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Identification of the Insulin-Like Growth Factor II Receptor as a Novel Receptor for Binding and Invasion by *Listeria monocytogenes*

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The gram-positive bacterium *Listeria monocytogenes* causes a life-threatening disease known as listeriosis. The mechanism by which *L. monocytogenes* invades mammalian cells is not fully understood, but the processes involved may provide targets to prevent and treat listeriosis. Here, for the first time, we have identified the insulin-like growth factor II receptor (IGFIIR; also known as the cation-independent mannose 6-phosphate receptor ^{CI}M6PR or CD222) as a novel receptor for binding and invasion of *Listeria* species. Random peptide phage display was employed to select a peptide sequence by panning with immobilized *L. monocytogenes* cells; this peptide sequence corresponds to a sequence within the mannose 6-phosphate binding site of the IGFIIR. All *Listeria* spp. specifically bound the labeled peptide but not a control peptide, which was demonstrated using fluorescence spectrophotometry and fluorescence-activated cell sorting. Further evidence for binding of the receptor by *L. monocytogenes* and *L. innocua* was provided by affinity purification of the bovine IGFIIR from fetal calf serum by use of magnetic beads coated with cell preparations of *Listeria* spp. as affinity matrices. Adherence to and invasion of mammalian cells by *L. monocytogenes* was significantly inhibited by both the synthetic peptide and mannose 6-phosphate but not by appropriate controls. These observations indicate a role for the IGFIIR in the adherence and invasion of *L. monocytogenes* of mammalian cells, perhaps in combination with known mechanisms. Ligation of IGFIIR by *L. monocytogenes* may be a novel mechanism that contributes to the regulation of infectivity, possibly in combination with other mechanisms.

The gram-positive bacterium *Listeria monocytogenes* is a human pathogen that causes listeriosis primarily in immunosuppressed individuals. Symptoms are flu-like, and yet disease may progress to severe complications such as meningitis, septicemia, spontaneous abortion, or listeriosis of the newborn (28, 39, 83). The number of cases of listeriosis range from 40 to 44 per year in Australia (3, 79) and average 100 per year in the United States (13). Although the incidence of listeriosis is low compared to that of other foodborne diseases, the associated mortality rate is high (approximately 30%) (39, 74, 83). Knowledge of the molecular mechanisms underlying the binding and internalization of *Listeria* spp. to host cells is limited. New antibiotic, vaccine, and diagnostic targets are needed to prevent and treat infection by *Listeria* spp., and understanding the processes of adherence and entry into cells is fundamental to defining appropriate preventative and therapeutic strategies.

Random peptide phage display has been successfully employed to identify new receptors and receptor ligands (44, 52, 53, 82) and in epitope mapping and mimicking of protein antigens and antibodies (16, 42, 61) and drug discovery (40, 52, 78). In this study we employed random peptide phage display in an attempt to identify novel surface antigens that may be used as therapeutic targets and diagnostic markers for *Listeria* spp. and *L. monocytogenes*. Subtractive panning of the phage display library was performed on immobilized *L. monocytogenes* cells, and a single peptide sequence was identified. The peptide sequence corresponds to a sequence found in repeat 3 of the insulin-like growth factor II receptor (IGFIIR; also known as the cation-independent mannose 6-phosphate [M6P] receptor ^{CI}M6PR or CD222), which is one of the binding sites for M6P in this receptor. Phage display results were confirmed by demonstrating that *Listeria* spp. specifically bound a synthetic peptide (M6Pbs peptide), designed to match the sequence of the M6P binding site, as well as the native receptor. The specificity of the interaction was further investigated by employing competitive binding and invasion assays using the M6Pbs peptide and M6P. We demonstrate a role for this receptor in adherence and invasion of mammalian cells by *L. monocytogenes*. This is the first time that the IGFIIR has been characterized as a receptor for *Listeria* spp.

MATERIALS AND METHODS

Bacteria and cells. (i) Bacterial strains. The bacterial strains used are described in Table 1.

(ii) Mammalian cell lines. MS9 and MS9II cells (L cells) and McCoy cells are mouse fibroblast cell lines. MS9 cells are deficient in IGFIIR expression, while MS9II cells are MS9 cells stably transfected with human IGFIIR, which is expressed by a cytomegalovirus vector (60, 64). Both cell lines were obtained

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TABLE 1. Bacterial strains employed in the study

Species	Serotype or strain	Source[a]	Property
L. monocytogenes	1/2a	CDC	Wild type, 1 of 3 strains commonly causing listeriosis
	1/2b	TT 1707	Wild type
	1/2c	CDC	Wild type
	3a	IMVS 0224	Wild type
	3b	IMVS 0270	Wild type
	3c	IMVS 1830	Wild type
	4a	TT 1706	Wild type
	4b	CDC	Wild type
	4c	ATCC 19116	Wild type
	4d	ATCC 19117	Wild type
	4e	ATCC 19118	Wild type
	EGD	K. Ireton[b]	Wild type
	EGD	K. Ireton	Mutant strain lacking *inlA*
	EGD	K. Ireton	Mutant strain lacking *inlB*
	EGD	K. Ireton	Mutant strain lacking *inlAB*
L. innocua	4	IMVS 1871	Wild type
	6a	ATCC 33090	Wild type, representative serotype
L. ivanovii	5	IMVS 1918	Wild type
		IMVS 1171	Wild type
		ATCC 19119	Wild type
		TECRA food isolate	Wild type
Bacillus cereus	ND[c]	TECRA food isolate	Wild type
Escherichia coli	ER2738	NEB	K-12; F′ $proA^+B^+$ $lacI^q$ $\Delta(lacZ)M15$ *zzf*::Tn*10*(Tet^r) *fhuA2 glnV* Δ(*lac-proAB*) *thi*-1 Δ(*hsdS-mcrB*)*5*

[a] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; TT, Toxin Technology, Sarasota, Fla.; IMVS, Institute for Microbiological and Veterinary Sciences, Adelaide, Australia; NEB, New England Biolabs, Beverly, Mass.; TECRA, TECRA International, French's Forest, Australia.
[b] University of Toronto, Canada (all strains were from reference 30).
[c] ND, not determined.

from J. Trapani (Peter MacCallum Cancer Research Institute, Melbourne, Australia) and used with permission of W. Sly (St. Louis University). McCoy cells were sourced from the ATCC.

Preparation of bacterial strains for coating and panning. Bacterial strains were grown overnight (o/n) (10 ml tryptic soy broth, 37°C, static conditions). An *L. monocytogenes* cocktail was obtained by combining 100-μl amounts of each serotype (1.1 ml total). An *L. innocua* cocktail (500 μl of each serotype) and an *L. ivanovii* cocktail (250 μl of each isolate) were similarly produced (1 ml total). Bacteria were washed four times (10 mM phosphate buffer [pH 8.1], 6,000 × *g*, 10 min), and the pellets were resuspended (1 ml of phosphate buffer with 0.1% sodium azide).

Preparation of bacterial cells for binding and invasion assays. Bacterial strains were grown o/n. Log-phase bacterial cultures were obtained by inoculating 0.5 ml of the culture into 9.5 ml of tryptic soy broth. Cultures were grown (37°C with shaking at 200 rpm) to an optical density at 600 nm of 0.5 to 0.7, washed four times (phosphate-buffered saline [PBS], 6,000 × *g*, 10 min), and resuspended (1 ml PBS).

Labeling of bacterial cells. O/n bacterial cultures were gently washed three times (10 mM phosphate buffer [pH 8.1], 1,400 × *g*, 10 min, 4°C) and resuspended. Bacteria (1 ml) were labeled by incubating with 5- (and 6-) carboxyfluorescein diacetate-succinimidyl ester (CSFE; Molecular Probes, Eugene, Oregon) (2 μl, 5 mM, 30 min, 37°C). Bacteria were washed, resuspended (1 ml PBS), and stored at 4°C for up to a week.

Binding of labeled peptides by bacterial cells. Peptides were synthesized by Auspep Pty. Ltd. (Parkville, Australia) and labeled with fluorescein isothiocyanate (FITC) during synthesis. Peptides were the identified peptide PLAQSGG SSYI (M6Pbs peptide), a control peptide with a randomized sequence of the M6Pbs peptide YPSGSILGSQA (M6Pbs control peptide), and a second control peptide corresponding to the IGFII binding site of the IGFIIR, FGQTRIS VGKA (IGFIIbs peptide) (27). Lyophilized peptides were resuspended (5 mg/ml) with 50% dimethyl sulfoxide (DMSO) (M6Pbs peptide and M6Pbs control peptide) or PBS (IGFIIbs peptide). Bacterial cultures were washed (1 ml PBS) and resuspended with each of the peptides (40 μl, 2 h, 4°C). Bacteria were washed, resuspended (1 ml PBS–0.1% sodium azide), and stored at 4°C for up to a week.

Preparation of mammalian cells for fluorescence-activated cell sorting (FACS) and invasion assays. MS9, MS9II, and McCoy cells were grown in serum-free supplemented Dulbecco's modified Eagle medium (DMEM) containing fetal calf serum (FCS; CSL, Parkville, Australia) (10%), HEPES (25 mM), L-glutamate (2 mM), sodium pyruvate (0.1g/liter), and penicillin-streptomycin (100 μg/ml). To maintain IGFIIR expression, MS9II cell medium contained methotrexate (SIGMA, Sydney, Australia) (3.2 μM). For invasion assays, cells were seeded into 24-well tissue culture plates (~10^5 cells/well) and grown until >90% confluent.

Phage display. Phage displays were produced using a Ph.D. 12mer random phage display peptide library (NEB, Beverly, Massachusetts). Panning was performed using Dynex Immunolon microtiter wells coated with *L. monocytogenes* cocktail (150 μl/well, diluted 1:100, in phosphate buffer, o/n, room temperature [RT]). After coating, wells were blocked (5 mg/ml bovine serum albumin [BSA], 300 μl/well, 2 h, RT), washed once (distilled water), and stored at 4°C until further use. Nonspecific phages were removed by absorbing the phage library sequentially on uncoated wells and wells coated with BSA (1 mg/ml, 30 min, RT, rocking). Selection of antigen-positive phages was performed by the addition of the absorbed phage library to immobilized *L. monocytogenes* cocktail (60 min, RT, rocking). Unbound phages were discarded, and wells were washed three times (Tris-buffered saline–0.5% Tween 20). Subtractive selection was carried out by the sequential application of *L. innocua* cocktail and *L. ivanovii* cocktail (100 μl, 1:1,000 in Tris-buffered saline) to wells (30 min, RT), with washing between steps. Low-affinity *L. monocytogenes*-specific phage peptides were removed by rapid exposure and removal of *L. monocytogenes* cocktail (100 μl). The wells were washed before incubation with *L. monocytogenes* cocktail (30 min, RT) to elute specific high-affinity bound phages. The phage eluate was titered, amplified, and panned twice more according to the manufacturer's instructions. DNA was prepared from single phage clones after two rounds of panning by phenol extraction (Tris-buffered phenol, 1:1) and ethanol precipitation before DNA sequencing (Newcastle Sequencing, University of Newcastle, Newcastle, Australia).

Binding assays using fluorescently labeled peptides. Microtiter wells were coated with bacterial preparations (1:100) and BSA (1 mg/ml, 150 μl/well, in phosphate buffer). M6Pbs peptide and M6Pbs control peptide were serially

diluted (100 to 6.25 μg/ml) in PBS–0.05% Tween 20 (PBS/T) containing 25% DMSO,while the IGFIIbs peptide was serially diluted in PBS/T. Binding was performed by incubating peptides with immobilized bacteria (100 μl/well, o/n, 4°C). Wells were washed five times (PBS/T), and distilled water (100 μl) was added before the fluorescence was read (excitation 485 nm, emission 538 nm) using a fluorescence enzyme-linked immunosorbent assay plate reader (Fluoromark; Bio-Rad).

Affinity purification of IGFIIR from FCS by use of magnetic beads coated with bacteria. Magnetic beads (Dynal, Victoria, Australia) (0.5 ml) were prepared as described in the manufacturer's instructions, coated with *L. monocytogenes* cocktail (1 ml, 1:100, 0.1 M phosphate buffer [pH 8.0], o/n, 4°C, rotating wheel) and washed five times (50 ml PBS) to remove unbound material. For coating of beads with cell surface protein extract, an o/n culture of *L. monocytogenes* (250 ml) was washed twice (50 mM phosphate buffer [pH 7.6], 1,400 × *g*, 4°C), resuspended (3 ml, 50 mM phosphate buffer [pH 7.6], 1% sodium dodecyl sulfate [SDS]), and incubated in a water bath (2 h, 70°C). Cell debris was removed (3,000 × *g*, 10 min), the cell surface protein extract was dialyzed (50 mM phosphate buffer [pH 7.6], o/n, 4°C), and magnetic beads were coated with the extract (1:100). Affinity purification of IGFIIR was performed by incubating coated beads with FCS (250 ml, o/n, RT, rotating wheel). Unbound material was removed by washing three times (50 ml PBS, 4°C), and bound material was eluted (1 ml, 0.1 M glycine–HCl, pH 2.5). Eluates were concentrated using microcon concentrators (molecular weight, 30,000; Millipore), and concentrates were made up to 0.25 ml (10 mM Tris–0.8% NaCl, pH 8.0) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Preparation of mammalian cells for protein analysis. Mammalian cells were harvested (10 ml, PBS–0.05% Triton X-100) and sonicated (10,000 Hz, 2 min), and the protein content of cell lysates was determined using a bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer's instructions. The concentrations of cell lysates were adjusted (0.5 mg/ml) prior to analysis by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. SDS-PAGE (47) was performed using 10%- or 4- to 20%-gradient polyacrylamide gels (Gradipore, French's Forest, Australia). Briefly, samples were heated in sample buffer and separated on gels, and proteins were visualized using Coomassie blue or a silver staining kit (Bio-Rad, Sydney, Australia). For Western blotting, gels were transferred onto nitrocellulose (Bio-Rad) in transfer buffer (12 mM Tris, 0.96 M glycine [pH 8.0], 20% methanol), and transfer was performed (o/n, 4°C, 60 V). The membrane was blocked (5% skim milk powder–PBS/T, 4 h, RT), and bovine IGFIIR was detected with monoclonal mouse anti-bovine IGFIIR antibody (Affinity BioReagents, Golden, CO) (1 μg/ml, 1% skim milk–PBS/T) as primary antibody (o/n, RT) and goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (SIGMA) (1:5,000, 1% skim milk–PBS/T) as secondary antibody (1.5 h, RT). Detection of human or murine IGFIIR was performed with goat polyclonal anti-human or anti-mouse IGFIIR, respectively (Affinity BioReagents) (1 μg/ml, 1% skim milk–PBS/T) and rabbit anti-goat immunoglobulin G horseradish peroxidase conjugate (SIGMA, 1:100,000, 1% skim milk–PBS/T). Blots were washed three times between steps (5 min, PBS/T, rocking). Bands were detected using Supersignal chemiluminescent substrate (Pierce).

FACS scanning. Fluorescent cells were detected with a FACScan flow cytometer and analyzed using CellQuest software (BD Biosciences). Bacterial binding was calculated by determining the relative fluorescent intensity (relative fluorescent intensity = geographical mean of the sample/geographical mean of the control).

Binding of FITC-labeled peptides by bacteria. FITC peptide-labeled bacteria (100 μl) were taken up in paraformaldehyde (900 μl, 1%), and control procedures were performed by incubating bacterial cells in 25% DMSO–PBS (as the control for the FITC-M6Pbs peptide and FITC-M6Pbs control peptide) or PBS (FITC-IGFIIbs peptide).

Effect of M6Pbs peptide on bacterial adherence. CSFE-labeled bacteria (50 μl, 10^9 to 10^{10} cells/ml) were incubated with M6Pbs peptide (40 μl, 5 mg/ml, 30 min, 37°C) or the control (25% DMSO in PBS). Unbound peptide was removed by washing three times (5 min, PBS, 6,000 × *g*), and bacteria were incubated with mammalian cells (400 μl, 10^6 to 10^7cells/ml, DMEM, 20 min, 37°C). Samples were washed three times (5 min, PBS, 700 × *g*) and resuspended (200 μl, 2% paraformaldehyde).

Effect of M6P on bacterial adherence. CSFE-labeled bacteria (50 μl, 10^9 to 10^{10}cells/ml) and mammalian cells (50 μl of 10^6 to 10^7cells/ml) were added to 400 μl of PBS, PBS containing 50 mM glucose-6 phosphate (G6P) (controls), or PBS containing 0, 10, 20, or 50 mM M6P (20 min, 37°C). Samples were washed three times with PBS, PBS containing 10 mM G6P (control samples), or PBS containing 10 mM M6P (M6P samples) (5 min, 700 × *g*) and resuspended (200 μl, 2% paraformaldehyde).

Invasion assay-gentamicin protection assay. Mammalian cell monolayers were washed three times (DMEM, 37°C) before the addition of log-phase bacterial cells at a multiplicity of infection of ~10:1 (1 h, 37°C) followed by the addition of gentamicin (20 μg/ml final concentration, 2 h, 37°C). Mammalian cells were washed three times (serum-free DMEM, 37°C), and cells were lysed by the addition of ice-cold 0.2% Triton X-100 (15 min, 4°C). Intracellular bacterial cells were recovered by scraping the wells vigorously and plating recovered bacteria (tryptic soy agar plates, o/n, 37°C). Bacterial colonies were counted, and the percentage of relative invasion was determined using the following equation: number of recovered bacteria × 100%/number of bacteria in inoculum.

Statistical analysis. Analysis of statistical significance for studies showing dose-dependent variations (Fig. 1; see also Fig. 6 and 8) was performed using the test Nptrend (23). The two-sample Wilcoxon rank-sum test was performed to determine statistical significance of differences observed between two groups (Fig. 1 and 2; see also Fig. 5 and 7).

RESULTS

Identification of a peptide bound specifically by *Listeria* spp. by phage display. A random peptide phage display was used to identify novel surface antigens on cells of *Listeria* spp. A 12mer phage display library was used in a protocol designed to select peptides on phages that were bound specifically and with high affinity by a cocktail of *L. monocytogenes* serotypes. Decreasing ratios of phage input/phage recovery indicated that enrichment of antigen-positive phage peptides occurred after each round of panning (Table 2). DNA from 10 single plaques was prepared and sequenced after the second round of panning. The amino acid sequence deduced for 9 of the 10 clones was TTS PLSQGSSYI. The NCBI database (http://www.ncbi.nlm.nih .gov:80/entrez/query.fcgi?db = Protein) was screened to show that the identified peptide had significant homology (underlined amino acids) with the peptide sequence PL(A)Q(S) (G)GSSYI found in the human IGFIIR (59) (NCBI accession no. 225752).

The synthetic peptide is bound by *Listeria* spp. Binding of the labeled synthetic M6Pbs peptide by *Listeria* spp. was confirmed by fluorescence spectrophotometry and FACS.

Binding of labeled peptides by immobilized bacterial cells. The identified peptide was synthesized, labeled with FITC (FITC-M6Pbs peptide), and used in binding assays to show that listerial cells specifically bound the peptide. The identified peptide was bound by all *Listeria* spp. but not by other bacterial species (such as the gram-positive species *Bacillus cereus* and the gram-negative strain *Escherichia coli* ER2738) or by BSA (for 100 μg/ml, $P < 0.001$; for 50 μg/ml, $P < 0.01$; and for 25 μg/ml, $P < 0.05$) (Fig. 1A). To further demonstrate the specific association between the M6Pbs peptide, *Listeria* cells, and the IGFIIR, two FITC-labeled control peptides were used in this study. One of the control peptides (M6Pbs-control peptide) had a randomized sequence of the M6Pbs peptide (YPSGSIL GSQA), and a second peptide (FITC-IGFIIbs peptide) corresponded to a sequence within the IGFII binding site of the IGFIIR (FGQTRISVGKA) (10). By contrast to the M6Pbs peptide results, neither of the control peptides were bound by *Listeria* spp. or other bacteria (for 100 μg/ml, $P < 0.001$; for 50 μg/ml, $P < 0.01$; and for 25 μg/ml, $P < 0.05$) (Fig. 1B and C).

Analysis of binding of labeled peptides by bacterial cells. Specific binding of the FITC-M6Pbs peptide by *Listeria* spp. was confirmed by FACS. All *Listeria* spp. bound this peptide at significantly higher levels than controls ($P < 0.01$). *L. monocytogenes* deficient in virulence factor InlA and/or InlB bound

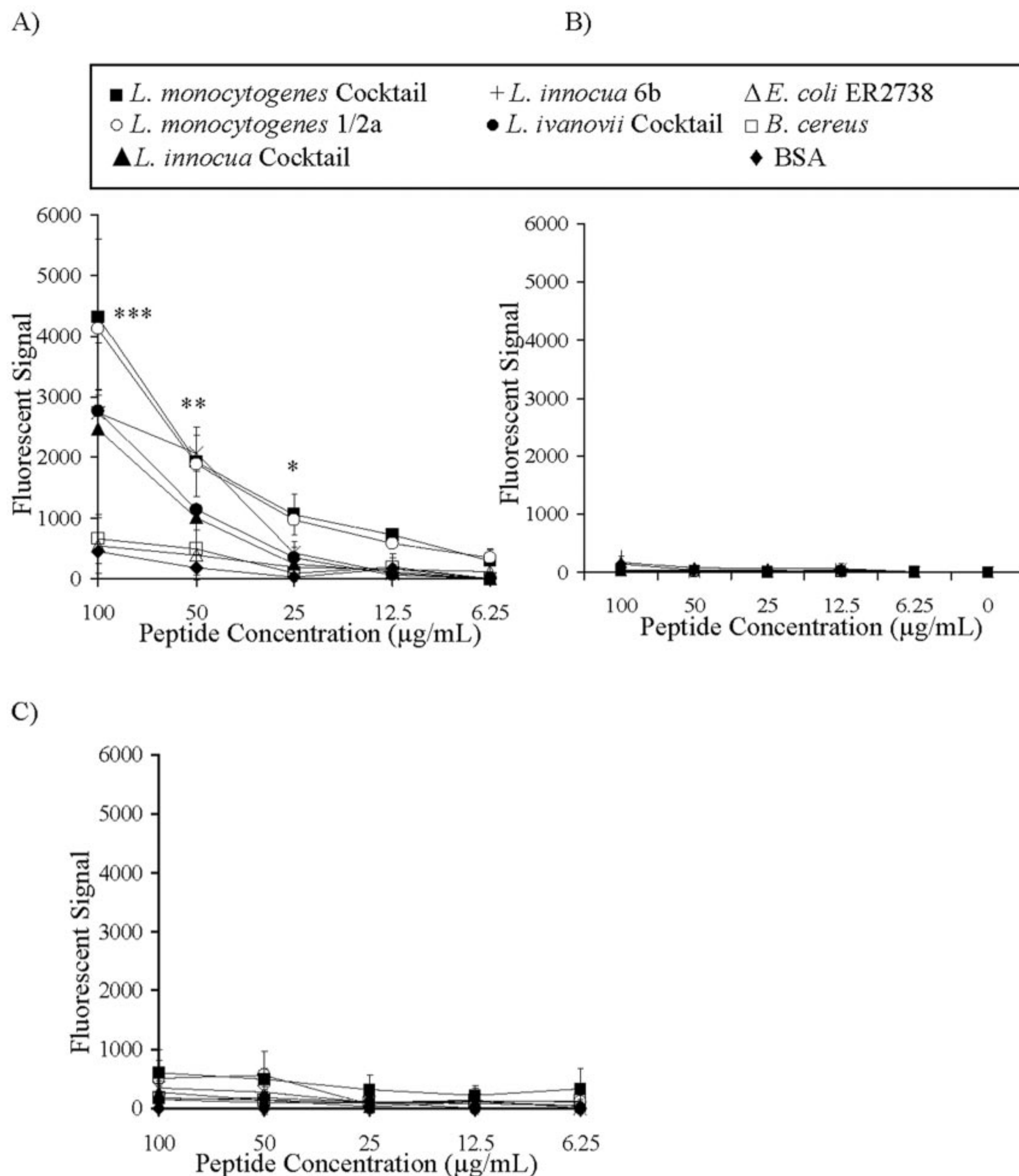

FIG. 1. Binding of FITC-labeled M6Pbs peptide to immobilized bacteria. Bacterial preparations were immobilized on microtiter wells, and binding of synthetic, FITC-labeled peptides was determined by measurement of fluorescence spectrophotometry. (A) Binding of the M6Pbs peptide by various *Listeria* spp. was dose dependent ($P < 0.01$) and was significantly increased compared to control results at 100 μg/ml (***, $P < 0.001$), 50 μg/ml (**, $P < 0.01$), and 25 μg/ml (*, $P < 0.05$). (B and C) Only background binding of the M6Pbs-control peptide (B) and control IGFIIRbs peptide (corresponding to a sequence within the IGFII binding site of the IGFIIR) (C) by immobilized bacterial preparations and BSA was observed in comparisons to binding of the M6Pbs peptide at 100 μg/ml (***, $P < 0.001$), 50 μg/ml (**, $P < 0.01$), and 25 μg/ml (*, $P < 0.05$). Data presented are means ± standard deviations ($n = 3$ in duplicate).

the M6Pbs peptide at levels similar to those seen with wild-type (wt) strains, demonstrating that loss of these virulence factors had no impact on the binding of this peptide. M6Pbs peptide did not bind to controls (*B. cereus* and *E. coli* ER2783) (Fig. 2A). To further determine that the binding of FITC-M6Pbs peptide by *Listeria* spp. was specific, binding of the FITC-labeled control peptides (M6Pbs-control peptide and IGFIIbs peptide) by cocktails of *L. monocytogenes*, *L. innocua*, and *L. ivanovii* serotypes was measured and compared to FITC-M6Pbs peptide binding by use of the same preparations. Binding of FITC-labeled control peptides by cocktails of *L. monocytogenes*, *L. innocua*, and *L. ivanovii* serotypes was significantly reduced compared to binding of FITC-M6Pbs peptide ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively) (Fig. 2B).

***Listeria* spp. can bind the native receptor.** To confirm that *Listeria* spp. can bind the native IGFIIR, whole bacterial cells and surface protein extracts were coated onto magnetic beads

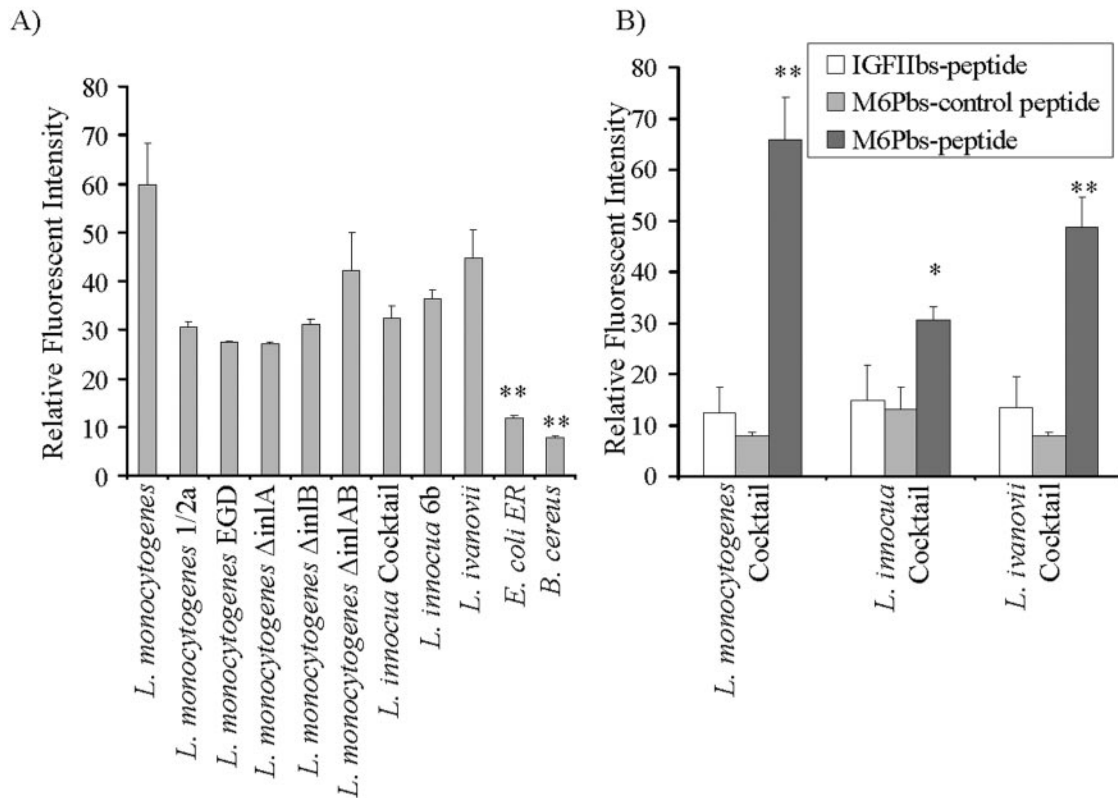

FIG. 2. Binding of FITC-labeled M6Pbs peptide by various bacterial strains. Binding of FITC-labeled peptides to *Listeria* spp. and controls was measured by FACS, and relative fluorescent intensity was calculated. (A) Levels of binding of the M6Pbs peptide by various *Listeria* spp. were significantly (**, $P < 0.01$) elevated compared to control (*E. coli* and *B. cereus*) results. (B) Binding of the M6Pbs-control peptide and IGFIIbs peptide (controls) by cocktails of *L. monocytogenes* (**, $P < 0.01$), *L. innocua* (*, $P < 0.05$), and *L. ivanovii* (**, $P < 0.01$) was significantly lower than binding of the M6Pbs peptide. Data presented are means ± standard deviations ($n = 2$ in duplicate).

and used to isolate the native receptor from FCS by affinity purification. Purified IGFIIR protein is not commercially available; however, a truncated form missing the cytoplasmic domain of the receptor is circulating in fetal serum and can be affinity purified from FCS by use of M6P affinity matrices (11, 49, 50). Although the M6Pbs peptide sequence used is homologous to that of the human IGFIIR, demonstration of binding of the bovine IGFIIR by *Listeria* spp. is relevant because this protein is highly conserved in mammalian species (38, 84). The IGFIIR was eluted from listerial preparations and detected by SDS-PAGE and Western blotting using a monoclonal anti-bovine IGFIIR antibody. A strong band of approximately 200 kDa, corresponding to the molecular mass reported for the circulating form of the receptor (49), was detected in affinity-purified material from *L. monocytogenes* and *L. innocua* preparations but not in materials purified from *L. ivanovii* or controls (Fig. 3). There were also some additional faint (119 kDa and below) bands detected in the Western blot (Fig. 3A) that may have resulted from the detection of breakdown products of the IGFIIR.

TABLE 2. Panning of phage-displayed peptides[a]

Panning round	Phage input (PFU)	Phage recovery (PFU)	Input/recovery ratio
1	1×10^{11}	2.5×10^{5}	4×10^{5}
2	1.3×10^{9}	3×10^{5}	4.3×10^{3}
3	1.3×10^{11}	3×10^{8}	4.3×10^{2}

[a] Panning was performed by incubating the 12mer peptide phage display library with immobilized *L. monocytogenes* cells from a cocktail of different serotypes. Phage input (number of phages for each pann) and phage recovery (number of phages after elution) were measured by phage titration, and the ratio of phage input to phage recovery was determined.

The IGFIIR is involved in binding of *Listeria* spp. to mammalian cells. A role for the IGFIIR in binding of *Listeria* spp. to mammalian cells was demonstrated. The receptor was shown to be present on MS9II and McCoy cells. Binding of *Listeria* spp. to these cells was inhibited by the M6Pbs peptide and the M6P.

Detection of the IGFIIR in mammalian cells. The presence of the IGFIIR in MS9II and McCoy cells but not in MS9 cells was confirmed by SDS-PAGE and Western blotting using antibodies against human or murine IGFIIR. A distinct band of approximately 300 kDa was detected in Western blots of cell lysates prepared from MS9II and McCoy cells but not in MS9 cells (Fig. 4). The detected band (300 kDa) corresponds to the reported molecular mass for this receptor in mammalian cells (84). Additional smaller-sized bands (75 kDa and below), which may have been due to breakdown products of the IGFIIR specifically interacting with the antibodies, were also detected.

Binding of *Listeria* spp. to mammalian cells in the presence of M6Pbs peptide. MS9 and MS9II cells were used to demonstrate that binding of *Listeria* spp. to the IGFIIR can be inhib-

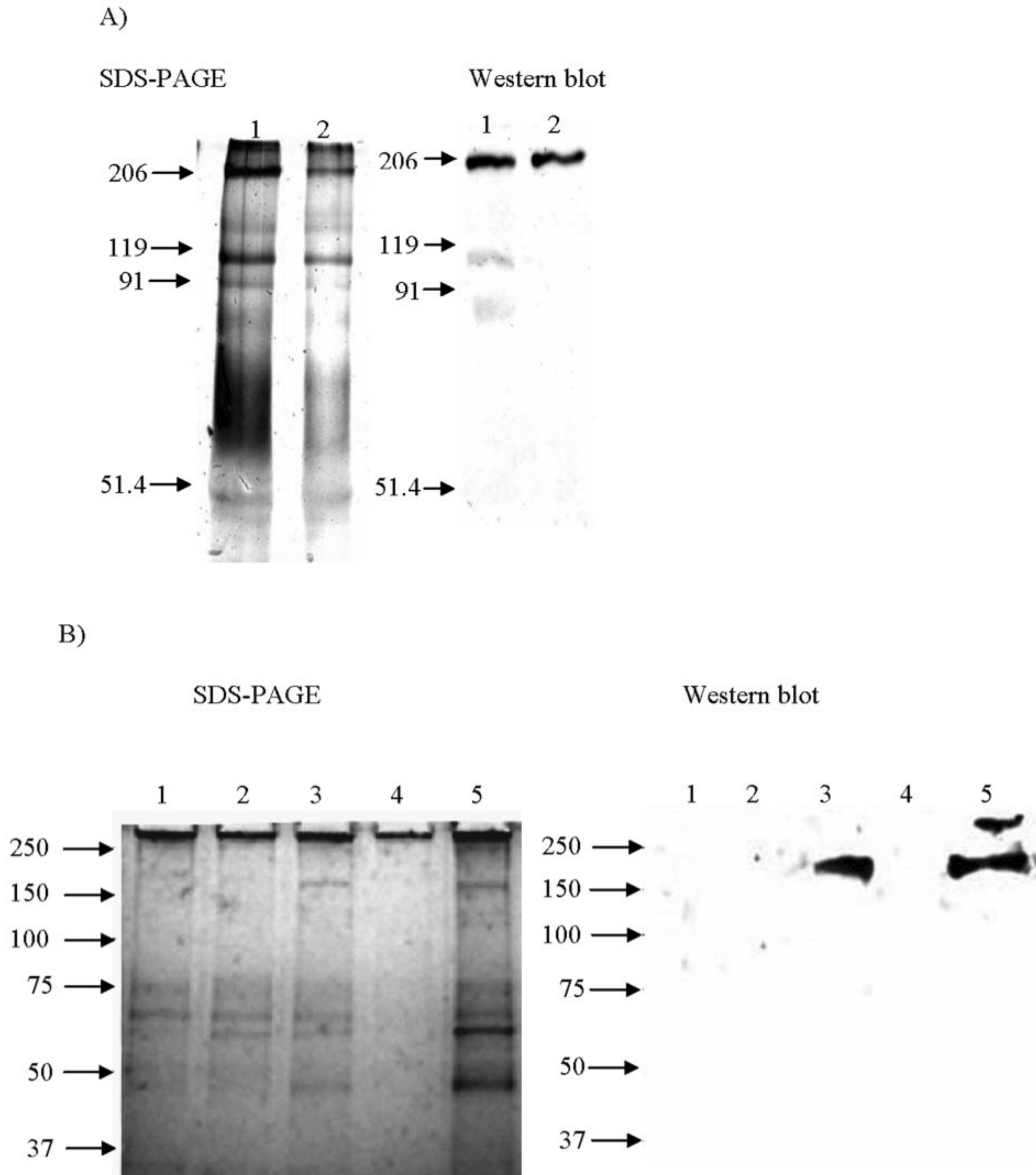

FIG. 3. Affinity purification of bovine IGFIIR by use of magnetic beads coated with various bacterial preparations. Magnetic beads were coated with bacterial preparations and incubated with FCS. Affinity-purified material was eluted, and binding of IGFRII was detected by SDS-PAGE and Western blotting. The Western blot was developed using a specific monoclonal anti-bovine IGFIIR antibody. Bovine IGFIIR (~200 kDa) was bound by *L. monocytogenes* cocktail cells (panel A, lane 1) and cell surface proteins (panel A, lane 2) and cells of *L. monocytogenes* serotype 1/2a (panel B, lane 5) and *L. innocua* cocktail (panel B, lane 3) but not by cells of *L. ivanovii* cocktail (panel B, lane 4) or controls (*E. coli* ER2738 [panel B, lane 1] or *B. cereus* [panel B, lane 2]). Standard molecular masses are represented in kilodaltons. The gels depicted are representative of duplicate experiments.

ited by the M6Pbs peptide. CSFE-labeled *L. monocytogenes* (cocktail and serotype 1/2a), *L. innocua* (cocktail), and *B. cereus* were incubated with M6Pbs peptide. Binding to MS9 and MS9II cells with or without prior incubation with the peptide was examined by FACS. There were no differences in binding of any of the bacterial strains to MS9 compared to MS9II cell results in the absence of peptide (Fig. 5). However, binding of *L. monocytogenes* (cocktail and serotype 1/2a) to

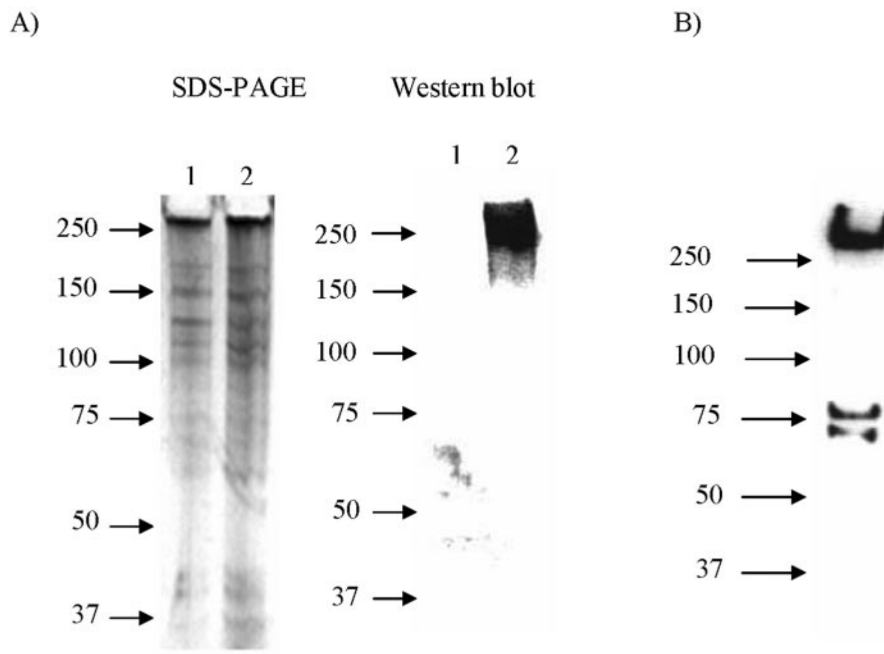

FIG. 4. Detection of IGFIIR in MS9, MS9II, and McCoy cells. Cell lysates of MS9, MS9II, and McCoy cells were prepared by sonication and separated on a 4- to 20%-gradient SDS-PAGE gel prior to Western blotting. The Western blots were developed using a monoclonal anti-human IGFIIR antibody for MS9 and MS9II cells and a polyclonal anti-mouse IGFIIR antibody for McCoy cells. The IGFIIR (~300 kDa) was detected in cell lysates from MS9II (panel A, lane 2) and McCoy (panel B, lane 1) cells but not MS9 (panel A, lane 1) cells. Standard molecular masses are represented in kilodaltons. The gels depicted are representative of duplicate experiments.

MS9II cells was significantly inhibited by M6Pbs peptide ($P < 0.01$ and $P < 0.05$, respectively) but no inhibition was observed for *L. innocua* (cocktail) or *B. cereus* (control). The M6Pbs peptide did not inhibit binding of any of the bacterial preparations to MS9 cells (Fig. 5).

Binding of bacterial preparations to mammalian cells in the presence of soluble M6P. The peptide sequence identified by phage display is located in repeat 3 of the IGFIIR, which has been identified as a carbohydrate binding site with specificity for M6P (11, 37). Binding of *L. monocytogenes* to McCoy cells was inhibited using soluble M6P as a competitive inhibitor. CSFE-labeled listerial preparations were incubated with mammalian cells, and adherence was measured by FACS (Fig. 6). Binding of *L. monocytogenes* (cocktail and serotype 1/2a) to McCoy fibroblast cells was significantly inhibited by M6P in a dose-dependent manner ($P < 0.05$ and $P < 0.01$, respectively). Surprisingly, adherence of *L. innocua* (cocktail and serotype 6b) to McCoy cells increased significantly in the presence of M6P ($P < 0.05$). To determine that inhibition was a result of M6P specifically inhibiting binding of *Listeria* spp. to the M6P binding sites of the IGFIIR, G6P was used as an inhibitor in control experiments. G6P is a closely related analogue of M6P, but it has no affinity for the M6P binding sites of the IGFIIR. Binding of *Listeria* spp. to McCoy cells was not inhibited in the presence of G6P (results not shown).

The IGFIIR is involved in invasion of mammalian cells by *Listeria* spp. A role for the IGFIIR in invasion of mammalian cells by *Listeria* spp. was demonstrated. Invasion of *L. monocytogenes* into MS9II cells was significantly increased compared with invasion of MS9 cells. Furthermore, invasion of McCoy cells by *L. monocytogenes* was significantly inhibited by soluble M6P.

Invasion of *Listeria* spp. into MS9 and MS9II cells. Invasion of wt *L. monocytogenes* (cocktail, serotype 1/2a, or strain EGD) into MS9/MS9II cells represented approximately 1% of the inoculum, but invasion was significantly (0.2 to 0.3%) reduced in strains lacking InlB ($P < 0.05$) compared to wt strain results (cocktail, serotype 1/2a, and strain EGD) (Fig. 7). Invasion of *L. monocytogenes* cocktail into MS9II cells was significantly greater than MS9 cell invasion, as determined using raw ($P < 0.01$) (Fig. 7) or normalized ($P < 0.01$) (not shown) data. Normalizing data is an accepted method of analyzing data (76). Results of invasion of *L. monocytogenes* serotype 1/2a and strain EGD and strains lacking *inlA*, *inlB*, and *inlAB* into MS9II cells compared to MS9 cell invasion were not significantly different, as determined using raw data (Fig. 7). However, significantly greater invasion into MS9II cells compared to MS9 cell invasion for *L. monocytogenes* serotype 1/2a ($P < 0.05$) and strains lacking *inlA* ($P < 0.01$), *inlB* ($P < 0.05$), and *inlAB* ($P < 0.05$) was determined by normalizing raw data (not shown). Normalized relative invasion values were obtained by setting relative values for invasion into MS9 cells at 1%, and relative invasion into MS9II cells was calculated relative to 1%. The noninvasive species *L. innocua* was used as a control and did not invade MS9 or MS9II cells (not shown).

Invasion of *Listeria* spp. into McCoy cells in the presence of soluble M6P. Invasion studies were performed by incubating log-phase listerial cultures with McCoy cells in the presence of various concentrations of M6P as a competitive inhibitor. Invasion of McCoy cells by *L. monocytogenes* (cocktail and sero-

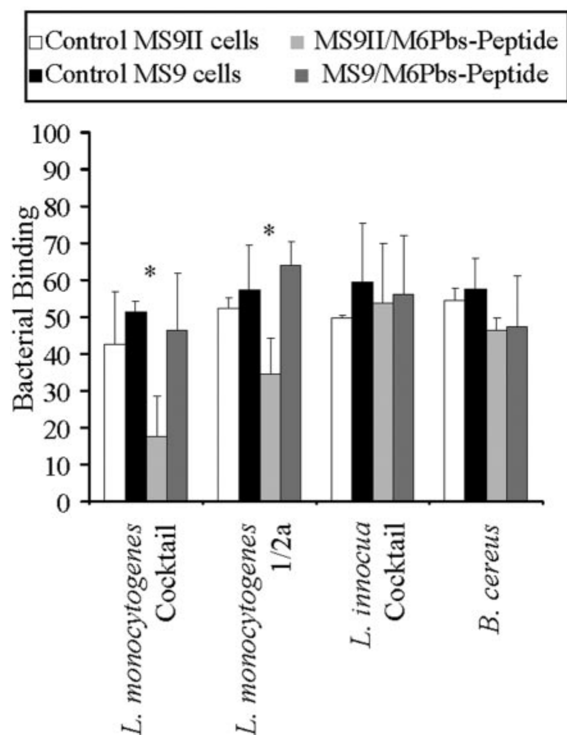

FIG. 5. Binding of *Listeria* spp. to MS9 and MS9II cells in the presence of M6Pbs peptide by FACS. Bacteria were labeled with CFSE and incubated with MS9 and MS9II cells. Bacterial binding was determined using FACS by calculating the relative fluorescent intensity. No differences were observed between binding to MS9II cells and binding to MS9 cells for any of the bacterial strains used. Binding was further investigated using inhibition of binding by the M6Pbs peptide. M6Pbs peptide was bound by CSFE-labeled bacteria, which were then incubated with MS9 and MS9II cells. Significant inhibition of binding to MS9II cells in the presence, compared to the absence, of the M6Pbs peptide was observed for *L. monocytogenes* cocktail (*, $P < 0.01$) and serotype 1/2a (**, $P < 0.05$) but not *L. innocua* cocktail or *B. cereus*. Data presented are means ± standard deviations ($n = 2$ in duplicate).

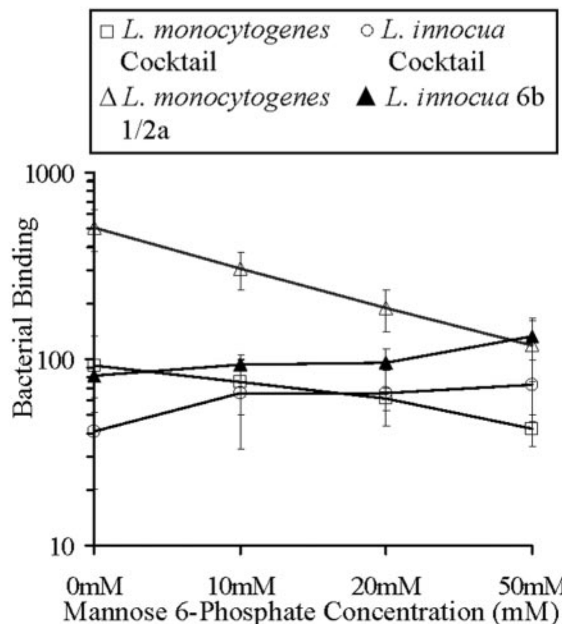

FIG. 6. The effect of M6P on binding of *Listeria* spp. to McCoy cells. CSFE-labeled *Listeria* spp. were incubated with McCoy cells in the presence of M6P (0 to 50 mM), and binding was measured by FACS. Bacterial binding was determined by calculating the relative fluorescent intensity. Significant dose-dependent inhibition of binding was observed for *L. monocytogenes* cocktail ($P < 0.05$) and serotype 1/2a ($P < 0.01$). There was a smaller but significant dose-dependent increase in binding for *L. innocua* cocktail and serotype 6b ($P < 0.05$). Data presented are means ± standard deviations ($n = 3$ in duplicate).

type 1/2a) was inhibited by M6P in a dose-dependent manner ($P < 0.01$). Inhibition of entry occurred at a concentration of 10 mM M6P (relative invasion reduced from 9.5% to 5%) but was most prominent at 50 mM (relative invasion reduced to 1.7%) (Fig. 8). Invasion of McCoy cells by *L. innocua* cocktail and serotype 6b significantly increased in the presence of M6P in a dose-dependent manner (Fig. 8).

DISCUSSION

In this investigation we demonstrated for the first time that *Listeria* spp. utilize the IGFIIR on mammalian cells during binding and invasion. Random peptide phage display was first used to identify binding of a peptide by *L. monocytogenes* with high affinity, and this peptide corresponds to a M6P binding site on the IGFIIR. Specific binding of the synthetically produced labeled peptide was confirmed and was independent of known virulence factors (InlA and InlB) (Fig. 1 and 2). *L. monocytogenes* and *L. innocua* (but not *L. ivanovii* or controls) also bound the native receptor protein (Fig. 3). A role for the receptor in binding and invasion of *L. monocytogenes* and *L. innocua* was demonstrated by competitive inhibition with the peptide and soluble M6P and by using mammalian cells transfected with the IGFIIR (Fig. 4 to 8).

Thus, all *Listeria* spp. bind the M6Pbs peptide; however, some differences were observed in the binding of the IGFIIR protein from different mammalian species by some *Listeria* spp. *L. ivanovii* bound the M6Pbs peptide (corresponding to the human IGFIIR sequence) (Fig. 2) but not the native bovine IGFIIR (Fig. 1 and 3).

The functions of the IGFIIR in mammalian cells are to bind and internalize insulin-like growth factor II (IGFII) (26, 36, 43, 75), to internalize and to sort lysosomal enzymes and other M6P-tagged proteins (11, 12, 24, 45, 46), and to regulate transforming growth factor β activity (33, 51). The majority of the receptor is found inside the cell, with only 5 to 10% located on the cell surface (45). The surface-associated IGFIIR is an important factor in the regulation of cell growth and in tumor suppression, because it removes IGFII from circulation and regulates transforming growth factor β activity (27).

The IGFIIR is made up of 15 cysteine-based repeats, a transmembrane domain, and a short, hydrophobic cytoplasmic domain (59). The peptide sequence identified in our studies is located in repeat 3 of the extracellular domain that is one of the three M6P binding sites of the IGFIIR (25, 68). Recently, the amino acid sequence in repeat 3 was determined (25, 38, 65); the identified (M6Pbs) sequence is located between two conserved cysteine residues (C334 and C375) and upstream of

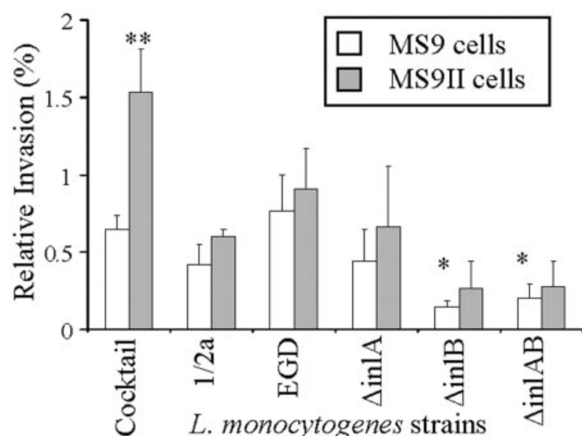

FIG. 7. Invasion of *Listeria* spp. into MS9 and MS9II cells. Log-phase cultures of *L. monocytogenes* at a multiplicity of infection of 10:1 were incubated with MS9 and MS9II cells, and gentamicin was added to kill extracellular bacteria. Intracellular listerial cells were released by lysis of mammalian cells and recovered by plating onto media. Invasion was further investigated using listerial strains deficient in known virulence factors InlA and/or InlB. Percent relative invasion was calculated using the number of recovered listerial cells $\times$ 100%/the number of cells in the inoculum. Invasion of *L. monocytogenes* cocktail was significantly (**, $P < 0.01$) increased in MS9II cells compared to MS9 cell results. Invasion of *L. monocytogenes* strains lacking InlB was significantly reduced compared to the wt EGD strain results (*, $P < 0.05$). Noninvasive *L. innocua* was used as a control and was not recovered from lysed mammalian cells (not shown). Data presented are means $\pm$ standard deviations ($n = 3$ in duplicate).

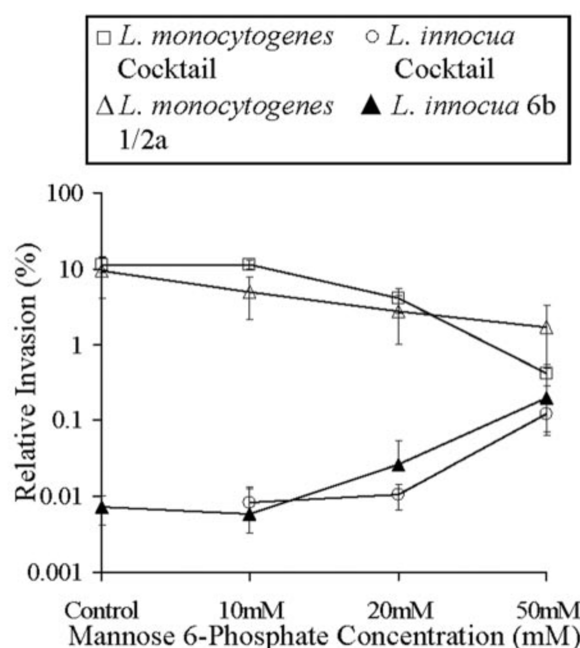

FIG. 8. The effect of M6P on invasion of *Listeria* spp. into McCoy cells. Invasion assays of *Listeria* spp. into McCoy cells were performed in the presence of M6P (0 to 50 mM). Significant inhibition of invasion of McCoy cells by *L. monocytogenes* cocktail ($P < 0.01$) and serotype 1/2a ($P < 0.01$) and increases in invasion by *L. innocua* cocktail ($P < 0.01$) and serotype 6b ($P < 0.01$) were observed. Data presented are means $\pm$ standard deviations ($n = 3$ in duplicate).

a conserved residue (Y368) which is essential for carbohydrate recognition (38, 65, 68, 84).

Adherence of *Listeria* to mammalian cells is known to occur through a wide range of different listerial virulence factors and cell surface components such as fibronectin binding proteins (31, 41), carbohydrates (15, 20, 22, 67), protein 60 (9, 72), the autolysin ami (58), lipoproteins (69), and teichoic and lipoteichoic acids (1, 34, 66, 67). These listerial cell surface components interact with a diverse range of host cell receptors, many of which have not yet been identified. Our results demonstrate that the IGFIIR is another such host cell receptor for *Listeria* spp. that is involved in both binding and invasion.

Binding of *L. monocytogenes*, but not *L. innocua*, to the receptor on MS9II cells could be competitively inhibited by the M6Pbs peptide but not the control peptides (M6Pbs-control peptide and IGFIIbs peptide). Furthermore, binding of *L. monocytogenes* to McCoy cells was significantly inhibited by M6P in a dose-dependent manner. Competitive inhibition studies using soluble M6P as an inhibitor of binding of other ligands are established techniques for the investigation of IGFIIR function (37, 62, 73). The M6P effects were specific, because G6P, a closely related analogue that does not bind to the IGFIIR (38), had no effect. These results demonstrate that the IGFIIR is involved in adherence of *L. monocytogenes*. Surprisingly, adherence of *L. innocua* strains to McCoy cells increased significantly in the presence of M6P. When considered together, these results suggest that the usage of the IGFIIR differs between different *Listeria* spp.

A fundamental role for the IGFIIR in invasion of *L. monocytogenes* is also evident by the demonstration that there was a trend to increased invasion into MS9II cells compared to MS9 cell invasion for all *L. monocytogenes* serotypes and mutants tested. Furthermore, invasion of McCoy cells by *L. monocytogenes* strains was significantly inhibited by M6P in a dose-dependent manner. Internalization of *L. monocytogenes* by mammalian cells is known to be mediated by the major virulence factors InlA and InlB through their interaction with specific host cells receptors E-cadherin and the hepatocyte growth factor receptor, respectively (17, 18, 56, 57). However, invasion into mouse fibroblast cells is not mediated by InlA, because the murine E-cadherin contains an amino acid substitution that abolishes interaction with InlA (48). *L. monocytogenes* strains deficient in InlA invaded MS9 and MS9II at approximately the same level as wt strains, confirming that invasion into murine cells is not mediated by InlA (48). Invasion of MS9II and MS9 cells by *L. monocytogenes* bacteria deficient in InlB and both InlA and InlB was significantly reduced.

Both of these observations indicate that internalization into murine fibroblast cells is primarily mediated by InlB (8, 19). The observation that invasion into MS9 cells by mutants lacking *inlB* and *inlAB* was not completely abolished also indicates that yet other receptors may be involved. The residual invasiveness of *L. monocytogenes* mutants lacking the major virulence factors InlA and InlB was also evident in other studies (4, 7, 29, 35) and suggests that there are a number of other host cell receptors involved in *L. monocytogenes* invasion (2, 34).

A significant increase in both binding and invasion of *L. innocua* of murine fibroblast cells was observed in the presence

of soluble M6P in a dose-dependent manner. A possible explanation for this observation is that, in contrast to pathogenic *L. monocytogenes*, the nonpathogenic species *L. innocua* cannot utilize hexose phosphate sugars (14, 70, 77). We speculate, therefore, that this organism may have nonspecifically incorporated the soluble M6P on its surface, which led to uptake of *L. innocua* through the IGFIIR. The M6P binding sites of the IGFIIR have been targeted previously to introduce M6P-coated foreign substances into mammalian cells by use of M6P-coated vesicles (63, 71) or albumin modified with M6P (5, 6). This provides evidence that the IGFIIR can function effectively to transport large particles from the extracellular space into the host cytoplasm.

Interactions of *L. monocytogenes* with mammalian cells, which involve binding of lectins or lectin-like molecules, including carbohydrate binding components and D-galactose- and glucosamine- or fucosamine-containing proteins, have previously been demonstrated (20–22, 80). Such components have been shown to play an important role in listerial pathogenicity and innate immune responses (32). However, there is as yet no evidence that *Listeria* spp. contain M6P, as a component of the cell wall, teichoic acids, lipoteichoic acids, or other virulence factors, that is available to interact with IGFIIR during pathogenesis. The carbohydrate binding site of the IGFIIR in domain 3 has been shown to recognize related structures such as phosphodiesters or mannose 6-sulfate (54, 55, 81), and the interaction of *Listeria* spp. with the IGFIIR may be based on such a related structure that is present on the cell surface of *Listeria* bacteria.

In conclusion, we have shown that binding and invasion of mammalian cells by *L. monocytogenes* may involve the IGFIIR and that this may be a novel mechanism of pathogenesis.

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