from phage display libraries constructed in a minor coat protein (pIII) by biopanning on immobilized integrins (corresponding references are given). Although PEP-3 has no RGD motif, it was demonstrated to bind selectively to $\alpha_5\beta_1$ integrin with no appreciable binding to $\alpha_{IIb}\beta_3$ integrin [28]. The amino acid sequence of inserted oligopeptides are shown in bold. The putative disulfide bonds are indicated.

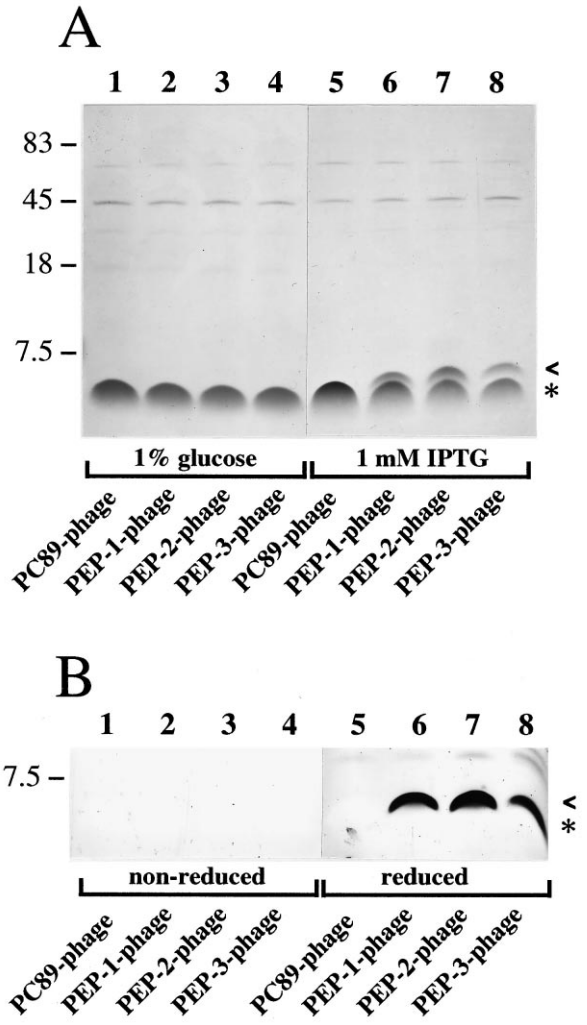

Fig. 2. Expression of the recombinant integrin-binding peptides in the phage coat. (A) Phage were grown in the presence of 1% glucose (lanes 1–4), or 1 mM IPTG (lanes 5–8) and subjected to SDS-PAGE followed by Coomassie blue staining. (B) Phage were grown in the presence of 1 mM IPTG and subjected to SDS-PAGE under non-reducing conditions. The proteins were then electrotransferred onto PVDF membrane, and half of the membrane was incubated in 2% (v/v) 2-mercaptoethanol in K-phosphate buffer pH 8.5 for 30 min (lanes 5–8), whereas the reduction step was omitted in the processing of the other half of the membrane (lanes 1–4). Free SH groups were visualized on both membranes using DIG Protein Labeling and Detection kit. Asterisks indicate the position of the wild-type protein VIII of bacteriophage. Arrowheads indicate recombinant protein VIII. Positions of molecular mass standards (kDa) are indicated.

filamentous phagemid pC89 (Fig. 1). Since the proteins comprising the phage coat can impose physical constraints on peptides expressed as fusions with pVIII [35,36], we examined the conformation of the displayed peptides and their accessibility for interaction with integrin receptors. The phagemid coat is comprised of pVIII molecules encoded by both the recombinant gene VIII of the pC89 phagemid, as well as the wild-type gene VIII of the helper phage M13KO7 that is necessary for the assembly of phagemid particles. Since the expression of phagemid gene VIII is controlled by the Lac promoter, the synthesis of the recombinant pVIII is repressed by glucose (Fig. 2A, lanes 2–4). In the presence of the inducer IPTG, the recombinant peptide-pVIII molecules are synthesized and can be resolved by SDS-PAGE as additional bands located slightly above the wild-type pVIII band (Fig. 2A, lanes 6–8).

To examine whether the displayed peptides form cyclic structures as depicted in (Fig. 1), we analyzed sulfhydryl groups of two cysteines flanking the integrin-binding motives (Fig. 2B). Free SH groups were not detected in the recombinant pVIII under non-reducing conditions (Fig. 2B, lanes 2–4), whereas

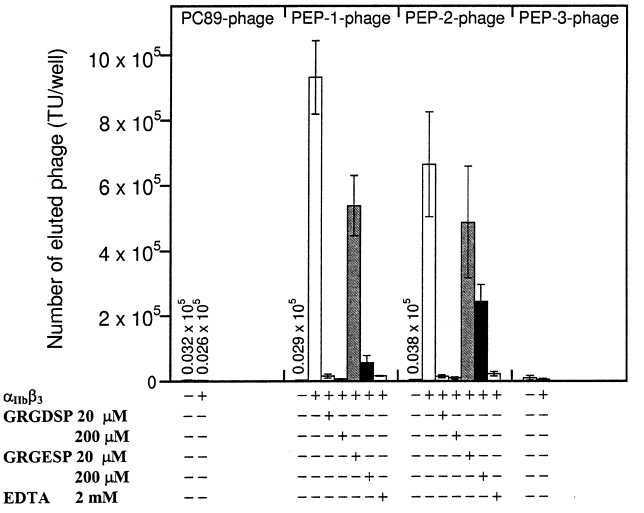

Fig. 3. Binding of wild-type and recombinant phage to $\alpha_{IIb}\beta_3$ integrin. Phage binding to microtiter wells coated with $\alpha_{IIb}\beta_3$ integrin and blocked with BSA was assayed in the absence or presence of synthetic peptides or EDTA as indicated. The control wells were blocked with BSA without prior coating with integrin. The phage input was 5×10^9 TU per well. The attached phage were eluted with a low pH buffer and quantitated by infection of bacteria. The data represent the mean ± S.D. of triplicate wells and are representative of two experiments. The numbers indicate the mean values where the phage yield was too low to be seen in the histogram.

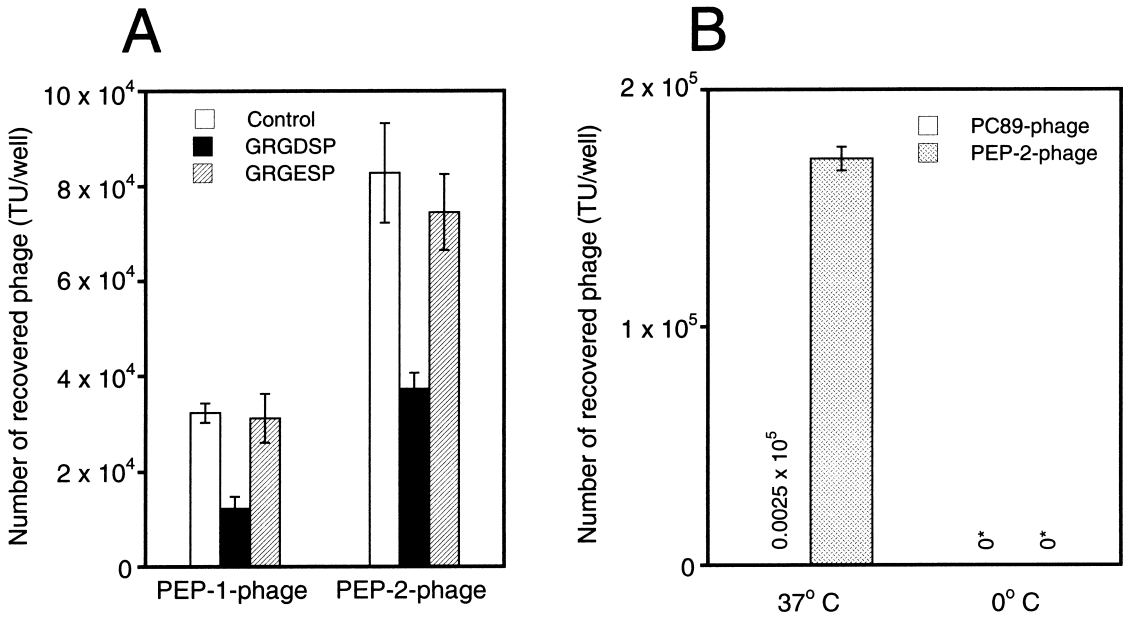

Fig. 4. Effect of GRGDSP peptide and incubation temperature on phage internalization by HEp-2 cells. (A) Inhibition of PEP-1-phage and PEP-2-phage uptake with GRGDSP peptide. Cells were incubated with phage (2×10^{10} TU/well) in the absence or presence of 0.2 mM GRGDSP or 0.2 mM GRGESP peptide for 2.5 h at 37°C. Internalized phage were assayed as described in Section 2. (B) Abolition of phage internalization at 0°C. pC89-phage and PEP-2-phage (2×10^{10} TU/well) were incubated with the cells at either 37°C, or 0°C for 2.5 h, and the number of internalized phage was determined as described in Section 2. The data are mean values from triplicate wells. The number in B indicates the mean value of phage yield which was too low to be seen in the histogram. 0* indicates that the number of recovered phage was less than the minimum detection limit which was 100 TU per well.

the reduction of disulfide bonds exposed free sulfhydryl groups in the recombinant pVIII (Fig. 2B, lanes 6–8), but not in the wild-type pVIII which lacks cysteine (Fig. 2B, lane 5). These data suggest that the integrin-binding peptides are expressed in the phagemid coat in a cyclic disulfide-bonded form.

Accessibility of the displayed peptides for interaction with integrins was evaluated in the binding assay using purified integrin $\alpha_{IIb}\beta_3$ (Fig. 3). Wild-type pC89-phage, and PEP-3-phage which display the peptide specific for $\alpha_5\beta_1$, but not $\alpha_{IIb}\beta_3$ integrin [28] demonstrated no specific binding with integrin-coated wells. In contrast, PEP-1-phage and PEP-2-phage showed highly selective attachment to integrin-coated wells versus the control wells coated with BSA. We observed a non-specific background binding of less than 1%. The binding of PEP-1-phage and PEP-2-phage to the integrin was strongly inhibited by a competitor peptide GRGDSP. The peptide GRGESP, which is generally used as a non-binding control, did in fact inhibit binding, but to a lesser extent, and at much higher concentration than GRGDSP. PEP-2-phage were less sensitive to the inhibitory action of GRGESP peptide than were PEP-1-phage. EDTA also inhibited phage attachment, consistent with the requirement of divalent cations for integrin receptor function [37,38]. These results demonstrate that integrin-binding peptides displayed by PEP-1-phage and PEP-2-phage are accessible for interaction with integrin receptors. However, the noticeable inhibitory effect of GRGESP peptide on binding of PEP-1-phage and PEP-2-phage to $\alpha_{IIb}\beta_3$ integrin suggests that the conformation or/and exposure of phage-displayed peptides may not be optimal for strong and specific interaction with integrin receptors. The effect of conformation of RGD-containing peptides on their affinity and specificity to integrin receptors has been described [39].

3.2. Recovery of internalized phage

We next determined whether integrin-binding pep-

tides displayed on phage can bind cell-surface integrins and mediate phage internalization. In order to quantitate the number of internalized phage, we used a protease, subtilisin, to inactivate extracellular phage. Subtilisin has been shown to proteolyze the phage protein pIII, thus destroying an adsorption complex of the phage and leaving the phage particles non-infectious [40,41]. Thus, we reasoned that treatment with subtilisin would effectively destroy the non-internalized phage, allowing the subsequent recovery and propagation of only the internalized phage.

To optimize the recovery of internalized phage, we also tested different detergents for cell lysis. SDS (0.5%) in buffers of different composition inactivated phage in 10 min at room temperature corroborating earlier observations [42], whereas 1% Triton X-100 or 2% DOCNa had no detrimental effect on phage infectivity (data not shown). Since 2% DOCNa solution (2% DOCNa, 10 mM Tris, 2 mM EDTA, pH 8.0) dissolved the cells more rapidly than 1% Triton X-100 and exhibited no inactivation effect on phage titer after 24 h incubation at 4°C, we selected this detergent for further experiments.

Our initial experiments demonstrated that exposure of phage to subtilisin (subtilisin 3 mg/ml in HBSS(−) containing 20 mM Tris, 2 mM EDTA, pH 8.0) at 0°C for 2 min decreased the titer of infectious phage from about 10^{12} to 10^{6} TU/ml (data not shown). In contrast, some phage that were incubated with the cells became protected from subtilisin action and inaccessible for elution by a low pH buffer (data not shown). The number of protected phage which can be recovered in cell lysates was about 200 times higher for PEP-2-phage than for pC89-phage suggesting that phage uptake was mediated by integrin-binding peptides displayed on phage. This suggestion was further supported in competition experiments using synthetic integrin-binding peptide GRGDSP (Fig. 4A). The number of subtilisin-resistant phage recovered in cell lysate was reduced by half when the cells were incubated with PEP-2-phage in the presence of 0.2 mM GRGDSP peptide, whereas the inert peptide GRGESP (0.2 mM) had no effect.

The inhibitory peptide GRGDSP was remarkably less potent in phage internalization assay (Fig. 4A) than in phage binding assay (Fig. 3). This difference

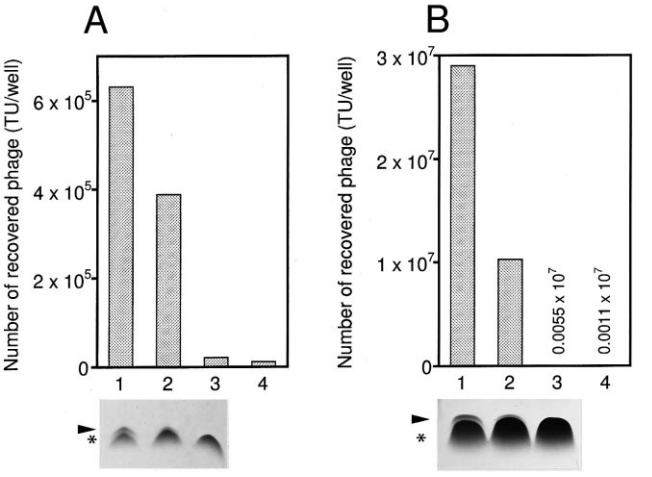

Fig. 5. The dependence of phage uptake on the density of integrin-binding peptide in the phage coat. PEP-2-phage (A), and Ech3-phage selected for high rate of internalization [52] (B) were grown in the presence of 1 mM IPTG (bars and lanes, 1), in the absence of IPTG (bars and lanes, 2), or in the presence of 1% glucose (bars and lanes, 3). The phage (2×10^{10} TU/well) were incubated with ECV304 cells for 2.5 h at 37°C and the number of internalized phage was assayed as described in Section 2. For comparison, the values of uptake of pC89-phage are also shown (bars 4). Data represent the mean of duplicate measurements. The phage proteins were analyzed by SDS-PAGE and the bands corresponding to wild-type (asterisks) and recombinant (arrowheads) pVIII are shown below the bars. Gels in A and B were loaded with different amounts of phage protein in order to obtain the best resolution of the recombinant pVIII. The numbers in B indicate the mean values where the phage yield was too low to be seen in the histogram.

in peptide activity may be due to different integrin receptors involved in phage binding in these assay systems. Alternatively, the apparently low activity of GRGDSP peptide in phage internalization assay may indicate that upon binding integrin receptors, phage particles become rapidly trapped into the forming endocytotic structures and thus are unavailable for dissociation. Essentially similar inhibitory concentrations of GRGDSP peptide in the range of hundreds μM were described in the cell binding assay for fd-tet phage displaying RGD-containing cyclic peptides [23]. Because PEP-2-phage consistently exhibited a higher rate of internalization as compared with PEP-1-phage, we selected PEP-2-phage for further experiments.

To estimate the number of extracellular phage that could survive subtilisin treatment and therefore might be wrongly scored as having been internalized,

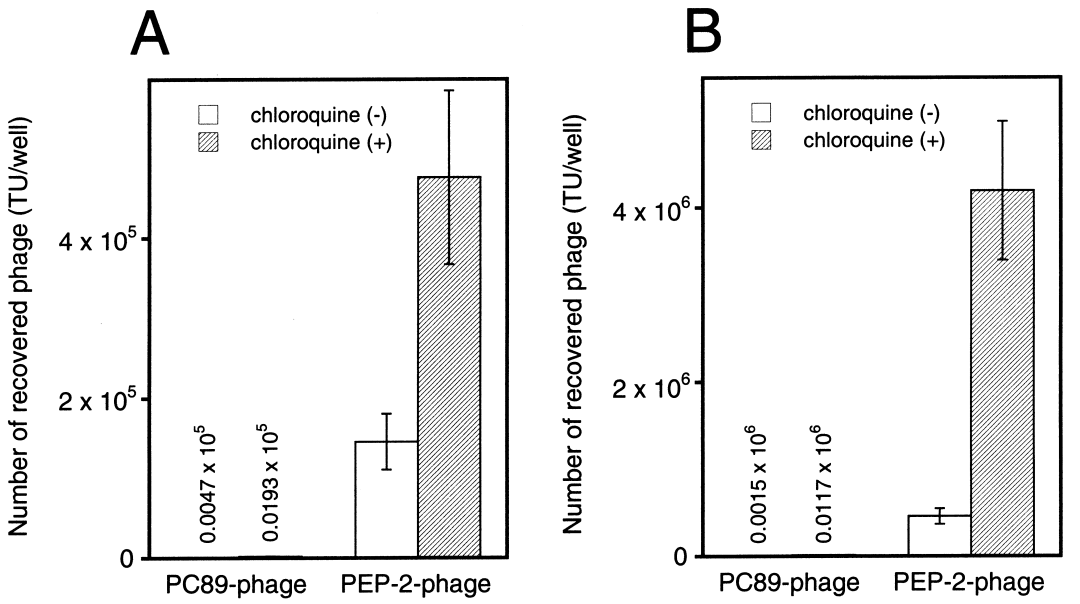

Fig. 6. Effect of chloroquine on recovery of internalized phage. HEp-2 cells (A) or ECV304 cells (B) were incubated with pC89-phage or PEP-2-phage (2×10^{10} TU/well) for 3 h at 37°C in the absence or presence of 100 μM chloroquine. The cells were then processed for the assay of internalized phage as described in Section 2. The data represent the mean ± S.D. from 6 or 8 wells and are representative of two experiments. The numbers indicate the mean values where the phage recovery was too low to be seen in the histogram.

we incubated the cells with phage at 0°C, thus providing conditions for phage binding to the cells, but prohibiting endocytic or phagocytic events. Under these conditions, no internalized phage were detected (Fig. 4B), indicating that the background of extracellular bound phage erroneously counted as having been internalized was less than 0.1%.

In order to evaluate whether a multivalent mode of peptide display is important for phage uptake, we obtained phage particles with different densities of integrin-binding peptides on their surface (Fig. 5). Visual comparison of serial dilutions of phage samples grown in the presence of 1 mM IPTG estimates the ratio of recombinant to wild-type pVIII to be approximately 1:5 for PEP-2-phage (Fig. 5A, lane 1), and 1:20 for Ech3-phage (Fig. 5B, lane 1). The total number of pVIII molecules comprising the coat of the phagemid pC89 with a genome of 3.5 kb can be approximately estimated as 1500. This estimate is based on the assumption that the wild-type phage with a genome of 6.4 kb contains approximately 2700 copies of pVIII [33], and that the number of pVIII molecules in the capsid is roughly proportional to the size of phage genome. Consequently, the number of peptides displayed per phage particle can be roughly estimated as 250 for PEP-2-phage, and 70 for Ech3-phage.

The number of internalized phage was dependent

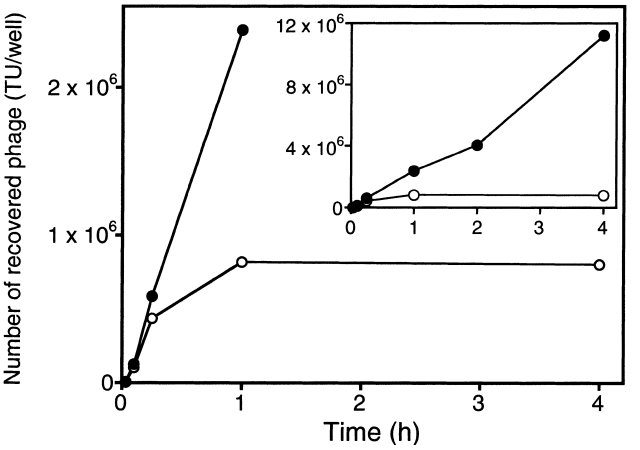

Fig. 7. Time dependence and the effect of chloroquine on phage recovery from ECV304 cells. The cells were incubated with PEP-2-phage (2×10^{10} TU/well) at 37°C for various time periods in either absence (○) or presence (●) of 100 μM chloroquine. Following the incubation, the cells were processed for quantitation of intact internalized phage as described in Section 2. The inset shows the data from the same experiment, but on a wider *y*-axis scale. The data are the mean values from two wells and are representative of two experiments.

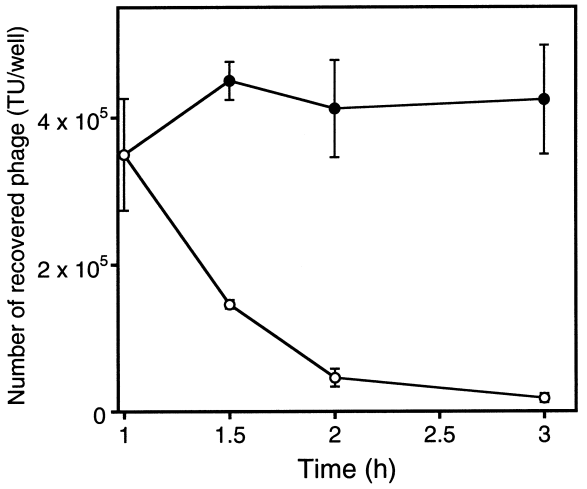

Fig. 8. Pulse-chase measurements of phage inactivation in ECV304 cells. The cells were either continuously incubated with PEP-2-phage (2×10^{10} TU/well) at 37°C for different periods of time (●), or incubated for 1 h with PEP-2-phage, and then washed three times with Medium 199 supplemented with 10% FBS, and incubated in the same medium at 37°C for various 'chase' periods (○). The number of internalized intact phage was determined in cell lysates as described in Section 2. The data represent the mean ± S.D. of triplicate wells and are representative of two experiments.

on the density of the displayed peptide. Phage grown in the presence of 1% glucose showed the lowest uptake which was, however, 2–5 times higher than that of the pC89-phage. Although no recombinant pVIII could be detected by SDS-PAGE in the phage grown in the presence of 1% glucose, some peptide might still have been displayed on these phage resulting in slightly increased uptake as compared with pC89-phage. These data suggest that peptides displayed in multiple copies are more effective triggering internalization.

3.3. Intracellular fate of internalized phage

In order to determine whether internalized phage reach and undergo inactivation in the acidified vesicular compartments such as lysosomes, we investigated the effect of a lysosomotropic agent chloroquine on the titer of internalized phage. Chloroquine is a weak base which rapidly diffuses across the plasma and lysosomal membranes. In the acidic environment inside lysosomes, chloroquine becomes protonated and accumulated because the lysosomal membrane is much less permeable to the

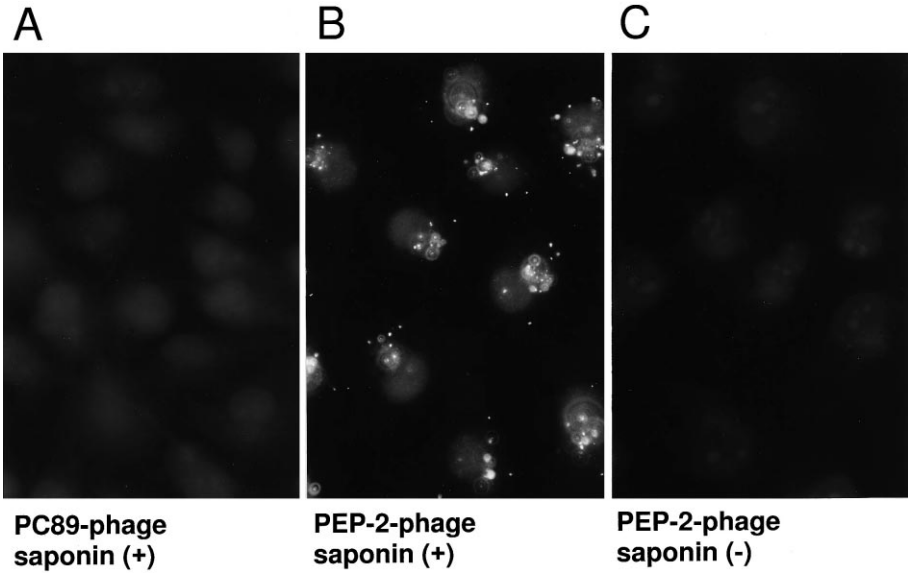

Fig. 9. Detection of internalized phage particles in ECV304 cells by immunofluorescence microscopy. The cells were grown on chamber slides and incubated with pC89-phage or PEP-2-phage (2×10^{9} TU/well) for 16 h. The slides were then processed for immunofluorescence microscopy as described in Section 2. Prior to incubation with antibodies, the cells were either permeabilized with 0.1% saponin (saponin (+)), or the permeabilization step was omitted (saponin (−)). In the saponin-permeabilized cells, both internalized and extracellular bound phage particles are visualized, whereas without saponin treatment, only extracellular phage are accessible for immunodetection. The background fluorescence reveals the nuclei of the cells.

protonated form of chloroquine than to the free base. The accumulation of chloroquine is associated with the increase of intralysosomal pH, inhibition of lysosomal hydrolases, and prevention of degradation of macromolecules in the lysosomal compartment [43].

Our experiments revealed a strong effect of the lysosomotropic agent chloroquine on the titer of internalized phage (Fig. 6). This suggests that internalized phage particles reach an acidified intracellular compartment where they lose infectivity. The kinetics of inactivation of internalized phage were studied mainly with ECV304 cells. Over the first 15 min of phage incubation with the cells, addition of chloroquine had no significant effect on the yield of internalized phage (Fig. 7). In experiments performed in the absence of chloroquine, the titer of internalized phage reached a steady state by the 1-h time point. In contrast, in the presence of chloroquine, the titer increased in an almost linear fashion during 4 h incubation period (Fig. 7). These data suggest that upon internalization, phage particles enter a non-acidified subcellular compartment in which they are predominantly located within 15 min after uptake. From this compartment, the phage are gradually translocated into an acidified compartment associated with phage inactivation. In pulse-chase experiments performed in the absence of chloroquine (Fig. 8), the titer of internalized phage dropped to approximately 5% of the steady state value after 2 h incubation without the influx of newly internalized phage. These data suggest that the phage pool identified in the non-acidified compartment likely represents an intermediate station (rather than a storage depot) on the pathway to an acidified compartment.

3.4. Immunolocalization of internalized phage in ECV304 cells

Immunofluorescence microscopy identified phage particles in the cells which were incubated with the peptide-presenting PEP-2-phage (Fig. 9B), but not in the cells incubated with the wild-type pC89-phage (Fig. 9A). No immunostaining associated with PEP-2-phage was detected in non-permeabilized cells (Fig. 9C), indicating that all PEP-2-phage particles detected in permeabilized cells (Fig. 9B) are localized inside the cells. Internalized phage demonstrated an uneven distribution within the cells and were predominantly clustered on one side of the cell nuclei (Fig. 9B).

4. Discussion

Internalization and recovery of the infectious phage form the key elements for using of phage libraries for systematic multi-round selection of the novel ligands targeting endocytosing receptors. Such ligands will likely be of particular interest in developing targeting systems for intracellular delivery of macromolecules.

Three findings emerged from the present study may be helpful in the design and optimization of the selection experiments. First, we demonstrated that display of receptor-binding peptides in a multivalent fashion is required for efficient phage internalization. This suggests that phage libraries constructed in vectors displaying multiple rather than single copies of peptides or other molecules should be used for selection of internalized ligands. The importance of multivalent interactions may be intrinsically related to the mechanism of phage uptake. The length of the phagemid pC89 (approximately 0.5 μm) appears to be too large for packaging into clathrin-coated endocytotic vesicles of ~100–150 nm in diameter [44]. Therefore, the phage particles are likely internalized via a process resembling phagocytosis, and involves the sequential recruitment of cell surface receptors for multi-point contact with the particle surface by a 'zipper' mechanism [45]. The necessity of multivalent phage–cell interaction for phage uptake would explain the lack of phage internalization in the experiments where the cell specific binding peptides were isolated from the random peptide libraries constructed in the minor coat protein pIII [46,47]. Our experiments showed that presentation of ligands as fusions with pVIII provides selective phage binding to cognate receptors. In both phage attachment to immobilized integrin and phage internalization assays, the background of non-specific binding was consistently less than 1% (Figs. 3 and 4B). Thus, the libraries constructed in pVIII-vectors might be an appropriate source of the novel cell targeting ligands for intracellular delivery of macromolecules. In addition to peptides, larger proteins including

antibody variable binding fragments [48,49] can potentially be selected from the appropriate libraries using this approach.

Second, our kinetic data on phage uptake and inactivation show that an incubation with the phage for 15–60 min is sufficient for isolation of internalizable phage-displayed ligands. It is conceivable, therefore, to perform in vivo selection of the ligands specifically internalized by endothelial cells. In this respect, filamentous phage have been shown to continue circulation in blood for many hours after injection [50]. Isolation of the ligands specifically internalized by angiogenic tumor vasculature would be of particular therapeutic importance [51].

Finally, specific strategies could be designed for the isolation of the ligands targeting various intracellular compartments. For example, selection of internalizable phage-displayed ligands in the presence of chloroquine may facilitate isolation of lysosomal targeting ligands. Alternatively, recovery of the internalized phage after a prolonged 'chase' period (as shown in Fig. 8) would favor isolation of ligands targeted to non-lysosomal compartments or those which undergo transcytosis.

Although the selected phage themselves will not likely be used for therapeutic purposes, the peptides targeting endocytosing receptors could potentially be transplanted into more advantageous vehicles for drug or gene delivery including drug conjugates, polycation–DNA complexes, and liposomes. In the companion report [52] we demonstrate the feasibility of exploiting multivalent phage display libraries for the selection of cell specific internalized ligands.

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